Design and Evaluation of a Flexible Automatic System for 3D Cell Cultivation

Dissertation Thesis

for obtaining the academic title

Doktor Ingenieur (Dr.-Ing.)

at the Faculty for Computer Science and Electrical Engineering

at the University of Rostock

Prepared at the Center for Life Science Automation (celisca)

applied at 31.05.2015

by

M.Sc. Ricarda Lehmann | Am Anger 10 |85072 Eichstätt

born on March 27th 1982 in Rostock

Dissertation - University of Rostock
Faculty for Computer Science and Electrical Engineering

Referee

Name	Institute	University
Prof. Dr.-Ing. habil. Kerstin Thurow	Institute of Automation	University Rostock
Prof. Dr.-Ing. Norbert Stoll	Institute of Automation	University Rostock
Prof. Dr. Richard A. Wysk	Edward P. Fitts Department of Industrial and Systems Engineering	North Carolina University

Date of the defense: 05.11.2015

For my family

Directory

List of Figures

List of Tables

List of Abbreviations

3D	three-dimensional
2D	two-dimensional
ADME	adsorption, distribution, metabolic and excretion
AK	Adenylate kinases
ASRS	Automatic Storage and Retrieval Systems
$CaCl_2$	calcium chloride
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DWP	deep well plate
ECM	extra cellular matrix
FACS	fluorescence automatic cell sorting
FCS	fluorescence correlation spectroscopy
FIDA	fluorescence intensity distribution analysis
FP	Fluorescence Polarization
FRET	Fluorescence resonance energy transfer
GAG	Glycosaminoglycan
GFP	green fluorescence protein
HaCaT	immortal human keratinocyte line
HCS	High content screening
HD	hanging drop
HeLa cells	cervix carcinoma cells
HEPA	High Efficiency Particular Airfilter
HepG2	human hepatocytes
HTRF	Homogeneous Time-Resolved Fluorescence
HTS	High throughput screening
Jurkat cells	T- leukemia cells
kD	kilo Dalton
LN-229	frontal parieto-occipital glioblastoma cells
Molt-4 cells	T- leukemia cells
MCF-7	breast adenocarcinoma cells
mM	Millimolar
MSC	mesenchymal stem cells
MTMOS	methyltrimethoxysilane
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nM	Nanomolar
PC-3	prostate adenocarcinoma cells
PCR	polymerase chain reaction
P/S	penicillin/streptomycin
PVAL	polyvinyl alcohol
PKH	Paul Karl Horan
RPMI	Roswell Park Memorial Institute medium
SCARA	Selective Compliance Assembly Robot Arm

SEM cells	B- precursor leukemia cells
Si-O-Si	siloxane
SW620	colorectal adenocarcinoma cells
TMOS	tetramethoxysilane
TRF	Time-resolved fluorescence
RS-4 cells	B- precursor leukemia cells
UV	ultra violett
WST	[2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium
WP	well plate
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide
μm	micrometer

1 Introduction

The demographic change associated with an increasing aging population is changing many things. Environmental factors or inherited predispositions combined with cell mutations during regular cell division processes increase the risk of cancer diseases over our lifetime [1]. The investigation of specific diseases, for example cancer and illness of the joints is gaining in importance. New strategies are required for the investigation and management of these diseases. In Germany, the second most frequent cause of death is cancer. In the last 30 years, fatalities due to cancer increased about 25% [2]. The standard therapy for cancer is a combination of radiotherapy, surgery and chemotherapy. The investigation of the cytotoxic effects of anticancer drugs is a popular research part of high throughput screening [3]. The second most important disease is Arthrosis of people over 60 years old. Currently no common therapy is available. Therefore, the investigation of human cartilage production for implantation in the human body is an important research area.

Research in life science for actual research areas in the human population has been ongoing in academia, biotechnological and pharmaceutical companies to evaluate biological or chemical processes with technology support in form of automation. The interest at life science research automation has increased in the last 15 years. Market growth increased about 10 % in the years 2000 to 2015 [4]. There are many manual methods to automate. Important is a flexible automation to adapt a variety of applications [5]. Automation enables: 1) an increased sample throughput, 2) reduction of human mistakes, and 3) guarantees the same procedure for all tests. Laboratory automation and the development of laboratory information systems started in the late 1970s and early 1980s. Advances in healthcare, clinical laboratory service, increasing age of the population and the creation of disease management companies, has resulted in the development of lab automation [6]. The research focuses for laboratory automation are: drug discovery, diagnostic, genome screening, including polymerase chain reaction, transfection or cloning. Further research includes colony and plaque picking, fermentation, gel electrophoresis, protein crystallography and also cell culturing [7, 8]. The automation of cell culture and bioscreening are complex processes. At present, these activities are limited and regarded separately. On the one side, there are different forms of bioscreening to evaluate cell processes, and automatic cell cultivation is situated on the other side, which is mainly limited to cell expansion of adherent cells. Papers published in the field of automatic cell cultivation have increased over the last 10 years.
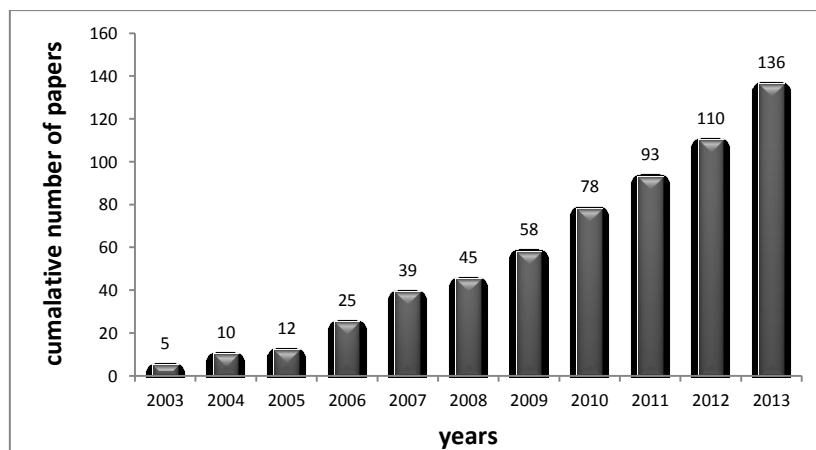
Figure 1-1 Cumulative number of papers in the field of automatic cell cultivation

Cell culture research covers different cell forms (adherent cells and suspension cells) and three dimensional (3D) cell-constructs. Normally, two dimensional (2D) cell cultures are used to investigate diseases and develop drugs for different cell lines. The plane monolayer in 2D cell cultures however cannot represent *in vivo* conditions [7, 9–11]. The solutions are three dimensional (3D) cell constructs to mimic the environmental situations and cell behavior in physiological tissue [12]. The drug development with 3D cell cultures enables the faster evaluation of drug candidates associated with simulation of *in vivo* conditions with the reduction of pitfalls, costs and time at the development of new medicines. Furthermore, these would support the ethical principles, which include reduction, replacement and advancement of animal experimentation [11, 13]. 3D cultures support the cell interaction, which is fundamental for proliferation, migration and apoptosis [11, 13]. Nevertheless, the 3D cultures offer the possibility to integrate proteins, adhesion molecules, growth factors and compounds for the stimulation of cells and mimicking of physiological conditions. The 3D cell culture models should be used for drug discovery and tissue engineering, but the limiting factors are burdensome as well as time consuming procedures for cultivation and manufacture. Therefore automation of these processes provides a variety of improvements. The greatest benefits are an increased efficiency, accuracy and reproducibility [7–9]. Continually optimized automatic processes enable a standardization of methods and a quality assurance. These cell cultures are a basis for forward-looking screenings, high content screening (HCS) and high throughput screening (HTS), stem cell research and tissue engineering.

The objective of this thesis is to design, evaluate and demonstrate an all-encompassing system for the automatic 3D cell cultivation associated with bioscreening for medical use. The automatic manufacturing of different 3D cell culture constructs and the automatic cell handling of suspension cells are integrated using the Biomek® Cell Workstation system associated with specific bioscreenings and compared with the traditional manual methods. Various cell lines have different requirements and different 3D cultures are needed to cover the whole cell spectrum. These cell culture systems should involve the cultivation of different cell lines (cancer cells, primary cells) to mimic *in vivo* conditions of the relevant medical aspects. Additionally, bioscreened cells are needed to evaluate the quality of the cell culture products, using quantification of cell-proliferation and toxicity. Furthermore, cell cultivation is regularly performed with antibiotic and the influence of antibiotic at the cell behavior, bioscreenings and interactions with compounds are neglected at the cell culture research. The analysis of this interaction is also objective of this thesis.

The aims of this thesis were realized using the instrumental reconfiguration of the Biomek® Cell Workstation and the specific SAMI® software programs [10]. The existing full functional Biomek® Cell Workstation for automatic adherent cell cultivation was modified for suspension and 3D cell culture handling cultivated in media with (+P/S) and without (-P/S) antibiotics. The adapted 3D cell culture systems used were alginate beads, pellet cultures and spheroids formed in hand hanging drops. The bioscreening was conducted using the existing High Throughput Screening System in the laboratory of the Center for Life Science Automation (celisca) at two detection dates. The proliferation was evaluated using the integrated EZ4U- proliferation assay which was adapted from 2D cell cultures to 3D cell cultures. The applications and requirements for the cell culture research are manifold. Further assays were integrated to evaluate the proliferation (WST-1 assay, DNA quantification) and cytotoxicity (AK assay). Basically, a new adapted cleaning and decontamination processes of the system guarantees the stable and contamination free cell cultivation and 3D manufacturing. Cross-contamination was excluded by the parallel cultivation of fluorescence dyed (PKH-26 and PKH-67) cervix carcinoma cells (HeLa) and the consequently fluorescence microscopy as well as fluorescence

activated cell sorting (FACS) analysis. Subsequently, four different suspension cell lines (Jurkat, SEM, Molt4, RS4) were stable cultivated, parallel disseminated and screened (WST-1 assay). 3D cell cultures were evaluated using microscopic analysis, histological staining (alginate beads, pellet cultures) and bioscreening of the proliferation (EZ4U assay, DNA quantification) and cytotoxicity (AK assay). Cervix carcinoma cells were investigated using two different 3D cell cultures - alginate beads and hanging drops. Human primary chondrocytes are used to manufacture pellet cultures. Again, the cultivation and detection of cell behavior in media without antibiotic was successfully implemented for the automatically cell handling and better mimicking of physiological conditions.

This thesis is sectioned in twelve main chapters to highlight all of the relevant components of the research. The first chapter provides an introduction in the topic of this dissertation, and a description of a flexible automatic system for 3D cell cultivation. Chapter two overviews the current state of the art of the different relevant topics (cell culture, three dimensional cell cultures, life science and automation, screening in biotechnology and software of the automatic systems).

The third chapter addresses the scientific problems and objectives of this thesis as well as the basic research method used. Chapter four provides the implementation as well as modification of the needed systems and processes of the Biomek® Cell Workstation system and the high throughput screening system to translate the manual processes to the full automatic solutions. The fifth chapter shows the manually and automatically generated results of 2D- and 3D cell cultures. For contamination free cultivation, microscopic analysis and bioscreenings of the proliferation (WST-assay, EZ4U assay, DNA quantification) as well as cytotoxicity (AK assay) were used. The performance of the automatic systems is described in chapter six for both the cell culture system and the high throughput screening system. Chapter seven addresses the discussion. Chapter eight presents the conclusions after that the outlook in section nine. The summary is provided in chapter ten. Chapter eleven contains the references followed by the supplements in chapter twelve.

2 Current State of the Art

2.1   Cell Culture Research in Life Science

In general, cell culture is the cultivation of cells with different origins (human, animal, botanical) out of an organism ("*in vitro*") under controlled conditions. Since the 1950s human cells have been used for the expansion and cultivation of cells for various reasons. Cells are required in a variety of research disciplines, for example in the biotechnology, omics-technology, gentechnology, pharmacology and toxicology, intracellular activity research, investigation of interaction between cell-matrix and cell-cell as well as in tissue engineering [12].

2.1.1   Categories of Cell Cultures

Adherent (anchorage-dependent) cells are the most popular type of cells. They attach themselves to hydrophilic and loaded surfaces. These cells only proliferate after they attach at the bottom of a cell culture vessel. Examples for anchorage-dependent cells are cartilage cells, epithelia cell, fibroblasts and cervix carcinoma cells (see figure 2-1; A). Non-adherent cells are suspension cell cultures. These cells float and proliferate in the media. Suspensions cells include lymphocytes, granulocytes and leukemia cells (see figure 2-1; B) [12].
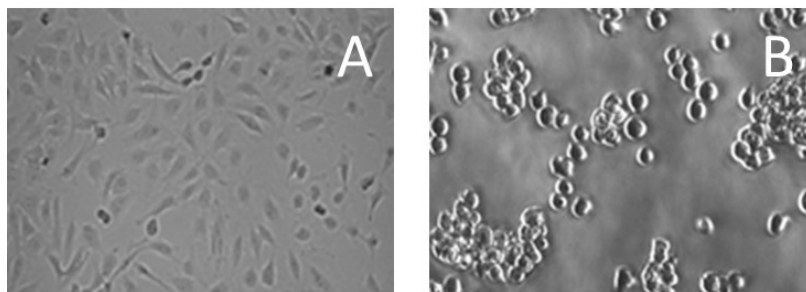
Figure 2-1 Different categorized cell lines: adherent HeLa cells (A), non-adherent Jurkat cells (B) [14]

2.1.2   Tissue Engineering

A variety of medical diseases and mechanical injuries cause tissue damage. The research part of tissue engineering unites many techniques such as cell culture, engineering, testing of biomaterials with suitable biochemical plus biophysical factors. Tissue engineering is a method for cultivating cells in a three dimensional form to produce tissue for artificial tissue as a substitute for transplantation. Transplants are helpful, if the defects are to huge or regeneration processes are disturbed. There are three different forms of translations. First, autologous transplantation uses material (cells) of the same patient. Second, allogeneic transplantation uses cells from another patient. Third, xenogeneic transplantation uses animal cells and tissue. Consequently in practice, cultivated tissue is used for defect repair of different tissues, for example bone, cartilage, skin and blood vessels [12, 15].

Tissue engineering contains several steps. Firstly, cells are biopsied of a donor. Then, these cells are isolated, cultivated and expanded in cell culture flasks. The cells might be cultivated under three

dimensional conditions with or without scaffold if enough vital cells are available. The transplant is transferred into the donor after generation of the graft.
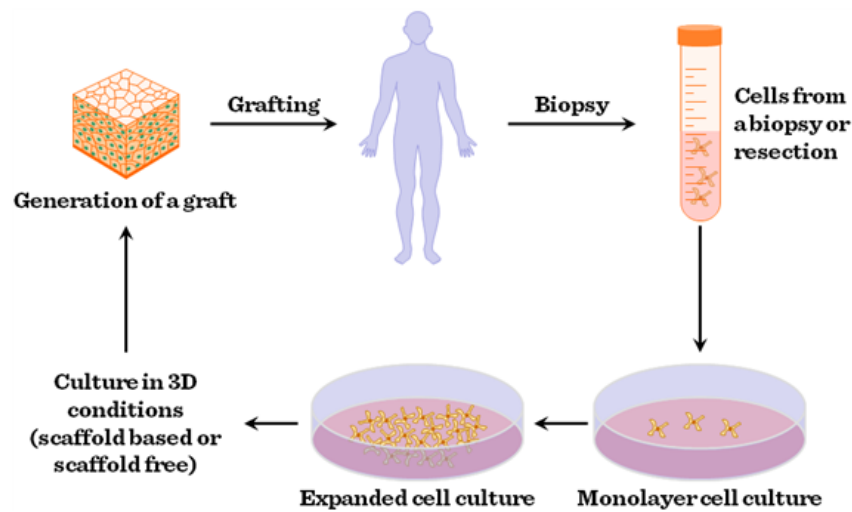
Figure 2-2 Basic principle for tissue engineering [15]

2.1.3 Cell Culture Processes

There are different methods used for cultivation, dependent of the cell type. Moreover, different cell lines and applications require different methods and culture conditions, cell culture flasks and technical equipment to cultivate homogenous cell populations [12].

2.1.3.1 Manual Cell Culture

In most laboratories, cell cultivation is carried out manually. Regarding this, cells must first be distinguished as adherent cells and non-adherent cells, which need different processing for cell cultivation (Figure 2-1).

Adherent cells proliferate at the bottom of cell culture flasks using hydrophilic and charged surfaces. The cells have to be enzymatically resolved because of strong cell-cell contacts and the attachment at the bottom of cell culture vessels. Normally, this enzymatically solution is trypsin which splits extracellular proteins and separates the cells. This reaction is stopped by adding media plus fetal bovine serum (FBS) to the solution. Often, the cell suspension is centrifuged. After removing supernatant cells are resuspended in fresh media and the cells are seeded in new cell culture vessels with applicable dilutions or cell count. The media change is performed using removing of the supernatant, and fresh media is added to the monolayer. Coated plastic cell culture vessels facilitate the attachment of the cells on cell culture vessels, for example with fibronectin, plasma or physically rough (see figure 2-3) [12].

The non-adherent cell forms are suspension cells. They do not have to be detached because these cells float in the media. The cell suspension has to be centrifuged. After removing the supernatant, the pellets have to be resuspended in new media for splitting and media change. The expansion of suspension cell is performed in regularly uncoated cell culture vessels, spinner flasks and stirrer flask [16, 17].
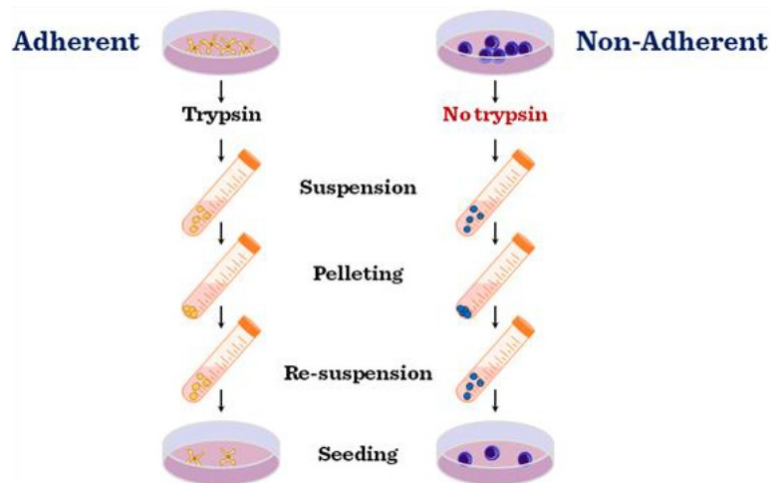
Figure 2-3 2D cell cultures [15]

2.1.3.2 Automatic Cell Culture

The automation of cell culture processes requires the translation of manual cell culture processes to large-scale production processes. The benefit of automatic cell culture processes are the performance and control of costs as well as reduction of manual process interventions, improvement of process control, constant cell culture quality and reduction of contamination [11]. Automatic cell culture processes are involved in many research fields. These include: antibody production, therapeutic protein production, vaccine production, stem cell research, high content screening and cell based assays as well as cell therapy and tissue engineering. There are many challenges for cell culturing such as human errors and standardization, sample tracking, constant culturing and production of cells, reproducibility and variables for multifactorial experimental designs, reduced throughput with shaker flasks, complexities of structure and physicochemical properties of antibodies and other therapeutic proteins or also time consuming process of formulation development as well as clone selection and evaluation [18].

2.2 Three Dimensional (3D) Cell Constructs

Cell cultivation in 3D constructs more closely reflects the *in vivo* environmental conditions, and cell behavior in physiological tissue instead of conventional two-dimensional cell cultures in petri dishes by the arrangement of cells like in the human body and the development of *in vivo* similar microenvironments [19]. Generally, 3D cultures can be used in multiple research fields. For example basic research, regenerative medicine as well as drug development and screening require cell cultures. However, these applications are still limited [20]. 3D cultures are being used for fundamental research of cancer or defect repair with tissue engineered grafts. *In vitro* cell cultivation in a three dimensional system enables the controlled 3D cell expansion and allows for the analysis of the cells using different assays. There are a large variety of 3D cultures available on the market. The methods used to produce 3D cell culture systems are 1) manifold, such as manufacturing of 3D constructs by carrier materials in form of biopolymers, and 2) acellular matrices; specific plates,

inserts, dishes and slides as well as dynamic systems or reactors. The self-production of 3D cell culture constructs can be replaced by ready to use micro tissues. The method used depends on application, cell line, laboratory equipment, scaffold free and scaffold-based (origin: natural, synthetic) as well as financial resources [20, 21].

2.2.1 Scaffold-based 3D Cell Cultivation

Generally, scaffold based 3D cultivation can be subdivided in two different methods:1) the cells are embedded in liquid gels followed with a polymerization (e.g. hydrogels, alginate) [16], and 2) cell seeding, which is performed on an acellular three dimensional matrix (e.g. Alvetex) [22]. The scaffolds are used as carrier material for the cells. These materials must be biocompatible to support the cell expansion and development of tissue similar constructs. The primary subdivision is according to materials containing biopolymers separately or associated with specific plates and acellular products/scaffolds.

2.2.1.1 Biopolymers

3D cell cultivation using biopolymers is considered a manifold process. The cells can be encapsulated in the matrix (e.g. hydrogel, alginate) or the cells may attach to the surface of microporous microcarrier (e.g. Cytodex 1, 3). 3D cultivation with microporous microcarrier need spinner vessels for the continuous gentle mixing [23], which makes automatic handling problematic, because of the continuous mixing required and the high volume. Fortunately, the encapsulation of cells has the potential for a fully automatic processing because only liquid handling steps are needed to embed cells, and the volumes are reducible to work with labware in a 96 well format. The production of bead cultures containing biopolymers with cells was rudimentarily performed automatically.

Encapsulating methods are well established methods with a wide range of applications, especially in pharmaceutical research [24, 25] and food industries [26, 27]. Encapsulating can be used for only cells, co-encapsulating with cells and nutrients, as well as encapsulating with cells and synergistic microorganism or agents. The benefits of cell-embedding using a beneficial microenvironment without stress factors (mechanical stress, UV-lights, temperature) results in a prolonged shelf life. Co-encapsulating of cells with a depot of nutrients improves the metabolic activity over a time period,and co-encapsulating with microorganism and agents enables the controlled target delivery and the protection of laboratory staff e.g. against harmful compounds [28].

Polymers differing in their physico-chemical properties (e.g. molecular weight, counter ions, gelation), costs as well as safety and quality (degradability, toxicity and batch) are currently used for manufacturing three dimensional cell constructs [28]. Popular encapsulating-materials are biodegradable polymers of natural polysaccharides, e.g. alginate, agarose, pectin [29], polypeptides like poly-L-lysin [30] or proteins like gelatin [29]. On the other hand, there are manifold synthetic polymers, e.g. polyurea, polyureathanes, polyamides or polyesters polyvenylalcohol [28].

The manufacturing of capsules is typically delivered by forming droplets from liquids by dripping through a nozzle. Some important dripping methods for the cell encapsulating are: the termal gelation, ionic gelation, spay drying, complex coacervation, LentiKats® and sol-gel immobilization (see figure 2-4). These methods are characterized by low cell quantity, large bead size depending on

the nozzle (1-4 mm) and limited bead production dependent on the dripping-speed. Thermal gelation is formed using warmed thermal dependent polymers (e.g. gelatin or agarose) with cells and dropping in cold solution. Ionic gelation is achieved by cells resuspended in Na-alginate or $CaCl_2$ and dropping into $CaCl_2$ or Na-alginate solution with consequently crosslinking and bead formation. Spray drying is conducted by dropping a polymer solution into a hot air stream and drying the water. Coacervation is performed using two dispersed water-soluble polymers. These create a coacervate phase, and a dilute equilibrium phase using opposite electric charges of the polymers. The LentiKats® method includes dropping a mixture of polyvinyl alcohol (PVAL)-hydrogel, cells and glycerol through the nozzle onto a conveyer to produce lens-shaped beads after rehydration. Sol-gel immobilization combines inorganic matrix materials with biomolecules and cells. Tetramethoxysilane (TMOS) or methyltrimethoxysilane (MTMOS) treatment supports the siloxane (Si-O-Si) polymere matrix formation with biomolecules and cells [28].
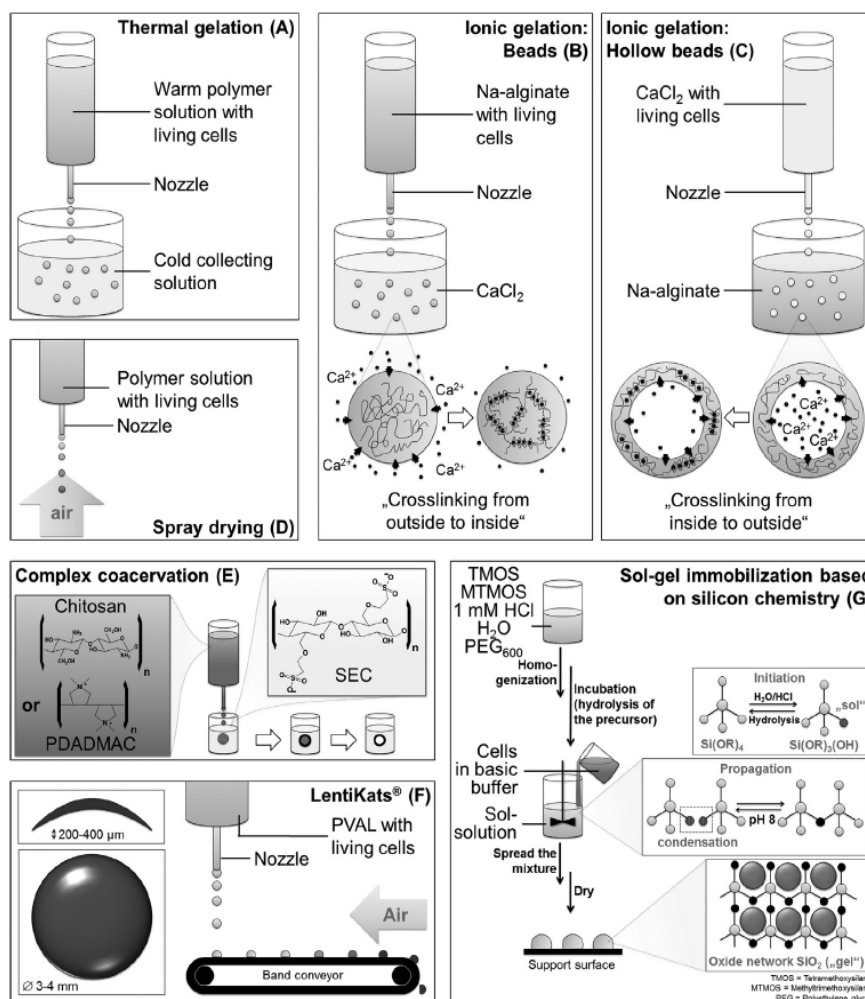
Figure 2-4 Encapsulating methods by dripping [28]

Hydrogels are water rich gels containing of a hydrophilic component and a polymer insoluble in water. These enable the development of 3D constructs by soaking in water among form maintenance. The 3D formation occurs by chemical bonding of the polymer chains or physically by electrostatically, hydrophobic and dipole-dipole interaction between the polymer chains. The characteristics of the hydrogel are variable by using of different monomers for the development of

the polymers with different forms of the cross-linking and cross-linking level. Synthetic hydrogels are based on polymethacrylic acids, polymethacrylat, polyvinylpyrrolidone and polyvinyl alcohol. The hydrogels are regularly biocompatible and used in the fields of biomedicine as well as pharmaceutical research [48].

The application and research fields of hydrogels are manifold.

Alginate (algenic acid) is a polysaccharide containing uronic acids. The 3D alginate constructs are formed by guluronic acid and mannuronic acid block polymers and are connected by (1-4)-glycosidic bonds. The main application field for alginates is biomedical research. Cells can be encapsulated in alginate by adding of calcium chloride and recovering the cells by complex formation of calcium chloride without enzymatic, thermic and mechanic impairment [50]. The cell encapsulation was performed manually with a syringe plus a needle and semi-automatic by encapsulator or droplet generator. Jonitz *et al.* used alginate beads to encapsulate human chondrocytes manually. The 3D cell culturing in the presence of several growth factors have led to a differentiation process of the cells, which was shown by the accumulation of ECM components like collagen type II, aggrecan and glycosaminoglycans [31]. Tagler *et al.* performed the manually encapsulation of mouse-follicle in alginate hydrogels in feeder-free (without mouse embryonic fibroblasts) culture environments and stimulated growth as well as survival [32]. Moshaverinia *et al.* embedded dental-derived mesenchymal stem cells (MSCs) manually in alginate beads to investigate injectable and biodegradable scaffolds. The microbeads (diameters 160.1 mm) showed viable cells after 4 weeks of incubation with a mineralization over the time [33].

The encapsulating of cells is mainly enabled using hydrogels- and alginate matrixes. There exist a variety of products at the market (see Table 2-1). The choice of the polymers depends on producer, application, cell line, needed format and financial background. The manufactures offer mainly kits to produce the 3D constructs. A miniaturization of 3D cell cultures in and on polymers is enabled using µ-fluidic systems, which are mainly used for cell based microscopy assays.

Table 2-1 Overview of different polymers for 3D cell encapsulating available on the market [34]

3D cell culture system	Product name/ manufacture	Characteristics
Hydrogels	**3D Life Hydrogel Kits /** BioCat	• hydrogel containing of biological inert synthetic polymer • modifiable with bioactive molecules (matrix proteins) • consistency of the gel variable/adjustable • costs: from 60 €
Continued on next page		

3D cell culture system	Product name/ manufacture	Characteristics
Hydrogels	**3D Life Hydrogel Kits /** Cellendes	• biomimetic, synthetic and transparent hydrogels • variable stiffness

3D cell culture system	Product name/ manufacture	Characteristics
		• partly cell and operator (cell harvesting) induced biodegradability • costs: 60 – 160 €
	Corning Matrigel / Corning Life Science	• gel to produce reconstituted extracellular matrix • reduced of growth factors

	Mimsys® / Iriosbiosciences	• hydrogels for 3D cell cultivation
		• applications + costs:
		  • embedding of cells and compounds; costs: from 91 € (Mimsys® G)
		  • embedding of cells in water-soluble marine glycosaminoglycan; costs: from 118 € (Mimsys® U)

	RAFT 3D cell culture system – starter kit / TAP Biosystems	• hydrogel containing of collagen based matrix • 96 well format automatable at Freedom EVO Workstations • format: 96 well plate (colorless, black), 24 wells, Inserts (24 wells) + plate heater, reagents • application: formation of complex 3D tissue models, co-cultivation with air-lift cultures • costs: 1440 € (96 wells), 850 € (24 wells), 820 € (Inserts)
	MAPTrix / Amsbio	• recombinant extracellular matrix mimetic • based on recombinant mussel adhesive protein • for the coating of different materials (plastic, glass, metal and biological materials) • costs: from 60 €
	Geltrex LDEV-Free (RGF) BME / Life Technologies	• extracellular substance for plating • tumor-mouse extract • 148 € (5ml)
Alginate	**Sodium Alginate** (Alginate 3D Cell Culture Kit) / Amsbio	• gelling polysaccharide in connection with calcium • application: embedding of cells for the production of 3D alginate beads • costs: 150 € (alginate, 25 ml), 555 € (Kit)
	AlgiMatrix 3D Culture System (kit) / Life Technologies	• lyophilized alginate as bio-construct • modulated hardness • for 6-96 wells • costs: 198 – 212 €
Continued on next page		

3D cell culture system	Product name/ manufacture	Characteristics
Further substances	**Cultrex** (BME 1, BME 2, BME 3) / Amsbio	• basement membrane extract (BME) • emulates reconstituted basal membrane (37°C) • applications: general (BME 1), organoid[1] cultures (BME

		2), matrix for xenografts[2] • from 35 € (1 ml)
	CTS CELLstart Substrate / Life Technologies	• substance for plating • for therapeutic application • applications: stem cells, neuronal cells, mesenchymal stem cells (MSCs) • costs: 278 € (2ml)
	3D Collagen Culture Kit / Merk Millipore	• Kit for 3D cell cultivation in collagen matrix • applications: microscopic analysis for investigation of angiogenesis, cell migration, apoptosis, proliferation and tissue formation • costs: 379 €
Microporous microcarrier	**Cytodex 1, 3** / GE Healthcare Europe	• 3D cultivation by microporous microcarrier beads containing of dextran matrix • coated with positive loaded groups (Cytodex 1), gelatin (Cytodex 3)
	Cytopore 1 & 2 / GE Healthcare Europe	• 3D cultivation by macroporous microcarrier beads containing of cellulose matrix • coated with positive loaded groups
Labware with biopolymers	**Corning Permeable supports** / Corning Life Science	• transwell- and insert systems • available in combination with extracellular matrix gel • application: co-cultivation of cells

Slides/ chambers	**µ-Slides** /
	ibidi
		• µ-Slide Chemotaxis 3D
		  • chemotaxis system for cells in 3D matrix (collagen type I gel)
		  • stable concentrations gradient about the whole matrix
		  • application: investigation of chemotaxis of leukocytes or cancer cells
		  • costs: 285 € (10 pieces) + 50 € (3D gel)
		• µ-Slide Angiogenesis
		  • chamber for 3 D cultivation and monitoring of single cells in gel (collagen type I)
		  • costs: 160 € (15 pieces) + 50 € (3D gel)
		• µ-Slide VI 0.4
		  • chamber for the investigation and monitoring of cells in 3D matrix (collagen type I gel) with interstitial flux
		  • costs: 125 € (15 pieces) + 50 € (3D gel)

[1] organoid: cell culture with different cell lines (e.g. tumor cells with basalmembrane cells)
[2] xenograft: animal origin (transplant)

2.2.1.2 Acellular Matrices

There are different methods used to form 3D cell culture systems with acellular products available on the market (see Table 2-2). The 3D arrangement can be supported using acellular matrices, printing of bioscaffolds as well as magnetic 3D bioprinting and levation. These 3D cultivation forms are cost intensive and dependent on the application and cell lines. The problem are mainly the expensive manufacturing (materials) and acquisition cost for the automatic manufacturing of 3D cell cultures in an industrial scale format.

The acellular matrices are scaffolds mainly containing polystyrene (e.g Alvetex scaffolds) most suitable for the application of personalized drug screening [20] and clinical cell research [35]. Acellular matrices are also is adapted for 2D cell cultivation in cell culture flasks. Caicedo-Carvajal *et al.* published the positive effect on the proliferation of lymphoma cells cultivated in polystyrene 3D scaffolds and co-cultivated with stromal dermal fibroblasts [35]. These matrices enable the cultivation of carcinoma cells (SW620, HepG2, HaCaT, PC-3, LN-229 and MCF-7) and tissue, as well as histological staining (haematoxylin/eosin) and bioscreenings (MTT-, XTT-, alamarBlue- and neutral red assay) using different formats (12-384 wells) [22].

Another method used to generate matrices for 3D cultivation is pressure driven printing of bioscaffolds (e.g. Gesim BioScaffolder 2.1). Cell seeding is performed on the surface of the bioscaffolds after the printing process. The optional piezoelectric microdosing of the Gesim bio-printer even enables the integration of proteins and cells in the bioscaffold during the printing process. The external unit enables the control of pressure and fluidics. The printable materials include: hydrogels, bio-polymers (e. g. collagen, alginate), bone cement paste, biocompatible silicones and polymer pastes [36].

Magnetic bio-printing is another method to cultivate cells using acellular products. The formats are predestined for automatic high throughput manufacturing and analysis of the 3D constructs because automatic systems primarily support the handling of well plate formats. The magnetic 3D products from n3D Biosciences enable the levitation (1 – 96 well formats) or printing (96 – 384 well formats) of 3D spheroids within 15 minutes and up to 6 hours by magnetization of cells after incubation with NanoShuttleTM-PL and aggregation by magnetic forces. Manifold applications for 3D cell cultures are: screenings, immunohistochemistry, RNA isolation and qRT-PCR [37, 38]. Timm *et al.* screened the toxicity of compounds (ibuprofen, sodium dodecyl sulfate) in a 96 well format using a high throughput 3D cell migration assay for embryonic kidney cells (HEK 293) and tracheal smooth muscle cells (SMCs) after magnetic levitation. The results correlated with the viability and migration of cells in 2D cultures [37]. Tseng *et al.* investigated organic lung co-cultures (endothelial cells, smooth muscle cells, fibroblasts, epithelial cells) after 3D formation by magnetic levitation in 1- and 24 well formats. The histological staining and immunohistochemistry visualized using a simulation of lung tissue through the four layers of the different cell types [39].

Table 2-2 Overview about different acellular matrices for 3D cell culture available on the market [34]

3D cell culture system	Product name/ manufacture	Characteristics
Acellular matrices/	**Alvetex scaffold /** Amsbio and Biozol	• porous polystyrene scaffold for cell ingrowth • 12-348 well plates + 6-12 well Inserts

scaffolds	Diagnostica	• dimensions: 200 µm thickness, 36-40 µm pore size with 14 µm connections • applications: co-cultures, histological sectioning, differentiation of cells, cell assays • costs: 98-700 € (Inserts), 85 € (starter kit), 88-8.069 € (12 well plate), 203-9.374 € (24 well plate), 254-11.747 € (96 well plate)
	Alvetex Strata / Amsbio and Biozol Diagnostica	• porous polystyrene scaffold for growth of intact tissue and cell aggregates upon the scaffold • 6-12 well Inserts • dimensions: 200 µm thickness, 13 µm pore size with 5 µm connections • applications: co-cultures, embryoid bodies (3D constructs of stem cells), tissue culture • costs: 195 € (12 Inserts), 350 € (48 Inserts)
Bioscaffold	**Gesim BioScaffolder 2.1 /** Gesim	• pneumatic 3D printer to produce bioscaffolds • four independent Z-drives • optional: heated microplates and - cartridge holder inclusive heating of dosing tips up to 100°C • optional: piezoelectric microdosing e.g. of proteins and cells • program to generate scaffolds with simple geometric figures with cavities
Magnetic bioprinting	**Magnetic 3D bioprinting/levation /** n3D Biosciences	• levitation (1 well – 96 well) or printing (96 – 384 well) of 3D spheroids in 15 minutes – 6 hours • magnetization of cells by incubation NanoShuttleTM-PL and aggregation by magnetic forces • formats: 1-384 wells • applications: screenings, immunohistochemistry, RNA isolation, qRT-PCR • costs: 235 € (1-6 wells), 392 € (24 wells) 470 € (2x96 well), 783 € (2x384 well) [38]

2.2.2 Scaffold-free 3D cell cultivation

There are several methods used for scaffold-free cultivation from 3D constructs without carrier material. For scaffold free 3D cultivation specific labware (e.g. plates, inserts, slides, chambers) and dynamic systems support the formation of 3D constructs. InSphero produced ready to use microtissue for this application. The 3D scaffold free cell cultivation is mainly used in cancer research and drug screening as well as tissue engineering for defect repair.

2.2.2.1 Specific Labware

Spheroid cultures are 3D cell constructs which can be produced by specific labware. These 3D cell cultures are self-aggregated clusters of cell colonies. The formation of spheroids is driven by the domination of cell-cell interaction over cell-substrate interactions [40, 41]. The benefits of spheroids formed in hanging drop plates are the uniform size and the 3D formation in an industrial scale format [19, 37]. Furthermore, this cell model supports cellular self-organization of appropriate 3D extra cellular matrix (ECM) [41]. Spheroids provide a good physiological tumor model because of the development without carrier material and the fast spheroid self-organization about aggregation of the cells by strong cell-cell contacts [40]. Current fields of research for spheroid cultures include structural and functional issues of 3D cultures, various primary or progenitor-like cell types with enhanced viability and functions as spheroids, development for models of solid tumors, components in bioartificial livers [42], cellular building blocks in tissue engineering, and embryoid bodies. Tumor spheroids allow us to study different cancer types growing as spherical aggregates in vivo, e.g. ascites in ovarian cancer [43]. Furthermore spheroid models enable the research of cancer stem cells, cancer metastasis, and invasion plus therapeutic screening [42, 44]. Additionally, spheroids support the study of cellular migration and tumor dissemination from 3D constructs, signaling and crosstalk between cells cultured in 3D environments or 3D cell–cell interactions and confrontational studies [43]. The mostly characterized 3D cell model is the spheroid formation due to its simplicity, reproducibility, and similarity to physiological tissues as compared to other methods involving extracellular matrix (ECM) scaffolds and hydrogel systems. There are limited culture forms and methods to manufacture spheroids: cell cultivation in non-adherent flasks or plates, spinner flask cultures, and rotary cell culture systems [45, 46]. The limiting factors for these spheroid formations are the problematic management, low performance, dissimilar spheroid-sizes and most importantly automation of liquid handling for high volume, autonomous systems and the flask format is difficult. The use of microfluidic systems reduces these limitations, cultivation time and device-drug consistency [47–50].

Spheroids can also be formed in hanging drops for low volume applications. Basically, hanging drops were formed by dropping a cell suspension on the underside of plate lids, where the cells arrested in a drop by surface tension and shaped at the boundary layer of air and medium, the tip of the drop [46, 51]. The manual produced hanging drops are used for cultivating human embryonic stem cells with an increased proliferation capacity of microcarriers for 10 days as well as the potential for automation [52, 53]. For a long-term (10-15 days) hanging drop cultures in a static format hollow spheres (HS) were used to form the embryonic bodies (EBs) with a multilineage differentiation. The large volume (500 ml) chambers were formed by dropping a liquid into a poly(dimethylsiloxane) mixture and generating cell culture chambers [50, 52]

3D cell cultivation by formation of pellet cultures is a simple and scaffold free method applied in basic research and tissue engineering [54]. The pellet formation is enabled by the predominant cell-cell contacts toward cell-matrix contacts. Basically, 15 ml centrifugation tubes are used for manually manufacturing pellet cultures. The pellet formation in 96 well plates enabled the miniaturization of this 3D cell cultivation with a reduction of the volume [55]. The manufacturing of pellet cultures is supported by high density cell solution in cell culture vessels with low cell attachment. Pellet cultures are primarily used for 3D cell cultivation of human mesenchymal stem cells and mainly for chondrocytes [56, 57]. Mesenchymal stem cells derived from adult bone marrow are a multi-

potential and self-renewing cell type. Furthermore, this cell type can be induced to differentiate along a variety of tissue-specific pathways, including cartilage (chondrogenesis) and bone (osteogenesis). During chondrogenesis, MSCs synthesize a cartilage-specific extracellular matrix (ECM) rich in glycosaminoglycan (GAG) and type II collagen and express cartilage markers. GAG is a sensitive and cost-effective measure of chondrogenesis [58].

Normally, 15 ml centrifugation tubes are used for manually manufacturing of pellet cultures [55]. Using this, Ong *et al.* differentiated human mesenchymal stem cells as pellet cultures into hepatocyte-like cells with growth factors because the spheroid cultures simulate the native liver lobule morphology and structure [56]. Chang et al. propagated the characteristically chondrogenic differentiation of human mesenchymal stem cells in the form of pellet cultures at three detection dates (day 7, day 14, day 21) associated with increased chondrogenesis markers (GAG content, type II collagen, aggrecan expression) [59].

There are various types of labware available on the market to manufacture 3D spheroid constructs (see Table 2-3). The labware is specific structured, coated with polymers or surface treated to suppress cell adhesion and results in the formation of spheroids. Some specific labware are equipped with honeycomb - and square patterned structures on the bottom and non-adhesive surfaces. Markovitz-Beshitz *et al.* generated uniform microtissues within every unit of the honeycomb structure [60]. Yoshii *et al.* investigated nanoimprinted plates with microcavities (1μm x 2μm). Nevertheless, the sizes of the microtissues were not similar but hypoxic cores were detectable in these microtissues, which are specific for 3D cell constructs [61].

The well format (96-384 well) of plates and inserts support automatic manufacturing, and results in high throughput bio screening. Furthermore, the costs for automatic manufacturing of 3D cell cultures by this labware are economically viable. The automatic formation of 3D cancer constructs was also one of the objects of this work and was realized with the Biomek® Cell Workstation.

Table 2-3 Overview about different labware for 3D cell culture available on the market [34]

3D cell culture system	Product name/ manufacture	Characteristics
Plates/ dishes	**Perfusion plate /** Amsbio	• perfusion system • standardized for 2D culture + perfusion • applications: medium circulation in combination with 3D cultures
Continued on next page		

3D cell culture system	Product name/ manufacture	Characteristics
Plates/ dishes	**Lipidure COAT plates /** Amsbio	• coated plates • 96 - 384 well plates (white, black, colorless); 60 mm and 90 ml dishes • costs: from 60 €

	NanoCulture plate (NCP) Kits / Amsbio	• plates with specific soil structure of micro-honeycomb • support development of similar shaped cell spheroids • applications: 3D cancer and tumor cultivation • costs: 290 €
	3D Biomatrix Plates / Biotrend Chemikalien	• plates to form efficient spheroids in hanging drop plates • formats: 96 wells, 384 wells • high throughput compatible • costs: 170 €
	Cell culture plate / Corning Life Science	• corning spheroid cell culture plate to form, cultivate and analyze spheroids • black – colorless plates with round button • formats: 96 well, 384 wells
	Mimetix plates / The Electrospinning Company	• removable delivery system produced by electospinning • formats: 6-96 (thickness 50 µm) wells • cell growth in fluid-air phase-interface • applications: microscopy analysis • costs: 85-95 €
	Cellstar cell culture dishes / Greiner Bio-One	• manufacturing of spheroid cultures • surface prevents the attachment of cells • application: formation of 3D stem cell aggregates
Inserts	3D Keratinocyte Starter Kit/ Cellntec	• Kit: 50 Inserts, with medium and cells to form 3D constructs with keratinocyte cells (epidermal equivalent) • Diameter: 0.6 cm^2 Millicell inserts, 0.47 cm^2 Nunc inserts for 60 mm dishes, 24 well plates • Support: histochemistry, immunohistochemistry and immunofluorescence analyses • costs: 850 €
	Millicell Inserts / Merck Millipore	• Inserts for well plates with microporous membrane • membrane enable transport of nutrients • formats: 24 well, 96 well • membranes variable: type and pore size • application: investigation of 3D explants by organotypic inserts
Continued on next page		

3D cell culture system	Product name/ manufacture	Characteristics
Inserts	AK-PolyFibers™ / AkronBiotech	• 3D scaffold inserts containing of electrospun polymeric nanofibers for simulation mechanical and physical structures of ECM • formats: 6-48 well inserts • applications: 3D cell formation, analysis (assays, gene

		expression)
		• costs: from 22 €
	3D Culture Matrix BME Coated 96 well plate / Biozol Diagnostica	• 96 well plates with hydrogel to support cell proliferation
		• applications: 3D formation of epithelial and endothelial cell lines
		• costs: 310 €
Chambers	**CytoCapture** / MoBiTec	• chambers for cell cultivation with different geometries microcavities of the chamber bases
		• general applications: microscopic analysis of single cells and 3D spheroids
		• formats: chambers, 96 well plates, dishes and slides
		• microcavities + applications:
		• square shaped structures (side length of 40 μm, depth of 15 μm) to support 3D formation of adherent cells
		• hexagonal structures (diameter of 250 μm or side length of 500 μm, depth of 100 μm) for cluster analysis of adherent and non-adherent cells (available with Low Cell Adhesion coating to support spheroid formation)
		• costs: 280 – 320 € (16 pieces)
	ChamberMatrix™ 16 Hydrogel / MicroStem	• slide with chamber system coated with hydrogel (highly cross-linked inert polyacrylamide)
		• applications: growth and imaging of suspension cells, 3D spheroid formation on the gel by prevention of cell attachment

2.2.2.2 Microtissues

The InSphero AG produces certificated, standardized, uniform and assay ready microtissues in a 96 well format in homo- or co-cultures with different cell lines (see Table 2-4). These cost intensive microtissues are produced in hanging drop plates. There are different types of microtissues available (liver microtissues, colorectal carcinoma microtissue, tumor microtissues). The applications for these microtissues are toxicity tests, compound screenings, and ADME-Toxicity studies. These microtissues are fixed with the specific cell types, and are not flexible for another cell lines and were thus not used [62].

Table 2-4 Overview about microtissues available on the market [34]

3D cell culture system	product name/ manufacture	characteristics
microtissues	**3D InSight Human Liver Microtissues (96x)** / InSphero	• 3D liver-microtissue containing of human primary hepatocytes (homo-culture) or with Kupffer cells
		• costs: 1.325 – 1.458 €
	3D InSight Rat Liver	• 3D liver-microtissue containing of primary rat

	Microtissues (96x) / InSphero	hepatocytes (homo-culture) or with non-parenchymal cells (NPC) • costs: 1.158 – 1.242 €
	3D InSight HepG2 Microtissues (96x) / InSphero	• 3D liver-microtissue containing of HepG2 cell line • costs: 825 €
	3D InSight human colorectal Carcinoma Microtissues (96x) / InSphero	• fluorescence tumor-microtissue • GFP-expressed HCT 116 tumor-microtissue • costs: 992 €
	3D InSight tumor Microtissues (96x) / InSphero	• different homo-typical tumor-microtissues • tumor cell line+NIH3T3 fibroblasts co-culture microtissue (908 €) • tumor cell line+MRC-5 human lung fibroblasts co-culture (950 €) • tumor cell line+NHDF human dermal fibroblasts co-culture (950 €)

2.2.3 Dynamic Systems

The formation of 3D cell cultures can also be performed within dynamic systems with firm specific software. The bioreactors can be separated by volume, format and application (see Table 2-5). There are systems available with a continuous nutrient supply. In general, these independent systems are designed with a small footprint for independent cell expansion under controllable conditions (temperature, CO_2, O_2) and for use in a production volume of up to 100 liters. The tubing systems and pumps provide the fluidic transfer (see figure 2-5) [63, 64].
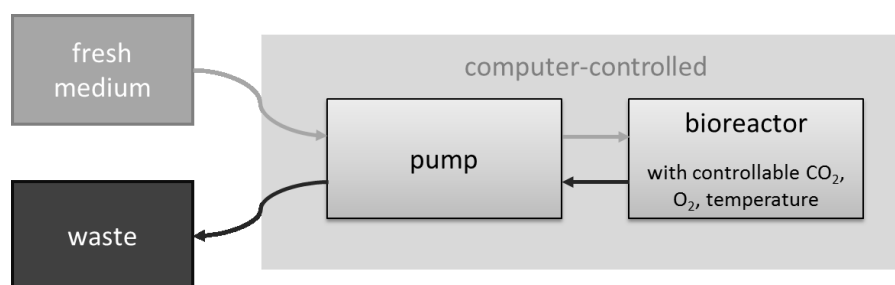
Figure 2-5 General setup of dynamic systems

The WAVE Bioreactor systems from GE Healthcare expand cells in a sterile and single-use cell bag with medium under rocking wave motions for continuous mixing [64]. The Minifor bioreactor of Lambda Laboratory Instruments is a stirrer tank bioreactor and fermenter. The controllable parameters are: stirrer speed, airflow and level control for continuous processing [63, 65]. These systems support the cultivation of batch cultures, fed-batch cultures, perfusion cultures and the continuous cultures. The TEB bioreactor of Ebers Medical technology is equipped with a CO_2 incubator, double-peristaltic pump system and a chamber system. These systems are useable for tissue engineering and biomaterial research [66]. The low-volume bioreactors are focused at a well plate format. The portable CellASIC® ONIX Microfluidic Platform of Merck Millipore enables cell

cultivation under hypoxic and dynamic conditions. A low-volume manifold connects the control system with the microfluidic plate. The control system includes 8 syringe pumps, a perfusion chamber apparatus, and a CO_2 incubator. The manifold is located on the plate to provides an air tight seal that supports pressure driven liquid flow. The microincubator supports the control of gas and temperature at the microincubation chamber among the microfluidic plate and the manifold. This system is designed for microscopic analysis, e.g. evaluation of morphology and viability. The embedding of cells in Matrigel connected with this platform enables 3D cell cultivation [67]. The Flexcell® bioreactor systems are computer controlled and enable 3D cell cultivation by simulating of *in vivo* conditions. The FX-5000C system allows for the cultivation by compressing 3D cell constructs (cyclic or static) between a piston and BioPress™ culture plates. The tissue engineering is also realizable with cells embedded in gel matrix with or without uniaxial tension by deforming of cell culture plates with positive air pressure [68–70]. The FX-5000TT system is used for the formation of 3D cell collagen gels within a 6 well tissue train loading plate. This system is equipped with a Trough Loader, and Arctangle Loading post to simulate human strain regimens (see figure 2-6) [71].
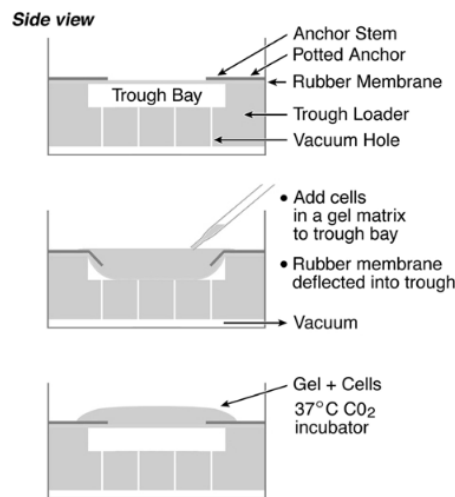
Figure 2-6 Production of cell-gel constructs in a Tissue Train Culture system [71]

The cost intensive bioreactors are autonomous systems and were therefor not integrated in the Biomek® Cell Workstation.

Table 2-5 Overview about different dynamic systems for 3D cell cultivation available on the market [34]

3D cell culture system	Product name/ manufacture	Characteristics
Reactors/ dynamic systems	**WAVE Bioreactors /** GE Healthcare Europe	• wave bioreactors • cell cultivation in a cell bag • single use • controllable: CO_2, O_2, temperature • culture volumes: 0.2 − 0.5 liter (system 2/10), 0.3 − 25

		liter (system 20/50), 10 – 100 liter (system 200), 50 – 100 liter (system 500/100) • applications: batch culture, fed-batch culture, perfusion culture and the cultivation of adherent cells [64]
	Minifor / Lambda Laboratory Instruments	• bioreactors for stem cell expansion • cell cultivation in a stirrer tank • re-usable and autoclavable • module system • controllable: stirrer speed, temperature, pH, oxygen, airflow, level control for continuous mode • volumes: 35 ml to over 6 liters • applications: cultivation of bacteria and stem cells [63, 65], • costs: cut of 9.998 €
	TEB / Ebers/Meintrup DWS	• flux-bioreactor • O_2- and CO_2-controlling • controllable temperature • multichannel pumps (outside of the chamber) • applications: tissue engineering, biomaterial research • costs: 20.500 € (TEB 505 table-top device), 45.000 € (TEB 1000) [66]
	CellASIC ONIX Microfluidic Platform / Merck Millipore	• microfluidic platform for dynamic cell culture and 3D cell culture in Matrigel-matrix • well plate format • chamber volume: 1ml • long-term cell cultivation by constant supply of nutrients and removal of metabolic products • applications: microscopic analysis [67]
	FX-5000 / Dunn Labortechnik	• bioreactor systems • 6 well plate format • applications: 3D cultivation in gel matrix with or without uniaxial tractive forces (FX-5000TT); 3D cultivation, stimulation and investigation of cyclic and static pressure (FX-5000C) [68]

2.2.4 Comparison of Different 3D Cultures to Automate

For commercial use 3D cell culture systems (bead-, spheroid-, pellet cultures) have to be automatic. The bead cultures and spheroid cultures enable the microtissue formation for all cell types, in particular from cancer cell lines and other primary cell lines. Pellet cultures are manufactured using 3D constructs, for chondrocytes and human mesenchymal stem cells. The cultivation time of the published 3D cell cultures depends on the cell line and culture system. The bead cultures and spheroid cultures form 3D constructs from the first day. Cells needed 7 days to form pellet cultures and the maximum cultivation period terminates at day 15 of spheroid cultures, day 28 of pellet cultures and day 35 of bead cultures. Only bead cultures require carrier material to form the 3D

constructs for embedding cells in a biopolymer. The spheroid cultures formed in hanging drops require specific hanging drop plates for the high throughput 3D formation. Pellet culture manufacturing is conducted in regular 96 well plates without surface treatment to prevent cell attachment at the bottom. However, many benefits for the 3D cultures have been published. Bead cultures enable the embedding of growth factors, biomaterials and compounds with cells in the matrix associated with simply microscopy analysis in the interior of the construct. The benefits of the spheroid cultures in hanging drops were the fast manufacturing of compact microtissues without carrier material. Furthermore, the hanging drops enabled high throughput 3D formation in plates of up to 384 wells. The research parts of the 3D cultures were multifarious. The bead cultures were used for tissue engineering, microenvironment -, vascular -, cartilage -, metastatic research as well as folliculogenesis associated with direct cartilage defect repair. The areas of research for spheroid cultures include tissue engineering, compound screening together with investigation of plate designs to realize high throughput. Pellet cultures are covered in research areas that include tissue engineering, regenerative medicine (chondrogenesis), skeletal development research, biomaterial research, and compound screening. They were limited primarily to chondrocytes and mesenchymal stem cells.

Table 2-6 gives a general overview of the characteristics of the published different 3D cultures.

Table 2-6 Overview of 3D cultures

Characteristics	Bead cultures	Spheroid cultures	Pellet cultures
Cells	every cell type (cancer cells, primary cells)	every cell type (cancer cells, stem cells, primary cells)	chondrocytes, human mesenchymal stem cells (hMSCs)
Cultivation time	1- 35 days	1-15 days	7-28 days
Carrier material	agarose, alginate	none	none
Benefits	inclusion of biomaterial, growth factors and compounds, possibility of inner microscopy	plate format (up to 384 well), none carrier material, fast compact microtissue production	none support material
Continued on next page			

Characteristics	Bead cultures	Spheroid cultures	Pellet cultures
Direct medical application	chondrocytes defect repairs	none	defect repairs of chondrocytes and intervertebral discs

Areas of research	microenvironment research, tissue engineering, vascular research, cartilage research, metastatic research, folliculogenesis	tissue engineering, cell plate design, compound screening	tissue engineering, regenerative medicine (chondrogenesis), skeletal development research, biomaterial research, compound screening

2.2.5 Automatic 3D Culture Systems

In the course of this thesis three different 3D cell culture systems were automatic produced using the Biomek® Cell Workstation. The integrated 3D cell culture constructs were scaffold based (alginate beads) and scaffold-free (spheroids in hanging drops plates, pellet cultures) to cover a wide range of applications and cell lines with tolerable manufacturing costs.

2.2.5.1 Bead Cultures

Bead cultures are round and stable 3D constructs with embedded cells. Different biopolymers (hydrogel, alginate) enable the formation of beads. This thesis addressed this topic of 3D bead formation by translation of the manually alginate bead production to closed automatic processes, including treatment with trypsin and the manufacturing of alginate beads. Furthermore, a new 96 well format was selected for high throughput bioscreening.

Hydrogel Beads

Kumachev *et al.* (2011) established a basis for the high-throughput generation of combinatorial microenvironments with different mechanical properties for cell studies by semi-automatic hydrogel bead formation with a self-produced microfluidic device, manufactured using a standard lithography procedure. They manually encapsulated two mouse embryonic stem cell lines in agarose microgels (size: 100 µm) with different elastic moduli. The microfluidic device was equipped with two independently controlled syringe pumps (Harvard Apparatus 33 Dual Syringe Pump, USA) for the different alginate solutions. The hydrogel beads were formed by two streams of agarose solutions with different concentrations at varying relative volumetric flow rate ratios and on-chip gelation of the precursor droplets [72].

Alginate Beads

Corward *et al.* published a paper on the semi-automatic encapsulation of HepG2 cells in alginate (diameter ~400 ± 17 µm diameters) [75]. First, the cells were manually treated with trypsin and mixed with 2% alginate. Subsequently, the beads were formed by dropping the cell suspension in a stirred solution (0.204 M $CaCl_2$ in 0.15 M NaCl buffer) through the syringe pump of an Inotech IER-20

encapsulator (Inotech, Dottikon, Switzerland) at a flow rate of 5 ml cell suspension/minute by a 200 mm nozzle vibrating at 1295 Hz. Consequently, the beads were cultivated in a rotary cell culture system (RCCS) with horizontal rotations [73–75]. Comparison of a rotary against static bead cultivations showed enlarged proliferation, protein synthesis and detoxification in the rotary system [73, 75]. In 2009, Corward *et al.* investigated HepG2 cells encapsulated in alginate within a bed bioreactor in normal and liver failure (LF) human plasma. The results were an increased cell viability, metabolic activity, protein synthetic and detoxification cultivated in LF plasma [74]. Rokstad *et al.* semi-automatically encapsulated serotonin (5-HT) producing intestinal neuroendocrine tumor cells (KRJ-1) in alginate beads. The cells were manually prepared with alginate (1.8%) followed by the bead production with an electrostatic droplet generator, 0.4 mm needles and a flow rate of 5–20 ml/h. The alginate beads with a size of 200-400 μm enabled reflected basal 5-HT secretion and reflect a suitable method for long-term cultivations (>30 days) [76]. Kim *et al.* investigated the semi-automatic embedding of human embryonic stem cells (hESCs) in alginate to support the differentiation to dopamine neurons. The cells were manually detached with accutase and mixed with 1.1% sodium alginate at a concentration of 1.25×10^6 cells/ml. The automatic bead formation was realized by dropping the alginate-cell suspension in a 100 mM CaCl2/10 mM HEPES solution using an air-driven droplet generator (Nisco Engineering Inc., Zurich, Switzerland) at a flow rate of 8 l/ml and a pressure of 100 kPa [77]. Xu *et al.* embedded hepatocellular carcinoma cells (HCC) semi-automatically in alginate to evaluate the metastatic capability and simulate conditions in tumor tissue inside of alginate beads. The results showed an increased gene- and protein expression of metastasis-related molecules as well as a higher invasion of cell derived from alginate beads. The cells were manually treated with trypsin and embedded in alginate (1.5%) itself produced electrostatic droplet generator [78]. Moshaverinia *et al.* evaluated dental-derived mesenchymal stem cells (MSCs) semi-automatically encapsulated in alginate (2%) hydrogel beads within TGF-β1 (growth factor) produced by a microfluidic device with a two-channel fluid jacket microencapsulator and a micropipette. The beads were formed by shearin soybean oil and collection in $CaCl_2$ solution. The results showed the expression of chondrogenic markers (Col II and Sox-9) and newly synthesized matrix [79]. Leslie *et al.* investigated the controlled release of rat adipose-derived stem cells (ASCs) from alginate microbeads by integrating alginate-lyase in the beads. They manufactured alginate microbeads semi-automatically with embedded adipose derived stem cells (ASCs) by a Nisco Encapsulator VAR V1 LIN-0043 (Nisco Engineering AG, Zurich, Switzerland) at a flow rate of 5 ml/h and nozzle (inner diameter of 0.175 mm) to generate 0.12 mm sized beads. The cell release was dependent on the ration of alginate and was associated with decreased cell viability over the time (12 days) [80]. Kim *et.al* semi-automatically produced injectable multifunctional alginate beads with embedded endothelial cells (OECs) and growth factors (VEGF-vascular endothelial growth factor, HGF-hepatocyte growth factor) using an electrospraying method. The device contained a high-voltage source, a syringe pump, a stainless-steel nozzle and a syringe needle (28-gauge) from NanoNC (NNC-ESP 200, Seoul, South Korea). The beads were characterized by increased cell viability and controlled release of the growth factors to improve vessel and tube formation *in vitro*. The *in vivo* studies demonstrated enhanced blood flow perfusion and enlarged the vessel density.
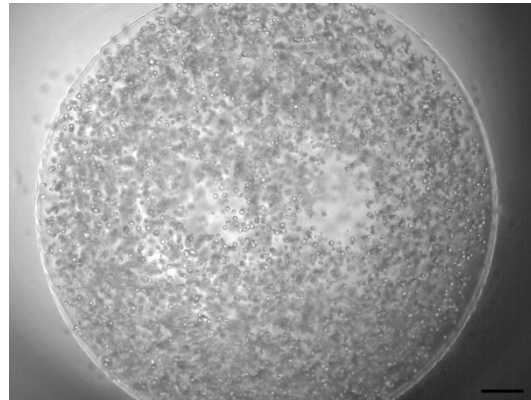
Figure 2-7 Cervix carcinoma cells automatically embedded in alginate matrix (bar 250µm)

2.2.5.2 Hanging Drops - Spheroid Culture

Specific hanging drop plates support high throughput procedures by standardized, reproducible and automatable solutions to manufacture and investigate microtissue in an industrial scale format, e.g. The Perfecta® 3D Hanging drops Plates and the Gravity Plus platform well plates are available on the market [46].
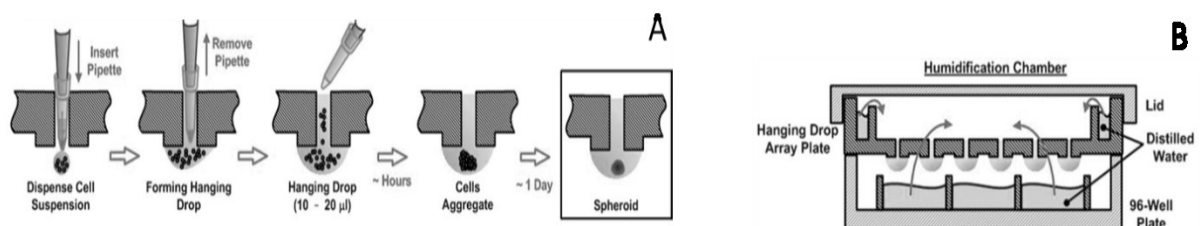
Figure 2-8 Hanging drop formation (A) in a specific hanging drop plate (B) [48]

The automatic production of hanging drops to generate spheroid cultures in a high throughput manner is a further development. Cavnar *et al.* formed spheroids with different cell types (MDA-MB-231 breast cancer cells, HeyA8 ovarian cancer cells, and a human mammary fibroblast cell line) in a 384-well hanging drop plate to evaluate a spheroid transfer and imaging (TRIM) plate downstreamed process (spheroid collection for flow cytometry, imaging and enzymatic studies) [52, 81]. Regarding the automatic hanging drop production, a commercial liquid handler (CyBi-Well, CyBio Inc.) was used for the cell seeding and hanging drop formation in a 384 hanging drop array plate [48] or only spheroid transfer [82]. In detail, Hsiao *et al.* demonstrated a 384 hanging drop array as fluorescence- and colorimetric-based assays to generate partly excellent robust Z´-Factor as a basis for high throughput screenings. Biomedical applications of spheroids cultivated in 384 hanging drop plates were demonstrated by the manufacturing of monocultures (mouse embryonic stem cells [mES-Oct4-GFP], HepG2 cells, DU145Luc prostate cancer cells, and HFOB human fetal osteoblasts) as well as co-cultures to form Janus spheroid (PC-3DsRed, HUVEC, and MC3T3-E1). Futher demonstrations were the addition of cells for concentric layer for patterning of different cell types and culture of a wide variety of cell types. An increased amount of dead cells in the inner circle of 3D –cultures was

visualized by cell cell viability testing with the LIVE/DEAD Viability/Cytotoxicity Kit (Life Technologies GmbH, Darmstadt, Germany) followed by fluorescence microscopy. The partly automatic transfer of spheroid was realized using a liquid handling robot (CyBi-Well, CyBio, Inc.) [82]. In a further paper Hsiao *et al.* published 2012 the development of the hanging drop method. They used stereolithography for the production of micro-rings structures to stabilize microdroplets for an extended cultivation of long term spheroid cultures in 384 hanging drop array plates and an injection molding tool for mass production of polystyrene 3D cell culture plates. Micro-rings stabilized the droplets against mechanical and chemical perturbations. These advances enabled long-term spheroid cultures up to 22 days inside the droplet array and revealed new prospects for high-throughput preparation of micro scale 3D cell constructs for drug screening and cell analysis. The authors designed the micro-rings to prevent a spread out of media in the top or bottom in the original hanging drop array. Droplet spreading on the bottom caused by centripetal acceleration was prevented by a bottom trench design with extra micro-ring feature of 0.25 mm width and 0.50 mm height. Extra micro-ring (extrusion) and trench (dent) design modifications around the top surface of the through holes enabled a rapid prevention of spreading out liquid on the top surface of the plate [83].

Tung *et al.* formed and investigated spheroid cultures in hanging drops with a 384 hanging drop array plate. They analyzed the negative proliferative effect of common anticancer drugs (5-fluorouracil, tirapazamine) the 3D cultures [48, 52]. Tung *et al.* described the production of spheroid cultures in a high throughput manner. They created a 384-well format hanging drop culture plate that enabled the efficient formation of uniformly sized spheroids, their long-term culture and drug testing using existing HTS instruments (i.e. liquid handling robots and plate readers) on the obtained 3D cellular constructs. The 384 hanging drop array generated 192 spheroid cultures by a commercially available liquid handler. Cellular viability was detected at 24, 48, 72, and 96 h of drug incubation using alamarBlue® (Life Technologies GmbH) to obtain fluorescence intensity readouts by plate reader. Moreover, these results were compared to the viability results gained by fluorescence microscopy imaging using live/dead stain. The results showed that the anti-proliferative anticancer drug 5-fluorouracil (5-FU) had a higher effect in 2D compared to the hypoxia activated drug tirapazamine (TPZ) which showed a higher anti-proliferative effect in 3D cultures [48].

2.2.5.3 Pellet Cultures

Automatic pellet production published by the improved manually formation of pellet cultures in a 96 V-bottom well plate and 300 µl medium/well was similar to pellet formation in tubes. The content of glycosaminoglycans (GAG)as published by Penick *et al.* and deoxyribonucleic acid (DNA) was increased in the plates and collagen type II and X were presented [55]. Schon *et al.* described a simple, manual, high-throughput pellet formation in a 96 well format. The pellet cultures showed after 28 days a regular size and shape. They also described the formation of pre-cultured pellets in a controlled manner into specifically designed 3D plotted porous scaffolds for cartilage tissue engineering constructs. The authors compared the conventional method (tubes) with a 96 well format and two different well geometries (round- and v-bottom plates). GAG/DNA analysis showed minor differences in tissue quality and size as well as the chondrogenic re-differentiation capacity of human chondrocytes. The simple production of large numbers of reproducible tissue of these pellets was possible since collagen type I and II production and collagen type I, II and aggrecan mRNA

expression were maintained in the 96-well plate format. This method also allowed the generation of labeled pellets [57]. Ibold *et al.* published the semi-automatic pellet formation of chondrocytes co-cultivated with synovial fibroblasts in a 3D pannus model in an uncoated, flat-bottom, 96-well tissue culture plate with a homogenous content of collagen type I, II and proteoglycans. The prior cell handling was performed manually. The automatic pellet production was carried out with cell seeding by the CyBi™-Disk workstation for liquid handling which is known for time saving, higher reproducibility and no differences of the extra cellular matrix of pellets in automatic processes [84]. Schon *et al.* showed potential for automating the fabrication method to produce large amounts of cell pellets [54, 57]. Huang *et al*. (2008) demonstrated a high-throughput screening assay for modulators of chondrogenesis of mesenchymal stem cells. The semi-automatic pellet formation was realized using the automatic dispensing of the cell solution by the Matrix Technologies Well Mate system in in 384-well conical plates. For high throughput screening the authors tested HTS campaign of the National Institute of Neurological Disorders and Stroke (NINDS) chemical library of small molecules (1,040 compounds). There were five potential inducers and 24 potential inhibitors of chondrogenesis. The test series included the pellet formation and library screening ensured automation in a 384 well format. Cells were dispensed automatically using especially a Matrix Technologies Well Mate system in 384-well conical plates. The pellet formation needed 7 days and then followed in-well papain digestion. Afterwards a robotic liquid handling system transferred the digestate to a new flat bottomed 384-well assay plate for performed GAG assay and DNA analysis through PicoGreen® reagent [58].
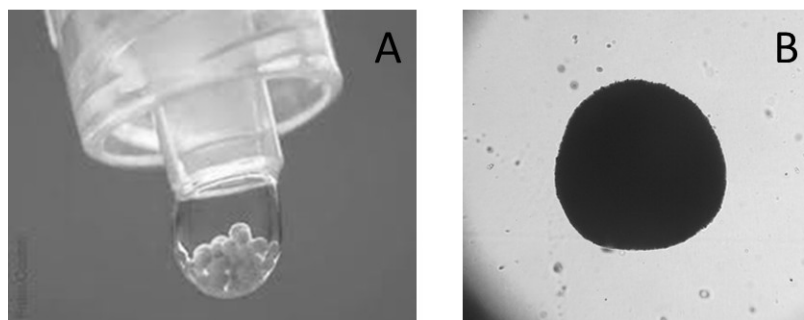
Figure 2-9 Pellet culture before transplantation (A) [85] **and automatically manufactured pellet culture (4x manifold) (B)**

2.2.6   Summary of Published Papers on 3D Cultures

The manual production of 3D cultures is time-consuming and complex processes. The fully automatic production resulting in different benefits (higher reproducibility, sterility, a consistent performance and saving of time and money [6–9]). The published production of the 3D constructs was performed either manually or semi-automatically. The semi-automatic methods include the manual cell preparation and 3D formation using liquid handler, dispenser or droplet generators.

The 3D constructs differ in the necessity of the carrier material, the former format, the cell lines including the consequent assays and the applications. The Table 2-7 summarizes the published automatic 3D cultivation forms.

Table 2-7 Overview about published 3D cultures

3D culture form	Production/format	Cell lines, tests/assays	Applications
Hydrogel beads (agarose)	semi-automatic, high throughput	embryonic stem cells; optical microscopy	microenvironment research, 3D tissue engineering [72]
Alginate beads	manual	dental-derived mesenchymal stem cells (MSCs) - PDLSCs and GMSCs bone marrow mesenchymal stem cells (hBMMSCs); fluorescence microscopy after live/dead staining, reverse transcription and polymerase chain reaction (RT-PCR), p-nitrophenyl phosphate assay, confocal laser scanning microscopy, scanning electron microscopy, histology and immunohistochemistry, *in vivo* studies	bone tissue engineering [86]
Alginate beads	manual	mouse-follicle; microscopy analysis	early stage folliculogenesis, fertility preservation [32]
Alginate beads	manual	chondrocytes; flow cytometry, DNA isolation and quantification, Blyscan™ glycosaminoglycans assay, histology and immunohistochemistry	3D cartilage tissue engineering [31]
Alginate beads	semi-automatic, high throughput	human liver carcinoma cells (Hep G2); Lowry assay (protein detection), ELISA, EROD assay (CYP450 activity), detection of glucose consumption by Analox GM-7 analyzer, confocal microscopy after FDA/PI-staining	microenvironment research, evaluation of cell behavior in alginate beads within a rotary system [73]
Alginate beads	semi-automatic, high throughput	neuroendocrine tumor cells (KRJ-1), MTT assay, Live/dead assay with confocal microscopy, Serotonin BAE-5900 ELISA	evaluation of neuroendocrine neoplasm cells for tumor model [76]
Continued on next page			

3D culture form	Production/format	Cell lines, tests/assays	Applications
Alginate beads	semi-automatic, high throughput	human liver carcinoma cells (Hep G2); confocal microscopy after FDA/PI-staining, lactate dehydrogenase (LDH) assay, detection of Glucose	evaluation of cell behavior in alginate beads within fluidized Bed Bioreactor [74]

3D culture form	Production/ format	Cell lines, tests/assays	Applications
		consumption by Analox GM-7 analyzer, alpha-fetoprotein (AFP) detection, bilirubin determination by a VITROS system, detection of CYP1A1/2 activity by a Cytofluor 2350, GC–MS analysis, sandwich enzyme-linked immunosorbent assay (quantification of albumin production)	
Alginate beads	semi-automatic, high throughput	human embryonic stem cells (hESCs); immunofluorescence staining, quantitative polymerase chain reaction (PCR) analysis, western blot analysis	differentiation of human embryonic stem cells to dopamine neurons [77]
Alginate beads	semi-automatic, high throughput	rat adipose-derived stem cells (ASCs); live/dead assay with confocal microscopy, p-nitrophenyl phosphate assay, TUNEL assay, real time PCR, histology	tissue regeneration (controlled cell release) [80]
Alginate beads	semi-automatic, high throughput	hepatocellular carcinoma cells (HCC); MTT assay, quantitative real-time PCR, histology (hematoxylin and eosin [H&E] staining), confocal microscopy after live/dead staining, zymography, *in vitro* invasion assay	metastatic research [78]
Alginate beads	semi-automatic, high throughput	dental-derived mesenchymal stem cells (MSCs) - PDLSCs and GMSCs bone marrow mesenchymal stem cells (hBMMSCs); RT-PCR, histology and immunohistochemistry	cartilage research, cartilage regeneration [79]
Alginate beads	semi-automatic, high throughput	endothelial cells (OECs); Alamar Blue assay, release profile of FITC-conjugated bovine serum albumin detected by fluorescence spectroscopy, *ex vivo* aorta sprouting assay, blood vessel induction plug assay, immunohistochemistry	vascular research [87]
Continued on next page			

3D culture form	Production/ format	Cell lines, tests/assays	Applications
Hanging drop – spheroid culture	semi-automatic, high throughput	breast cancer cells (MDA-MB-231), ovarian cancer cells (HeyA8), human mammary fibroblast cells; fluorescence microscopic analysis,	tissue engineering, design of cell culture plates [81]

	(384 well)	FACS analysis	
Hanging drop – spheroid culture	semi-automatic, high throughput (384 well)	murine embryonic stem (mES) cells (ES-D3), hepatocellular carcinoma cells , (HepG2), african green monkey kidney fibroblast cell line (COS7); human prostate cancer cells (DU145^Luc), human fetal osteoblast cells (HFOB), prostate cancer cell (PC-3D sRed), human umbilical vein endothelial cells (HUVEC), mouse osteoblastic cells (MC3T3-E1); microscopic analysis, fluorescence microscopic analysis, colorimetric-based assay, live/dead viability/cytotoxicity staining with fluorescence microscopic analysis	tissue engineering, mono- and co-cultures [82]
Hanging drop – spheroid culture	semi-automatic, high throughput (384 well)	prostate cancer cell (PC-3D sRed), human umbilical vein endothelial cells (HUVEC), mouse osteoblastic cells (MC3T3-E1);fluorescence microscopy	tissue engineering, design of cell culture plates [83]
Hanging drop – spheroid culture	semi-automatic, high throughput (384 well)	African green monkey kidney fibroblast cells (COS7), murine embryonic stem (mES) cells (ES-D3), human epithelial carcinoma cell that stably express mesothelin (A431.H9); Live/dead viability/cytotoxicity staining with fluorescence microscopic analysis, AlamarBlue viability assay	tissue engineering, design of cell culture plates, compound screening [48]
Pellet culture	manual (15 ml tube)	mesenchymal stem cells (MSC); Live/Dead Viability/Cytotoxicity staining with confocal microscopy, real-time PCR, semi-quantitative PCR, Urea assay, Albumin ELISA	tissue engineering, hepatocyte research [56]
Continued on next page			

3D culture form	Production/ format	Cell lines, tests/assays	Applications
Pellet culture	manual (15 ml tube)	human mesenchymal stem cells (hMSCs); histology and immunohistochemistry, Blyscan Assay (GAG analysis), DNA quantification after Hoechst dying	tissue engineering, cell differentiation research [59]

Pellet culture	manual (15 ml tube , 96 well)	bone marrow-derived human mesenchymal stem cells (hMSCs); histology/collagen immunohistochemistry, glycosaminoglycan quantification, DNA quantification after Hoechst 33258 dying	tissue engineering for in vitro chondrogenesis studies [55]
Pellet culture	manual (15 ml tube , 96 well)	primary chondrocytes; histology and immunohistochemistry, real-time reverse-transcription PCR, GAG (dimethyl-methylene blue dye) and DNA (Cyquant dye kit) assay	tissue engineering, biomaterial research [57]
Pellet culture	semi-automatic (96 well)	primary chondrocytes; histology and immunohistochemistry, real-time PCR	tissue engineering [84]
Pellet culture	semi-automatic (384 well)	bone marrow-derived human mesenchymal stem cells (hMSCs); real-time PCR, GAG assay, DNA assay	chondrogenesis research, compound screening [58]

2.3   Screening in Biotechnology

Screenings play an essential role for the evaluation of biotechnological processes. A distinction is made between high throughput screening and high content screening.

2.3.1   High Throughput Screening (HTS)

In the last 10-15 years high throughput technology has developed. This technology is principally used in the fields of pharmacology and biology. Main sectors are drug discovery and biopharmaceutical screening [87]. High throughput screening is defined as a scientific experimentation method often used for drug discovery and also relevant for fields of biology and chemistry. High throughput screening enables a fast and parallel analysis of a high number of chemicals in biochemical, genetic or pharmacological tests. These procedures permit rapid identifications of genes, antibodies, active compounds and biological processes for example with the aim to modulate these processes [88]. The miniaturized assay, automatic transfer, liquid handling steps, automatic qualitative and quantitative readouts are basic components of high throughput screening. The field is marked by advanced developments in robotics and engineering for the automation of assays, especially by miniaturization of cellular und biochemical assays. Based on this technology, it is possible to detect more complex cellular phenotypes in a high throughput format and screen for more complex biological information [87]. Advances in high-throughput screening (HTS) instrumentation have led to an enormous reduction of costs (e.g. of pipetting stations) and to the development of smaller instruments for automation of day-to-day routines in small research laboratories [89].

The cumulative number of published papers in the field of high throughput screening shows a continued growth In the years 2003-2013 the number of papers amounted to 29919. This shows an increased interest regarding high throughput screening.

2.3.2 High Content Screening (HCS)

High content screening unifies the aspects of high throughput format and cellular imaging to generate quantitative data of complex biological processes. Likewise high content screening has a great impact on drug discovery, for example in primary compound screening, in post primary screening to support structure-compound relationships, detection of combined toxicity-ADME (absorption, distribution, metabolism and excretion) processes, in stem cell research and genome analysis [90, 91]. The high content formats are based on more complex biological and phenotypic screens used as assay endpoints. These screens are image based to detect subcellular biological processes with the assistance of automatic microscopy and image analysis [87]. The complex quantitative information from each cell can be identified with increased screening speed, objective and statistical power by multiplexing detection [91].

2.4 Life Science and Automation

Life science is defined as the investigation of biological processes and structures of living organisms. The basis for understanding, mimicking and influencing these processes is the research of life science. Investigations in life science research can be supported by high throughput screenings and high content screenings.

Drug development in pharmaceutical companies is critically associated with the reduction of costs. The costs for drug development significantly increased over the years. The costs to develop a new drug amounted $54 million in 1976 and increased up to $800 million in 2000 [92]. The market evaluation displayed the total spending in biological screening at about $8.5B in the year 2001 which include only $1.7B for products and services in the field of high throughput screening. The market size and growth increase in respect of the expenses in the areas of reagents, consumable and equipment over the years. In 2006, the spending projections predicted to reach US$2.4B [93]. Consequently, the increased interest and budget demonstrated the need to develop and improve automatic processes.

The translation of manual procedures into automatic processes is associated with a lot of benefits.

In general, the lab automation for high throughput screening has many pros and contras:

Table 2-8 Pros and contras for automation and high throughput screening

Pros	Contras
decrease of time [9]	cost [9]
precision [9]	retrofitting [9]
increase economic survival [89]	error source: liquid handling [9]
reduction of operating costs [89]	

improve quality service [89]	
decrease of costs: reduction of staff [89]	
high reproducibility [89]	
automatic processes [94]	
liquid handling: nanoliter [94]	
boosting reproducibility [89]	

The improvement of laboratory services is established on the implementation of the correct automation technology. The design of this technology is based on the required functionality. Automation design unites hardware- and software based approaches. Hardware and software are independent systems. The interface between the laboratory automation system and the laboratory information system is a basic element. The trend in automation moves from total laboratory automation and hardware driven to a modular and process control approach. In particular automations develop from one-of-a-kind novelty towards a standardized product and from an in vitro diagnostics innovation to a marketing tool [8].

2.4.1 Automation Systems and Components

In the 1980s the first robots for the laboratory were developed. These robots were especially used for liquid handling of a huge number of samples. The biotechnology and development of pharmacy are main workstreams for lab robots to advance lab processes. Further systems are necessary for labelling, managing, opening, analyzing, closing and storage of samples. Consequently, lab robots are essential for modern high throughput screening of agents. The laboratory robots realize complex workflows like manufacture, preparation till analyze of chemical and pharmaceutical products [8]. The bioscreening can be separated in high throughput screening and high content screening. The high throughput screening (HTS) allows the investigation of a large number of samples. In contrast to the high throughput screening (HTS) the high content screening (HCS) enables the detection of cellular morphology and macromolecular subcellular localization. In the high content screening (HCS) especially ensued the detection of biological macromolecules function in densely packed, highly organized and specifically localized structures within cells which possible influences many aspects of biological chemistry, including protein folding and binding affinity [95, 96]. The main high content screening techniques are performed using confocal- and wild field microscopy.

2.4.1.1 Detection Systems - for High Throughput Screening

The increasing number of samples in high throughput screening requires capable detection systems. They were introduced in the late 1990s. The benefit of these readers is their versatility since they combine multiple measurement technologies (fluorometer, luminometer, and spectrometer/ spectrophotometer) in one compact instrument for endpoint and kinetic measurements [97, 98]. The microplate readers mainly enable the biological investigation by specific detection modes, which

include absorbance, fluorescence intensity, fluorescence polarization (FP), time-resolved fluorescence (TRF) and luminescence.

In the following paragraph, the different detection modes are described in detail. The absorbance (optical density [OD]) ensues when light pass through the sample. Thereby, atoms/molecules take up a photon with following reduction of the light transmission. The filter defines the specific wavelength. The non-absorbed light is recorded at the detector on the opposite of the light source. The concentration of a sample is distinguished by the amount of absorbed light based on the Lambert-Beer law [99]. Investigations with fluorescence depend on the detection of emitted light in nanoseconds upon emmission by a fluorophore (fluorescent molecule). The quantification is performed in Relative Fluorescence Units (RFU). A detector filtered, collected and measured the emitted light at the evaluation of the fluorescence intensity [100]. The fluorescence polarization is realized using polarizing filters which define the extinction of polarized light with a following emission of fluorescent molecules. The emission of polarized- and non-polarized light is defined by molecule size and mobility [101]. Time resolved fluorescence (TRF) is supported by fluorophores with a long-lifetime, e.g. Europium, Terbium, Samarium and Dysprosium. The long-lasting light emission is performed within microseconds after extinction to reduce background noise [102, 103].

The luminescence includes the detection of emitted light after chemical or biochemical reaction caused of enzymatic reactions. There is no extinction needed. The reactions can be separated in flash and glow luminescence. The glow luminescence emits the week intense signal for a time period of several minutes, while the flash luminescence emits the intensive signal for second [104].

The multimode readers cover different well formats (1 to 3456 wells) with flexible wavelength selection. The ranges of the wavelength are located in the area of 190-1000 nm. The visible ranges of excitation/emission are 230-850 nm [105–108]. The devices and manufactures are, for instance, SpectraMax® of Molecular Devices [108], Envision® of PerkinElmer [107], DTX 880 of Beckman Coulter Inc. [105] and Pherastar of BMG Labtech [106].

2.4.2 Automatic Cell Culture Systems

The diverse cell types require a variety of conditions for cultivations such as different cell culture flasks. The systems designed for automatic cell culturing are both plate-based and flask-based to house small- and large-cell volumes. The robotics for cell culture automation can be separated in two parts. On the one hand, there are only the basic components required for an assay, such as the dispenser, washer and reader. On the other hand there are large, motion-controlled tabletop systems that incorporate several robotic components which can perform multiple washings, incubations and readings all in one run. The trend moved to the replacement of large automation platforms with smaller workstations that are individually manageable. This enables the shift of research strategies, for example from shotgun approaches with large libraries to screening with smaller and more targeted libraries [109].

For years, the development of automatic cell culture systems has been promoted in the areas of the industry and university. The main working steps are: expansion of cells and cell productions (sub-culturing, cell seeding, harvesting). There are previous systems, e.g. MACCS[TM] of Matrical bioscience, SelectT[TM] and Cello[TM] of Tap Biosystems. At present, the following systems have been established on the market or were developed from research groups for specific applications:

Table 2-9 Automatic cell culture systems [110]

Cell culture systems	Features	Benefits	Disadvantages	Applications
CompacTSelecT (The Automation Patnership)	• compact and flexible system • industrial robot arm for labware handling • integrated items: cell counter, medium pump, flask decapper and holder • liquid handling by serological pipettes and 1 dispensing channel • footprint: 2.75m x 1.1m • sterile conditions: housing, HEPA-filter	• usable for regular cell culture flasks (T75) and well plates (6 up to 384 wells) • small footprint • allows handling with serological pipettes	• manual neutralization of the detachment process • industrial robot arm • horizontal arrangement • liquid handling with higher contamination risk (remove media by decantation, dispensing-channel by tube without disposable)	• regular cell handling (passaging, seeding, cultivation) • expansion of adherent human Caucasian osteosarcoma cells [111]
BioCel Systems (Agilent Automation)	• compact and expandable system • sterile conditions: filter systems with ultra-low penetration air (ULPA) filters, unidirectional airflow, positive pressure • devices integrable: Microplate Handler, Agilent Microplate Centrifuge, Agilent Microplate Labeler and Microplate sealing	• microplate based protocols • different options for environment control available	• central robotic arm • only proprietary components available	• cell expansion • cell biology (compound management, PCR sample preparation, cell-based assays, ADME-toxicity assays, high-throughput screening) [113]
Continued on next page				

Cell culture system	Features	Benefits	Disadvantages	Applications

Cell culture system	Features	Benefits	Disadvantages	Applications
Cellerity™ (Tecan)	• flexible and modified platforms • based on Freedom Evo® liquid handler • flasks: Corning® RoboFlask™ • able to handle 6- to 1536-well plates • flask flipper • cell counter: CEDEX cell analyzer • integrated incubator • Revco refrigerator • sterile conditions: housing + HEPA Filter • footprint: 5x3 meters • CellGEM software	• consumable and liquid logistics (up to 80l) • intuitive software • 8 re-useable tips	• vertical cell handling because exclusively Corning® RoboFlask™ handling and flask flipper • robotic manipulator arm to handle plates and flasks	• adherent cell cultivation and transfection to produce HIV pseudovirus [113] • cell handling (cultivation, transfection, colony picking, cell separation, cloning, flow cytometry) [114]
AI.CELLHOST (Hamilton Robotics)	• flexible system • Microlab STAR liquid handler • Cellavista cell counter • MICROLAB SWAP robot arm connects devices (cell counter, incubator, stacker) • HAMILTON plate lifter simulate manual handling • HAMILTON Cell[task] and Cell[track] software to manage and control the methods • integrated florescence imager [6] for HCS • sterile conditions: flow chamber, HEPA-filter	• large Hamilton star deck for manifold stations and modules • 8 re-useable tips	• robot arm • vertical arrangement • two HEPA filter needed	• cultivation of adherent cells (SH-SY5Y, BE(2)M17 and HEK293Tcell lines) [112]
Continued on next page				

Cell culture system	Features	Benefits	Disadvantages	Applications

Cell Culture System (reported by Kino-oka *et al.*)	• three main units (culture module, liquid handling, control unit) + constant temperature (37°C) and sterile air (5% CO_2) • programming on LabVIEW software • moveable CCD camera • Incubator integrated (culture module) • liquid transfer by electrical pinch valves tubing pumps and tilting of the growth chambers	• programmable with LabVIEW software • cell culture flasks: 75 cm^2 T-flasks and 632 cm^2 container	• transfer cell solution by tilting the growth chamber • manually cell counting • no centrifugation available	• serial cultivation of adherent myoblasts [113]
Cell Culture System (reported by Kato *et al.*)	• compact system • footprint (70 cm x 60 cm x 86 cm) • moveable arm with CCD camera integrated • supply unit with medium, trypsin, PBS • collection unit with one waste (1000 ml) and tube • warming solutions by a heater • liquid handling about pumps and valves	• small footprint	• sample solution must be inserted by a syringe • only three different solutions available • no integrated cell counter, incubator • CO_2 sensor but no constant 37°C • one culture dish (diameter 25 cm and volume 150 ml) • low waste volume (1000ml) • no flexible system	• cultivation of adherent cells (fibroblasts and bone marrow stem cells) from gingival tissue and bone marrow aspirate[112]

2.5   Software

Software for process control provides repeat testing, reflex testing, and transportation management, and overall computer-integrated manufacturing approaches to laboratory automation implementation are rapidly expanding areas [112]. Laboratory automation software enables hits on a combination of instruments from numerous hardware vendors. This supports best equipment for unique lab automation requirement and assays. The software allows operators to run, monitor and visualize complex processes over the time [95, 109]. The main benefits of software for lab automation are that it is mostly designed for large automatic systems, for majority finding of schedulable solution, for elimination of operator intervention and for an open software architecture. It increases the instrument value and the throughput of a robotic system while integration costs and development time of new products are reduced. Additionally it is fully compatible with Windows and Linux platforms.

Selected examples (Genera, OVERLORD[3], VWorks Automation Control, SAMI® EX) for the lab automation software are explained in detail. The Genera software of RETISOFT Inc. includes a dynamic scheduler with look-ahead feature. This software displays the instrument status and support the reactive scheduling. The Genera software records all the commands, response messages and instrument errors. The seamless integration of third-party software is possible [113]. The OVERLORD[3] software of paa is a multiple document interface with an intuitive and multi-threaded application. This software enables the parallel processing on multiple instruments and advanced run-time error checking and recovery [114]. The VWorks Automation Control software of Agilent Technologies includes multiple simultaneous protocols, error-handling library, and system state editor, and import and export tools. This software is time restricted with a data- and event driven control [115]. The SAMI® EX software of Beckman Coulter Inc. is used for the automatic cell handling by the Biomek® Cell Workstation because it display an open architecture software for editing and visualization of runtime environment. The benefits of this software are the graphical intuitive method editor, the optimization of schedules, the dynamic rescheduling, the rigid and flexible timing of steps, the tip tracking and the operating environment (Microsoft Windows 7) [120].

2.5.1   SAMI® Process Management Software

In detail, the SAMI® Process management software is used for the organization of parallel and interleaved processes. This software includes a calendar view to simplify laboratory resource management and a view to simplify laboratory resource management. The SAMI® Process Definition Editor allows to graphically define complex processes that are composed of manifold tasks. The software support a simplified access for more detailed reports and more flexibility for the operators to process optimization and configuration in each single step. Data management includes for example tracking, logging and reporting. Additional benefits of this system are optimization of pipetting actions, a sample database and labware reports. The optimization of pipetting actions allows specific processes for different cell types, liquid class or labware types. The sample database and labware reports support the availability of every single plate and sample at any point of time, especially the definition and optimization of tasks ensued through SAMI® EX optimization scheduler. Through this scheduler individual tasks at any cell culture processes can be defined and run. The development of new tasks takes place in a simulation system without encroaching existing processes. The SAMI® Process Definition Editor allows determining individual tasks in detail such as planning, process definition and optimization. There is an interface for intuitive use and map tasks in the whole

process [11, 109]. At Beckman Coulters automating cell culture processes with integrated Biomek® Workstations guarantee the integrated SQL data-base integrity of all process steps. The SQL viewer also supports an easy LIMS connectivity. The Data Acquisition and Reporting tool (DART) is an integral part of the Biomek® Cell Workstations. This system safeguards data integrity between tasks and utilizes the combined SQL database. DART enable tracing of every plate in the system at any time [10, 18, 96, 116, 117].

3 Problems and Concepts

3.1 Scientific Problems and Objectives

The demographic change is associated with an increased age of the population. This results in the enlarged number of diseases like cancer and arthrosis. Cell-based research and high throughput bioscreenings are essential for the development of new drugs against cancer and the treatment of diseases of the joint apparatus. Therefore cell culture and screening in a high throughput manner in correspondence with medical application is an important assignment. The 3D cell culture models should be used for drug discovery and tissue engineering. But the limiting factors are the burdensome as well as time-consuming procedures for cultivation and manufacture. Consequently, automation of these processes would be a great benefit for the generation of 3D cultures. A combination of automatic 3D cell culture associated with high throughput screening would introduce a novel form of investigation. The automatic production of different 3D cultures would improve cell culture research by adjustment of the 3D cell culture for the specific cell line, cultivation time, application and bioscreening and the financial background availably for research processes.

In order to cover all the demands three different 3D cell culture constructs have to be integrated at the Biomek® Cell Workstation. These are alginate beads, pellet cultures and spheroid cultures formed in hanging drops. These three cultivation forms were chosen to cultivated cancer cells and primary cells in 3D constructs for different subsequent applications, e.g. bioscreenings and possible medical applications by direct defect repair. The wide range of applications is supported by the 3D cell cultivation in scaffold based and scaffold free cell cultivation forms. In light of future applications enable the scaffold based 3D cultivation within alginate beads offer the possibility to integrate proteins, adhesion molecules, growth factors and compounds for the stimulation of cells and mimicking of physiological conditions. The scaffolds can be adapted in form and structure to the necessary conditions [13, 20]. The benefits of scaffold free 3D cultures include no cross reaction of compounds with the carrier material and the reduced risk of immune response of the body after transplantation.

In detail, the formation of alginate beads with embedded cells is a common 3D cell cultures formed by manual processes. The great benefit of this cultivation is the application for a wide range of cells, i.e. cancer models or chondrocytes and cardiomyocytes cultivation for medical therapies and screening methods. The problem of bead cultures can be the loss of cells after the lysis of the beads for further investigations and screenings. Hanging drops are the most adapted form of 3D cultures. By this time 96 and 384-Well Hanging Drop Plates facilitate the production and handling of spheroid cultures in an automatic manner. The benefit of this cultivation method is that no carrier material has to be used and simple co-cultivation of different cells types is supported. The disadvantage of spheroid cultures is the low stability of the droplets and consequently the loss of 3D cultures. Pellet cultures are formed without carrier material and this 3D constructs are ideally suited for tissue engineering. They enable the production of a stable cartilage construct by 3D cultivation of human chondrocytes. Pellet cultures are produced manually. The published automatic methods of pellet cultures production are semi-automatic processes in which only the cell seeding was performed using a liquid handler. The strong cell-surface interactions can be problematic at the pellet formation and might prevent pellet formation.

There are several research approaches:

Table 3-1 Research approaches of 3D cultures in high throughput processes

3D cultures	Approaches	Benefits	Disadvantages
Alginate beads	• allows many screening methods (i.e. cell viability, toxicity, staining) **New:** • unpublished alginate bead production in a high throughput manner	• useful for variety cell lines • 3D research model • for medical therapies	• support material
Spheroid cultures	• 3D cell culture in 96 and 384 well format • compound screening **New:** • medical application	• no support material • fast 3D formation • creation of cancer constructs	• most researched 3D culture model in high throughput manner • stability of hanging drops
Pellet cultures	• 3D cell culture in 96 well format • GAG analysis • DNA analysis • microscopic analysis: sectioning + confocal microscopy **New:** • high throughput pellet production for patients -service	• direct arthroscopic defect repair possible • easy handling • no support materials	• Co.don® (manual pellet production for medical application) • high cell amount needed • formation dependent of donors

The limitations of other published systems are manifold and address the sterile working, labware, liquid handling and limited handling of cell cultures.

In general, cell culture automates are explicitly provided and programmed for specific cell lines and applications. The cell culture systems are equipped with different devices but no system meets all requirements to enable the needed flexible cell handling.

- **Sterile Working**

In detail, sterile conditions are the basis for cell cultivation processes. But the general equipment of cell culture systems with housing and filter systems cannot disinfect the surfaces and air. There are no standardized cleaning protocols available to disinfect the tubing systems.

Neglected forms in the field of automatic cell cultivation are suspension cells, e.g. leukemia cell lines and blood cell lines. This cells floating in the media and require specific process steps. Regarding this, the cells have to be separated from the supernatant for cultivation and dissemination processes,

which can be realized using a centrifuge. Standardly, automatic cell culture systems do mainly not include a centrifuge. No published automatic cell culture system enables the cultivation of suspension cells.

- **Labware**

Moreover, no system supports the handling of AutoFlasks™ (greiner) for a horizontal cell handling associated with low mechanical stress and prevention of dry out. These flasks also support the sterile transport of the cells.

- **Liquid Handling**

The supply of different media or solutions supports also the flexibility of cell culture system. The published systems enable the dispensing of a maximum of 3 different solutions.
The liquid transfer of the cell culture systems is realized using different ways. Automated systems enable liquid transfer by simple decanting of flasks, serological pipettes, 8-resuseable tips or one dispensing channel. But all these solutions do not cover the flexibility of steel cannulas which are used in the Biomek® Cell Workstation. In concrete terms, this means that the liquid handling solutions do not enable the penetration of the resealable septum for sterile cell handling, the removed of the supernatant, the aspiration and dispensation with one channel, the parallel cell dissemination and the mixing of cell solutions.

- **Handling of Cell Cultures**

At present, automatic systems for cell cultivation processes realize cell expansion methods of adherent cells or the semi-automatic formation of 3D cell cultures e.g. by droplet generators or liquid handlers. There is no system published which includes the cell detachment, dissemination and consequently 3D formation for the investigation of cell cultures by bioscreenings with flexible readouts (absorbance, fluorescence and luminescence). Adequate bioscreenings are needed to investigate the specific 3D cell cultures because the different characteristics require dissimilar screenings.

The goal of this thesis was the development of a cell culture system coverins all requirements. This system should enable the expansion of different cell types, for example adherent cell and suspensions cells. The system should allow the production of different 3D cell cultures with and without carrier material. The Biomek® Cell Workstation (celisca) was configured to fulfill all prerequisites. The resulting high throughput bioscreening allows the investigation of cells and cell constructs in simulated *in vivo* conditions. Comparative investigations were performed using the manually and automatic 3D formation as well as consequently bioscreening with manual and automatic methods. The quality, reproducibility and performance of the automatically produced 3D cell cultures had to be evaluated by comparison with manual manufactured cell constructs. The automatic processes enable an increased quality and reproducibility by formation of 3D cell constructs under the same conditions and handling steps independent of the laboratory staff.

A further additional aspect is that antibiotics are used in regular cell cultivation to prevent contaminations [118]. The routinely application of antibiotics can encourage the emergence of resistance, aseptic working and cryptogenic contaminations [119, 120]. The most widespread antibiotics in cell cultures are a combination of penicillin and streptomycin. The β-lactam penicillin is also a common antibiotic agent, which affects the cell wall syntheses of dividing bacteria [121]. However, streptomycin belongs to the group of aminoglycosides and influences the protein biosynthesis and cell death caused by accumulation of inoperative proteins [122]. A main question in automatic processes is whether antibiotics are required for the automatic cultivation of cells [73].

3.2 Concepts

The investigation of the automatic cell cultivation needed different objectives and prior considerations which had to be realized (see figure 3-1). The main tasks can be separated into the cell cultivation by the Biomek® Cell Workstation followed by the analysis with the HTS (High Throughput Screening) system. For the first, the cell lines had to be selected with the format for the dissemination and production of the 3D cell cultures. Then, the system had to be modified to realize the processes. These were the cultivation and dissemination of the cell forms (adherent cells, suspension cells) and the evaluation of the right 3D cell constructs for the different cell lines. The cancer cells are cultivated mainly in alginate beads and spheroid cultures to form 3D constructs. Instead, primary chondrocytes are used preferably to form pellet cultures. Lastly, the improving of the sterile cell handling associated with the exclusion of cross contamination are further objectives to support the sterile and parallel cell handling. These objectives had to be realized using the programming of the Biomek® liquid handler- and the SAMI® software to perform the processes. The modification of pipetting templates supported the cultivation and manufacturing processes. Afterwards, contamination-free cell cultivation followed using the workstation with improved conditions for sterile cell cultivation and the parallel cell cultivation to exclude cross-contaminations. The next task should be the automatic cell cultivation of the different cell lines (adherent cells, suspensions cells) in relation to the cell handling, cultivation and dissemination of the exact cell number. Lastly, the 3D cell constructs had to be manufactured by automatic processes. Subsequently, the analyses of the cell cultures had to be performed. On the one hand microscopic analyzes were used. This can be fluorescence microscopy and regular light microscopy during the cultivation as well as after sectioning and histological staining which should specifically be chosen for the different cell cultures. The feasibility and stability of the cultures and the intention, e.g. evaluation of cross contamination, for the microscopic evaluations have to be considered. On the other hand, quantifications by bioscreening are required. The formats, assays with specific regents, compound treatment and the resulting readouts (absorbance, fluorescence and luminescence) had to be selected. The screenings had to be optimized for a robust signal strength, high throughput and reproducibility. In the case of low signal strength for one 3D cell culture/well, pooling of the cell cultures can increase the signals. These whole objectives and prior considerations must be fulfilled for the successful automatic cell cultivation and 3D formation.
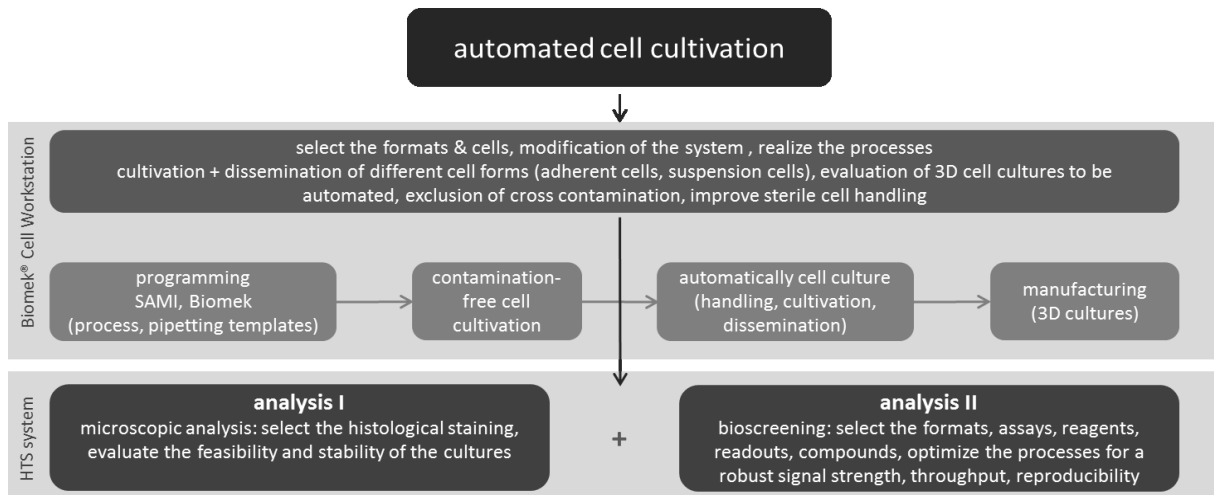
Figure 3-1 Overview about tasks for the automatic cell cultivation

3.2.1 Requirements of the Processes and Systems

There are many important aspects (realization of all process steps, flexibility in process steps, simulating manual handling) to be considered for the translation of manual methods into automatic processes. The production and screening of different 3D cultures is a challenge, especially for automatic processes.

The most important aspect is the realization of all process steps. Adherent cells in form of a permanent cell line or a primary cell line are the basis for the 3D cultures. The cells have to be seeded with a specific cell count to produce 3D constructs. Next to the regular liquid handling, cells have to be separated from the supernatant after a centrifugation step.

The second most important aspect is the flexibility in the process steps. Each cell line and 3D culture system includes different steps for the production and handling. The automatic cell culture system has to realize these steps and conditions. There are different cell culture well plates, viscous liquids and cell types to handle.

The third aspect is the simulating of the manual handling. The specific manual cell flask handling has to be realized using special devices. The regular manual used T75 cell culture flasks have no 96 well format, which is necessary for the automatic handling of cell culture flasks. The Biomek Cell® Workstation as well as other automatic systems supported the handling of 96 well format because the devices and automatic laboratory positioners (ALPs) are designed for this formats. The transfer of solutions is performed via a lateral screw cap with a hydrophobic membrane for the gas exchange. This cannot be handled by the Biomek® Cell Workstation. The automatically used cell culture flasks need a cavity covered by a re-occlusive septum, which enables the penetration by the liquid handler to transfer solutions into the flasks and guarantee a sterile transport of the cells. A hydrophobic membrane is needed to ensure the gas exchange. The CELLSTAR®AutoFlasks™ (greiner) are adapted at the Biomek® Cell Workstation and address these challenges. The adherent cells have to be dissolved from the bottom of cell culture flask. This is manually realized using knocking and pivoting the AutoFlask™. The cell suspension has to be resuspended before cell seeding. Additionally, specific labware is needed to store solutions during the cell culture methods. These are 15 ml and 50 ml tubes at the manual handling. The storage of solution during the automatic processes is realized using modular reservoirs which are available for different volumes. Differences of manual and

automatic processes occur at labware and the liquid handling. The cells are cultivated in T75 flasks for the manual handling and in AutoFlasks™ for the automatic cell culture. The manual liquid handling is performed with serological pipettes associated with a pipette help (> 1ml) and Eppendorf pipettes with disposables. The automatic liquid handling is performed using the Biomek® NX with the span-8 pipetting head and steel cannulas, which enable the liquid handling of more than 10 µl up to 5 ml per time. The cell amount and examination of the viability are essential for the dissemination and production of 3D cell constructs. Normally, the manual investigation of the cells is performed using trypan blue staining and cell counting with a Neubauer counting chamber. The automatic solution of the Biomek® Cell Workstation is the automatic trypan blue staining and cell counting by the Vi-Cell® (Beckman Coulter). For the purpose of maintaining comparability, the manually and automatically treated cells were counted by the Vi-Cell® (Beckman Coulter). The whole aspects have to be considered for the automatic generation and subsequent screening of 3D cell culture systems. Chapter Implementations shows the realization of these processes.

In general the different two dimensional (2D) cell lines (adherent cells, suspension cells) as well as three dimensional (3D) cell constructs require altered devices, ALPs and processes (see figure 3-1) for the automatic cell dissemination besides formation (3D). The most 3D cell cultures have different requirements. The challenge is the evaluation of an all-encompassing and flexible system to handle the whole spectrum of cell cultures. The processes to be automatic should be similar to the manual cell handling and manufacturing of the 3D constructs, apart from the labware (flasks, reservoirs) and volumes.

The concepts for the automatic processes visualized that the main differences of cell cultivation processes occur between adherent cells and suspension cells (see figure 3-2).
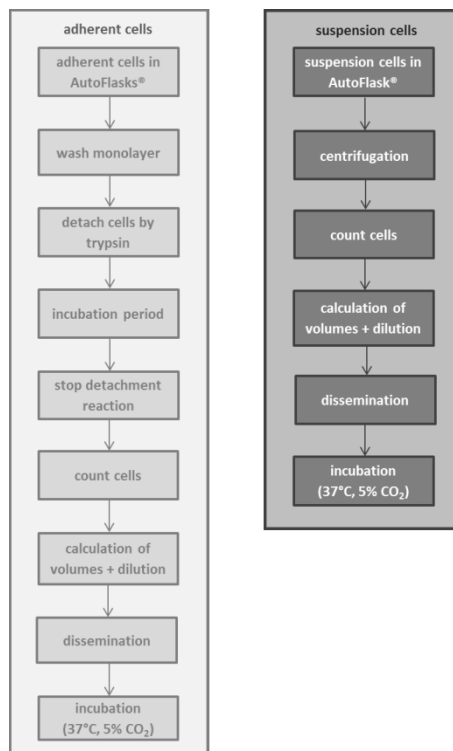
Figure 3-2 Concepts of automatic dissemination of adherent cells and suspension cells [123]

The adherent cells are located at the bottom of cell culture vessels and flask. The adherent cells have to be treated by enzyme (trypsin) to detach the cells within an incubation period. This reaction has to be stopped for the subsequent cell counting and viability testing. After the cell dilution follows the dissemination with the consequently incubation (37°C, 5% CO_2). Instead, the dissemination of suspension cells is difference in cause of the floating cells in the medium, whereby the manual and automatic process steps are similar. The cell dissemination started with the centrifugation to separate the cells from the consumed media. Subsequently, the cells have to be counted, calculated and diluted with fresh media. Then, the suspension cells are disseminated in well plates followed of the incubation and screening.

The concepts of the automatic process steps visualized that the adherent cell lines used for 3D cell cultures. The detachment process is similar to adherent cells until the calculation of the cell number. The fewer process steps require the formation of spheroid cultures formed in hanging drops (see figure 3-3).
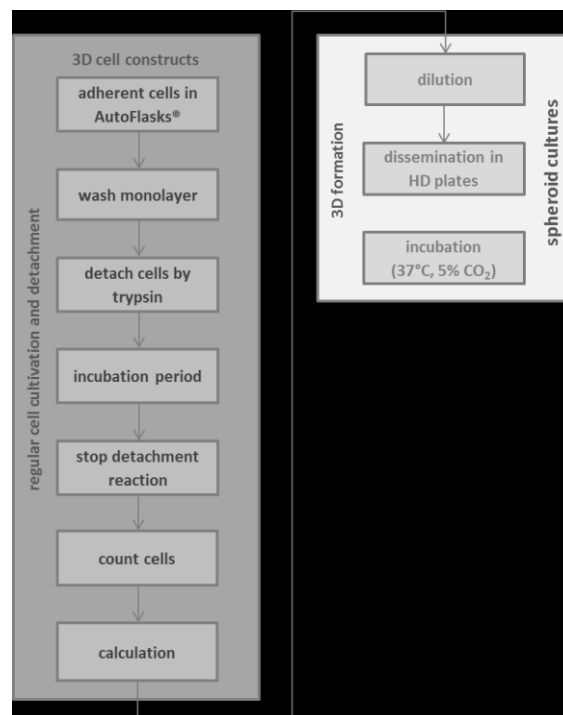
Figure 3-3 Concept for automatic formation of spheroid cultures [123]

After the dilution of the cell suspension follows the dissemination in hanging drops plates with the consequently incubation (37°C, 5% CO_2). The high throughput production of spheroid cultures is realizable by hanging drop plates. The Perfecta3D® Hanging Drop Plates (384 wells) are a suitable labware, which has to be integrated in the software with specific pipetting template. Additionally, the size of the cavities (1.5 mm) requires an explicit ALP to manufacture the hanging drops.

The pellet formation needs conceptually more process steps (see figure 3-4).
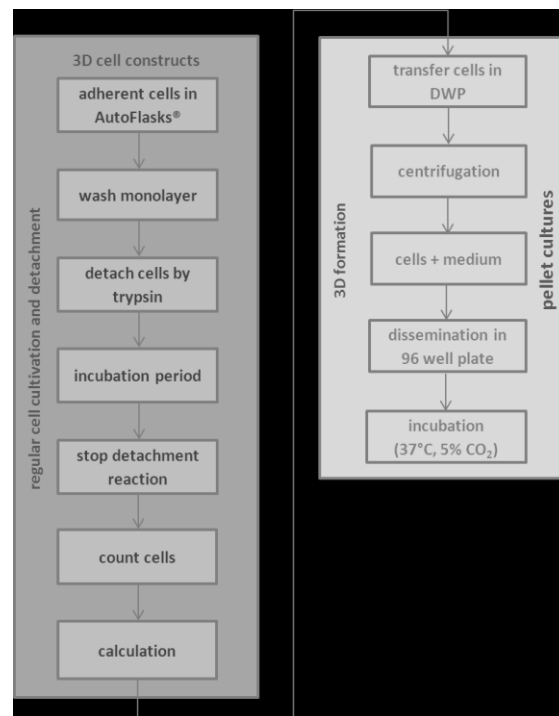
Figure 3-4 Concept for automatic formation of pellet cultures [123]

There is a high cell amount needed to form pellet cultures (500,000 cells/culture). The cell concentration will be increased by centrifugation and re-suspending of the pellets in a lower volume. For the regular manual pellet formation the cells are collected in a tube for centrifugation. At present, this is not realizable with the Biomek® Cell Workstation. There are no capping and decapping system, holder and centrifuge integrated for tubes. A centrifuge of the 96 well format is to be joined to the workstation. Afterwards, the automatic concentration of the cell suspension is performed using transferring the cell solution into a deep well plate (DWP) after the calculation with consequently centrifugation to resuspend the pellet in a low volume of medium plus growth factors. Subsequently, the cell suspension is transferred in a 96 well plate followed of the incubation (37°C, 5% CO_2). For the production of pellet cultures are only suspension cell plates needed to support the pellet formation, which are available in a 96 well plate format. The challenge displays the human chondrocytes. This primary cell line needs a sensitive cell handling and the pellet formation is greatly dependent on the donor. The medium for the pellet formation includes cost intensive growth factors and the liquid should be handled with minimal loss.

The highest number of process steps is required for the manufacturing of alginate beads (see figure 3-5).
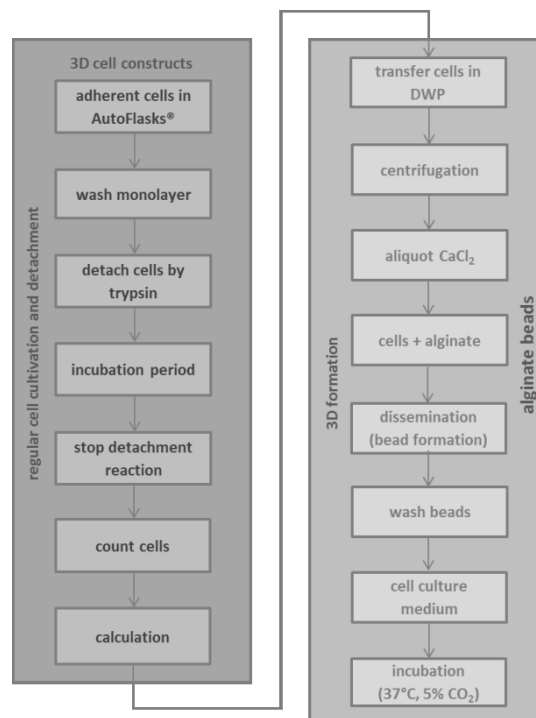
Figure 3-5 Concept for automatic formation of alginate beads with encapsulated cells [123]

Basically, the manual alginate bead formation is performed using the collection of the whole cell suspension in a tube for the centrifugation. The pellet has to be resuspended in alginate. The cell-alginate solution is dropped into 20 ml $CaCl_2$ solution in a pivoting beaker by a syringe and a cannula. After the incubation period, the alginate beads are transferred into a filter to wash the 3D constructs with NaCl. Then, the beads are aliquoted in a 6 well plate with medium. For the automatic alginate bead production a reduction of the volumes is necessary, for the bead formation in a 96 well format. This is realized by transferring the calculated number of cells into a DWP followed by a centrifugation step to remove the media and resuspending the pellet in alginate. Dropping a cell-alginate solution into a $CaCl_2$ solution (1 bead/well) form the beads. The beads are formed by dropping has to be performed with a syringe and a cannula (manual) or the liquid handler (automatic). The beads are incubated after the washing steps and addition of fresh medium.

The whole concepts are summarized in figure 3-6:
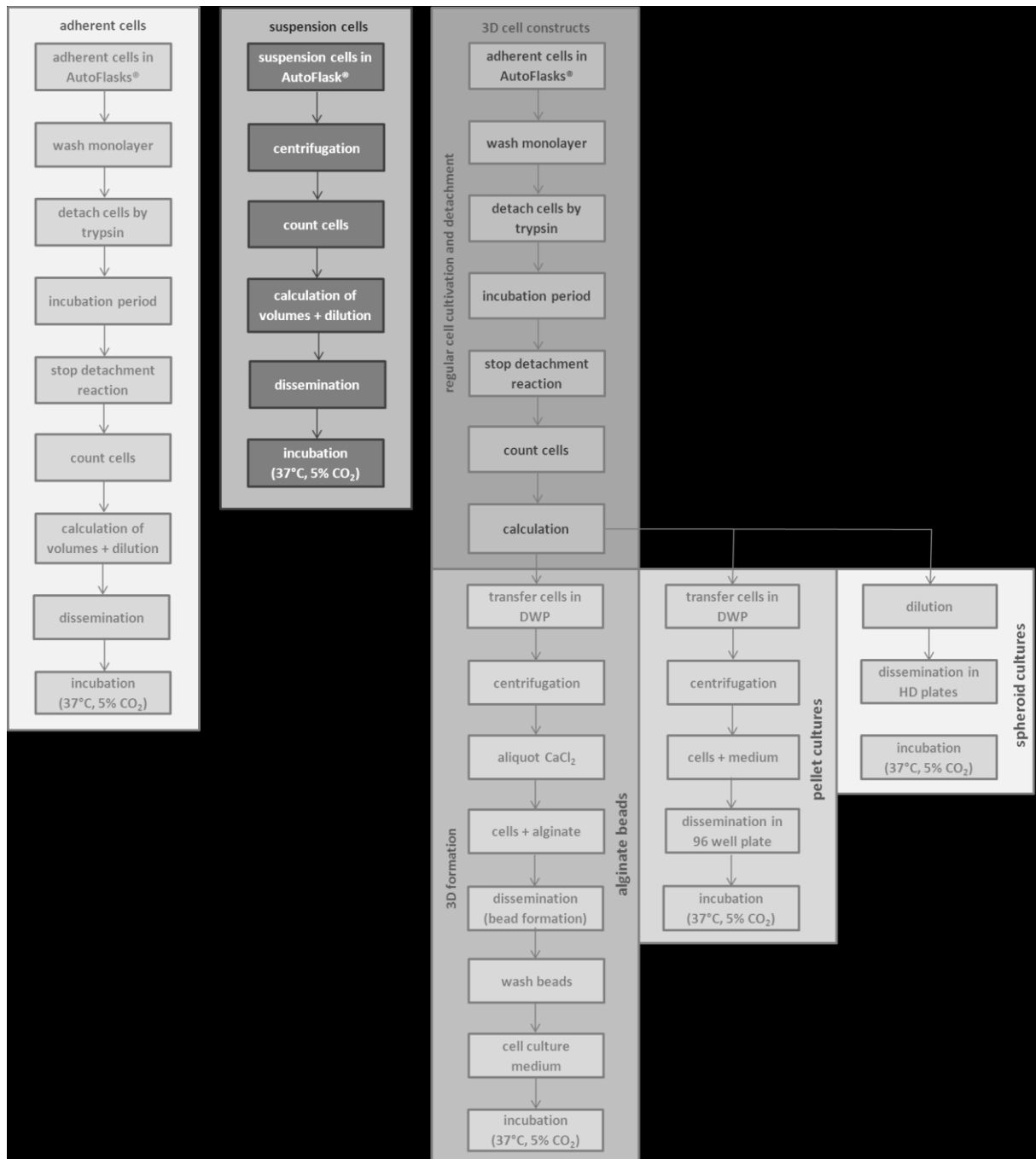
Figure 3-6 Summarized concepts of the general steps of automatic processes [110, 123]

3.2.2 Principle Description of the Biomek® Cell Workstation

Originally, the Biomek® Cell Workstation with the liquid handler deck (see figure 3-2, A and B) has been developed to cultivate different adherent cell lines (HeLa, HEK 293, Tis-10, CHO-K1) under sterile conditions [10]. The Workstation was basically equipped with a housing (1), a HEPA-Filter (2; Camfil, Stockholm, Sweden), a Biomek® NX liquid handler (3; Beckman Coulter, Krefeld, Germany), a lift (4), a Cytomat (5; Thermo Scientific, Schwerte, Germany), a computer (6), a cool box (7; WEMO-Geräte AG, Schlatt, Switzerland) and a Vi-CELL™ XR (8; Beckman Coulter) (see figure 3-2; A). The liquid handler deck of the Biomek® NX includes basically on deck incubators (a; inheco, Martinsried, Germany), a barcode reader (b; Microscan Systems, Inc., Renton, USA), the plate lift station (c), 3D tilt racks (d, e; AIG, Rostock, Germany), placeholder ALPs (f-j, n; Beckman Coulter), a shaker (m; Thermo Scientific), wash stations (k, l; Beckman Coulter) and a waste (o; Beckman Coulter). The ViCell-connection is integrated on a placeholder ALP (h) (see figure 3-2, B).
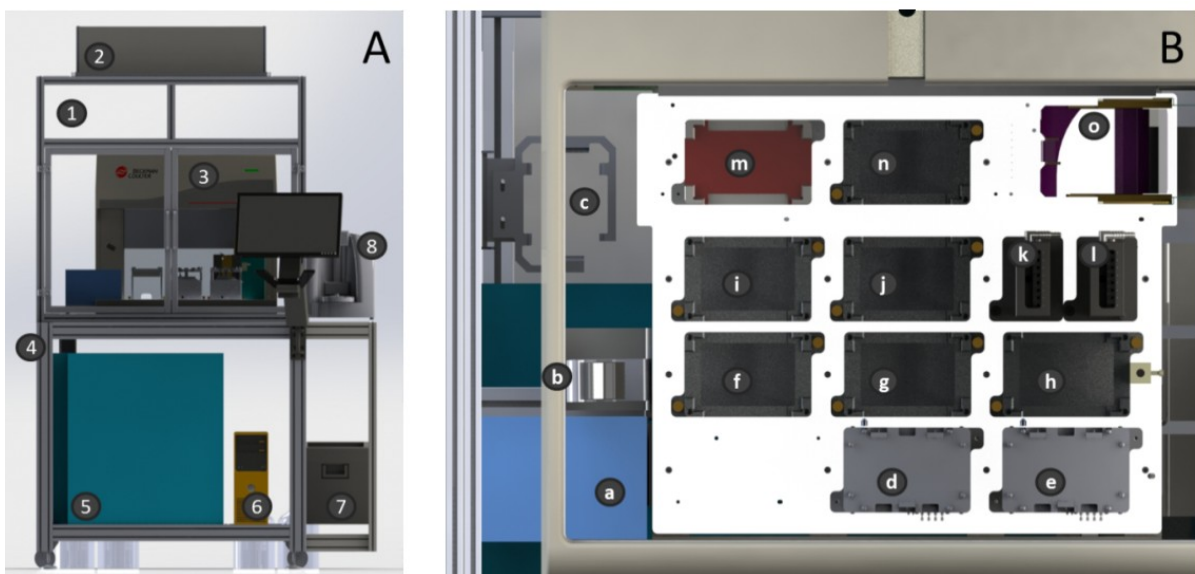
Figure 3-7 The basically equipment of the Biomek® Cell Workstation (A) and the liquid handler deck (B)

These basic components of the Biomek® Cell Workstation enable the cultivation and handling of one adherent cell line per time. This ensues because the steel cannulas and gripper are located at one rail to handle cell culture flasks. The cooling down of the cell cultures and short incubation periods e.g. at the treatment with trypsin need a fast addition of medium to stop the detachment process as well as to prevent damage to the cells. Thus, from a temporal perspective the cell cultivation should be quickly joined to the system.

3.2.2.1   System Improvements

Different improvements of the system are needed to advance sterile cell handling and realize the handling of different cell forms (adherent cells and suspension cells) as well as manufacture of different 3D cell constructs:

UV-lights

For a longer cultivation periods the sterile conditions have to be improved by UV-lights for superficial killing of microorganism. These have to be equipped with the housing to standardly cover the whole surface of the liquid handler deck. The UV-lights have to supply the exact wave length to kill a wide spectrum of microorganism. Specific cleaning steps of the tubing systems can support the disinfection and decontamination of the system.

Port selection valve

A flexible cell culture system has to provide different media to support the parallel cell cultivation of different cell lines. The port selection valve is associated with different solutions which should be dispensable by the liquid handler about pumps and a tubing system.

Centrifuge

A flexible system for automatic cell cultivation should be equipped with a centrifuge.
The integrated centrifuge would enable the separation of consumed medium from the cells and concentration of cell solutions. This is essential for the cultivation of suspension cells and the production of 3D cell cultures (alginate beads, pellet cultures). The centrifuges have to allow direct access from the liquid handler deck to support the sterile cell handling. The needed formats are 96 well plates to realize the automatic labware handling which are required by the automatic system.

Specific Automatic Laboratory Positioner (ALP) and Device

Additionally, the liquid handler deck needs two ALPs to support the 3D formation of spheroid cultures in hanging drops (Positive Position ALP) and alginate beads (Static Peltier Device).

Positive Position ALP

The Perfecta3D® 384 Hanging Drop Plates (3D Biomatrix, Ann Arbor, USA) enable the high throughput formation of spheroid cultures. An exact definition of the plate-position is required because of the plate design with small cavities (1.5 mm) to form hanging drops by fixing of the plates can be realized using the Positive Position ALP (Beckman Coulter).

Static Peltier Device

The tempering of solution is also an important aspect at the automatic cell cultivation. Especially, the alginate solution is a high viscose solution and has to warm up for an exact liquid handling. The suitable device for the warming of solution in automatic systems is the Static Peltier Device (Beckman Coulter).

Next to the hardware, specific programs and process steps (see figure 3-1) are needed to support the different cell handlings. The chapter implementation includes strictly the new integrated components to realize the flexible cell cultivation and the process steps.

4 Implementation

4.1 Cell Culture System

A flexible system for the automatic cell cultivation should enable the handling of different cell forms. The Biomek® Cell Workstation was basically configured for the sterile cell cultivation of adherent cell lines. These adherent cells proliferate at the bottom of cell culture flask. The detachment process with enzymes enables the cultivation and dissemination of the cells.

Suspension cells are another form of cell lines. These cell lines are floating in the medium and have to be centrifuged for the cultivation and dissemination processes. The different cell types of adherent and suspension cells require different handling steps. The system for automatic cell cultivation should cover the new strategy for cell cultivation processes, 3D cell cultures. These constructs, produced with and without carrier material, enable the mimicking of *in vivo* conditions to simulation physiological tissue. An automatic handling requires 96- and 384-well formats for the consequently high throughput screening. This thesis addresses these topics by automatic production of different 3D cell cultures with consequently bioscreenings.

The following chapter includes the technical realization associated with the automatic process steps.

4.1.1 Architecture of the Biomek® Cell Workstation

The Biomek® Cell Workstation (see figure 4-1) is a compatible and flexible system for automatic cultivation of various cell types. This complex system is vertically arranged and moveable with a small footprint of 1.49 m/1.64 m/2.63 m, in contrast to existing systems. Therefore, the devices are arranged one above [110].
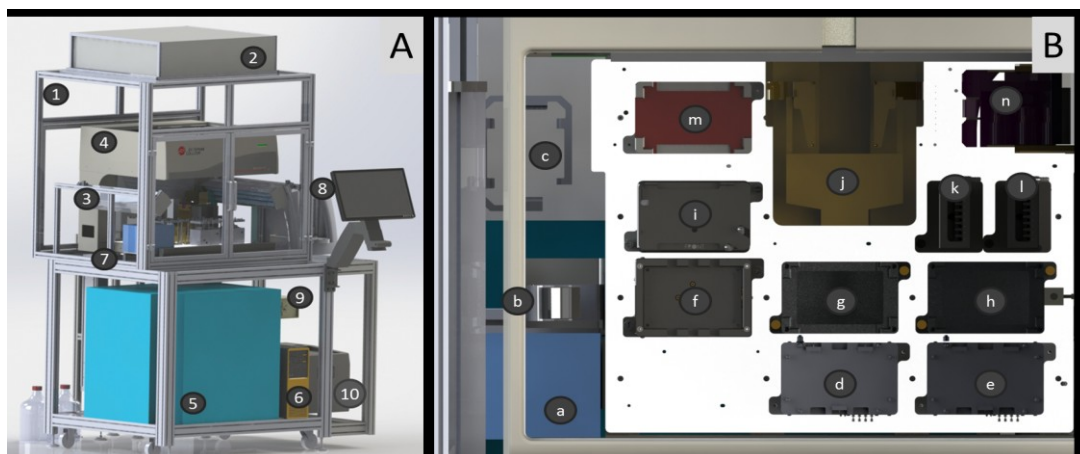
Figure 4-1 Biomek® Cell Workstation

Frontal view: The central components of the Biomek® Cell Workstation (A); the main components are: housing (1), HEPA-Filter (2), UV-lights (3), Biomek NX liquid handler (4), Cytomat (5), computer (6), lift (7), ViCell (8), port selection valve (9), cool box (10); an overview of ALPs of the liquid handler deck (B); visualized component are: on deck incubators (a), barcode reader (b), lift station (c), 3D tilt rack-2 (d), 3D tilt rack-1 (e), Static Peltier Device (f), placeholder ALPs (g, h), Positive Position ALP (i), Vspin™ (j), wash stations (k, l), shaker (m), waste (n), barcode readout is possible on ALP (f) and the ViCell connection is integrated on placeholder ALP (h).

The central element of the system is a Biomek® NX (Beckman Coulter, Krefeld, Germany) for all liquid handling processes. These processes are aspiration, dispensing and transport of solutions. The Biomek® NX (Beckman Coulter) is equipped with a span-8 pipetting head and has an integrated gripper at one rail. The one to eight pipetting channels are independent. The gripper positioned the cell culture flasks and well plates. The Biomek® NX enables the liquid transfer by disposables or steel cannulas. These are associated with syringe pumps. The pumps of channel one to four are associated with steel cannulas having maximum capacities of 1 ml. The pumps from channel five to eight enable the transfer of maximum 5 ml in one process step. Channel 7 is associated with a peristaltic pump and the port selection valve. This combination allows the transfer of larger volumes and a change between different media for parallel cell cultivation of different cell lines. System fluid (sterile water) is always in the houses for the transfer of exactly volumes [124].

The liquid handler deck is equipped according to the requirements of automatic cell cultivation (see figure 4-1, B). There are automatic laboratory positioners (ALP) and devices available for specific handling of cell culture flasks or as placeholders. A microplate shaking ALP is integrated on the liquid handler deck (VARIOMAG® Teleshake; Thermo Scientific, Waltham, USA). This is an active ALP for mixing solutions and cells in microplates or flasks with an adjustable shaking motion. The ALP is a magnetic shaking system for well plates or cell culture flask in a 96 well format with 100 rpm up to 2000 rpm movements and amplitude of 2 mm. This device has a small footprint (high: < 40 mm), minor transference of vibrations and self-centering to the zero position. The microplates (96- and 384-well) or cell culture flasks (greiner) can be added to the Orbital Shaker ALP by a lab technician or the gripper. For short incubation periods of the cell culture flasks at 37 °C, two on deck incubators (inheco, Martinsried, Germany) are available. These can directly be accessed from the liquid handler deck. The shaking periods are adjustable. Two washing stations (Beckman Coulter) have been included on the liquid handler deck for cleaning and disinfection of the steel cannulas. One wash station is associated with autoclaved water and the other one with isopropanol for active and passive washing steps. There are two bottles with the specific fluids connected with the wash stations and the waste is collected in one bottle. The steel cannulas are cleaned in two ways. The pumps of the Biomek® NX deliver water or isopropanol to the cannulas and system fluid will be dispensed into the wash station. Otherwise, steel cannulas can dispense systems fluid into the wash station. Thus, the solution washes inside the cannulas and the level of the wash fluid rises with increased speed into the cleaning wells to clean the outside of the cannulas positioned in the wash station [18]. For simulating the manual handling of the cell flasks, two 3D-tilt racks (AIG, Rostock, Germany) are integrated. The 3D-tilt rack (AIG) is a proprietary component for the automatic handling of cell culture flasks. The rack enables the tilting of the flasks in x, y or x/y axis, pivoting and knocking the flask. The tilt angle and speed are adjustable. The flask is fixed on the rack by deadlocks for pipetting steps through the septum [10]. The Positive Position ALP enables the fixing of well plates. Lastly, the Static Peltier Device allows the warming of solutions.

For the culturing and incubation of the cells, an incubator has been integrated into the system. The Cytomat® Automatic Incubator (Thermo Scientific, Schwerte, Germany) allows the cultivation of cells at 37 °C and 5 % CO_2. The temperature and oxygen concentration are controllable. Inside, there are nine storage modules with 21 places, each are integrated for flasks or sheets on a turntable. The plate transfer of the Cytomat to the lift takes place by a plate shuttle system and an X/Y/Z moveable handler through a heated automatic access door [125]. Since the system is organized in a vertical way in order to realize a small footprint, a transfer system between the incubator and the liquid handler deck had to be realized. The proprietary lift enables the transport of one well plate as well as

flask per time to bridge a height of 480 mm. The transport of the labware starts with the movement of the labware by the plate shuttle system from the back through the automatic access door of the Cytomat to the lift travelling stage and a rotation of 90 degrees by a pneumatic rotation mechanism with adjustable speed. The vertical movement to the liquid handler deck is delivered by the linear spindle drive with a time of 10 seconds. Then, the gripper of the liquid handler is able to locate the labware at the specific positions. The whole process, to move the labware from the Cytomat to the positon of the liquid handler deck, requires a time of 35 seconds. The Vspin™ (Velocity 11, Palo Alto, USA) enable the centrifugation steps of cell culture vessels in a 96 well plate format. Cell viability and cell count are evaluated using a ViCell™ XR analyzer (Beckman Coulter, Brea, USA). This device is associated with the liquid handler deck over a conduit and has an analysis time of 2.5 minutes. The cells will be evaluated by the trypan blue method. The death cells are marked red because the trypan blue stains the cells dark blue. The membranes of living cells exclude trypan blue actively. These cells are marked green. The viability range of 0-100 % is detected in a sample volume of 0.5 ml with the image technologies of the auto-focus CCD array and firewire camera by the video image through a quartz flow cell. The size range of the cells is 2 μm till 70 μm [18].

All subsystems including the Biomek® NX liquid handler (Beckman Coulter), Vspin™ centrifuge (Velocity 11), barcode reader, lift and a conduit to the ViCell XR (Beckman Coulter) [10] are covered by a housing. The housing is associated with a HEPA-filter (Camfil, Stockholm, Sweden) and UV-lights (Vilber, Eberhardzell, Germany) to guarantee sterile conditions. The HEPA-filter system works in continuous operation whereas the UV-lights are used for a time period before and after the cultivation processes.

The integration modules enable the automatic control of the different devices of the Biomek® Cell Workstation to be controllable with the automation software (SAMI® Software) about the firmware. The devices (for example ViCell) supported by Beckman® Coulter have integration modules. However, external devices (for example shaker and incubator) required new proprietary integration modules [110].
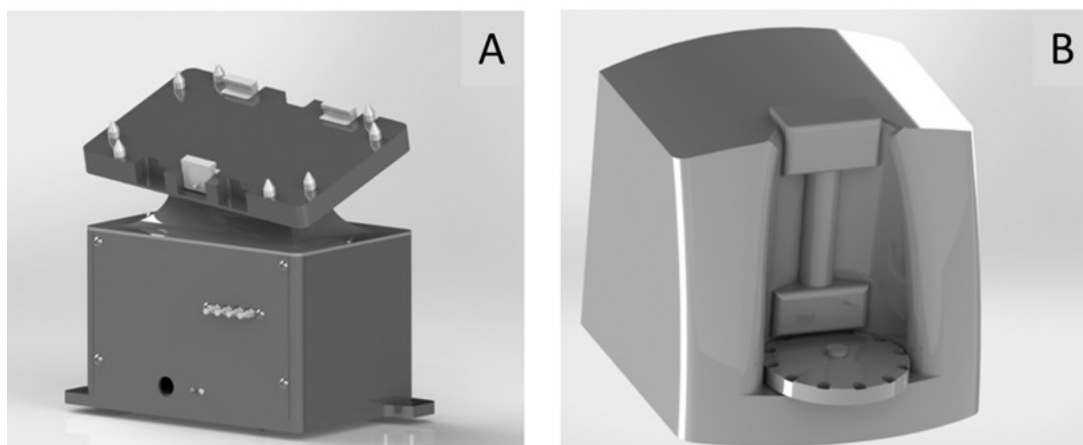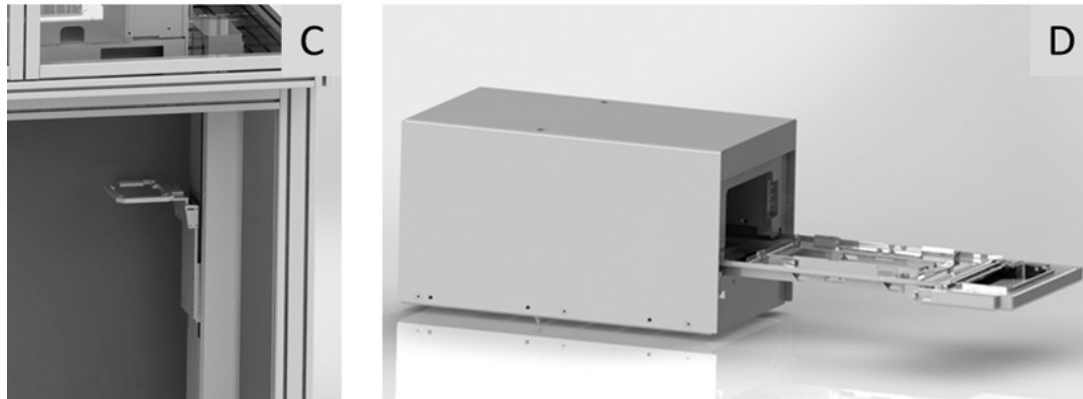
Figure 4-2 Basic components for the automatic cell cultivation

The basically important components for the cell cultivation are the 3D tilt racks for the automatic handling of cell culture flasks (A), ViCell XR for the cell counting (B), the lift to connect the Cytomat with the liquid handler deck (C) and the on deck incubators for short incubation periods at 37 °C (D).

4.1.2   System Enhancement

The Biomek® Cell Workstation had to be modified and enhanced to realize the parallel cell cultivation of suspension cell lines and formation of 3D cell constructs (alginate beads, pellet cultures, spheroid cultures) regarding the integrated devices and modification of the liquid handler deck (see figure 4-3, A and B). Moreover, the sterile cell handling had to be supported by specific program steps and additional equipment.

The cell culture automate was further equipped with subsequent devices: UV-lights (A, 1), the port selection valve (A, 2) and the Vspin™ centrifuge (A, 3; B, c). The UV-lights support the sterile cell cultivation and the disinfection before cultivation processes. The port selection valve permits the liquid handling of six different solutions. The centrifuge enables the separation of the cells from the supernatant. Additionally, two further ALPs (automatic labware positioners) had to be integrated: the Positive Position ALP (B, b) and a Static Peltier Device (B, a). The Positive Position ALP fixes plates and the Static Peltier Device is used for warming solutions.

The additionally developed and integrated components of the Biomek® Cell Workstation are described in more detail in the following chapter.
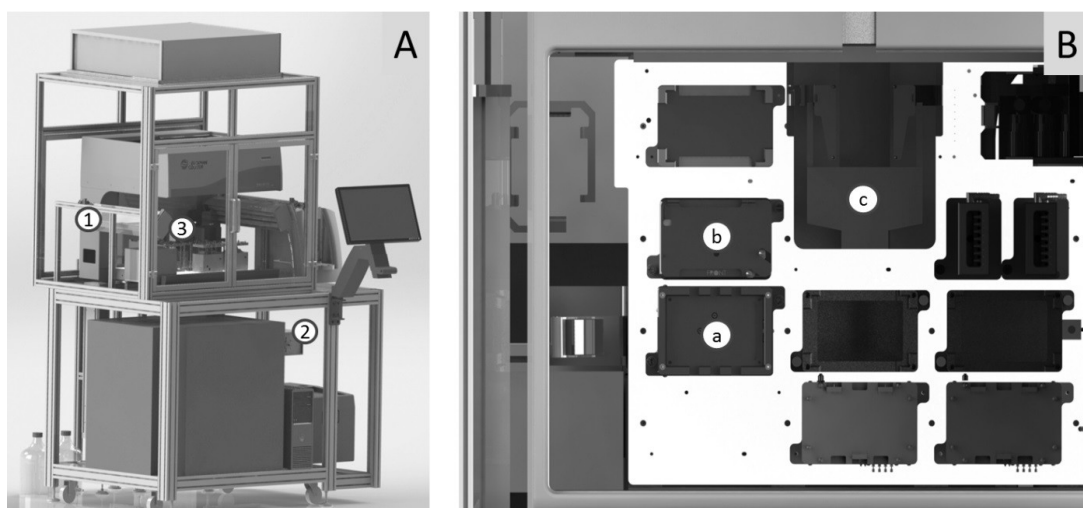
Figure 4-3 Changes in setup of the Biomek® Cell Workstation

Frontal view: The Biomek® Cell Workstation (A); the changed devices are: UV-lights (1), port selection valve (2) and Vspin™ (3); the changes setup of the liquid handler deck (B) are: Static Peltier Device (a), Positive Position ALP (b) and the direct access to the Vspin™ (c).

4.1.2.1 *Positive Position ALP*

The Positive Position ALP (Beckman Coulter, Krefeld, Germany; see figure 4-4) is a removable ALP for variable positions of the liquid handler deck. This ALP is specifically required to fix the plates for spheroid formation because the cavities only have a diameter of 2 mm to form hanging drops. This ALP is located on positon 1 (P1) on the liquid handler deck of the Biomek® Cell Workstation.

This active ALP enables the exact positioning of labware by fixing and allows high precision of the liquid handling in the wells by different operations modes (clamping, unclamping, recognition of labware by a sensor). The fixed labware positioning guide in association with the Positive Position ALP clamp realizes the fixing of the labware. The integrated sensor of the Positive Position ALP detects the presence of labware on the ALP. The accepted labware have to be in a microplate format. The Biomek gripper on the ALP can load this. The ALP is fastened on the liquid handler deck using thumbscrews. This ALP is controllable within an automatic method by the Biomek® Software about device action steps. The manual control outside of a method is enabled by the Advanced Manual Control [126].
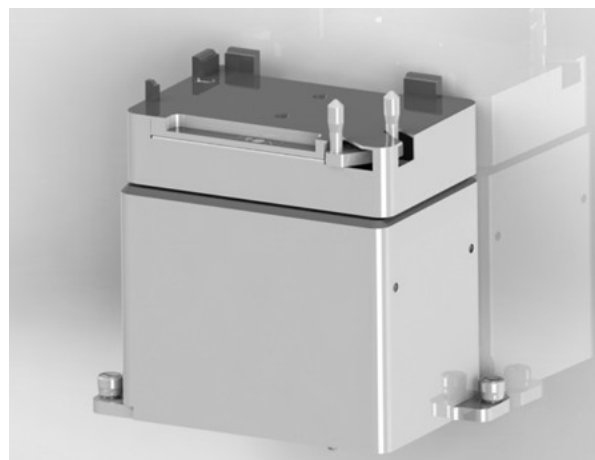
Figure 4-4 Positive Position ALP

4.1.2.2 *Static Peltier Device*

The heating and cooling of media is essential for special application realizable of the Static Peltier Device (see figure 4-5), which has been specifically integrated for the 3D cell cultivation. Especially heating of media and solutions is important in cell cultivation. The temperature optimum for cells is 37 °C simulating *in vivo* conditions. This device is essential for the alginate bead production because lower temperatures of alginate increase the viscosity. It is located at position 2 on the liquid handler deck of the Biomek® Cell Workstation.

The Static Peltier Device is fixed on the deck by thumbscrews. The connected controller enables the cooling and heating of fluids. The temperature range is 4-50 °C. However, the solutions located in reservoirs only reach a temperature of 37 °C at the maximal controller temperature. The needed format of labware is 96- or 384-well microplates. Black stand (adapter) contained of anodized aluminum enables the tempering of solutions within the modular reservoirs. The Biomek® Software enables to control of the peltier steps during the automatic method. Instead, the manually control without software is realized using the Watlow Controller [127].

Figure 4-5 Static Peltier Device

4.1.2.3 Vspin™ Centrifuge

Various cell culture steps include the separation of cells from the supernatant. This is specifically required for the cultivation and dissemination of suspension cells as well as formation of alginate beads and pellet cultures. Usually, the published automatic cell culture systems do not include centrifuges. The centrifugation at the Biomek® Cell Workstation is realized using the integrated Velocity11 Vspin™ (Velocity 11, Palo Alto, USA; see figure 4-6). This centrifuge is designed for automatic process steps. Within the thesis, the centrifuge was integrated in the cell culture system with a direct access from the liquid handler deck integrated in the housing to guarantee sterile conditions. This Vspin™ (see figure 4-6) enables the centrifugation of two cell culture flasks or deep well plates in one step with a 96-well plate format. The liquid handler places the well plates on the Access2 plate transport station in front of the centrifuge, which is part of the Vspin™. This enables the transport of the microplates in and out of the centrifuge. The liquid handler placed the microplates on the bracket and the gripper of the centrifuge moves the plates into the Vspin™. The spindle speeds are 0-3,000 rpm of cell culture flasks with maximal 250 g (each plate). The time for acceleration and deceleration is 7.5 second for 0-3,000 rpm [128].
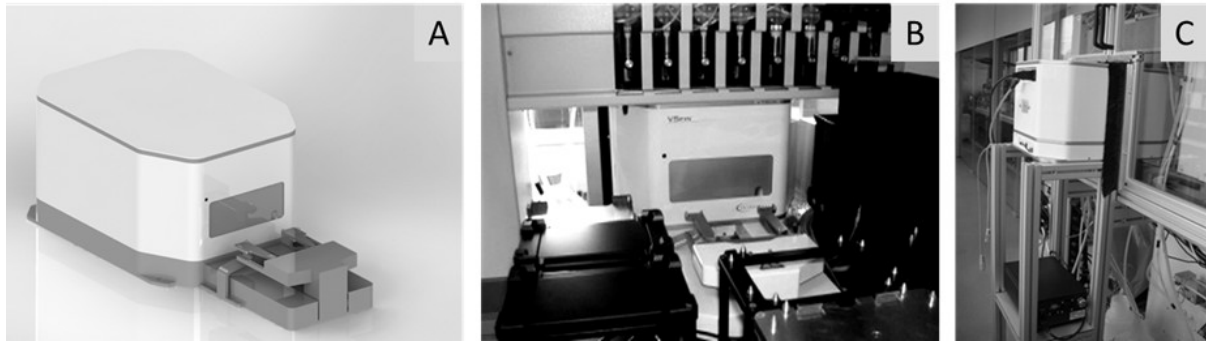
Figure 4-6 Vspin™ centrifuge (A), centrifuge integrated at the Biomek® Cell Workstation (B, C)

The Vspin™ centrifuge can be controlled in the Biomek® and SAMI® software by an action configuration dialog. The centrifuge has to be initialized before using. For plate loading the centrifuge must be open and plates have to be moved from load to defined position in the Vspin™ controlled and programmed about the Biomek software during the automatic process. The percentage acceleration, deceleration and speed settings can be selected by the action "centrifugation". Furthermore, time of centrifugation has to be distinct. The "time estimate" shows the time of the process.

4.1.2.4   Port Selection Valve

The integrated liquid handler of the automatic cell culture system is able to transfer lower volumes up to 5 ml. Regarding this, the liquids are submitted in reservoirs on the liquid handler deck. The Biomek® NX allows single channel up to eight-channel pipetting steps. A bulk dispense media system is required for transferring higher volumes into automatic cell cultivation. This is needed for the automatic parallel cell cultivation and detachment process of human primary chondrocytes.

The integrated port selection valve at the Biomek® Cell Workstation links 6 different media or solutions in flasks with safety caps with a volume up to 2 l. The port selection valve is associated with a low pressure injector and switching valves with a bore size of 1.5 mm (Valco Instruments, Schenkon, Switzerland). It is connected with a Reglo digital tubing pump from Ismatec (IDEX Health & Science, Glattbrugg, Germany) and the cannula of channel 7 of the liquid handler, which enables the transport of solutions about a tubing system (see figure 4-7). This combination allows sterile changing of liquids for parallel cultivation, cleaning and disinfection. These liquids are water, isopropanol and 4 different media. This system is able to deliver 50 µl to 150 ml. The speed of the liquid dispensing is 200 µl/s. The Biomek® software follows the control and selection of the channel.
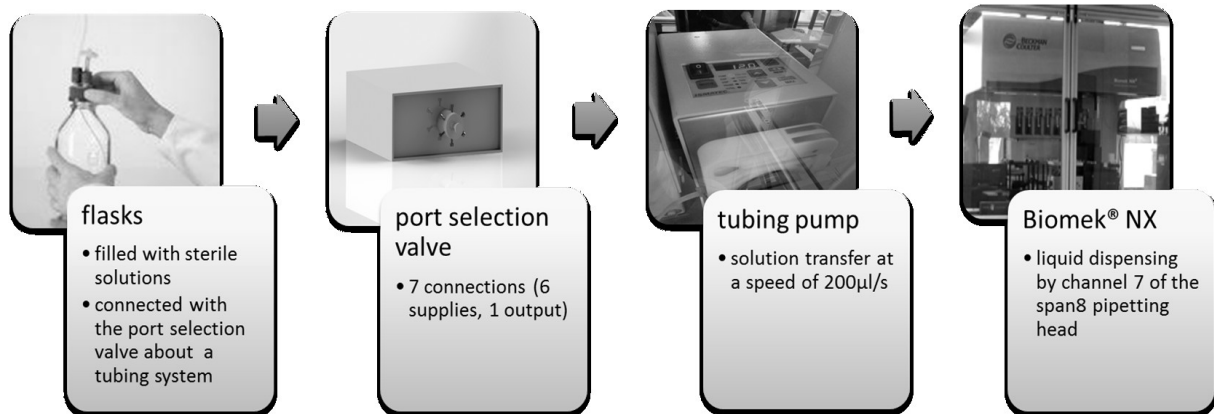
Figure 4-7 Solution transport by the port selection valve

4.1.2.5   UV Lights

The main forms of contamination in cell cultures are microorganism. This can be bacteria and fungi. Reasons for this contamination are negligence of aseptic working and spores in the air. Bacterial contaminations are combined with a decrease of the pH level, turbidity of media and mortify of the cells [119, 120].

UV light is a common solution for the reduction of germ counts in room air and at surfaces. Short wave UV radiation has an antibacterial effect. The optimum efficiency will be achieved at 254 nm. Firstly, bacteria are killed. The radiation tolerant fungal spores require a vastly superior radiation. The wavelength of 254 nm leads to the development of formation of thymine dimers. Thereby errors of the DNA polymerase effect in mutations in deletions. The result is the termination of the replication. Finally, cells are no longer accomplished for proliferating and persisting. There are three different UV-radiations important in biology (Table 4-1). Hereof UV-C is most important for disinfection [12].

Table 4-1 Overview about UV-radiation and biological impact

UV-radiation	wavelength	biological impact
UV-A	320 - 400 nm	low
UV-B	280 – 320 nm	middle
UV-C	200 – 280 nm	high

Two UV lights have been integrated into the automatic cell culture system at the sides of the housing (see figure 4-8; A, B). The disinfection by UV lights take place 15 minutes before cell handling. On the left side, a trestle allows the pivoting of the UV light (see figure 4-8, A). The UV-light on the right sight is directly associated with the housing due to lack of space. Consequently, following the disinfection of the whole deck, the steel cannulas and air above the liquid handler deck.
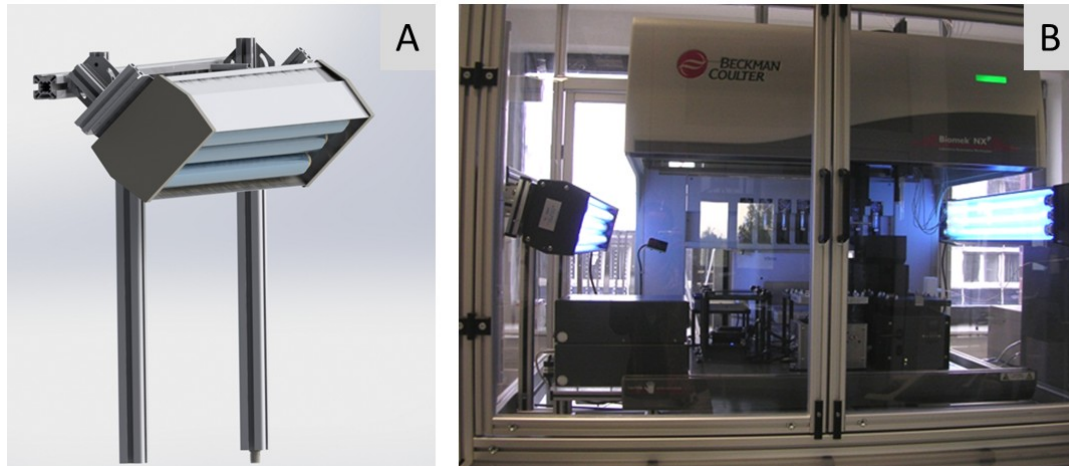
Figure 4-8 UV-lights in the cell culture system

4.1.3   Translation of the Manual Processes to Full Automation

The translation of manual processes into fully automatic operations is a great challenge because a one to one translation is frequently not possible. The automatic processes often need subsidiary steps. Special flasks and devices are needed for simulating the manual handling.

4.1.3.1   Labware and Solution Handling

The systems for automatic cell cultivation have to handle special labware. The grippers of liquid handler or robot arms transfer the labware. These labware are cell culture flasks, well plates and lids. Generally, the size corresponds with a well plate format. But there are differences between producers. The labware size has to be transferred into the labware type editor (see figure 4-10) of the Beckman software. This is programmable and expandable by the user. The labware type editor is part of the Biomek® software and contains the exact names and dimensions of the labware, which can be used at the automatic processes. This is essential for the transfer of labware or solutions by the liquid handler. Hereof, the exact labware has to be used for the programming and realization of the automatic methods.

Cell culture vessels

The automatic processes require specific vessels for the cell cultivation and the storage of solutions and media (see figure 4-9).

There are different cell culture stackable flasks for the automatic cell handling available on the market (see table 4-2). These are CELLSTAR AutoFlask™ (greiner, Frickenhausen, Germany), Corning® RoboFlasks® (Corning Incorporated Life Sciences, Lowell, USA) and the BD Falcon™Automatic Cell Culture Flasks (BD Bioscience, Bedford, USA). The flasks for automatic cell handlings are available in an automatic friendly microplate format and consist of the same material, polystyrene (PS). The footprint of 96-well plates is an essential aspect for the standardization of different automation processes and enables the automatic stacking. The growth areas ($83.6 – 94$ cm^2) are larger than for

manually operating cell culture flasks (75 cm^2). They are equipped with a septum for liquid transfer, a membrane to guarantee the gas exchange and barcode for the flask identification. The septum of the Corning® RoboFlasks® (Corning Incorporated Life Sciences) and the BD Falcon™Automatic Cell Culture Flasks (BD Bioscience) is lateral located. Herein, the flasks must be tilted for the liquid transfer. This means mechanical stress for the cells and a longer drying period for the monolayer. Instead, the septum of the CELLSTAR AutoFlask™ (greiner) is located on the top-right corner and permits the direct horizontal handling of the cell cultures. The membranes of all automatically handled cell culture flasks are located on the top. Primarily, the flask for the automatic handling designed for adherent cell lines. Only the CELLSTAR AutoFlasks™ (greiner) are available for adherent and suspension cell lines. The specific surface-treatment increased the attachment of adherent cells and the hydrophobic surface supports the cell growth of suspension cells. The Corning® RoboFlasks® (Corning Incorporated Life Sciences) and the BD Falcon™ Automatic Cell Culture Flasks (BD Bioscience) enable the automatic cell cultivation of the Tecan® Cellerity™ Cell Maintenance and Assay System. Instead, the Biomek® Cell Workstation support the CELLSTAR AutoFlask™ (geiner) because the integrated 3D tilt racks enable the automatic cell handling [10, 129–131].

Junginger et al. investigated the different cell culture flasks for the automatic handling. The cervix carcinoma cells (HeLa) and the Chinese hamster ovary cells (CHO) showed different cell proliferation in the diverse flasks. The cell counts were lowest in CELLSTAR AutoFlask™, followed of Corning® RoboFlasks® and highest within BD Falcon™ Automatic Cell Culture Flasks. However, the HeLa cells in AutoFlasks™ showed a significant increased cell count at the fourth approaches. The cell count of CHO cells cultivated in AutoFlask™ was similar to the CellStar flask (greiner) for the manual handling and amount 60x10^5/ml. Instead, the other flasks from other producers showed greater variation [10].

Table 4-2 Cell culture flasks for automatic handling available on the market

Parameter	CELLSTAR AutoFlask™	Corning® RoboFlasks®	BD Falcon™ Automatic Cell Culture Flasks
Material	polystyrene (PS)	polystyrene (PS)	polystyrene (PS)
Growth area	83.60 cm^2	92.6 cm^2	94 cm^2
Location of the septum	top-right corner	lateral	lateral
Barcoded	yes	yes	yes
Location of the membrane	top-left	top-left corner	top-left
Cell forms	adherent cells, suspension cells	adherent cells	adherent cells
Continued on next page			

Parameter	CELLSTAR AutoFlask™	Corning® RoboFlasks®	BD Falcon™ Automatic Cell Culture Flasks
Automatic handling by these systems	Biomek ® Cell Workstation	Tecan® Cellerity™ Cell Maintenance and Assay System	Tecan® Cellerity™ Cell Maintenance and Assay System

The Biomek® Cell Workstation has to be equipped with a specific device for supporting the RoboFlasks® (Corning Incorporated Life Sciences) and the BD Falcon™ Automatic Cell Culture Flasks (BD Bioscience) next to the CELLSTAR AutoFlask™ (greiner). The septa of the RoboFlasks® and BD Falcon™ Automatic Cell Culture Flasks are positioned at the side and require a flipping of the flasks to an upright manner about 90° for the liquid transfer. This is realizable by the proprietary flipping device (celisca, Rostock, Germany). The flipping device is an automatic labware positioner (ALP) for the liquid handling by the Biomek NX Span-8 and turns e.g. two RoboFlasks® per time by switching of pneumatic cylinders [10]. Nevertheless, the hydrophobic membrane of the CELLSTAR AutoFlask™ (greiner) is polytetrafluoroethylene (PTFE) coated and prevent from wetting. The comparability of the cell counts in flasks for the manually and automatically handling is a further benefit of the CELLSTAR AutoFlask™ (greiner). Only the AutoFlask™ is equipped with a centrifugation pocket for media exchange. But the greatest advantages of the AutoFlasks™ are the availability for different cell lines (adherent cells, suspension cells) and the clemency horizontal cell handling. This is unique for this format and labware. The CELLSTAR AutoFlasks™ (greiner; see figure 4-10, A) were chosen to realize the flexible automatic cell handling.

The solutions, cell suspensions and buffers are presented in sterile common modular reservoirs (Beckman; see figure 4-9; B) or bottles with air filters (see figure 4-9; C). The re-usable reservoirs for the automatic handling consist of polypropylene. The reservoirs placed on Static Peltier Device enable tempering of solutions. The reservoirs are located on specific positions of the liquid handler deck and provide media (up to 40 ml) for the automatic liquid transfer of the span-8 pipetting head. The glass bottles are associated with the port selection valve to transfer higher volumes of media.

The cells are seeded in 96-well plates (greiner) for the consequently bioscreening. The well plate format allows seamless automatic processes with the screening system [110].
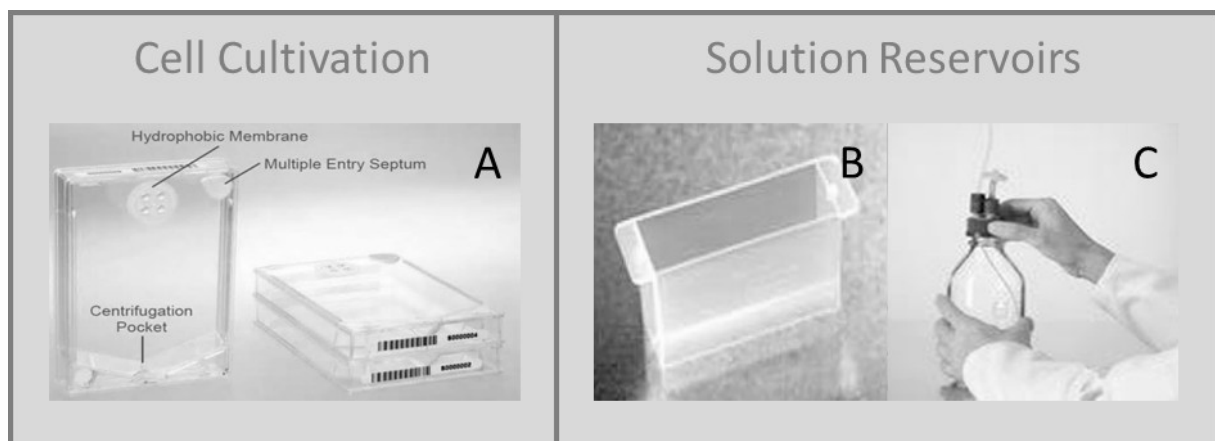
Figure 4-9 The specific flasks and reservoirs for automatic cell cultivation by the Biomek® Cell Workstation [110]

The cell culture AutoFlask™ can be used for the automatic cell cultivation of adherent or suspension cell through the 96-well format, the hydrophobic membrane and the entry septum for liquid transfer (A) [132]. The solutions are stored in modular reservoirs (up to 40 ml) (B) [133] and bottles with air filter (up to 2000 ml) (C) [110].

4.1.3.2 Modifications in the Control Software

The different cultivation forms require specific programs to realize the manufacturing procedures. Generally, the methods are separated in a detachment process of the cells and the following 3D formation method. Basically, new labware had to be defined using the Biomek® software. Then, the realization of the liquid handling steps followed using the Biomek® software. New pipetting templates had to be evaluated. The Biomek® software enables the combination of the liquid handling, cell calculating, centrifuging and rinsing steps. The SAMI® software enables the combination of the whole process consisting of different liquid handling methods.

Firstly, the only new to integrated labware were the HDP1384 Perfecta3D®384-Well Hanging drop plates (3D Biomatrix) about the Labware Type Editor of the Biomek® liquid handling software, named with "hanging_drop 1384" (see figure 4-10). The plates contain a tray, the hanging drop plate and the lid. The accurate dimension has to be integrated for an optimal hanging drop formation. The basic information of the plate had to be programmed with following data: span 12.776 cm (X) and 8.574 cm (Y) as well as a height of 1.125 cm. The well offset (X: 1.175 cm; Y: 0.97 cm), well count (X: 24; Y: 16), well spacing (X: 0.45 cm; Y: 0.45 cm), maximum volume (30 µl) and well configuration (shape: round; upper radium: 0.2 cm; lower radius: 0.08 cm, height: 0.675 cm) had to be edited.
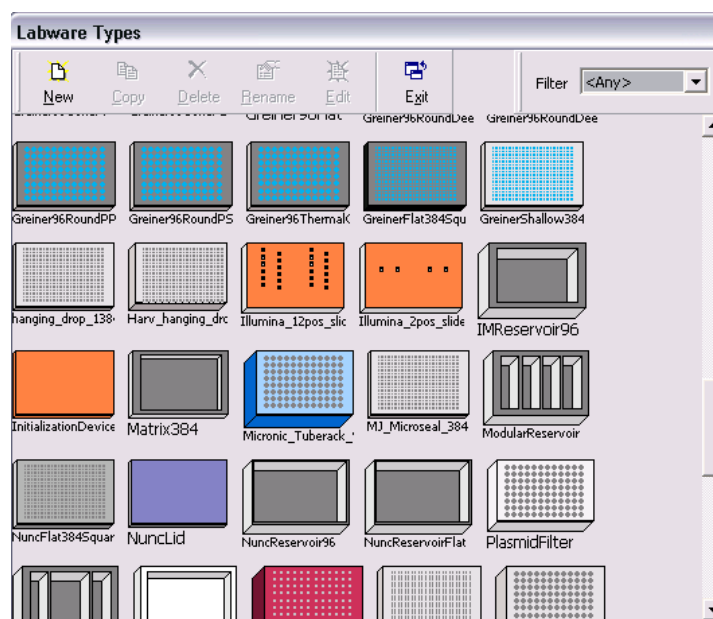
Figure 4-10 Labware Type Editor

The next programming step includes the specific pipetting steps using the pipetting template Editor. The Pipetting Template Editor allows exact definition of the aspiration, dispense and mix pipetting steps, for example of piercing location, time, height, speed and volume (see figure 4-11). This is immensely important for the production of 3D cell cultures.
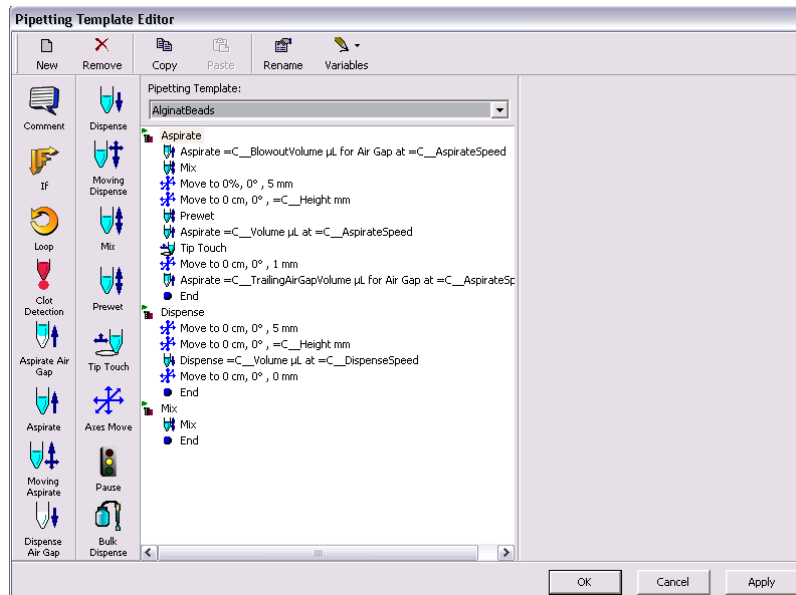
Figure 4-11 Pipetting Template Editor

Alginate beads needed specific pipetting templates because the viscose solution requires a slower aspiration and dispense for prevention of air gaps. The cell-alginate solution should drop in the middle of the well with a defined speed so that drops fall into the solution.

The spheroid cultures needed a detailed template for the hanging drop formation under 2 mm cavities in the hanging drop well plates.

The pellet cultures required a pipetting template for the carefully cell handling to prevent cell loss.

The following table summarizes the changes aspects at the pipetting templates related on the 3D cell culture:

Table 4-3 Changes in pipetting templates related on the 3D cell cultures

3D cell cultures	Process steps	Changed aspects
Alginate beads	remove the supernatant (DWP) of the cell pellets after the centrifugation	steel cannula: aspirated 3 mm above the bottom at a speed of 750 µl/s
	drop cell alginate solution in the wells	steel cannula: dropped at the cell center from a height of 1.5 cm from the top with a speed of 208 µl/s
Spheroid cultures	form hanging drops with the HD plate	steel cannulas: moved to the well center dispensing speed: 50 µl/s at a height of 6 mm from bottom
Pellet cultures	remove the supernatant (DWP) of the cell pellets after the centrifugation	steel cannula: aspirated 3.1 mm from bottom at 45° from the well center aspirating speed: 50 µl/s

The SAMI® Workstation EX Editor software is used for controlling of the complex applications (see figure 4-12). The cell cultivation and 3D formation was realized using object-oriented programming. The required labware has to be chosen with home positions, volumes of reservoirs in association with the Biomek® programs. Every cell cultivation and 3D formation needed different labware and liquid handling steps. Additionally, this software contains an optimizing scheduler for pre-validated schedules. Generally, the cell culture processes started and end with disinfection methods (Biomek methods), whereby steel cannulas are incubated 3 minutes with isopropanol and rinsed with water. The other process steps are subdivided in separately Biomek liquid handling steps.
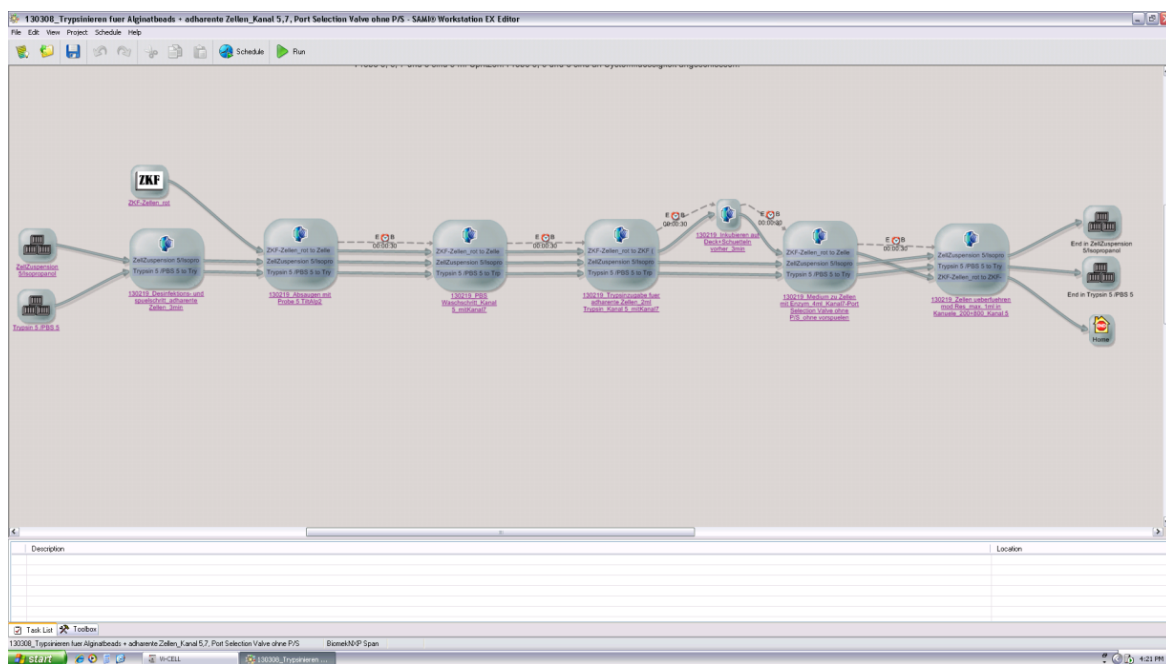
Figure 4-12 SAMI® software: detachment process of cells

4.1.3.3   Evaluation of Cross-Contamination

The increasing interest and demand for 3D-cell culture and human tissue, which consists of more than one cell line, is in contrast to the problem of cross-contamination. The fluorescence labeling with PKH dyes (Sigma Aldrich) is a new way for the evaluation of cross-contamination of cells in automatic cell culture processes. The membrane labeled cells were automatically cultivated and then evaluated by microscopy and fluorescence automatic cell sorting (FACS).

The evaluation of the cross-contamination required 7 days (see figure 4-13). Basically, adherent cells have to be expanded and PKH-stained manually. Before starting the automatic process, the Biomek® NX was set back, cleansed and disinfected. The liquid handler deck was decontaminated with UV-radiation. The channel 5 pipetted exclusively for red-marked (PKH26) cells and channel 6 for green-marked (PKH67) cells. Channel 7 was set for a bulk dispense added with a port selection valve plus dispensing pump and acted as a medium donor. The deck reservoirs were kept separately to prevent contamination through this action. The cell suspension reservoir was changed after each family for the red- and green labeled cells. A family contented the cell cultivating of two AutoFlasks™ (greiner),

first run with PKH26 fluorescent cells, the second with PKH67 fluorescent cells. Subsequently, the cells were investigated using a fluorescence microscope (Leica, Wetzlar, Germany) and manually detached for the FACS analysis using a FACSCalibur Cytometer (Becton and Dickinson, Heidelberg, Germany) [134].
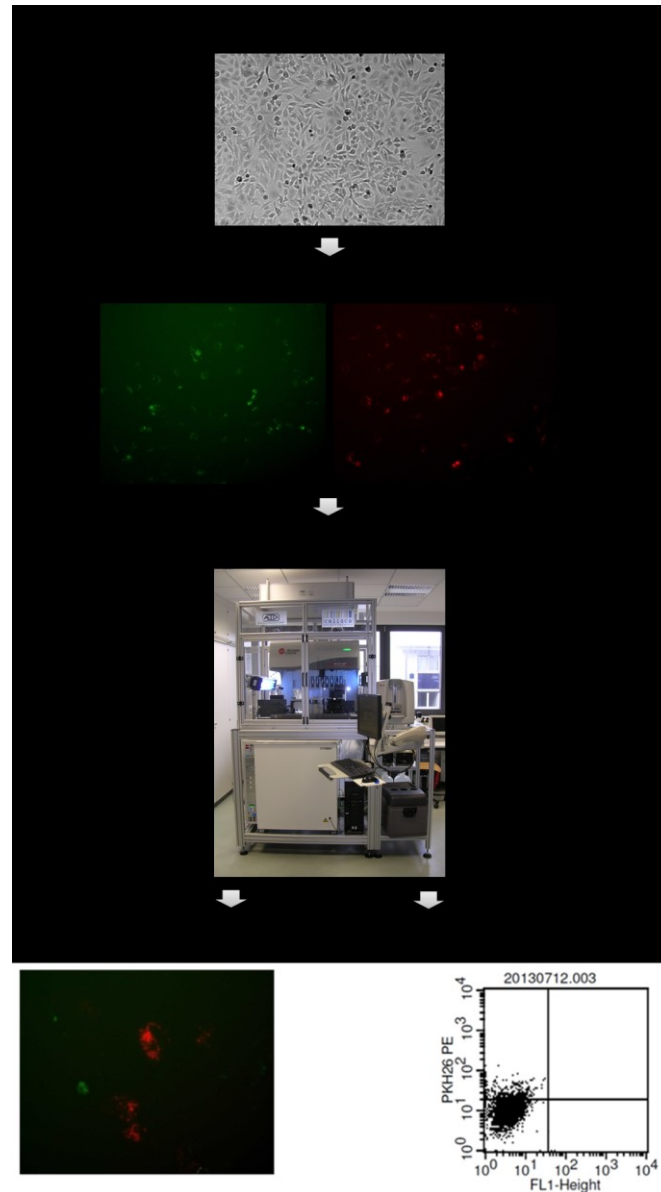
Figure 4-13 Cross- contamination investigation modified from [134]

4.1.3.4 Processes in Automatic Cell Cultivation of Suspension Cells and 3D Constructs

Often, the exact one to one translation of manual methods in automatic processes is not possible. New solutions have to be evaluated for automatic cell processing. Next to the different cell culture vessels, the cell cultivation may include changed steps.

The right location of the labware is essential for the cultivation processes. The different positions of the liquid handler deck are visualized in figure 4-14. The position 7 is directly located on the on deck

incubators (position IT, IB). There are two 3D tilt racks available (position 5, Tilt3D). The two placeholder ALPs are located on position 4 and 6, which is supplemented with a connection to the ViCell XR. The Static Peltier Device is located on position 2 and the Positive Position ALP is located on position 1. The lift position is located next to the shaker (position TShake). The centrifuge is position (VS). The waste ALP (TR1) is for disposables. The two wash stations are located above position six to remove media and rinse the steel cannulas.
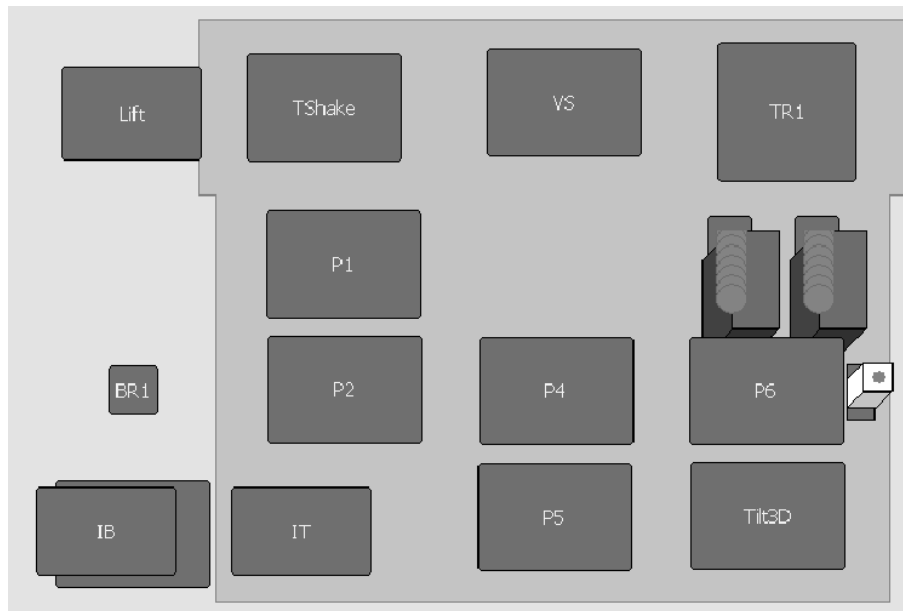
Figure 4-14 Biomek® software: different positions of the liquid handler deck

Suspension Cell Dissemination

The published papers with automatic systems for cell cultivation address so far exclusively monolayer cultures of adherent cell lines. Suspension cell lines are another cell type, which are essential for the research, especially for the investigation of leukemia. However, suspension cells floating in the media and require different methods for cell handling, devices for the cultivation and dissemination of cells in the automatic way compared to adherent cells [110]. A specific challenge is the automatic parallel cell handling of 4 different suspension cell lines. Each of these cell lines needed another medium (Jurkat: IMDM; SEM: RPMI 10 % FCS; Molt-4: MEM alpha; RS-4: RPMI 20 % FCS).

The SAMI® program is subdivided in five Biomek® liquid handlings steps to disseminate the suspension cells. The process started with the dissemination of Jurkat cells, followed of SEM cells, Molt4 cells and consequently RS4 cells at the specific methods by the Biomek® NX liquid handler. The process closed with the Biomek® NX disinfection method to decontaminate the steel cannulas. The needed labware for SAMI® programming are the reservoirs and well plate with lid. The dissemination of suspension cells is described in detail in the following section.
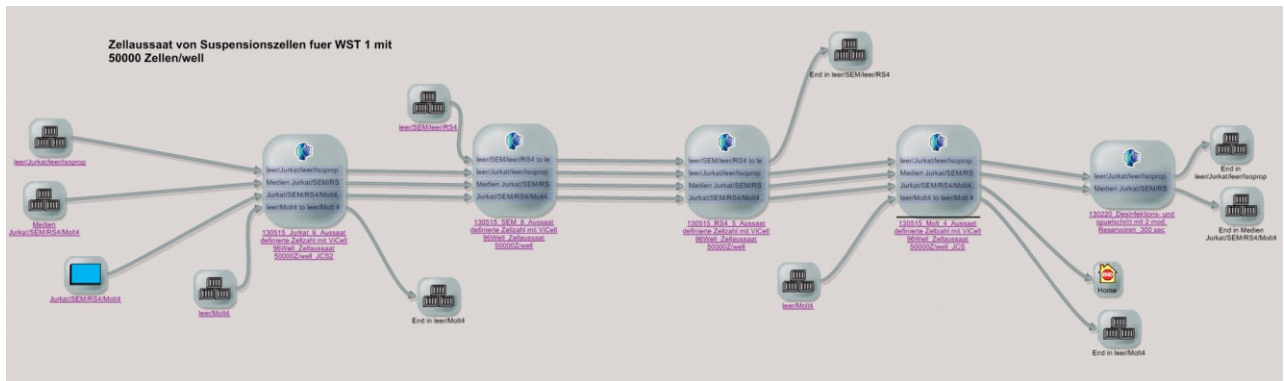
Figure 4-15 SAMI® program: dissemination of suspension cells

The basically steps for the manual and automatic dissemination of suspension cells are the same:

[1] centrifugation and removing the supernatant
[2] resuspend cell pellet in fresh media
[3] dilute cell suspension
[4] cell seeding (50,000 cells/well)
[5] incubation at 37 °C and 5 % CO_2

The direct translation of the manual handling (manual) of suspension cells into automatic processes (automatic) could not be executed. The cell culture flasks, storage of solutions, centrifugation, cell counting and cell seeding were different. The suspension cells are cultivated into T25 flask (manual) and AutoFlask™ (greiner) to realize the automatic procedure. The solutions are stored in tubes (manual) and reservoirs (automatic). The centrifugation is performed using tubes in the manual method, and deep well plates to support the automatic separation of the cells from the supernatant by the Vspin™. The cell amount is detected with a Neubauer chamber (manual) and ViCell™ XR counter (automatic). Lastly, the cell seeding is completed with Eppendorf pipettes plus disposables (manual) and Biomek® NX liquid handler with steel cannulas.

Regularly, the suspension cells are cultivated in T25 cell culture flaks (Sarstedt) at upside position. The cells are polled, centrifuged and diluted in 50 ml tubes. Mainly, the manually cell counting is performed with 10 µl cell-trypan blue solution by the Neubauer chamber after trypan blue staining. The cell seeding is enabled using a one-channel Eppendorf pipette or a reservoir with an 8-channel Eppendorf pipette into a 96 well screening plate, which supported the consequently automatic bioscreening.

The automatic dissemination of four different suspensions cells (Jurkat, SEM, RS4, and Molt4) differ from manually cell handling, concerning of the cell culture vessels and liquid handling. The automatic parallel medium transfer was realized using the port selection valve and channel 7 of the liquid handler. Therefore, the tubing system was rinsed with isopropanol (15 ml), water (15 ml) and the specific medium (15 ml) before handling a new cell line. The cell suspensions were stored within modular reservoirs on the liquid handler deck (cell culture process: position 6). The automatic separation of the cells from the conditioned medium was performed using a centrifugation step of the cell solution with a deep well plate. The storage and dilution of the cells was performed in separate reservoirs. The cell proliferation was investigated after an incubation period of 24 hr.

The following sheet visualized the automatic dissemination process of suspension cells (see figure 4-16).

The cell culture process contains two different automatic main processes. First, the regular automatic cell cultivation is done; whereby two cell lines are handled per time to transfer the cell suspension into the modular reservoirs. The Cytomat (Thermo Fisher) included the AutoFlasks™ (greiner) with cells (Jurkat: hotel 1, position 1; SEM: hotel 2, position 1; Molt 4: hotel 3, position 1; RS4: hotel 4, position 1) and the 96 well plates (greiner; hotel 1; position 3). The deep well plates with lids were located on position 1 (cells) and 3D tilt rack (counter weight). The cell culture flask with the suspension cells and the 96 well sheets are transferred to the liquid handler deck. The 3D tilt rack in combination with the liquid handler allowed the transfer of 2 ml/well cell suspension into a deep well plate (DWP; greiner). The same volume (water) is transferred in a counter weight. The Vspin™ centrifuged the cell suspension (2,250 rpm, 5 min). The liquid handler removed the supernatant and water (2 ml) as well as consequently resuspended the cell pellets in fresh media (0.5 ml/well). Finally, the cell suspension is pooled in a modular reservoir (Beckman Coulter) located on position 6.

The automatic dissemination of the four different suspensions cell lines followed in the second main process step. The ViCell XR (Beckman Coulter) detected the viability and counted the cell number. The dilution of the cell lines was performed in a separately reservoir. After calculation of the exact cell number (50,000 cells/well $\triangleq$ 3.4x10^5 cells/ml) medium and subsequently cell suspension were transferred into the 96 well plate (position 4) with a total volume of 150 µl. The liquid handler and the lift transferred the well plates with cells to the home positions. They will be incubated by the Cytomat (37 °C, 5 % CO_2) [110].
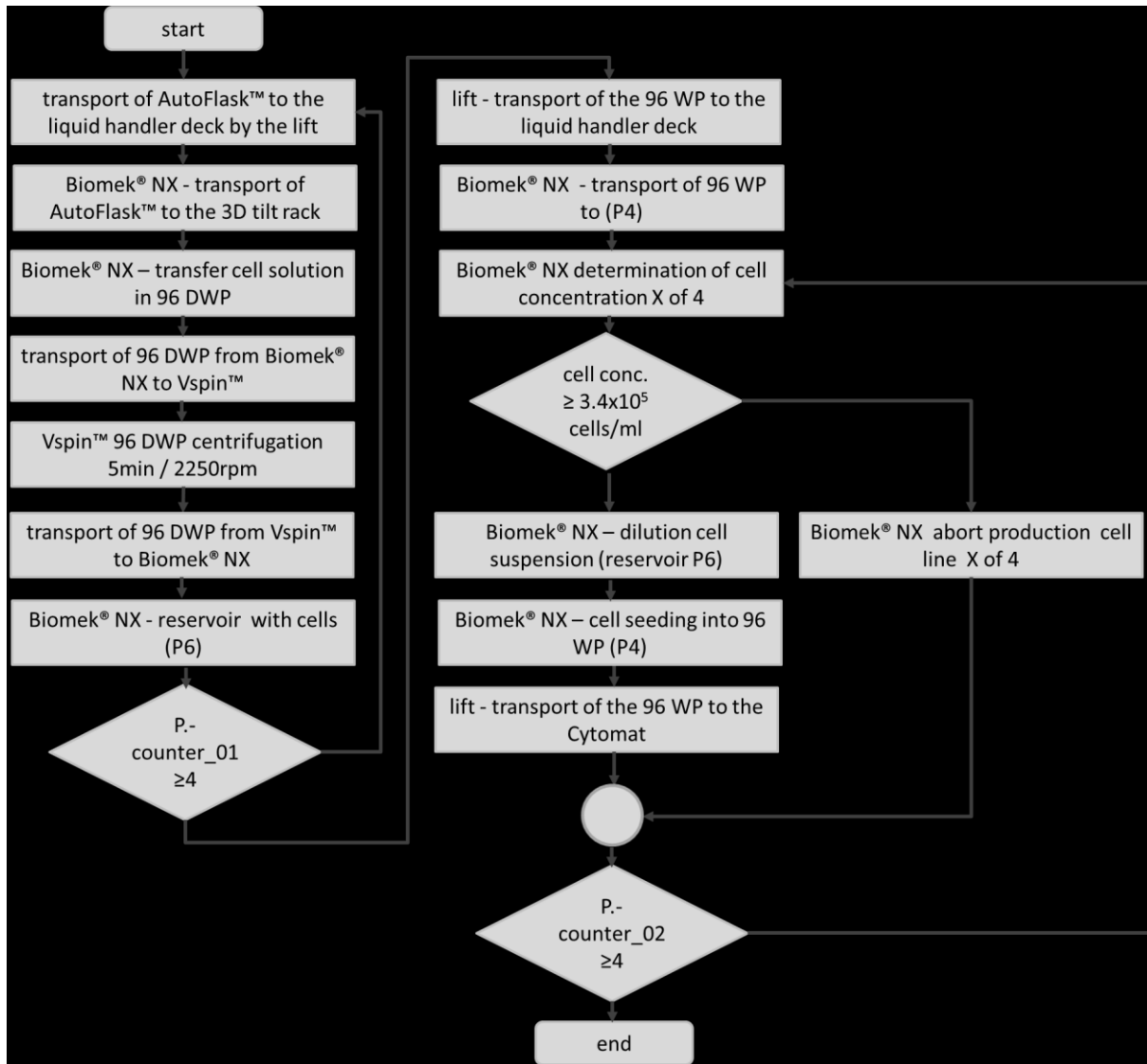
Figure 4-16 Flow sheet of the dissemination of suspension cell lines

Manufacturing of Alginate Beads

A popular manual method to form 3D constructs are alginate beads. The cells are encapsulated in biocompatible alginate after the polymerization process. These 3D cultures are suitable for a variety of adherent cell lines e.g. primary chondrocytes and cancer cells. The integrated pores enable the transport of nutrients and metabolic products to a certain extent, which is associated with a hypoxic core. Therefore, alginate beads are a suitable form to simulate physiological tissue and the *in vivo* environmental conditions. The applications of alginate beads can be separated in bioscreenings and the direct medical use.

The SAMI® program is subdivided in four Biomek® liquid handlings steps to manufacture alginate beads (see figure 4-17). The process started with a disinfections step of the steel cannulas with isopropanol for three minutes followed of the manufacturing of alginate beads. Then the rinsing method with NaCl and the adding of medium followed. The production process closed with a

disinfection method to decontaminate the steel cannulas. The needed labware for SAMI® programming are the DWPs, lids, reservoirs and well plates with lid. The manufacturing of alginate beads is detailed described in the following chapter process steps.
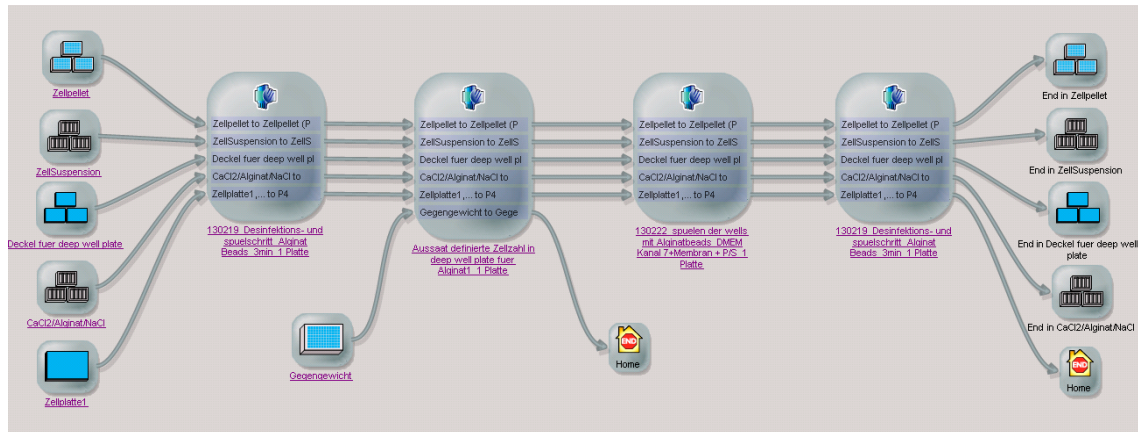
Figure 4-17 SAMI® program: manufacturing of alginate beads

The main steps of the alginate beads production in manual and automatic handling have to be the same:

[1] cell suspension with defined cell count $2x10^6$ cells/ml alginate

[2] centrifugation and removing the supernatant

[3] resuspend cell pellet in alginate

[4] drop alginate-cell solution in a vessel with $CaCl_2$

[5] wash beads with $NaCl_2$

[6] incubation in media at 37 °C and 5 % CO_2

A direct translation of the manual manufacturing (manual) of alginate beads to an automatic production (automatic) was not possible because the storage of the solutions, the centrifugation and the bead formation are different. In general, the solutions are stored in tubes (manual) and modular reservoirs (automatic). The cell solution is centrifuged within tubes (manual) and deep well plates at the automatic procedure. The beads are normally formed within a tumble with 20 ml $CaCl_2$ solution at the manual method and a 96 well plate (automatic).

In detail, for the regular manually handling the solutions are stored in sterile 50 ml tubes. The manual bead formation is performed using dropping cell alginate solution with a syringe and cannula into a pivoting tumble with 20 ml $CaCl_2$ solution. Previously, the cell suspension has to be centrifuged in a 15 ml tube to resuspend the pellet in a calculated volume of alginate for a concentration of $2x10^6$ cells/ml. Subsequently, beads are washed with $NaCl_2$ solution through a filter and aliquoted in a 6-well plate.

The automatic bead production differs from manual process in cell handling by a liquid handler, volumes and cell culture vessels. For the automatic method, microplate formats and the reduction of the volumes are required. Firstly, other volumes have to be used in the automatic method involving different vessels, plates and devices which are integrated in the automatic cell cultivation system to

realize a manageable process steps at the automatic manufacturing and the consequently bioscreening. Prior trials have resulted the successful manufacturing of 1 bead/well filled with 200 µl $CaCl_2$ solution. In the automatic process steps the solutions are stored in modular reservoirs on position 6 (P6; cell solution, isopropyl alcohol) and at the Static Peltier Device (P2; $CaCl_2$, NaCl, alginate). The cell count of $2x10^6$ cells/ml alginate has to be the same. The Vspin™ centrifuge only allows the handling of vessels in a multi well plate format. Thus, the cell suspension has to be aliquoted in a 2 ml deep well plate for centrifugation with a cell count of $1x10^6$ cells/. The water is parallel transferred in a deep well plate with the same volume and in the same wells like the deep well plate with cell suspension. The supernatant is removed by the liquid handler at a height of 3 mm above the bottom at a speed of 750 µl/s. The cell pellet is diluted and resuspended with 500 µl alginate/well. The most serious difference in the automatic method is the reduced volume of $CaCl_2$ to generate alginate beads. Regarding this, $CaCl_2$ solution (200 µl/well) is transferred in a 96 well flat bottom well plate for the polymerization of one alginate bead per well. The automatic process allowed the dropping of cell-alginate solution from a constant height of 1.5 cm from the top with a speed of 200 µl/s to form beads with consistent size.

The cells embedded in alginate are analyzed after a defined proliferation period (14 d and 35 d). A subsequent high throughput screening is facilitated by the fact that alginate beads are cultivated in 96-well plates.

The following flow sheet visualizes the automatic manufacturing of alginate beads (see figures 4-18, 13-2) realized with the Biomek® Cell Workstation (Center for Life Science Automation). The required solutions (isopropanol,water [P6]; PBS, trypsin [P4]; $CaCl_2$, NaCl, alginate [Static Peltier Device, P2]) were stored within modular reservoirs on the liquid handler deck. The cell culture media were provided by the port selection valve combined with a peristaltic pump associated with channel 7 of the Biomek® NX liquid handler. The regular dispensing steps are performed with a speed of 200 µl/s. The CELLSTAR® AutoFlask™ and the well plate (both: greiner) were stored in the Cytomat (37 °C, 5 % CO_2). The lift transported the labware from the Cytomat to the liquid handler deck. The gripper of the liquid handler robot moved the AutoFlask™ to different positions within the workstation. For the detachment process, the AutoFlask™ with 90 % confluency monolayer have to be transported to the 3D Tilt rack. The 3D Tilting-ALP enables the homogenous distribution of PBS through different tilting angles. Cells were detached by incubation with Trypsin/EDTA (Sigma Aldrich) using an on deck incubator. The enzyme was neutralized with medium and the cell suspension was collected into a modular reservoir at position six (P6).

The cell count was measured with the Vi-CELL™ XR (Beckman Coulter) and the cell suspension was transferred into a deep well plate (DWP, greiner) located on position 4 (P4) with a concentration of $1x10^6$ cells/well well ≙ $0.5x10^6$ cells/ml. This production phase I (detachement process and alginate bead formation until calculation step; see figure 12-2) was aborted because to low cell count. The production phase II (alginate bead formation from cell transfer into the DWP until incubation; see figure 12-2) started at sufficient cell count. The same volume of water was parallel transferred into the counter weight located on 3D tilt rack-1. The gripper of the Biomek® NX transferred lids on the deep well plates to guarantee sterile cell handling, stacked on position 7 at the liquid handler deck directly above the on deck incubators. Then, the DWPs are transferred to the Access2 plate transport station by the liquid handler. The gripper of the station transported the DWP one after the other into the Vspin™ for the centrifugation step (1,150 rpm; 5 min). Parallel the $CaCl_2$ solution (200 µl/well) was aliquoted into the 96-well plate by the liquid handler. After the location of the DWPs at their

specific positions the supernatant had to be removed. The specific pipetting template realized the removing of the supernatan at 3 mm from button to minimize the cell loss. The cell pellets were resuspended six times with alginate (500 µl/well) without bubbles, which was warmed at 37 °C on the temperable device to realize a exact liquid transfer. The prevention of bubbles was performed using solution mixing without air gap and the slow aspirat of the cell alginate solution (speed of 25 µl/s) by steel cannula of channel 6. Similar sized beads without bubbles are formed after the pipetting of 15 µl cell-alginate solution in the well of the deep well plate followed of the dispensing of 20 µl cell-alginate solution/well (16 wells) at a constant height of 1.5 cm from the top to form round beads. Then, the 96-well plate has to be covered with sterile and gas permeable foil to prevent the loss of 3D cultures at the wasching steps. Alginate beads were washed three times with NaCl (1x200 µl, 2x 130 µl) and 200 µl fresh media was added. The well plate was covered with the lid. Consequently, the labware was transferred to the home positions (hotel 1, position 1: 96 MWP, hotel 3, position 1: AutoFlasks™) at the Cytomat (37 °C, 5 % CO_2) by the lift [73].
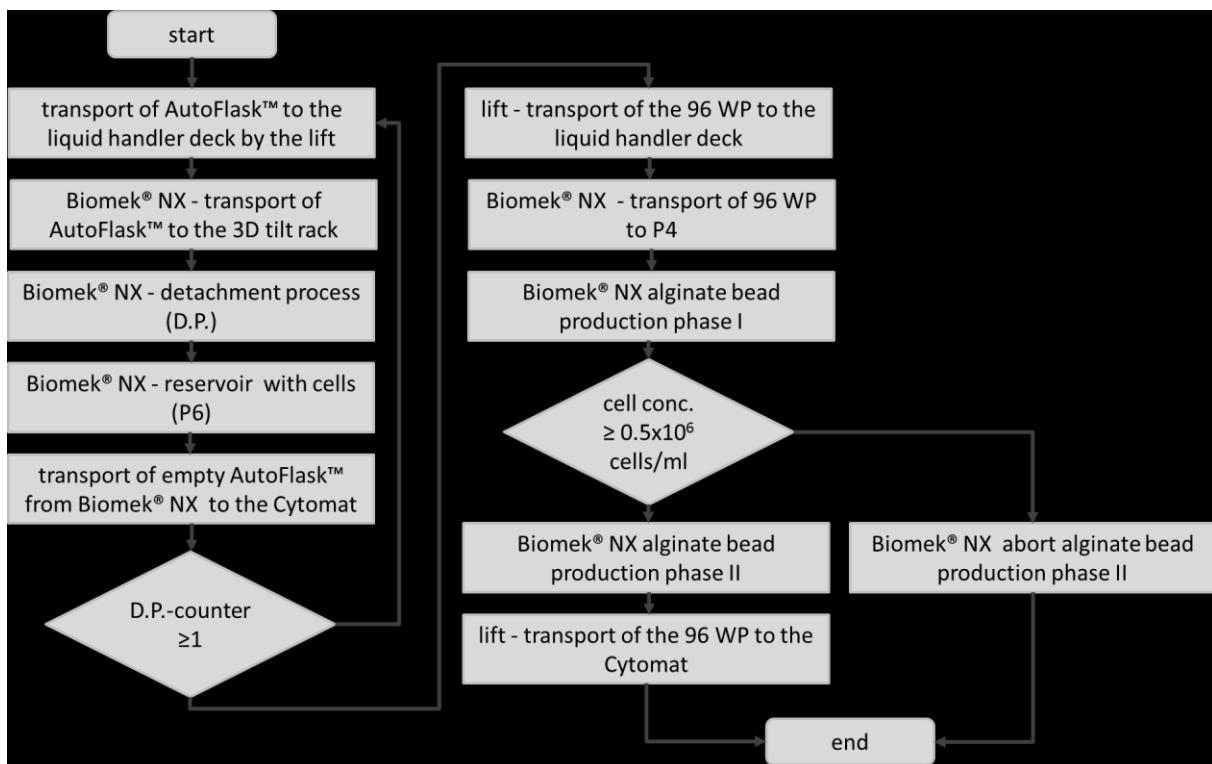
Figure 4-18 Flow sheet of the automatic manufacturing of alginate beads

Spheroid Cultures in Hanging Drops

The most common form to produce 3D cell constructs are spheroid cultures formed in hanging drops. The automation is supported by hanging drop well plates in a 96 - 384 well plate format. The benefits of spheroid cultures are the formation of 3D constructs without carrier material within a short time period. These cultures are predestined for the consequently bioscreening to perform compound screening or different assays (proliferation assays, toxicity assays). Primarily, the 3D formation in hanging drops is used with cancer cell lines to form tumor similar constructs.

The SAMI® program to produce spheroid cultures automatically by Hanging Drop Plates is subdivided in 3 Biomek® liquid handling methods (see figure 4-19). The manufacturing process starts and ends with the disinfection steps for 3 minutes to disinfect and rinse the used steel cannulas. The main liquid handling program is the seeding of the cells in the hanging drop plate. This process is described in detail in the following chapter: process steps. The needed labware are modular reservoirs for the storage of solutions and the hanging drop plate with lid.
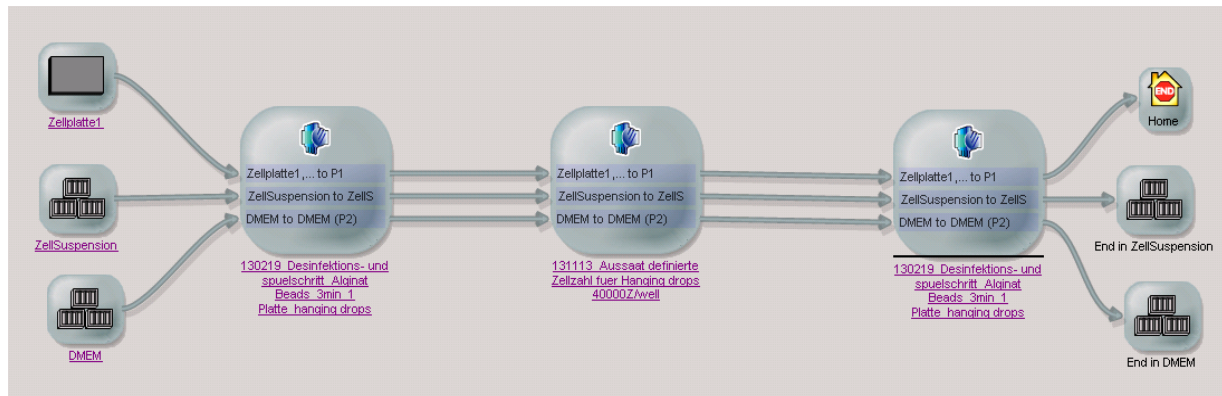
Figure 4-19 SAMI® program: manufacturing of spheroid cultures by Hanging Drop Plates

The HDP1384 Perfecta3D® 384-Well Hanging Drop Plates (3D Biomatrix) are designed for automatic production of hanging drops. These plates contain 3 different components (lid, tray with a hanging drop plate). In cause of that fact, the steps for manual and automatic hanging drop production are similar:

[1] dilute cells for 40000 cells/30 µl with media
[2] transfer of 30 µl cell suspension in the access holes (196 wells)
[3] incubation at 37 °C and 5 % CO_2

A direct translation of the manual manufacturing (manual) of hanging drop to an automatic solution (automatic) was mainly possible. It was not possible with respect to the storage of solutions and the forming of the hanging drops. The solutions are stored in tubes (manual) and modular reservoirs for the automatic processes. The manual formation is performed with a pipette and the hanging drops were produced with the Biomek® NX liquid handler (two hanging drops per time).

The storage of solutions at the manual process was performed in 50 ml tubes. The cell seeding was manually performed with an Eppendorf pipette (30 µl cell solution/hanging drop). The difficult and careful hanging drop formation allowed only the cell seeding for one hanging drop per time.

Mainly important was the integration of the Positive Position ALP at the liquid handler deck for fixing the plate. The low diameter of the cavities (1.5 mm) needs a precise alignment of the plate to the steel cannulas to transfer the cell solution into the cavities of the Hanging Drop Plate (see figure 4-20, A). The automatic manufacturing was only realizable at this ALP because the hanging drops are formed by transferring the cell solution in the cavities under the hanging drop plate, 6 mm from bottom with a speed of 50 µl/s. Therefore, the plate had to be right oriented. The cell seeding was performed using the channels 1 and 2 of the Biomek® NX liquid hander, consisting of steel cannulas combined with syringe pumps. (see figure 4-20, B).
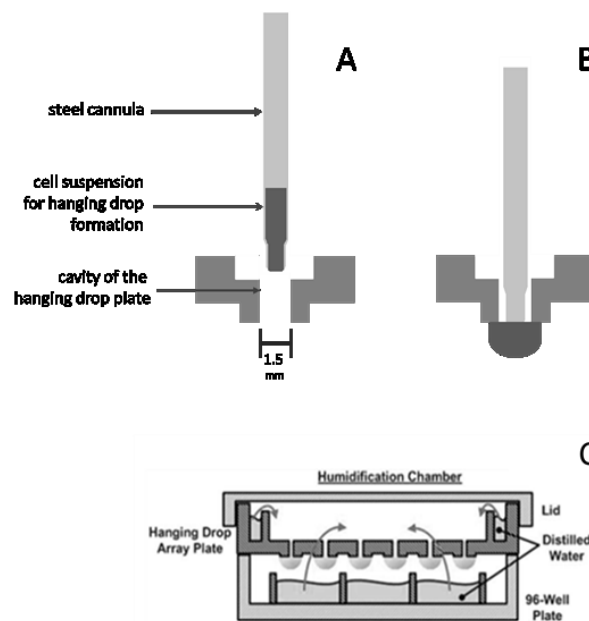
Figure 4-20 Cavity of the hanging drop plate (A), hanging drop formation (B) and hanging drop plate (C) [69]

The spheroids were analyzed after a defined proliferation period (14 d and 35 d).

The flow sheet shows the automatic manufacturing of spheroid cultures in Hanging Drop Plates (see figures 4-21, 13-3) using the Biomek® Cell Workstation (Center for Life Science Automation).

The required solutions for the hanging drop formation are stored on the liquid handler deck (isopropanol: position 6; PBS, trypsin: position 4). The AutoFlasks® (greiner; hotel 3, position 1 [P1]) and the Perfecta3D® 384-Well Hanging Drop Plate (3D Biomatrix, hotel 1, position 1) are located at the Cytomat (Thermo Fisher Scientific). The lift transported the labware to the liquid handler. The integrated gripper of the liquid handler positioned the labware on the deck (AutoFlasks™: 3D Tilt rack; Hanging Drop Plate: Positive Position ALP). Initially, the detachment process (D.P.; 2 families) of cervix carcinoma cells was realized using the 3D tilt racks fixe, pivot and angle the flasks for the liquid transfer. The transfer of solutions was performed using steel cannulas, which were penetrated through a septum. The consumed medium (15 ml) was removed and pipetted to the waste (isopropanol). The monolayer was rinsed with PBS (Sigma Aldrich). The cells were detached with trypsin/EDTA (Sigma Aldrich) by a defined incubation period in the on deck incubator (inheco). Subsequently, this process was stopped with medium (Sigma Aldrich), which was aliquoted by channel 7. This is associated with the port selection valve. The medium transfer is realized using a dispensing pump at a speed of 200 µl/s. After pivoting of the AutoFlask™ by the 3D tilt rack, the cell suspension was transferred into a modular reservoir (Beckman Coulter) located on position 6 of the liquid handler deck. The AutoFlasks™ are transported to the Cytomat by the lift and arrested at their home positions. Additionally, the formation of hanging drops started by the transport of the Perfecta3D® 384-Well Hanging Drop Plate (3D Biomatrix, hotel 1, P1) to the liquid handler deck. The gripper of the liquid handler positioned the Hanging Drop Plate at position 1 (P1), the Positive Position ALP. Then, the plate had to be fixed and the gripper transferred the lid to position IB. The needed cell concentration was 40,000 cells/30 µl $\triangleq$ 1.4x10^6 cells/ml. Therefore, the cell suspension

was counted by the ViCell™ (Beckman Coulter) within 2.5 minutes. The position 6 of the liquid handler deck is associated with the ViCell™ about an adapter. The liquid handler had to transfer 500 µl to the adapter and the cell solution is sucked about a tube. The sufficient cell count was followed by production phase II (phase I: detachment process and manufacturing of hanging drops, until cell calculation; phase II: manufacturing of hanging drops, from dilute cells until incubation; see figure 12-3) by dilution of the cell suspension in the modular reservoir (P6) by adding of medium. The cell solution was transferred into the Perfecta3D®Hanging Drop Plates by channel 1 and 2 of the liquid handler in parallel at every second well to guarantee stable drops over the incubation period. The exact orientation of the steel cannulas and sensitive liquid handling is essential for the hanging drop formation. The channels have to be located at the center of the plates and dispensed the cell solution at 6 mm from the bottom with a speed of 50 µl/s. After covering the hanging drop plate with the lid, the lift transported the plate to the home position in the Cytomat (37 °C, 5 % CO_2) [52].
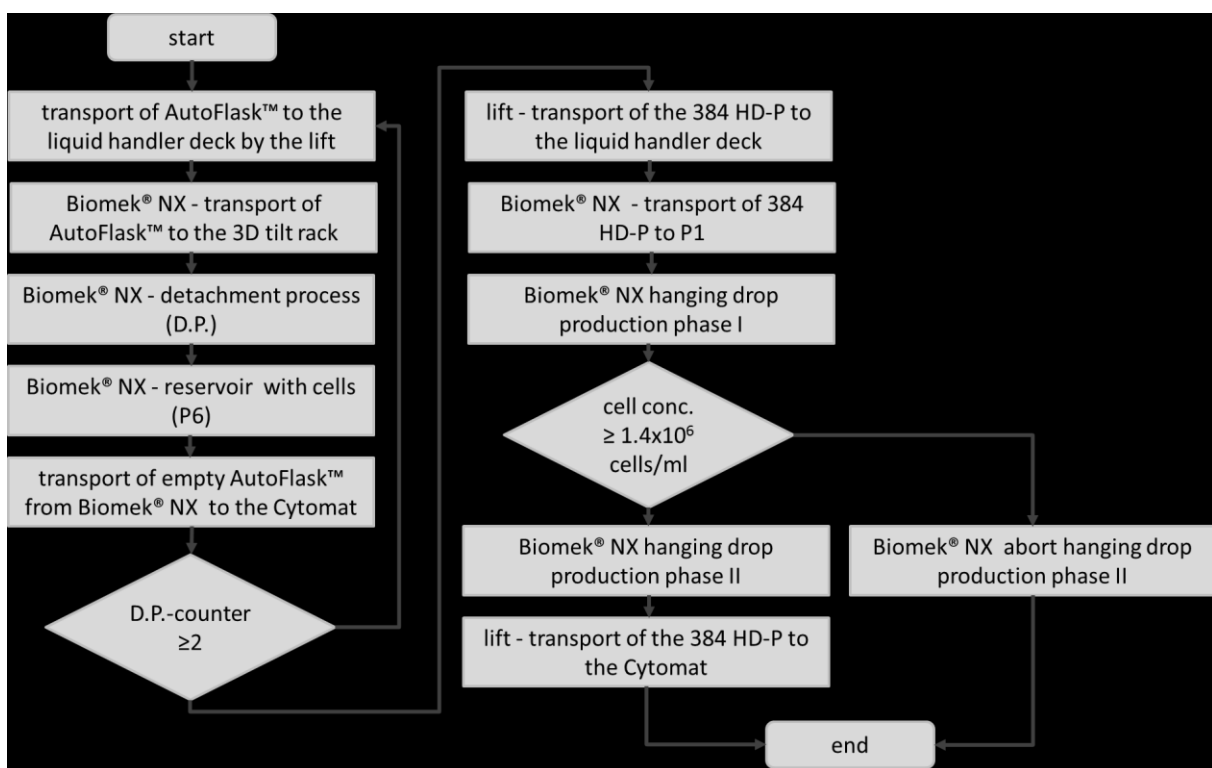
Figure 4-21 Flow sheet of the automatic manufacturing of spheroid cultures in Hanging Drop Plates

Pellet cultures

Specific forms of 3D cell culture systems are pellet cultures. The 3D formation is realized using strong cell-cell contacts and low cell-matrix contacts without carrier material. 96-well plates for suspension cells support the formation and low cell-matrix contacts. Especially suited for this 3D cell cultivation are human chondrocytes, a primary cell line. They enable the *in vitro* development of cartilage after a specific incubation period with growth factors.

The SAMI® program for the automatic pellet formation has one Biomek® liquid handler method (see figure 4-22). This includes the disinfection steps and the cell seeding processes, which are completely

defined in the following chapter process steps. The required labware are DWPs, lids, reservoirs and the well plate with lid.
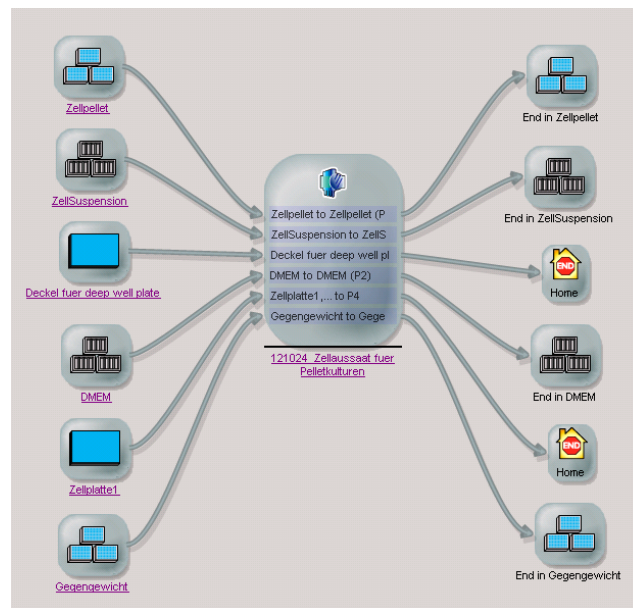
Figure 4-22 SAMI® program: manufacturing of pellet cultures

The main steps for manual and automatic pellet culture production are similar:

[1] concentration of cells for $5x10^5$ cell/pellet culture with media plus growth factor
[2] transfer of cell suspension in not surface treated vessel
[3] incubation at 37 °C and 5% CO_2

A direct translation of the manual manufacturing (manual) of pellet cultures to an automatic procedure (automatic) was in most cases possible. The automatic process needed other labware, liquid handling and process steps for cell seeding. The storage of solutions was performed in tubes (manual) and reservoirs (automatic). The centrifugation was realized within tubes (manual) and deep well plates (automatic). The resuspension of the cell pellets in medium with growth factors was performed in the tubes (one pellet/tube, manual) and deep well plates (up to 96 pellets/DWP; automatic).

The manually generated pellet cultures can be generated in 15 ml tubes or 96 well plates for suspension cells. The 3D formation within 15 ml tubes is realized by aliquoting of the cell solution directly in the tubes ($5x10^5$ cells). Then, the centrifugation followed with the consequently resuspension in medium with growth factors. For the 3D formation in 96 well plates, the cells are detached and pooled in a sterile 50 ml tube. After the centrifugation the cell pellet is resuspended with a specific volume of medium with growth factor for the concentration of $5x10^5$ cells/well (200 µl). The cell seeding of the high-density cell suspension is performed using an Eppendorf-pipette. The subsequently, incubation was performed under standard culture condition (37 °C, 5 % CO_2)

For the automatic pellet formation, the solutions are stored in modular reservoirs and the 3D formation performed using 96-well plates for an automatic handling and the consequently bioscreening. The automatic high-density pellet formation was realized by transferring the cell

suspension ($5x10^5$ cells/well) into a DWP after the detachment process. The supernatant had to be removed slowly from the right side of the well center at 50 µl/s to prevent cell loss. Then, the pellet was resuspended in the media with growth factors and transferred into the 96 well plate, every well one after the other. Flushing steps have been programmed to prevent the transfer of cells.

The pellet cultures were analyzed after time periods of 14 days and 35 days.

The subsequent flow sheet showed the automatic manufacturing of pellet cultures (see figure 4-23, 13-4) by the Biomek® Cell Workstation (Center for Life Science Automation). The AutoFlasks™ (greiner) and the well plate are stored at the Cytomat (37 °C, 5 % CO_2). The deep well plates with lid are located at position 1 (cells) and 3D tilt rack 1 (counter weight). The required solutions are stored at the liquid handler deck (isopropanol, PBS, trypsin: P4; medium with growth factors: P2). The labware transport on the liquid handler deck is realized by using an integrated gripper of the Biomek® NX. The media for the detachment process is stored in glass vessels and transferred over the port selection valve with the dispensing pump and channel 7 of the Biomek® NX. For the high density 3D pellet cultures, eight AutoFlasks™ were necessary for a 96 well plate, which was strongly influenced by the patient and cell proliferation. For the detachment process, the AutoFlasks™ were transferred by the lift to the liquid handler deck. The integrated gripper of the liquid handler positioned the AutoFlasks on the 3D tilt rack. Chondrocytes were washed in PBS (Sigma Aldrich), which was homogenously distributed with a 3D tilt rack. The detachment of the cells in CELLSTAR® AutoFlask™ was realized using Trypsin/EDTA (Sigma-Aldrich) and an incubation of the cells for 10 minutes in an on deck incubator. Fresh DMEM media (Life technologies) was added (channel 7) for neutralization and the cell suspension was collected into a modular reservoir located on position 6 (P6, Beckman Coulter). The lids of the DWP were stacked on position IB. The cells were counted by the Vi-CELL™ XR (Beckman Coulter) and at sufficient cell number ($0.5x10^6$ cells/well ≙ $2.5x10^5$ cells/ml) followed production phase II (phase I: detachment processes and production of pellet cultures, until cell calculation; phase II: production of pellet cultures, from cell transfer into the DWP until incubation; see figure 12-4). Hereby, the cell solution was transferred into a deep well plate located on position 1 (greiner). Parallel the same volume of water was transferred to the counter weight (position 3D tilt rack-1). After the covering with lids (position 7 [P7]) the liquid handler moved the DWPs one by one to the Access 2 plate transport and the gripper transferred the plates into the Vspin™. The centrifugation of the deep well plate was performed at 2,250 rpm for 5 minutes. After positioning the DWPs on the liquid handler deck and detaching the lids, the supernatant had to be carefully removed by a specific pipetting template. The steel cannula of channel 6 removed the media at 3.1 mm from the bottom at 45° from the well center with a speed of 50 µl/s. Then, the pellet was resuspended with temperate medium plus growth factors (200 µl/well). The cell solution (200 µl/well) was transferred into a 96 well microtiter plate using the liquid handler (Beckman Coulter). The well plate was moved to the Cytomat (37 °C, 5 % CO_2; Thermo Fisher Scientific) by the lift. The pellet formation was promoted by a shaking period of half an hour by a thermal mixer (Eppendorf) at 37 °C [54].
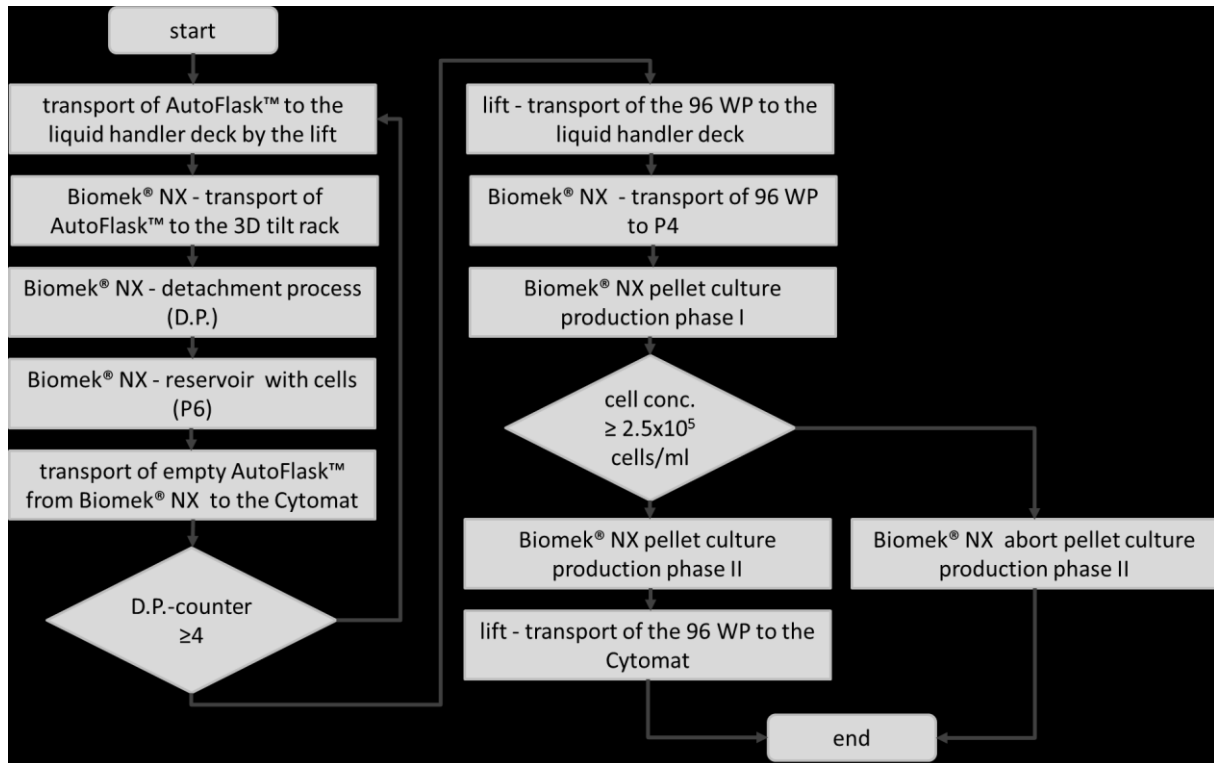
Figure 4-23 Flow sheet of the automatic manufacturing of pellet cultures

4.2 High Throughput Screening System

The quality and usability of 3D cultures for further investigations have to be evaluated. The quantifications of biological processes can be evaluated by bioscreenings. These screenings can be used for the detection of multiplying, cell death and metabolic products. The combination of bioscreenings with compound testing is also a very important aspect. Furthermore, it must be investigated which bioscreening is useable for the specific 3D cell culture.

4.2.1 Architecture of the High Throughput Screening System

The High Throughput Screening system involves a variety of components for a wide range of bioscreenings (see figure 4-24; A, B). The control work station (computer) and the Biomek® liquid handler NX and FX are external of the housing. The Biomek® FX (Beckman Coulter) is equipped with a 96-well head next to the gripper, which are integrated at one rail. The liquid handler deck of the Biomek® FX (Beckman Coulter) includes 15 passive placeholder ALPs (Beckman Coulter), a loading station for disposables, a wash station (Beckman Coulter) and a refill reservoir (Beckman Coulter) for continual replenishment of solutions. The Biomek® NX (Beckman Coulter) liquid handler includes a Span 8 pipetting head for disposable tips and gripper at one rail. The liquid handler deck is equipped with 5 placeholder ALPs (Beckman Coulter), and 3 VARIOMAG® Teleshake ALPs (Thermo Scientific), which enable flexible shaking motions up to 2,000 rpm for microplate formatted labware. Furthermore, a trash for disposables and a wash station (Beckman Coulter) complete the Biomek® NX liquid handler deck. The rail-mounted robot Motoman (Yaskawa, Fukuoka, Japan) is associated

with the liquid handlers about shuttles, connects the whole system and transports the well plates as system integrator. Included in the housing are 3 readers (PHERAstar Plus, a FLUOstar galaxy reader, NOVOstar microplate reader; BMG Labtech, Orthenberg, Germany) for the detection of absorbance, luminescence and fluorescence. Different assays and readouts are possible by the flexible filter modules of the readers. These devices enable the readout of 6- to 1,536-well plates. Two types of a Cytomat are integrated. The Cytomat Hotel (Kendro, Langenselbold, Germany) stores boxes filled with pipette tips and well plates. The second Cytomat (37 °C and 5 % CO_2, Thermo Fisher Scientific) incubator encamps well plate with cells or plates for incubations periods. There are nine hotels with 21 storage positions included, which are positioned on a turntable. The integrated handler enables X/Y/Z movements for an automatic transfer of plates through a heated access door. The barcode reader and printer (Beckman Coulter) is elementary for well plate identifications in high throughput bioscreenings. The plate sealer and peeler (HJ-Bioanalytik GmbH, Moenchengladbach, Germany) are able to cover and recover plates with foil during incubation periods. Lids are handled at the lid station. A plate washer is also associated in the system. A centrifuge (Sigma Aldrich) allows the separation of cells and solutions. This can be loaded with 4 well plates per time for centrifugation steps up to 15,000 rpm. The temperature, spindle speed and time are adjustable.

The assays and devices are controllable as well as manageable by the Biomek® Software and the SAMI® Software [73, 135].

Figure 4-24 Overview: High Throughput Screening System (A) with integrated components (B) [135]

The different devices of the High Throughput Sheening: Biomek® NX (1) associated with a shuttle (3), Biomek® FX (2) associated with a shuttle (4), Cytomat Hotel (5), Cytomat incubator (6), Pealer (8), Sealer (12), Barcode printer and reader (9), PHERAstar Plus (14), FLUOstar galaxy reader (7), NOVOstar microplate reader (10), lid station (11) centrifuge (13), computer (15), Motoman (16) and washer (17).

4.2.2 Translation of Manual Processes to Automatic Bioscreening

4.2.2.1 Processes in Automatic Bioscreening

The cells and 3D constructs were evaluated for quantification of biological processes (see figure 4-25). These processes contained proliferation and cytotoxicity, which cover the basic quantification assays for investigating cell behavior. Determination of cell proliferation was evaluated by different assays: tetrazolium salt dependent assays (EZ4U, WST-1) and DNA (deoxyribonucleic acid) quantification. Furthermore, EZ4U assay enabled the detection of the living cells after compound treatment. The cytotoxicity was quantified by the adenylate kinases (AK) assay. The goals were the integration of new assays at the High Throughput Screening System and the investigation of the most suitable assays for the different cell types as well as 3D constructs. These assays should represent the basis for further investigations e.g. compound screening.
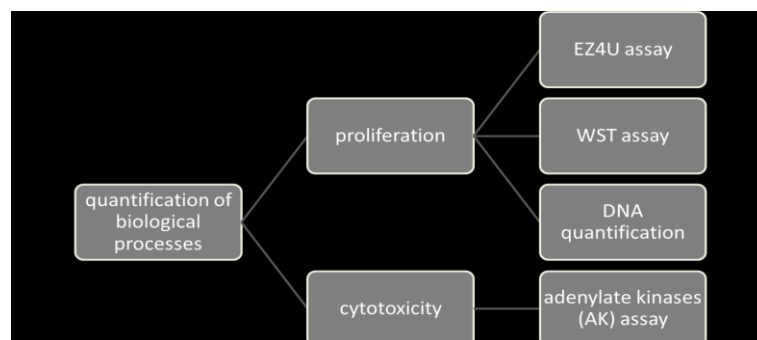
Figure 4-25 Quantified biological processes with specific screening forms

The exact location of the labware and solutions within reservoirs is necessary for automatic processes. Therefore, the liquid handler deck positions of the Biomek® FX and Biomek® NX at the High throughput screening system are visualized in figure 4-26 (A, B).

The Biomek® FX liquid handler deck is equipped with 16 placeholder ALPs (P1- P16). The shuttle position enabled the transfer of the labware to the specific positions on the deck. Next to the shuttle position a wash station and a refill reservoir are located (Top1). On the other side of the shuttle station a loading station (TL1) for disposables is positioned.

The Biomek® NX is equipped with six placeholder ALPs (P1-P3, P5-P8), three shakers (S1, S2, S3). The wash station (W1) is located next to the waste (TR1) for disposables. In the front, the shuttle position (C1) is placed.
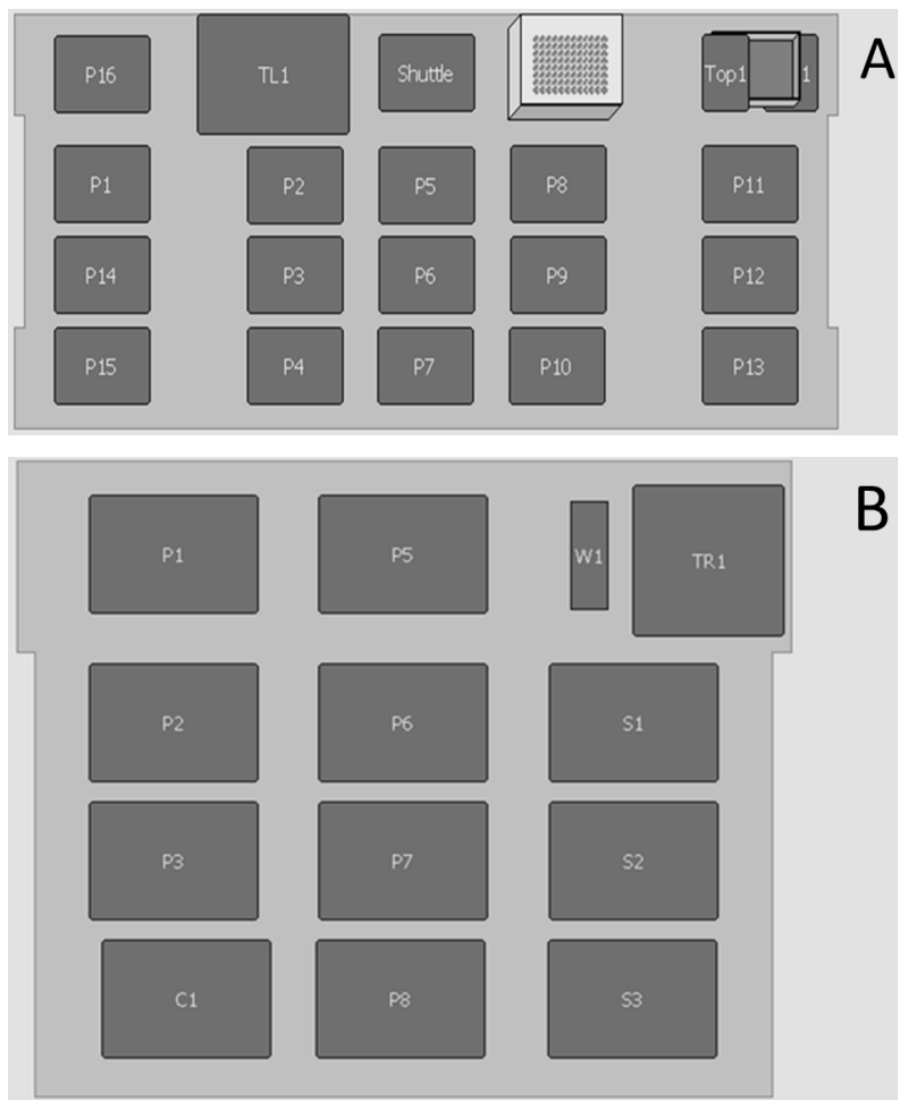
Figure 4-26 Biomek® software: different positions of the Biomek® FX (A) and Biomek NX (B) liquid handler decks at the High Throughput Screening System.

WST-1 Assay

The adapted assay to investigate the proliferation of suspension cell lines is the WST-1 assay [110]. This assay (Roche, Berlin, Germany) bases on the conversation of tetrazolium salt to colored formazan and consequently detection of the absorbance.

The WST-1 assay was newly adapted at the High Throughput Screening System of celisca.

The programmed SAMI-method is visualized in figure 4-27. The bioscreening, using the WST-1 assay kit, includes one liquid handler step at the Biomek® FX, whereby the reagent was transferred into the well plate with cells. The required labware are a low volume reservoir, a tip box as well as the well plate with cells and lid. The shaking period and moving of the lid is performed at the Biomek® NX. The read out is realized with the PHERAstar. The method ends with moving the labware to their home positions.

The process steps are described in detail in the following section.
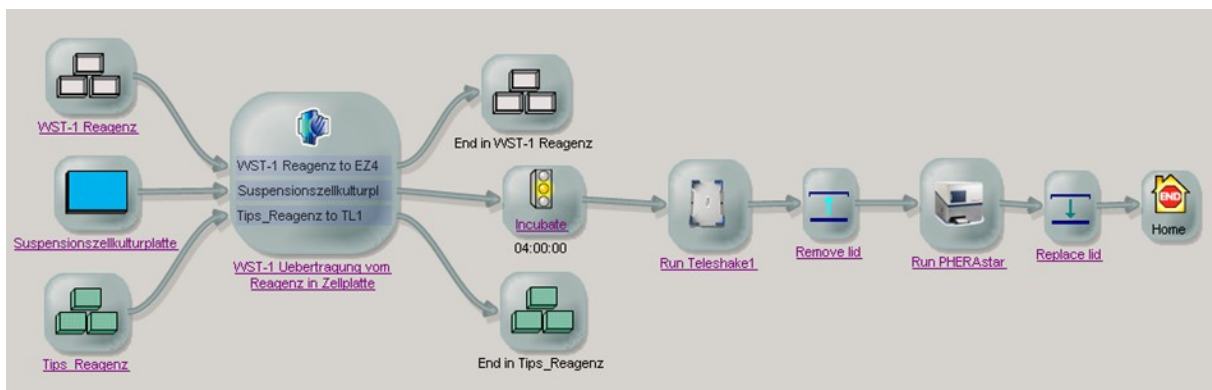
Figure 4-27 SAMI® software: programed WST-1 assay

The main process steps for the manual and automatic bioscreening by the WST-1 assay are similar:

[1] adding of reagent (15 µl)
[2] incubation period in the Cytomat (37 °C, 5 % CO_2)
[3] detection of the absorbance using the PHERAstar

The direct translation of the manually processes steps into the automatic process steps was mainly possible. Dissimilar were the storage and liquid transfer of the reagent. The automatic liquid transfer was performed with the Biomek® FX. This device enabled the transfer of 96 wells per time by the 96 well pipetting head. The reagent had to be stored in a low volume reservoir. However, the reagent at the manual process is stored into a reservoir and transferred with an 8 channel Eppendorf pipette row by row.

The following flow sheet visualizes the automatic WST-1 assay (see figure 4-28). The cells within a 96 well sheet are located in the Cytomat (37 °C, 5 % CO_2; hotel 1, position 1). The reagent (2.5 ml) is stored in a low volume reservoir on position 2 (P2) at the Biomek® FX liquid handler deck. The tip box is located at the Cytomat hotel (hotel 8, position 1). The Motoman moved the tip box to the Biomek® FX, which positioned the box on the loading station. Then, the Motoman conveyed the well plate to the shuttle of the Biomek® FX liquid handler. The integrated gripper positioned the sheet on position

3 (P3) and putted the lid to position 4 (P4). The reagent was transferred into the well plate (15 µl). The well plate with lid was moved to the Cytomat (37 °C, 5 % CO_2) by the Motoman and incubated for 4 hours. Subsequently, the plate was moved to the Biomek® NX (S1) for a shaking period of 1 minute as well as 100 rpm and removing the lid to position 2 (P2). The Motoman transported the plate to the PHERAstar for read out of the optical density (OD) at 450 nm (reference wave length 630 nm). Afterwards, the Motoman moved the plate to its home position (Cytomat) after moving of the lid on the well plate at the Biomek® NX.
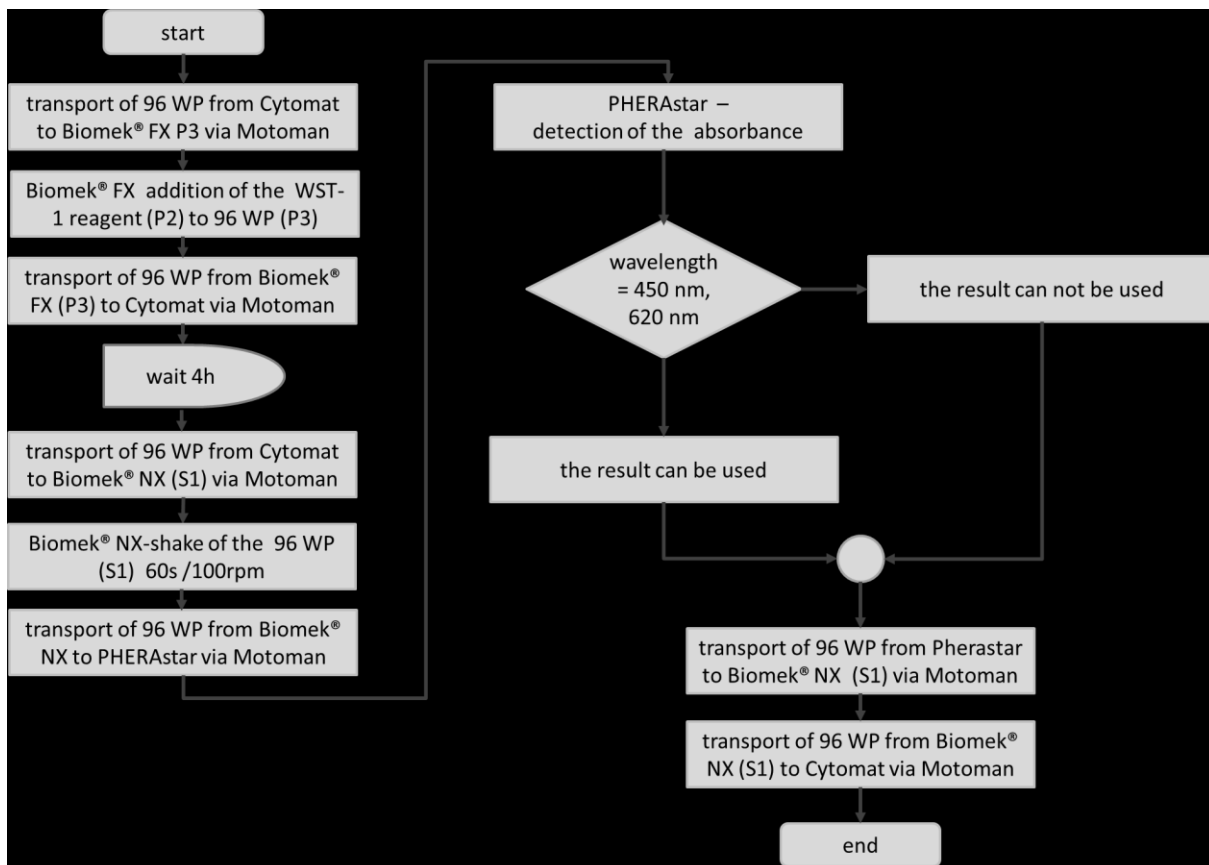
Figure 4-28 Flow sheet: automatic WST-1 assay

EZ4U assay and Adenylate Kinases (AK) Assay

The bioscreening of the proliferation (EZ4U assay) and cytotoxicity (adenylate kinases assay) was realized using the High Throughput Screening System (celisca). The bioscreening processes were combined in one method. This is justified by the fact that the adenylate kinases is detectable in the supernatant and the EZ4U reagent is converted by living cells in the 3D constructs.

The detection of the cell proliferation within 3D constructs was realized with the EZ4U assay kit. This is a non-radioactive proliferation and toxicity assay based on the reduction of tetrazolium salt to colored formazan in living cells. The absorbance is detectable after a specific incubation period. The EZ4U assay has been adapted and automated for the investigation of proliferation of adherent cell lines. Nevertheless, it has never been applied to bioscreening processes of 3D constructs.

Adenylate kinase is released of death cell in consequence of membrane disruption resulting in a protein detection in the supernatant. The interruption of the cell membrane is a result of cell death

and cytotoxicity. In cause of this adenylate kinase is fast released in the media. The adenylate kinase is a ubiquitous protein located in all eukaryotic and prokaryotic cells. The bioluminescence detection of adenylate kinase is measured in one step that is separated in two chemical reactions. Firstly, adenosine diphosphate (ADP) is converted into adenosine triphosphate (ATP) catalyzed by adenylate kinase. Secondly, light is released due to the reaction of ATP and luciferin. The investigation of the cytotoxicity by the adenylate kinases assay was newly integrated in the High Throughput Screening System of celisca.

The programmed SAMI-method is visualized in figure 4-29. The automatic program contains seven different process – and liquid handler steps. The adenylate kinases assay is performed using the steps 1, 3 and 4. The EZ4U assay is completed using the steps 2, 5, 6 and 7. Firstly, a specitic volume of supernatant was transferred into a white 96 well plate whereas the rest of the supernatant was discarded into the waste (step 1.). Secondly, media was pipetted to the 3D cultures (step 2.). Then supernatant was diluted with the reagent (step 3.). After an incubation period, luminescence was detected by a reader device (step 4.). Fourthly, the EZ4U-reagent was transferred into the plates with 3D cultures (step 5.). After the incubation period, the supernatant was transferred into a new plate (step 6.) and absorbance was detected (step 7.).

The needed labware are the 96 well plate with 3D cultures, screening plates for the assays (EZ4U assay: clear, flat button; AK assay: white, round button), different reservoirs (AK reagent, EZ4U reagent, waste and medium) and tip boxes for the supernatant, reagent as well as medium. The absorbance and the luminescence were detected with the PHERAstar by different optic modules. The different process steps are described in detail in the following section.
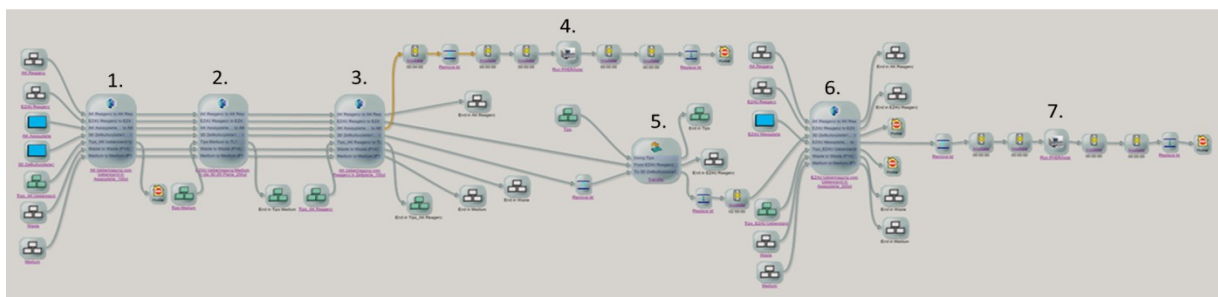
Figure 4-29 SAMI® software: programmed combination of the EZ4U assay and AK-Assay

The general process steps for the manual and automatic bioscreening by the EZ4U assay and AK assay are analogous and conformed to the manufactures requirements.

The adenylate kinase (AK) assay is subdivided in following single steps:

[1] transfer supernatant into a white screening plate (100 µl)
[2] adding of reagent (100 µl)
[3] incubation period at room temperature
[4] detection of the luminescence using the PHERAstar

The EZ4U assay is sectioned in subsequent single steps:

[1] addition of fresh media to the 3D constructs (250 µl)
[2] adding of reagent (25 µl)
[3] incubation period in the Cytomat (37 °C, 5 % CO_2)
[4] detection of the absorbance using the PHERAstar

The straight translation of the manual method into automatically performed processes was mainly possible. Next to the storage of the solution, the liquid handling was the main difference. The liquid transfer in the manual methods was enabled using Eppendorf pipettes row by row. Firstly, the liquid transfer was enabled by the Biomek® FX liquid handler, which enabled the solution transfer for 96 wells per time. Subsequently, the pipetting templates and liquid handling steps have to be optimized for 3D constructs to guarantee the integrity of the 3D cell cultures. The solutions were aspirated at 330° from the well center at 0.1 – 4 mm from bottom with a speed of 80 % (100 µl/s). Thus, the solutions were right-sided aspirated. The solutions were dispensed at the well center at 2 mm from bottom with a speed of 80v% (100 µl/s).

The following flow sheet shows the automatic EZ4U assay in combination with the AK assay (see figure 4-30). The Cytomat (37 °C, 5 % CO_2) stored the different plates: 3D cell cultures (hotel 1, P1- one family), AK assay plate (hotel 2, P1- one family) and the EZ4U screening plate (hotel 3, P1 – one family). The solutions were manually positioned at the Biomek FX liquid handler deck: medium (P15), waste (P14), EZ4U reagent (P2) and AK reagent (P1). The bioscreenings started with the transfer of the well plates containing the 3D cultures and the AK assay plate as well as tip box (200 µl) to the shuttle of Biomek® FX one by one. The gripper positioned the plates on the Biomek® FX liquid handler deck (3D cell culture plate: P6; AK assay plate: P3) and removed the lids (3D cell culture plate: P7; AK assay plate: P4). The conditioned medium of the 3D cultures was aspirated (330° from well center, 0.1 mm from bottom at 80 % speed) using the loaded tips to transfer 100 µl into the waste and 100 µl in the AK assay plate. The tip box was transported to the home position and a new box was moved to the Biomek® FX to dispense (at the well center, 2 mm from bottom at a speed of 80 %) fresh media (250 µl) into the 3D cell culture plate for preventing dehydration. The AK assay was continued by the addition of the reagent (100 µl) to the AK screening plate by fresh tips. The incubation period was performed on the liquid handler deck at room temperature for 4 minutes 10 seconds covered with a lid. Then, the gripper removed the lid and the Motoman transferred the plate from the shuttle to the Biomek® NX. The re-grip of the plate was required for the right location of the plate. After the detection of the luminescence by the PHERAstar and the re-grip at the Biomek® NX by the Motoman, the plate was transferred to the Biomek® FX to cover the plate with the lid. Lastly, the Motoman moved the plate to the Cytomat. The EZ4U assay was continued with the 3D assay plate after the addition of fresh media (250 µl). Subsequently, new tips were loaded and transferred (at the well center, 2 mm from bottom at a speed of 80 %) the EZ4U reagent (25 µl/well) into the 3D cell culture plate. The plate was covered with the lid and moved to the Cytomat (37 °C, 5 % CO_2) by the Motoman. After the incubation period of 3 hours, the plates (3D cell culture plate, EZ4U assay plate) were transferred to the Biomek® FX by the Motoman. The gripper located the plate from the shuttle to its specific positions (3D cell culture plate: P6, EZ4U assay plate: P9) and removed the lids (3D cell culture plate: P7, EZ4U assay plate: P10). The 96 well pipetting head loaded fresh tips and aspirated (4 mm from bottom at a speed of 80 %) supernatant from the 3D cell culture plate and dispensed the liquid to the EZ4U screening plate (4 mm from bottom at a speed of 80 %). The re-grip at the Biomek® NX was performed using the Motoman. The proliferation was quantified by detection

the absorbance (OD 450 nm). The screening plate was transported to the Biomek® FX by the Motoman. The plates were covered with the lids and the whole labware was transported to their home positions.
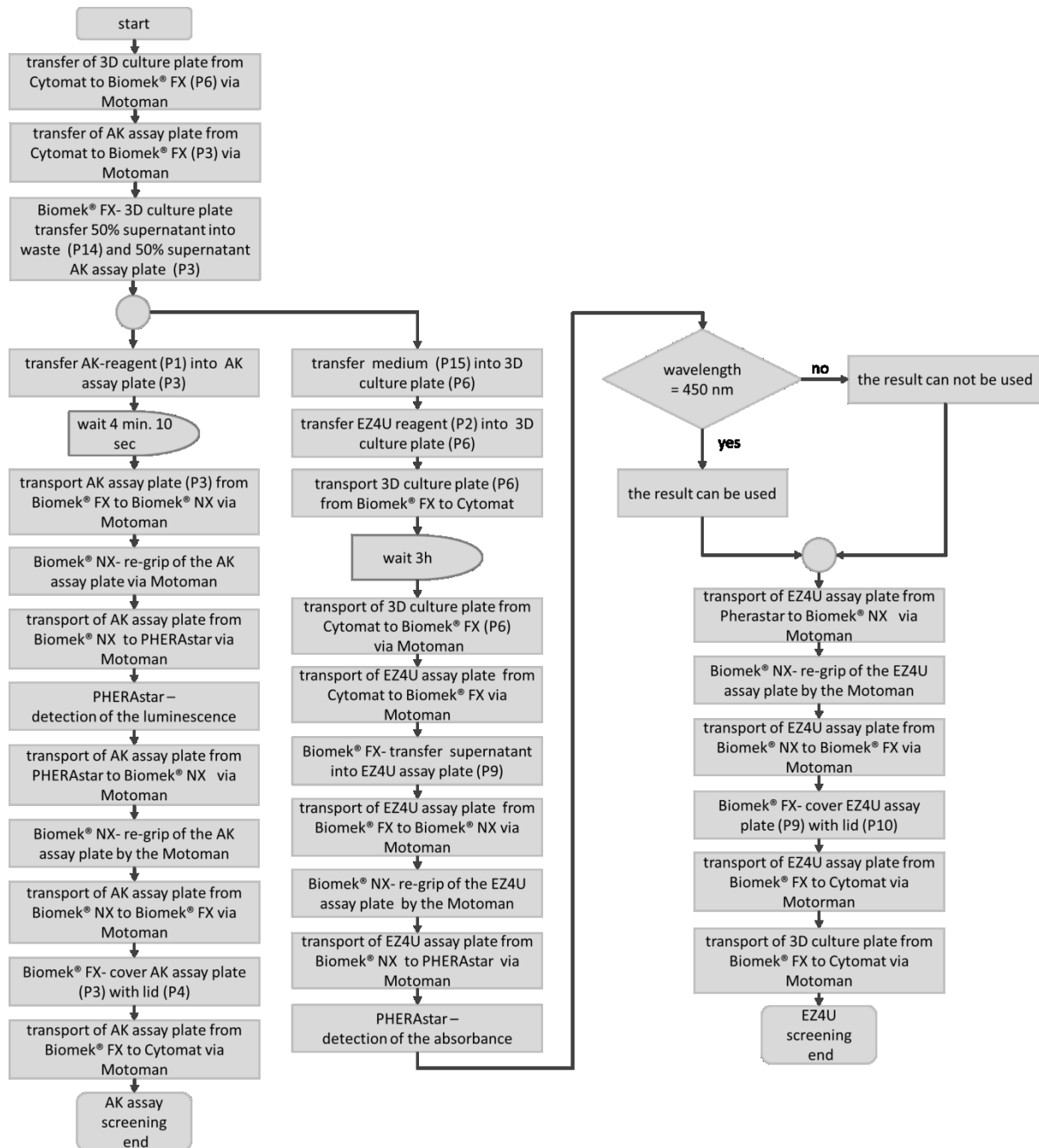
Figure 4-30 Flow sheet: AK assay and EZ4U assay

DNA Quantification

Quantification of DNA is important for biological research. This includes e.g. the quantification of DNA amplification products, the detection of DNA fragments in drug discovery and the purification of DNA for subcloning or library production with synthesized cDNA. The Quant-iT™ PicoGreen® reagent

is an ultrasensitive fluorescent nucleic acid stain. This reagent is used for quantification of double strand (ds) DNA in liquids with amounts from 25 pg/ml to 1,000 ng/ml.

The DNA quantification by the Quant-iT™ PicoGreen® reagent kit (Life technologies, Regensburg, Germany) was a new adapted proliferation screening by the High Throughput Screening System at celisca.

The programmed SAMI-method is visualized in figure 4-31. The automatic program has one liquid handling step by the Biomek® FX. The investigation of ds DNA is carried out with the reader device (PHERAstar) using fluorescence detection. A standard curve allows the quantification. The required labware are the black 96 well plate with diluted DNA samples and standards, the tip box as well as the reagent within a low volume reservoir.
The different process steps are described in detail in the subsequent segment.
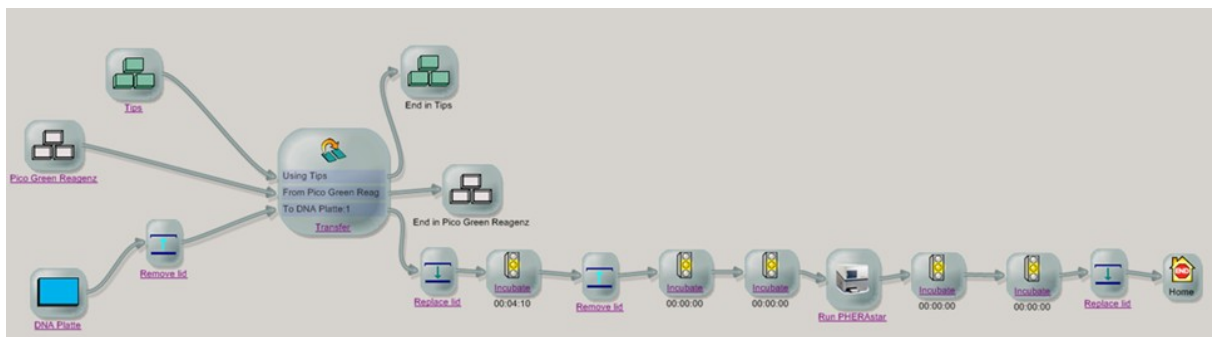
Figure 4-31 SAMI® software: programed DNA quantification

The general process steps for the manual and automatic bioscreening by the PicoGreen® assay kit are similar and followed to the manufactures requirements.

The assay is sectioned in following single steps:

[1] adding of reagent (1:2 dilution) to the samples
[2] incubation period at room temperature (prevention of light)
[3] detection of the fluorescence using the PHERAstar

The exact translation of the manual procedure to the automatically completed procedures was only basically possible. Regarding the DNA quantification, the volumes had to be reduced for handling with 96 well plates. The manufactures requirements appear the manually DNA quantification within disposable cuvettes or test tubes at a total volume of 2 ml. Moreover, for the automatic bioscreening by fluorescence detection black screening plates are needed. The incubation period for the PicoGreen® assay have to be performed without light because of the loss of reagent stability. The well plate was covered with aluminum foil at the manually procedure. The solution for the automatic process was a brown lid covered with self-adhesive aluminum foil to protect the reaction from light.

The following flow sheet shows the automatic DNA quantification (see figure 4-32). The DNA samples (100 µl/well) within the black 96 well plate and lid are located in the Cytomat (37 °C, 5 % CO_2; hotel

1, P1). The PicoGreen® reagent (12 ml) is manually positioned in a low volume reservoir on P2. The Motoman transported the tip box in the Cytomat hotel (hotel 8, P4) to the Biomek® FX and the gripper positioned the box at the loading station. Then, the sample plate was transferred and positioned on the Biomek liquid handler deck (P6). The lid was located on position 7 (P7) by the gripper. The 96 well pipetting head loaded the tips and dispensed the reagent (100 µl/well) to the samples by a liquid handling step (well center, 2 mm from bottom at a speed of 80 %). The gripper covered the plate with the lid for an incubation period of 4 minutes 10 seconds prevented from light. At the same time, the tip box was moved to their home position. The Motoman transported the screening plate to the Biomek® NX for the re-grip process after removing the lid (P7) and to the reader device (PHERAstar). The detection of dsDNA was carried out by the PHERAstar and fluorescence extinction (480nm) and emission (520 nm) wavelengths. After a further re-grip process, the Motoman transferred the plate to the Biomek® FX where the plate was covered with the lid. Finally, the sample plate was moved to the Cytomat.
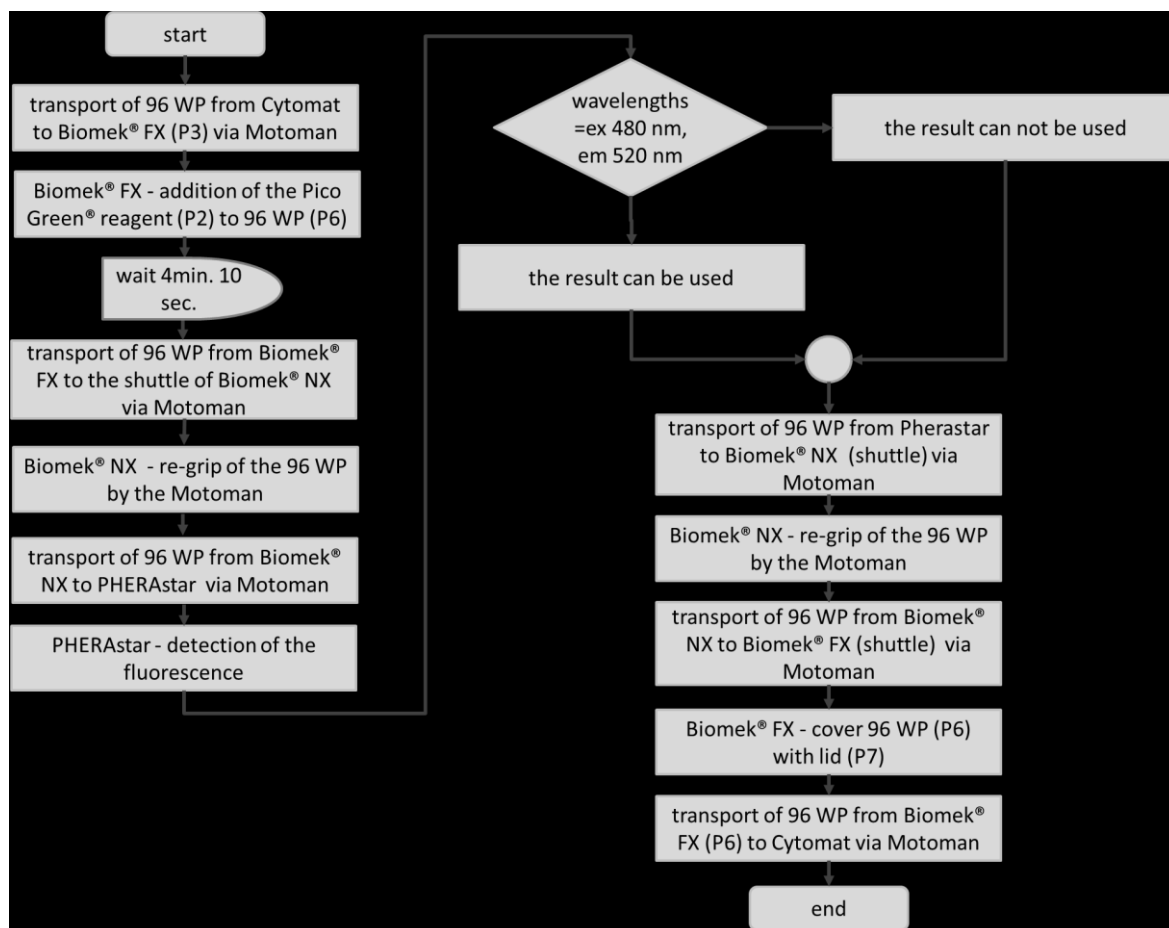
Figure 4-32 Flow sheet: DNA quantification

5   Results

Initially, the Biomek® Cell Workstation was used for the automatic cultivation of 2D cell cultures. These are adherent cell lines and suspension cell lines, which require different cell culture processes and bioscreenings (proliferation). The adherent cells are the basis for the manufacturing of 3D cell constructs. The quality of automatically produced 3D constructs was quantified with different

bioscreening processes (toxicity, proliferation) and compared with the manually manufactured 3D cultures.

The cells cultivated in media with and without antibiotic were investigated to exclude possible influences on cell viability. Generally, antibiotics are a common supplement of cell culture media to suppress contaminations by microorganism [118]. Nonetheless, there are a lot of disadvantages of using antibiotics. These are cryptogenic contaminations, development of resistance and reduction of aseptic working [119, 120]. The most common antibiotics are a combination of penicillin and streptomycin. The β-lactam penicillin disrupts cell wall synthesis of gram-positive bacteria [121]. Streptomycin belongs to the group of aminoglycoside and influences gram-negative bacteria. Regarding this increase, the intracellular inoperative proteins are impaired by the translation of undamaged mRNA-tRNA complexes [122]. Moreover, protein expression is influenced by regular penicillin and streptomycin concentrations. This might influence the cytoskeleton, chaperone and protein utilization [135].

The disinfection and cleaning steps showed a successful automatic cell cultivation without antibiotic.

5.1 Statistical Analysis

The significances were calculated by the student's t-test. Differences were regarded as significant if $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). The used t-test evaluated, how the mean values of two populations are related to each other, using the means of two independent samples. The following formula visualized the evaluation of the t-test [136–138]:

$$t = \frac{x_1 - x_2}{\frac{x_1^2 - \frac{x_1^2}{n_1} + x_2^2 - \frac{x_2^2}{n_2}}{n_1 + n_2 - 2} \times \frac{1}{n_1} + \frac{1}{n_2}}$$

Legend:

x_1 = arithmetic average of sample 1

x_2 = arithmetic average of sample 2

n_1 = number of elements in sample 1

n_2 = number of elements in sample 2

x_1^2 = sum about squared elements of sample 1

x_2^2 = sum about squared elements of sample 2

$x_1{}^2$ = square of the sum about the elements of sample 1

$x_2{}^2$ = square of the sum about the elements of sample 2

5.2 2D cultures

5.2.1 Contamination-free Cultivation

Various forms of contamination are a basic problem of cell cultivation. Microorganisms as well as other cell lines can cause these contaminations. Main causes for the contaminations in manual cell

cultivation are e.g. unsterile working. The prevention of contaminations is especially important for automatic cell cultivation because of the tubing system, high number of batches and cell cultures. Different sterilization and decontamination processes are applied. The sterile working by the automatic cell culture system is ensured with the housing with HEPA (High Efficiency Particular Airfilter) Filter, UV-lights, flushing and decontaminations steps in cultivation processes.

5.2.1.1 Microorganism-free Cultivation

Bacteria, fungi and spores in the air, on surfaces and within cannulas or tubing systems can cause contamination of cell cultures. The tubing system can provide the growth of microorganism. Different disinfection processes are needed to support microorganism-free automatic cell cultivation. The Biomek® Cell Workstation supports sterile working by various methods; an overview is given in figure 5-1.
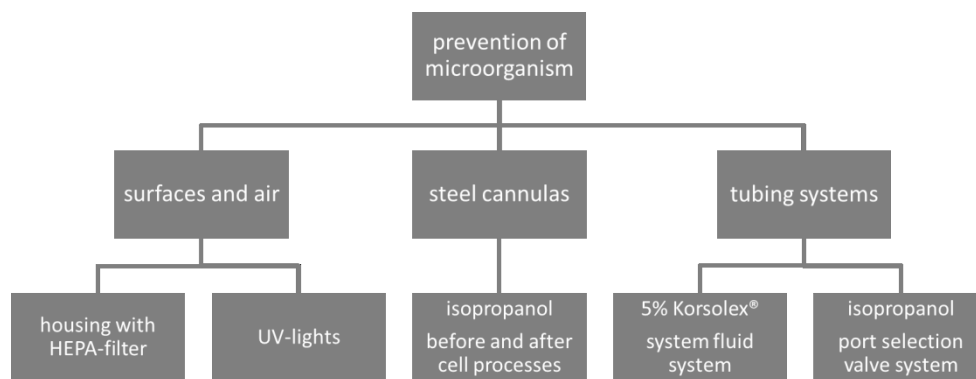
Figure 5-1 Provisions against microorganism of the Biomek® Cell Workstation

Basically, the Biomek® Cell Workstation is equipped with a housing combined with a HEPA Filter. For sterile working, the cell culture system is equipped with horizontal air flow. Hereby, air is guided inward through a high efficiency particular air filter. The filter is installed plane perpendicular to the user. Sterile conditions will be achieved through horizontal laminar displacement flow [12]. The filter works in a continuous mode.

The housing has to be opened to equip the liquid handler deck with sterile reservoirs and solutions. In this case, a larger exchange of air possibly containing microorganism could proceed. The UV-lights enable the decontamination by UV-radiation and consequently killing of the microorganisms in the air and on the liquid handler deck surface. The UV-lights have to be turned on 15 minutes before and after the daily cell culture processes.

The liquid transfer at the automatic cell culture system is realized using two tubing system. One system is associated the syringe pumps (channels 1-6, 8) and steel cannulas of the Biomek® NX. It enables the general liquid transfer of volumes up to 1 ml (channels 1-4) and 5 ml (channels 5, 6, 8). The system fluid is sterile water. The prevention of contamination by microorganism is realized by rinsing with 5 % Korsolex® solution. Due to the broad microbicide effect and its good compatibility with materials, Korsolex® is used in hospitals and practices. This tubing system is rinsed with 5 % Korsolex® once times the week or if the system is not used for longer than two days to prevent

contamination. Therefore, the 5 % Korsolex® solutions stays in the system. The following cell culture processes started with a long permanent purging period (10 minutes) of the tubing system with sterile water to wash away the disinfection solution.

The second tubing system is the port selection valve equipped with a dispensing pump and the channel 7 of the Biomek® NX liquid handler. This system enables the liquid transfer of six different solutions of higher volumes at a flow velocity of 200 µl/s. The solutions are stored in a cool box in sterile flask (mainly up to 500 ml) with air filter. The solutions are four different media, water and 70 % isopropanol. Before cell cultivation and media transfer, the used channel is rinsed with 26 ml 70 % isopropanol, 39 ml water and 13 ml medium. After the cell cultivation processes the channels have to be rinsed with 26 ml water and 26 ml 70 % isopropanol. Therefore, the flasks have to be manually changed. The 70 % isopropanol solution stays in the tubing system after finishing cell cultivation.

Cleaning and disinfections steps are required during the cell cultivation processes. Therefore, the cell cultivation methods started and ended with disinfections processes. The steel cannulas have to be disinfected for 15 minutes before and after daily cell culture processes and for 180 seconds before and after single cell cultivation methods with 70 % isopropanol. The disinfection of the steel cannulas was performed using aspirating 1 ml or 5 ml isopropanol (P6) followed by an incubation period (180 sec., 15 min.) and dispensing of 1 ml or 5 ml isopropanol. Consequently, the steel cannulas are rinsed with system fluid.

5.2.1.2 Cross-contamination free Cultivation

Another form of contamination is cross contamination. This represents the insert of foreign eukaryotic cell in an adapted cell linage. The result can be an overgrowing of the faster multiplying foreign cell line. The main risk is that such contamination could be remained undiscovered resulting in distort research results over a long time [139]. The increased use of human cell lines in biomedical research increases the risk of interspecies contamination. This contamination can be evaluated by DNA fingerprinting and short tandem repeat profiling [140].

The automatic cell culture system uses permanent steel cannulas. Different cell lines are cultivated with different channels at parallel cultivation to exclude cross contaminations. Channel 7 is used for every cell line to dispense the media. In the experiment with two different marked cells, one serious possible contamination danger was evaluated by using channel 7 as a medium donor for both probes. The channel 7 was programmed to dispense 4 ml medium in the waste before pipetting new medium for the other cell line and to wash the needle passively. To determine possible contamination, cervix carcinoma cells were dyed with PKH-26 and PKH-67. These kits are used for general in vitro membrane labeling of cells. Further, these stainings are for long term cell tracking and proliferation investigations [134, 141]. The cross-contamination was evaluated by two test series. Firstly, the different labeled cells were parallel automatically cultivated and microscopically analyzed the next day. Secondly, the cross-contamination was quantified using the fluorescence activated cell sorting (FACS) analysis after automatic cell cultivation. The cell cultivation with PKH-26 labeled cells followed the cultivation with PKH-67 dyed cells. Thus, possible cross contaminations (PKH-26 labeled) have to be detectable in the second cultivated cells line (PKH-67 dying).

Cells were parallel cultivated with regular automatic cell culture steps. First series of investigation cells carried out with the microscope 24 hours after cultivation to evaluate cross contaminations between PKH-26 and PKH-67 dyed cells at a scheme for classification separated in five different areas

(1x1 cm) in the cell culture flask. No cross contamination was proven at the first series of investigation (n=7) by microscopy (see figure 5-2) [134].
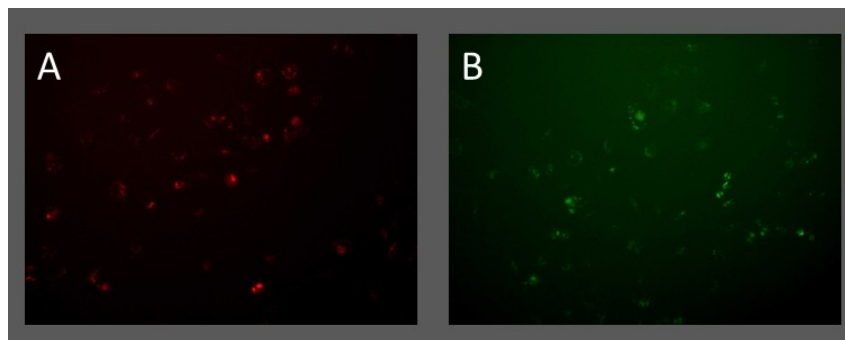
Figure 5-2 PKH-26 (A) and PKH-67 (B) stained cells after parallel cultivation

Second series of investigation cells were again parallel cultivated by the automatic cell cultivation system (n=6). For the detection of acute cross contamination, cells were analyzed on a laser FACSCalibur™ (Becton Dickinson) the next day. The FACS-analysis confirmed that 80 % dyed cells are labelled with PKH-67. Hereof, no sample showed cross contamination. The unmarked cells amounted 19.2 %. The mean of the cross-contamination was amounted to 0.015 %. A contamination is given at an amount of more than of 0.1 % (see table 5-1) [134].

Table 5-1 Overview of FACS results [134]

Sample	Contamination (%)	Unmarked (%)	PKH-67 (%)
1	0.00	17.49	82.28
2	0.02	22.21	77.48
3	0.01	18.18	81.48
4	0.00	24.14	75.53
5	0.05	19.16	80.44
6	0.01	17.24	82.47
Mean	**0.02**	**19.24**	**79.95**
Standard deviation	0.02	2.81	2.83

5.2.1.3 Summary

Microorganism or another cell lines can cause the contaminations of cells lines.

The basically equipped and new adapted disinfection processes guarantee the sterile and cross-contamination free automatic cell cultivation. The prevention against microorganism is a combination of housing with HEPA filter and UV-disinfection as well as decontamination methods of the tubing systems using Korsolex® and isopropanol.

The following table summarizes the processes and methods to prevent contaminations with microorganism.

Table 5-2 Overview about processes to prevent contamination with microorganism

Processes	Explanations
Housing with HEPA filter	• basic equipment • promotes microorganism - free cell cultivation • duration: continuous mode • supports sterile working by horizontal laminar displacement flow
UV-lights	• new integrated • promote microorganism - free cell cultivation • duration: 15 min before and after cell processes • decontamination of the air and liquid handler deck surface to kill microorganism by UV-radiation
Rinse tubing system of system fluid	• new adapted as a regular process • promote microorganism - free cell cultivation • tubing system associated with the syringe pumps and steel cannulas of the Biomek® NX (channel 1-6, 8) • disinfection with 5 % Korsolex® solution (once times the week or not used of the system for longer than two days); stay within the system • rinsed by 10 minutes permanent purging process with sterile water
Rinse tubing system of the port selection valve	• new integrated • promote microorganism - free cell cultivation • tubing system associated with the dispensing pump and steel cannula of the Biomek® NX (channel 7) • disinfection with isopropanol (when using the liquid transfer by port selection valve); stay within the system • before cell culture process: rinsing with water (39 ml) and media (13 ml) • after cell culture process: rinsing with water (26 ml) and isopropanol (26 ml)
Continued on next page	

Processes	Explanations

Rinse steel cannulas at the cell culture processes	• new integrated
	• promote microorganism - free cell cultivation
	• disinfection of steel cannulas of the Biomek® NX
	• disinfection with isopropanol by aspirating and dispensing 1 ml or 5 ml:
	   • for 180 seconds (before and after single cell culture processes)
	   • for 15 minutes (before and after daily cell cultivations)

Parallel cell cultivation processes and / or the contamination with another cell lines can cause possible cross-contamination. A system was created with a safe minimum of disinfection time and lower costs by avoidance of disposable tips and using stainless steel needles. It can be summarized that the field of cell automation is still in progress. Cross contamination leads to vulnerability in this section of technology although it has the highest potential in laboratory work. Therefore, it is important to further research the field of contaminations to detect error sources and solving those to ensure purity of the cell product [134]. A new method to exclude cross-contamination in automatic cell cultivation processes is the parallel cell cultivation of membrane labeled cells (PKH-26, PKH 67). There was no cross contamination detectable by microscopic investigations and quantification using FACS analysis. The decontamination processes and automatic parallel cell handling guarantee pure cell lines.

5.2.2 Screening Processes

Generally, screening processes are used to quantify cell behavior. The evaluation of cell proliferation is a basically bioscreening process to get statements about cell growth and effects of compounds.

5.2.2.1 Proliferation

Mainly, bioscreenings to quantify the cell proliferation bases on the reduction of tetrazolium salt to colored formazan by living cells. The proliferation can be quantified by different assays to investigate different cell forms (adherent cells, suspension cells) and 3D constructs. The 3D cell cultures cover different characteristics and applications. Regarding this, the optimal bioscreening has to be evaluated to visualize the cell growth.

EZ4U-Assay (adherent cells)

The EZ4U assay is an adapted and non-radioactive proliferation assay for adherent cell lines quantified using a High Throughput Screening System at celisca. The adherent HeLa cells were automatically and manually (n=6) detached as well as disseminated and examined in three different test groups (manual/sterile, automatic/sterile, manual/unsterile) to investigate the role and necessity of antibiotics in cell culture processes (see figure 5-3).

These groups were cultivated with and without antibiotics. The test groups automatic/sterile and manual/sterile showed a higher proliferation in media without antibiotics up to 6,000 cells/well. Automatic and sterile treated cells without antibiotics showed a higher proliferation rate compared to cells cultivated with antibiotic. The difference between the different media of automatic/sterile cells comprised 5 % at 1,000 cells/well up to 9 at 8,000 cells/well. Differences of proliferation rates for manually and sterilely cultivated cells were highest at 1,000 cells/well with 26 % and decreased to 6 % at 6,000 cells/well. The highest percentages and significant differences were detected in test group manual/unsterile. In this test group, cells, treated without antibiotic, showed a higher proliferation at all initial cell counts than cells with antibiotic. The major difference between media conditioned with and without antibiotics showed test group manual/unsterile. Variations of proliferation range from 39 % at 2,000 cells/well to 11 % at 8,000 cells/well. Altogether, the

proliferation of automatically/sterilely disseminated cells were slightly lower for all cell counts. In a comparison of media with antibiotic and media without antibiotics, the test groups handled under sterile conditions showed a higher proliferation without antibiotics. Contrary was the result of the manual and unsterile test group. Here, a higher proliferation was detected for all cell counts. The variability between the sterile and antibiotic-free test groups decreases from 26 % at 1,000 cells/well down to 1 % at 8,000 cells/well. In contrast, increasing differences were noticed from 4 % at 1,000 cells up to 20 % at 6,000 cells/well for cells with antibiotics [142].
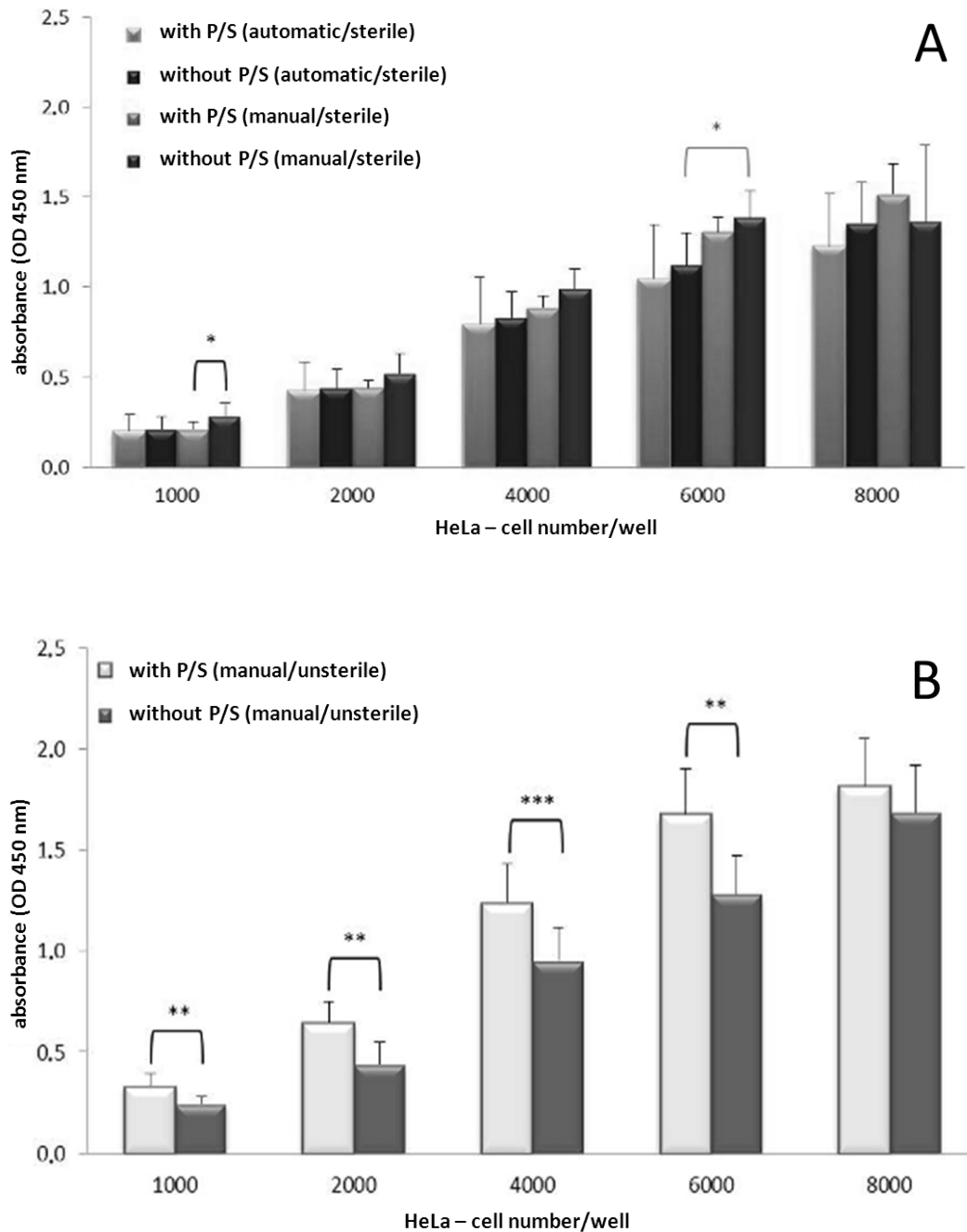
Figure 5-3 EZ4U assay of three test groups (A: automatic/sterile, manual/sterile; B: manual/unsterile)

The cells were cultivated in media with and without P/S with a cell count from 1000 cells/well to 8000 cells/well. Data ere presented as mean values + standard deviation (n=6; significances by T-test: *p<0.05; **p<0.01; ***p<0.001) [142].

Growth curve + EC$_{50}$ (adherent cells)

The standard cytotoxic agent for mama carcinoma is cyclophosphamide. The adherent cervix carcinoma cells were treated with cyclophosphamide in the range of 0.27 mmol/l to 35 mmol/l (see figure 5-4). The controls were used for non-cell death (medium) and 100 % cell death (dimethylsulfoxide [DMSO]). The growth curves of the three test groups (manual/sterile, automatic/sterile, manual/unsterile) cultivated in media with and without antibiotics were investigated resulting in a similar curve shapes of all groups (see figure 5-4; A, B; n=6).

Growth curves can also be used to evaluate the half-maximal effective dose (EC$_{50}$) of compounds [142].
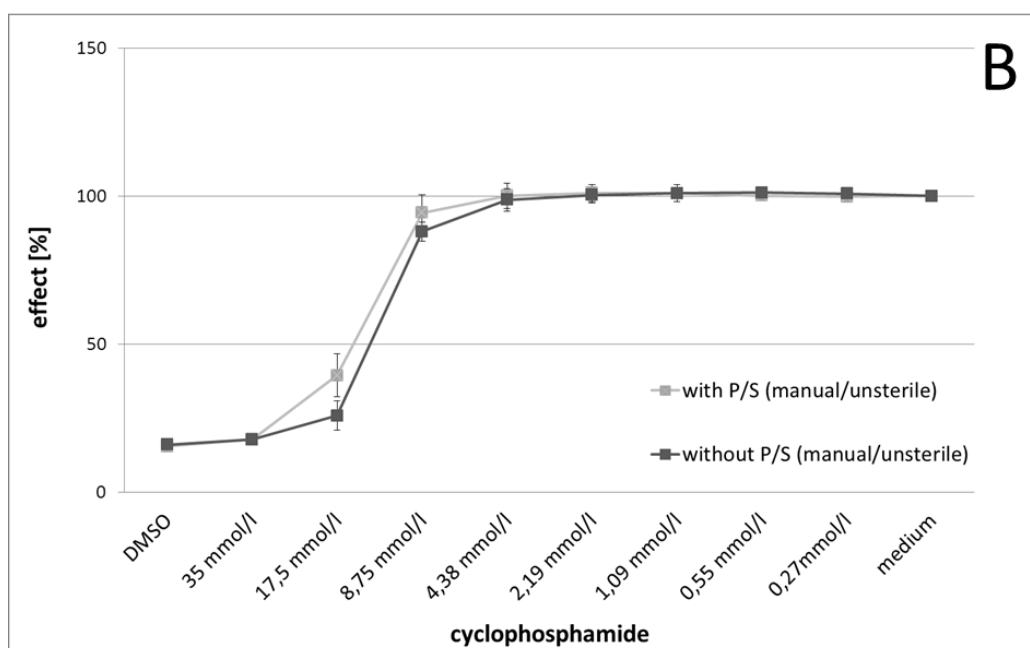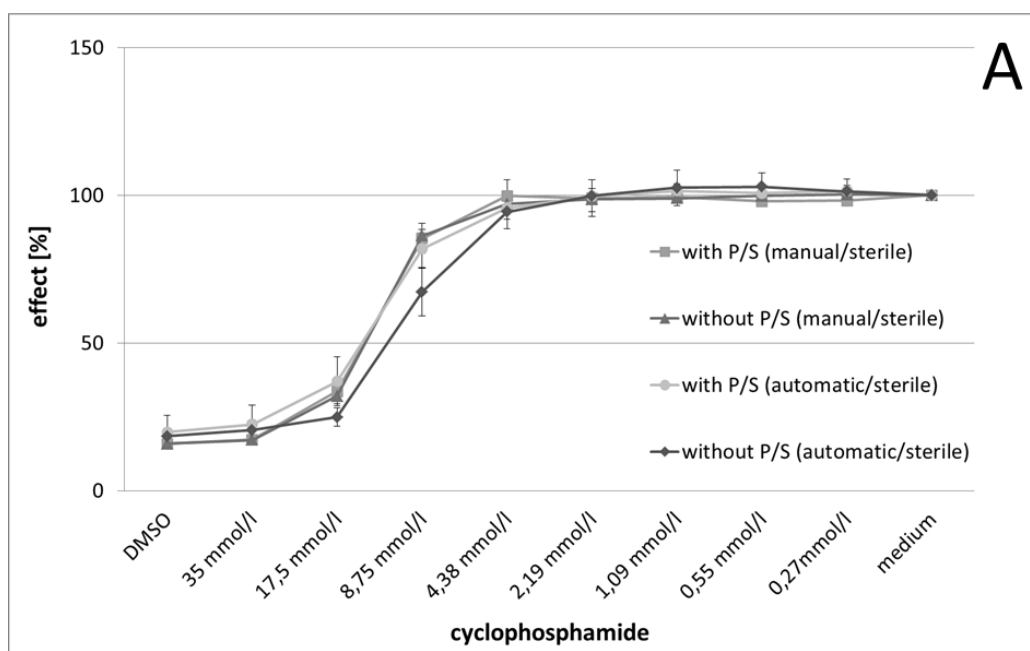
Figure 5-4 growth curves of three test groups (A: automatic/sterile, manual/sterile; B: manual/unsterile)

The cells were cultivated in media with and without P/S after treatment with cyclophosphamide. Data are presented as mean values + standard deviation (n= 6; no significances detectable) [142].

Generally, the EC_{50} values (n=6) of cells cultivated in media with P/S were higher than those of cells in media without P/S [142]. The half-maximal concentrations of cells cultivated in media without antibiotic were identified in the range of 12.31 - 14.59 mmol/l and of cells in media with antibiotic from 14.72 - 15.80 mmol/l (see table 5-3).

Table 5-3 Half maximal effective dose (EC_{50}) of cyclophosphamide at HeLa cells in 3 different test groups (n=6)

	EC_{50} concentrations (in mmol/l)	
Test groups	with P/S	without P/S
Manual/sterile	14.72	14.59
Automatic/sterile	14.97	12.31
Manual/unsterile	15.80	14.09

WST-Assay (suspension cells)

The Biomek® Cell Workstation allows the handling of suspension cells next to the cultivation of adherent cells. The cultivation and dissemination of four different suspensions cells (Jurkat, SEM, RS4, and Molt4) was performed by the Biomek® Cell Workstation to visualize the comparability of automatic processes with the manual methods.

The proliferation was evaluated by the WST-1 assay (Roche; see figure 5-5). This is a new adapted proliferation bioscreening based on the reduction of tetrazolium salt. The absorbance of manual handled suspension cells were compared with automatic processed cell lines.

The results visualized the highest proliferation rate of Jurkat cells, followed by SEM cells, Molt 4 cells and RS4 cells. The proliferation of automatically processed cells is non-significant decreased (Jurkat 13 %, SEM 12 %, RS4 17 %, and Molt4 16 %) compared to the manual treated cells. A non-significant decrease of the proliferation might be associated with stress to cells caused by sheer forces and consequently increased cell death and cell loss during the automatic transfer steps. The proliferation-standard error of the automatic method is mainly lower compared to the manual process (Jurkat 20 %, RS4 12 %, and Molt4 14 %). The SEM cells showed the same standard error (0.3 units) [110].
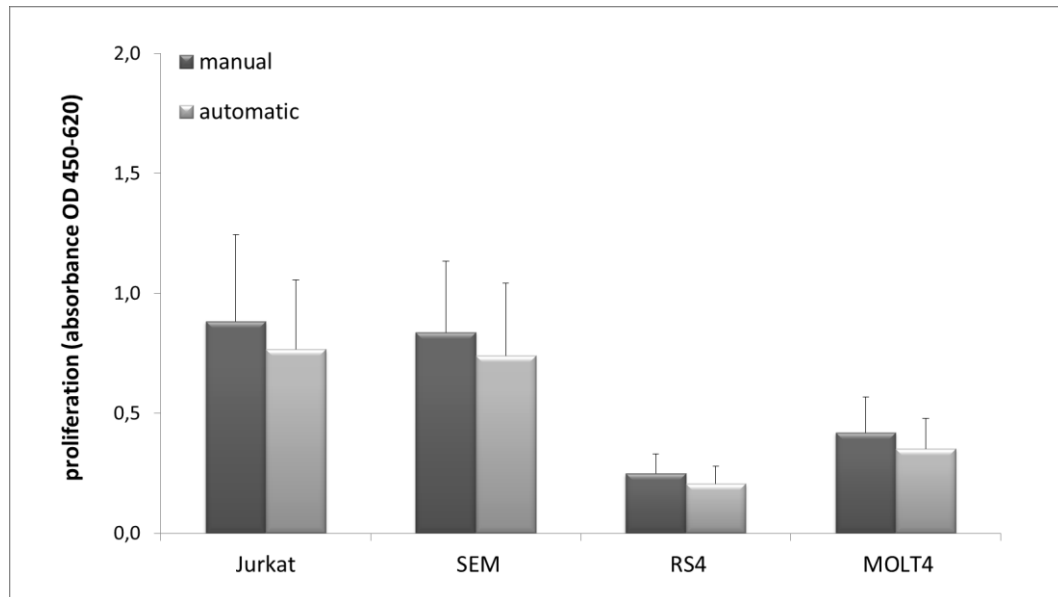
Figure 5-5 WST assay of suspension cell lines [142]

Proliferation of manually and automatically seeded suspension cell lines (Jurkat, SEM, RS4, MOLT4) evaluated by the WST assay. Data are presented as mean value ± standard error. No significances were observed (n= 6).

5.2.3 Summary

Initially, automatic cell cultivation processes were performed with the Biomek® Cell Workstation and compared with the standard manual methods. The proliferation was quantified by tetrazolium salt dependent assays.

The adherent cells with different cells counts (1,000 cells/well – 8,000 cells/well) were investigated in three different test groups (manual/sterile, automatic/sterile, manual/unsterile) cultivated in media with and without antibiotics. Afterwards, the proliferation rates of the groups were quantified by EZ4U assay. The increased cell count per well was associated with higher cell proliferation units. Mainly, the proliferation of sterile handled cells, cultivated in media without antibiotics, was increased. Instead, the cell proliferation of unsterile treated cells, cultivated in media without antibiotic, was almoet significantly decreased. The proliferations of automatically disseminated cells were mainly decreased compared to the manual handled cells. Growth curves were recorded to find EC_{50} values for cells treated with the chemosensitivity agent cyclophosphamide. The shape of the growth curves was the same of all test groups. Minimal higher concentration of cyclophosphamide is needed for cells cultivated in media with antibiotics to reduce the viability to 50 % of cervix carcinoma cells [142].

The cell cultivation and parallel dissemination of suspension cell lines (Jurkat, SEM, RS4, Molt4) as well as the proliferation bioscreening by WST-1 was new integrated. The results showed a non-significantly decreased proliferation of automatically treated suspension cells combined with a lower standard error of the automatic method [110].

5.3   3 D cultures

5.3.1   Microscopic Analysis

The formation process of manually and automatically manufactured 3D cultures were documented from week one until week five with an optical microscope (Leica, Wetzlar, Germany). The 3D constructs were cultivated in media with antibiotics (+P/S) and without antibiotics (-P/S).

5.3.1.1 Evaluation over the Time

Alginate Beads

The microscopic documentation visualized cell-containing alginate beads cultivated in media with antibiotics (+P/S; see figure 5-6, A) and media without antibiotics (-P/S; see figure 5-6, B; n=6). Alginate beads in both cultivation media showed the same results. In the first wees, embedded cells appeared isolated with a consistent cell distribution. In the second week, the growth of the cells is visualized by a formation of cell aggregates. From the third until the fifth week, the cell-aggregates developed and sizes of beads increased. The cells embedded in alginate are cultivated in a stable three dimensional form about five weeks [73].
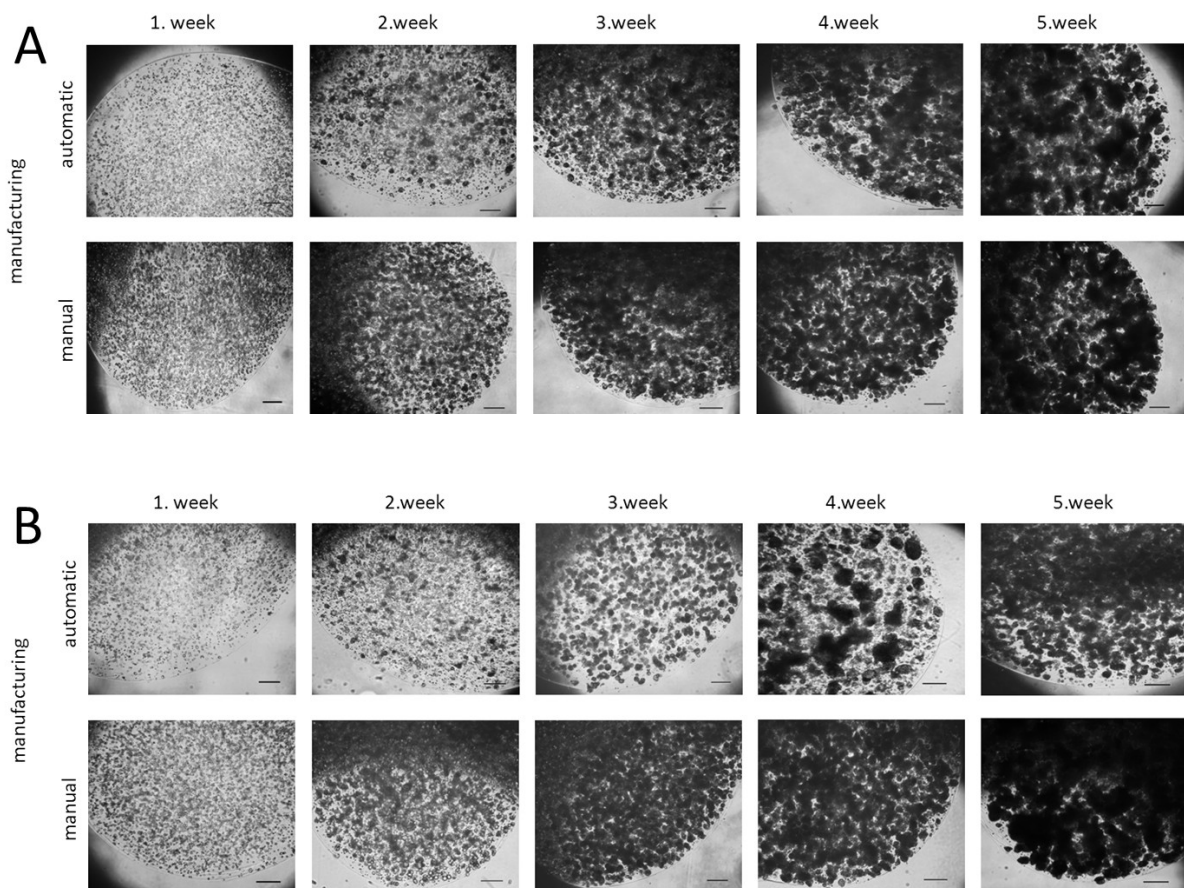
Figure 5-6 HeLa cells embedded in alginate matrix cultivated in media with (+P/S, A) [73] **and without antibiotics (-P/S, B)**

Development of automatically and manually manufactured HeLa cells encapsulated in three dimensional alginate matrix in media with penicillin/streptomycin about five weeks of cultivation (n=6). Pictures were taken with an optical microscope using an objective with a 4-fold magnification (bar= 200 μm).

Spheroid Cultures

The cervix carcinoma cells (HeLa) shaped spheroids were cultivated as hanging drops in media with (+P/S) (see figure 5-7, A) and without (-P/S) antibiotics (see figure 5-7, B; n=6).

The formation of spheroids (manual and automatic production) in both media was largely identical. The spheroids were already formed in the first week and showed an increased size until week 2. During the third week, the size of spheroids slowly decreased associated with loose and isolated cells in the hanging drops [52].
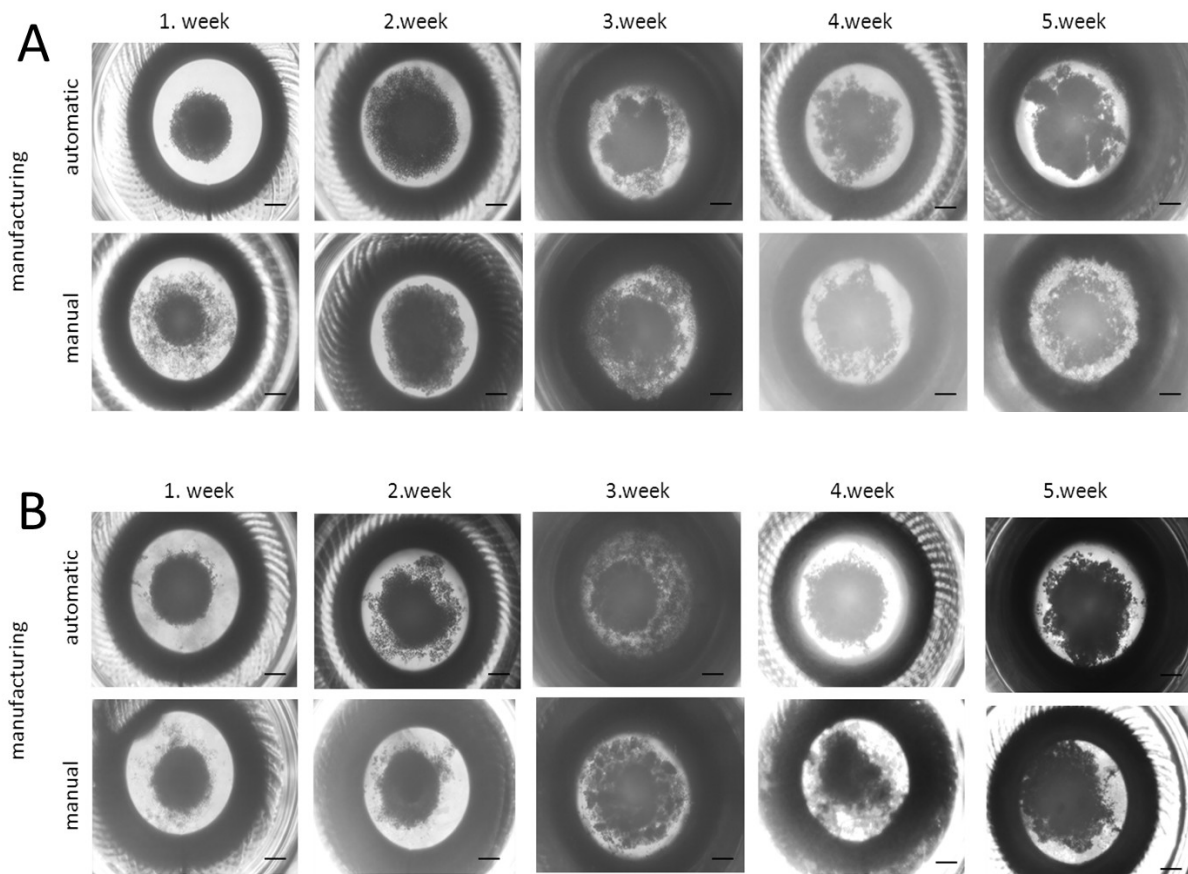
Figure 5-7 The formation of spheroids in handing drops cultivated in media with (A) [54] **and without antibiotics (B)**

The development of manually and automatically produced spheroid cultures in Perfecta3D® Hanging Drop Plates during the five weeks of cultivation (n=6). The images were taken with an optical microscope using an objective with a 4-fold magnification (bar= 200 µm).

Pellet Cultures

The pellet formation of human chondrocytes was performed in conditioned media with (+P/S; see figure 5-8, A) and without (-P/S; see figure 5-8, B; n=6) antibiotics as well as growth factors for chondrogenic stimulation.

The formation of manually and automatically manufactured pellet cultures in different media (+P/S, -P/S) was almost similar. The compaction of the pellet cultures was conducted from week one until week three. During the next two weeks, the increase of size of the 3D pellet cultures was followed by the proliferation and division of human chondrocytes [54].
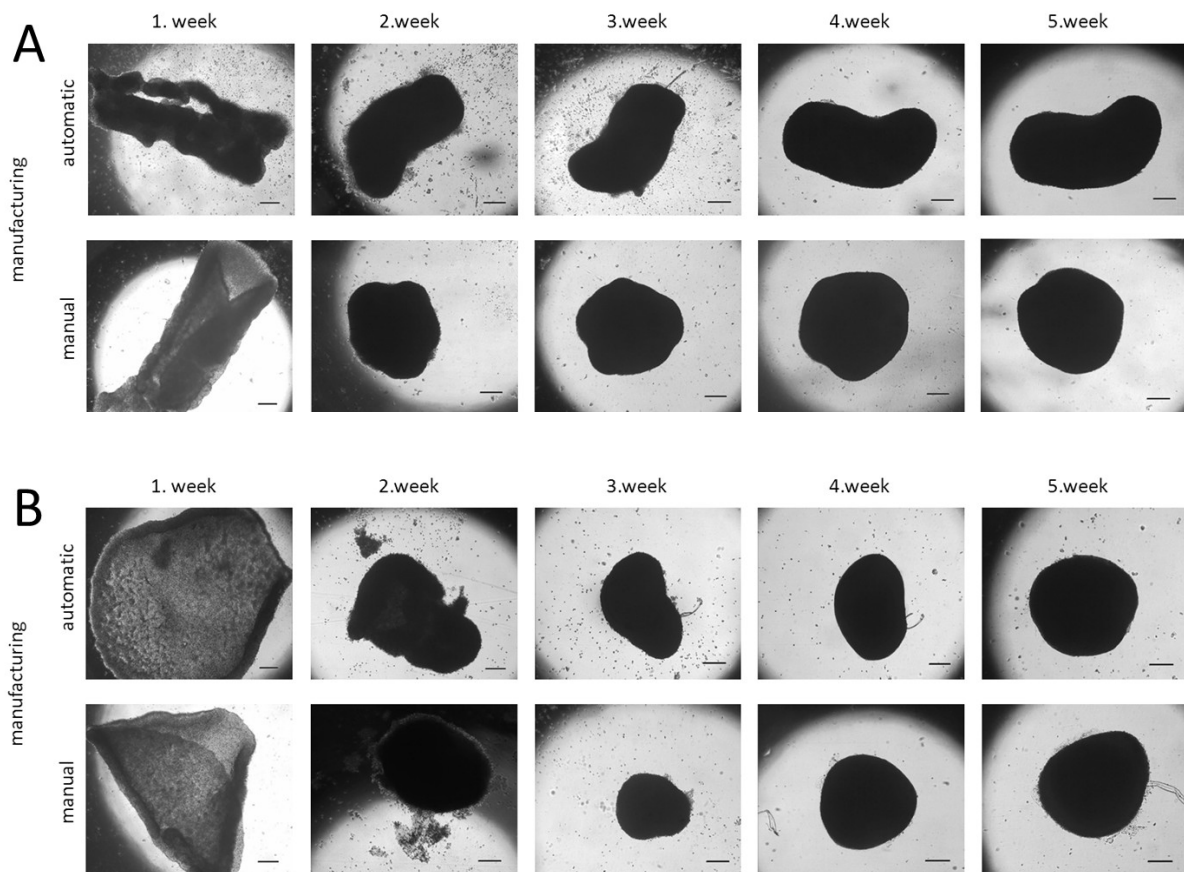
Figure 5-8 The formation of pellet cultures cultivated in media with (A) and without (B) [54] **antibiotic**

Development of automatically and manually manufactured pellet cultures containing human primary chondrocytes in media with or without penicillin/streptomycin after 35 days of cultivation (n=6). Pictures were taken with an optical microscope using an objective with a 4-fold magnification (bar= 200 µm).

Summary - Development of 3D cultures during the incubation time

The formation of 3D constructs of alginate beads, spheroid cultures and pellet cultures was visualized by microscopic documentation with an objective at 4-fold magnification from week one until week five. The results are summarized in the following table:

Table 5-4 Summary of the microscopic documentation about five week of 3D cultivation

Features	Alginate beads	Spheroid cultures	Pellet cultures
Cell lines	• cervix carcinoma cells	• cervix carcinoma cells	• human chondrocytes
Development over the time	• manual/automatic and +/- antibiotics similar • cells isolated (1st week) • formation of cell aggregates (2nd week) with increasing size until 5th week	• manual/automatic and +/- antibiotics similar • formation during 1st week • increasing size until 2nd week • decreased size + diffuse cells until 5th week	• manual/automatic and +/- antibiotics similar • compaction until 3rd week • size growth until 5th week

5.3.1.2 Size Detection

Due to the round shape the sizes of alginate beads and spheroid cultures was detectable by the Image-Pro Plus software (Media Cybernetics, Warrendale, USA). The diameters and areas of manually and automatically manufactured 3D constructs cultivated in media with (+P/S) and without (-P/S) were evaluated.

Alginate Beads

The manually and automatically produced alginate beads were evaluated by a microscopic investigation one day after manufacturing to document their maximum size (see table 5-5).
The sizes of automatically produced beads were only slightly larger than manual manufactured alginate beads in both media. The diameters of manually produced beads amounted 3,083.8 µm (+P/S; n=8) and 3,171.4 µm (-P/S; n=7). The diameters of automatically produced beads amounted 3,195.6 µm (+P/S; n=10) and 3,185.4 µm (-P/S; n=6). Compared to beads in media with antibiotics the diameter of manually produced alginate beads cultivated in media without antibiotic was larger (about 87.6 µm). Instead, the automatically produced beads in media without antibiotics were smaller (about 10.2 µm) compared to beads in media with antibiotics.

Table 5-5 Diameter (in µm) of automatically (n≥6) and manually (n≥7) produced alginate beads cultivated in media with (+P/S) and without (-P/S) antibiotic [54]**.**

	Diameter (µm) of alginate beads	
Antibiotic	**manual manufacturing**	**automatic manufacturing**
+ P/S	3,083.8 µm (± 10.1)	3,195.6 µm (± 51.2)
- P/S	3,171.4 µm (± 58.4)	3,185.4 µm (± 46.2)

Notes: Data are presented as mean value ± standard error. Statistical analysis was done with students T-test. No significances were observed.

The area of the single cell aggregates of histological sectioned and stained (Papanicolaous) slides of alginate beads was evaluated on day 14 (manual: n≥10; automatic: n≥8) and day 35 (manual: n≥9; automatic: n≥6). Here, the sizes of the cell aggregated increased over the time (see figure 5-9). From day 14 to day 35, the cell aggregate areas increased non-significantly (about 1,201 µm (manual, +P/S), 1,094.3 µm (manual, -P/S), 1,095.1 µm (automatic, +P/S) and 918.1 µm (automatic, -P/S)). The cell aggregates cultivated in media with antibiotic (+P/S) exhibit larger aggregates (day 14, 35). The aggregates of the manual produced beads (+P/S) have a 20 % larger area compared to aggregates in media without antibiotic (day 14, 35). The differences of aggregate areas of automatically produced beads are less than 6 % (day 14, 35). The areas of cell aggregates of manually produced beads are approximately9 % larger compared to automatic produced beads cultivated in media with antibiotic (day 14, 35). In contrast, aggregate areas (-P/S) of automatic produced beads are about 11 % (day 14) and 2 % (day 35) larger [73].
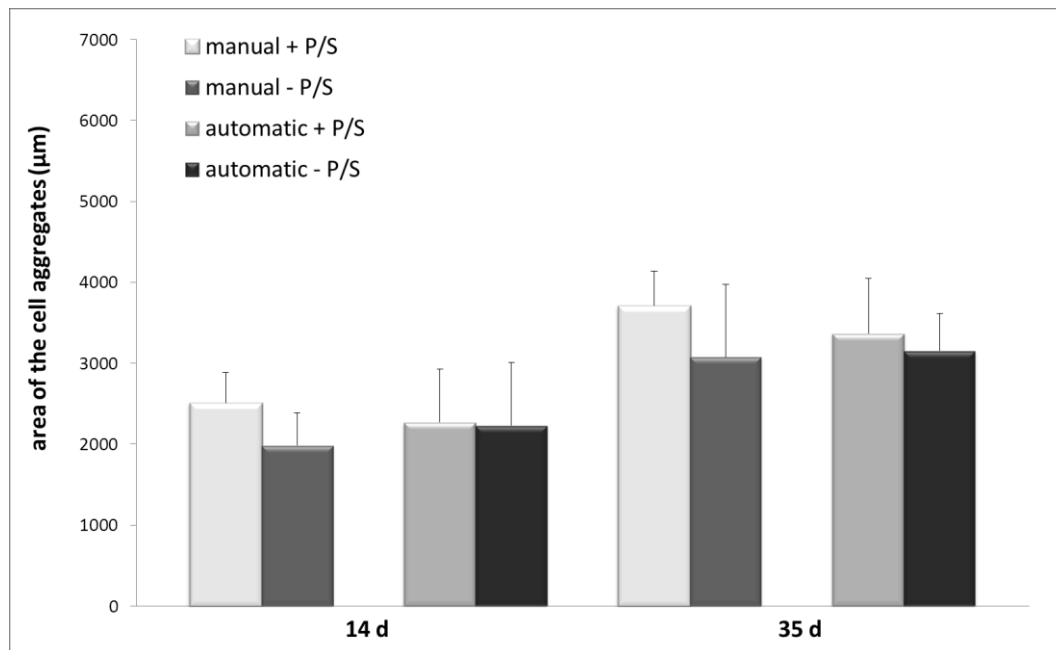
Figure 5-9 The area (µm) of cell aggregates consisting of alginate-encapsulated HeLa cells

The cell-aggregate area of automatically (n≥6) and manually (n≥9) produced HeLa cells embedded in alginate matrix cultivated in media with and without penicillin/streptomycin on day 14 and day 35. Statistical analysis was done with student's t-test. Data are presented as mean value ± standard error. No significances are observed. The manually manufactured alginate beads in media with antibiotics were chosen as controls.

Spheroid Cultures

The manually and automatically manufactured spheroid cultures formed in Perfect® 3D hanging drop plates were evaluated by microscopic investigations on day 14 (manual: n≥7; automatic: n≥6) and day 35 (manual: n≥10; automatic: n≥8) after manufacturing (see table 5-6). The diameter (µm) of the manually and automatically manufactured spheroids (+P/S; -P/S) reached in the average size of 356.1 µm (day 14) and 301.1 µm (day 35). The sizes decreased from day 14 to day 35 about 55 µm. In detail, the diameter (µm) of manually produced spheroids were mainly increased (day 14, +P/S and -P/S; day 35, +P/S) about 24.3 µm. The spheroids in media without antibiotics were increased compared to spheroids in media with antibiotic (day 14, manual: 11 µm; day 14, automatic 14.8 µm; day 35, manual: 20.9 µm; day 35, automatic 65.1 µm) [52].

Table 5-6 Diameter (in µm) of automatically (n≥6) and manually (n≥7) produced spheroid cultures cultivated in media with (+P/S) and without (-P/S) antibiotic [52]

		Diameter (µm) of cell aggregates	
Antibiotic	**days of cultivation**	**manual manufacturing**	**automatic manufacturing**
+ P/S	14d	363.9 µm (± 32.1)	335.4 µm (± 47.0)
- P/S	14d	374.9 µm (± 29.3)	350.2 µm (± 29.6)
+ P/S	35d	289.5 µm (± 28.5)	269.7 µm (± 26.8)
- P/S	35d	310.4 µm (± 43.3)	334.8 µm (± 8.7)

Notes: Data are presented as mean value ± standard error. Statistical analysis was done with students T-test. Significance is based on manually manufactured alginate beads in media with antibiotic. No significances are observed.

Next to the diameter the overall area was detected on day 14 and day 35 (see figure 5-10; n≥6). From day 14 to day 35, the spheroid cultures are one whole cell aggregate with a decreasing area of 47,939.5 μm (manual, +P/S), 11,068.9 μm (manual, -P/S), 34,253.5 μm (automatic, +P/S) and 2,040.1 μm (automatic, -P/S). The automatically produced spheroid significantly ($p<0.05$) decreased compared to the standard and control (manual, +P/S, 14 d).

The cell aggregates cultivated in media with antibiotics (+P/S) showed greater aggregates about 16.8 % (manual) and 6.8 % (automatic) on day 14. Instead, the spheroid aggregates in media with antibiotics (+P/S) displayed smaller aggregates about 18.8 % (manual) and 29 % (automatic) on day 35.

The areas of manually manufactured spheroid aggregates (+P/S, -P/S) are larger about 19.2 % (+P/S, day 14), 9.5 % (-P/S, day 14), 13 % (+P/S, day 35) and 0.5 % (-P/S, day 35) compared to automatic produced spheroid cultures at both detection time points.
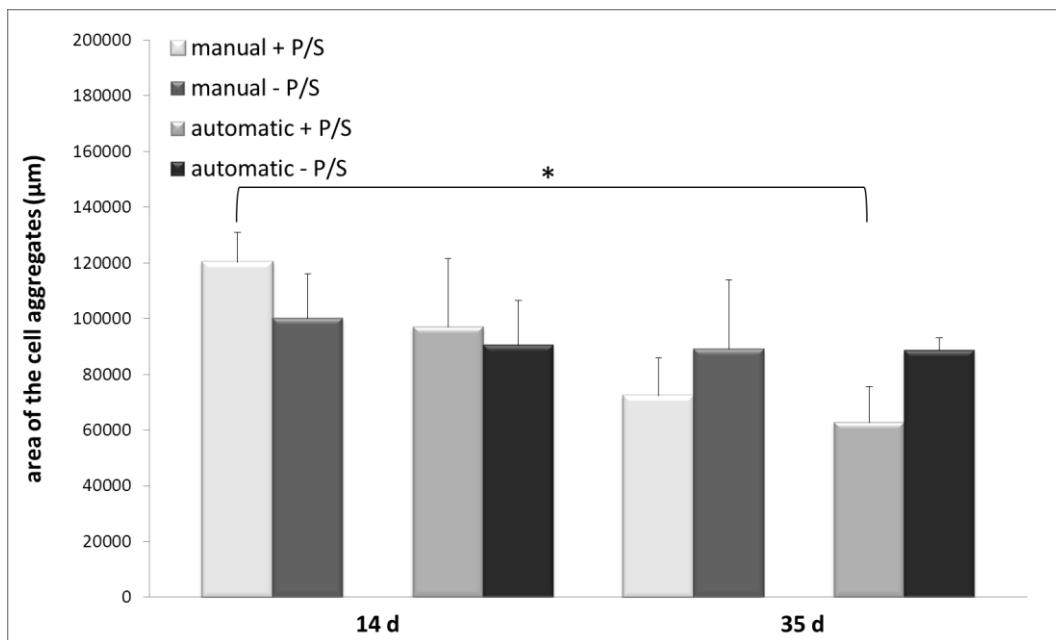
Figure 5-10 The area (μm) of spheroid aggregates containing HeLa cells

The area of automatically (n≥6) and manually (n≥6) produced spheroid cultures cultivated in media with and without penicillin/streptomycin (P/S) on day 14 and day 35. Statistical analysis was done with student's t-test. Data are presented as mean value ± standard error. Significance is based on the manually produced spheroids cultivated in media with antibiotic on day 14: ∗p < 0.05.

Summary - Size Detection

The diameter and aggregated areas of alginate beads and spheroid cultures cultivated in different media (+P/S, -P/S) were detected at different time points. The results are summarized in the following table:

Table 5-7 Sizes of alginate beads and spheroid cultures

Features	Alginate beads	Spheroid cultures
Diameter	• manual/automatic and +/- antibiotics: minor differences • manual/day 1: 3128 µm • automatic/day 1: 3191 µm	• manual/automatic and +/- antibiotics: minor differences • decrease from day 14 to day 35 (+P/S, -P/S) • day 14: 356.1 µm • day 35: 301.1 µm
Aggregate area	• manual/automatic and +/- antibiotics mainly similar • increased area of cell aggregates from day 14 tol day 35 • increased area of cell aggregates in media with antibiotics (day 14, 35)	• decrease from day 14 to day 35 (+P/S, -P/S) • increase area of manually produced spheroid cultures compared to automatically manufactured spheroids (day 14, 35) • day 14: increased area of spheroids in media with antibiotic • day 35: decreased area of spheroids in media with antibiotics

5.3.1.3 Histological staining

Alginate beads and pellet cultures allowed the sectioning and histological staining to visualize and characterize the cells inside the 3D constructs. The 3D constructs were manually and automatically produced as well as cultivated in media with (+P/S) and without (-P/S) antibiotics. At day 14 and 35, the 3D constructs were fixed, paraffin-embedded and sectioned (7µm). Consequently, the sections were stained specifically.

Alginate Beads

The Papanicolaous-staining is used for cytological investigation of gynecologic samples especially for cervix carcinoma cells. The cell nuclei of dead cells shows a dark violet staining.

The alginate beads cultivated in media with (A) and without (B) antibiotics of both manufacturing procedures (automatic, manual) visualized an increase of condensed cell aggregates from day 14 to day 35. On day 35 the size and compactness of the cell aggregates were increased (see figure 5-11; n=6). On day 14, the manually produced beads showed augmented cell aggregates. However, the amounts of cell aggregates, their size and distribution within the beads of manually and automatically manufactured beads were similar. Nevertheless, the cell aggregates of alginate beads cultivated in media with antibiotics appeared larger, were almost increased and more compact on day 35 [73].
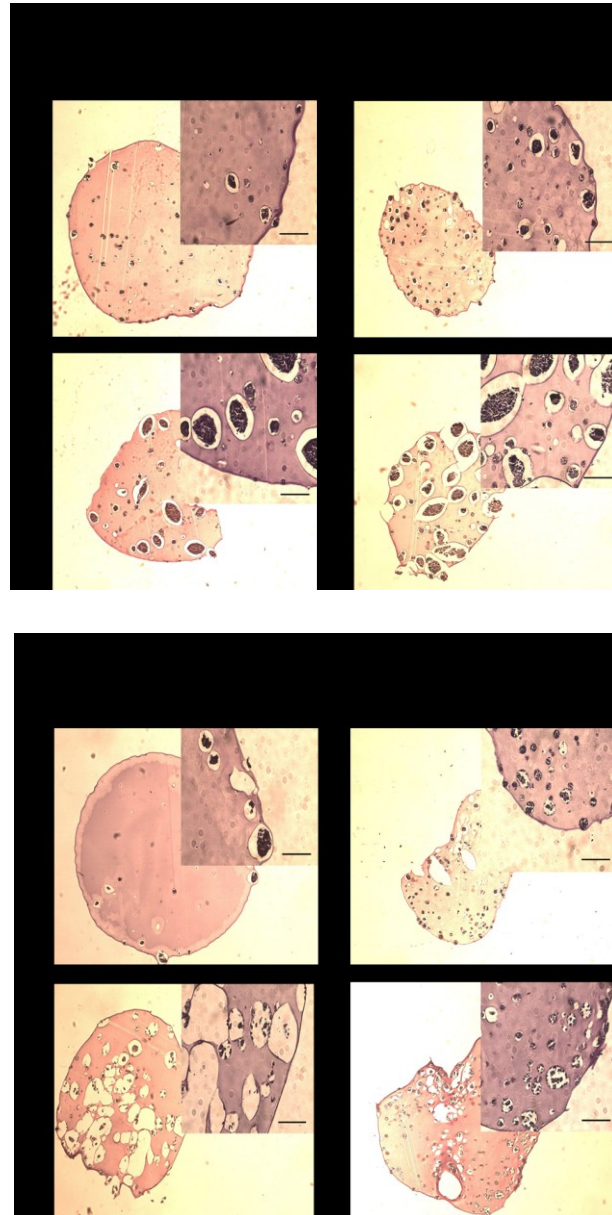
Figure 5-11 Papanicolaous staining of histological sections of HeLa cells embedded in alginate matrix

Histological analysis (Papanicolaous staining) of HeLa cells embedded in alginate after 14 and 35 days of incubation with (A) [73] and without (B; n=6) antibiotics. Pictures were taken with an optical microscope using an objective with a 4- and 20 fold magnification (bar= 100 µm).

Pellet Cultures

The new synthetized matrix within pellet cultures can be visualized by Alcian blue stainings. This staining is used to visualize acid mucosubstances. These glycosaminoglycans are part of the extracellular matrix. The nuclei are stained light red, the background colored rose and the mucosubstances are stained blue.

The Alcian blue stained sections (day 14, 35) of automatically and manually produced pellet cultures cultivated in media with (A) and without (B) antibiotics are shown in figure 5-12 (n=6). The pellet

cultures (manual, automatic) in both media visualized increased level of mucosubstances from day 14 to day 35.
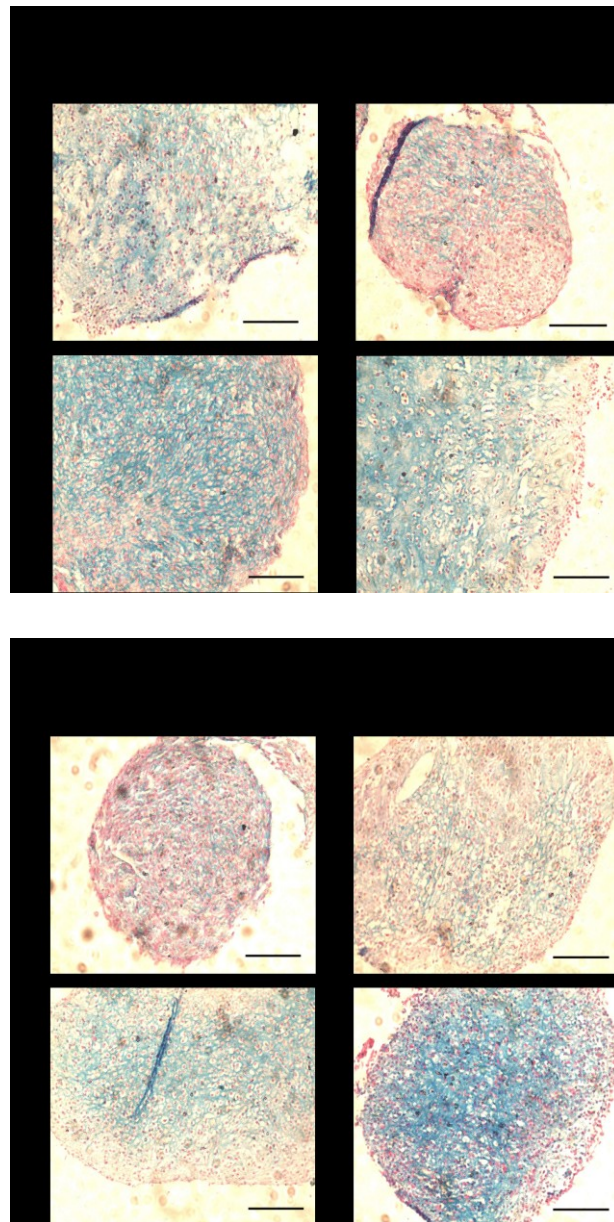
Figure 5-12 Alcian blue-stained histological sections of pellet cultures

Histological analysis (Alcian blue staining) of human chondrocytes cultivated in pellets after 14 and 35 days of incubation with (A) [49] and without (B; n=6) antibiotics. Pictures were taken with an optical microscope using an objective with a 4- and 20 fold magnification (bar= 100 µm).

Pellet cultures can be stained with Safranin-O to visualize new synthesized matrix (collagen) within the constructs which were cultivated in media with (A) and without (B) antibiotics (see figure 5-13; n=6). The proteoglycans represent the basic substances of cartilage, joints and tendons. The accumulation of proteoglycans is represented by the increased orange color of the sections.

The histological stainings of manually and automatic produced pellet cultures cultivated in different media (+P/S, -P/S) with chondrogenic growth factors were performed on two time points (day 14, day 35). After 14 days of cultivation, no synthesized ECM could be determined. In contrast, on day

35, manually and automatic produced pellet cultures showed a marked rise of new synthesized matrix in all culture conditions [54].
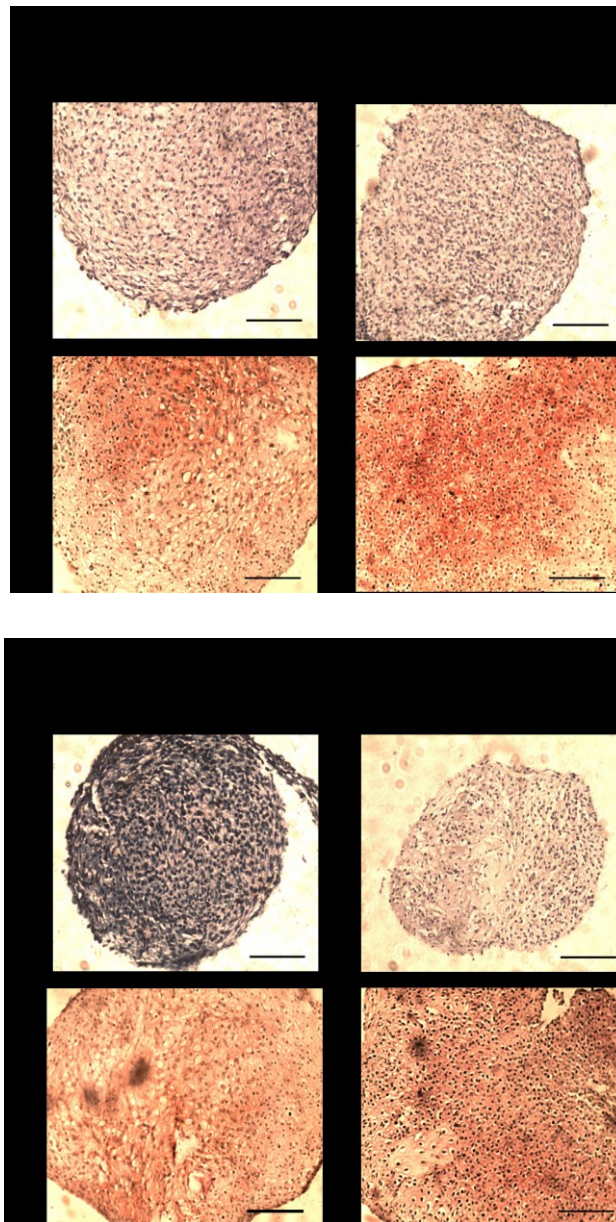
Figure 5-13 The Safranin-O stained histological sections of pellet cultures

Histological analysis of collagen of manually and automatically manufactured pellet cultures on day 14 and 35 days of incubation with (A) and without antibiotics (B) by Safranin-O staining (n=6). Pictures were taken with an optical microscope using an objective with a 20 fold magnification (bar= 100 µm).

Summary – Histological Staining

The culturing of cells in alginate beads and pellet culture allows the possibility of histological evaluation.The Papanicolaous-staining of cervix carcinoma cells in alginate matrix allowed the visualization of dead cell nuclei. Furthermore, freshly synthesized extracellular matrix of pellet

cultures cultivated in different media (+P/S, -P/S) was detected on day 14 and day 35 by specific histological stainings. The results are summarized in the following table:

Table 5-8 Summary of histological staining

Features	Alginate beads	Pellet cultures
Histological staining	Papanicolaous staining: • increased number of cell aggregates from day 14 to day 35 • day 14: size and distribution of the cell aggregates (+P/S, -P/S) similar • day 35: increased size and compactness of cell aggregates, specific for beads in media + P/S	Safranin-O staining • visualize proteoglycans of the ECM[1] (collagen) • day 7: no ECM[1] • day 35: marked rise of ECM[1] Alcian blue staining: • visualize acid mucosubstances of the ECM[1] • increased level from day 14 to day 35

Notes: ECM[1] - extracellular matrix

5.3.2   Screening Processes

The different 3D constructs comprise a varity of characteristics and applications. The bioscreening processes are used to quantify the quality of the 3D constructs. Here, the proliferation and cytotoxicity were directly analyzed. The proliferation was detected by the EZ4U-assay and DNA quantification. The cytotoxicity was detected by the adenylate kinases (AK) assay. The three screening arrangements were new adapted for 3D cell constructs. The bioscreenings were performed using manual and automatic procedures.

Skilled laboratory staff performed the manual bioscreening. The automatic screenings have been performed using a High Throughput Screening System.

5.3.2.1 Proliferation

EZ4U Assay

In order to analyze the proliferation in three dimensional alginate matrices, proliferation, the EZ4U assay was performed. This assay based on the reduction of tetrazolium salt into colored formazan by living cells.

Alginate Beads

Generally, the results (manual and automatic screening) displayed the reduction of the proliferations from day 14 to day 35 for cells cultivated in media with (+P/S) and without (-P/S) antibiotics (see figure 5-14; manual bioscreening: n≥13 [manual], n≥12 [automatic]; automatic bioscreening: n≥6 [manual], n≥7 [automatic]; in eight replicates).

In general, on all detection dates (day 14, 35), the proliferation of cells encapsulated in alginate and cultivated in media without antibiotics were almost higher compared to cells cultivated in media with antibiotics in both (automatic, manual) detection forms. The proliferation of automatically encapsulated cells (+P/S) was significantly (p<0.001) decreased about 36 % (manual screening) and 23 % (automatic screening) compared to the standard and control (day 14, manual manufactured, +P/S) on day 35. Only the automatically screened manual manufactured beads showed a significantly decreased (p<0.05) proliferation rate about 25 % on day 35 compared to the control day on day 14 (manual manufactured, +P/S).

On day 14, the comparison of manually screened beads cultivated (± antibiotics) visualized an increased proliferation rate of cells in media without antibiotics (-P/S) about 23 % (manually produced) and 19 % (automatically produced). However, the proliferation rate of the automatically produced beads (-P/S) was increased about 15 % (manual screening) and 20 % (automatic screening, p<0.01) compared to the proliferation rate of encapsulated cells in media with antibiotics.

The manual bioscreening procedures revealed a reduced cell proliferation rate after automatical production (± antibiotics) of alginate beads compared to manually manufactured ones which was in contrast to the automatic screening procedure. The cell proliferation (manual screening) of the automatically produced beads (+P/S, - P/S) was lower compared to the manual manufactured beads (7 % (day 14, +P/S), 10 % (day 14, -P/S), 34 % (day 35, +P/S; p<0.05) and 25 % (day 35, -P/S)). Instead, the proliferation rate (automatic screening) of cells in automatically manufactured alginate beads was only higher at day 14 (30%, -P/S; p<0.01).

Certainly, the signal strength of automatically screened beads was approximately one third increased compared to the manually screening [73].
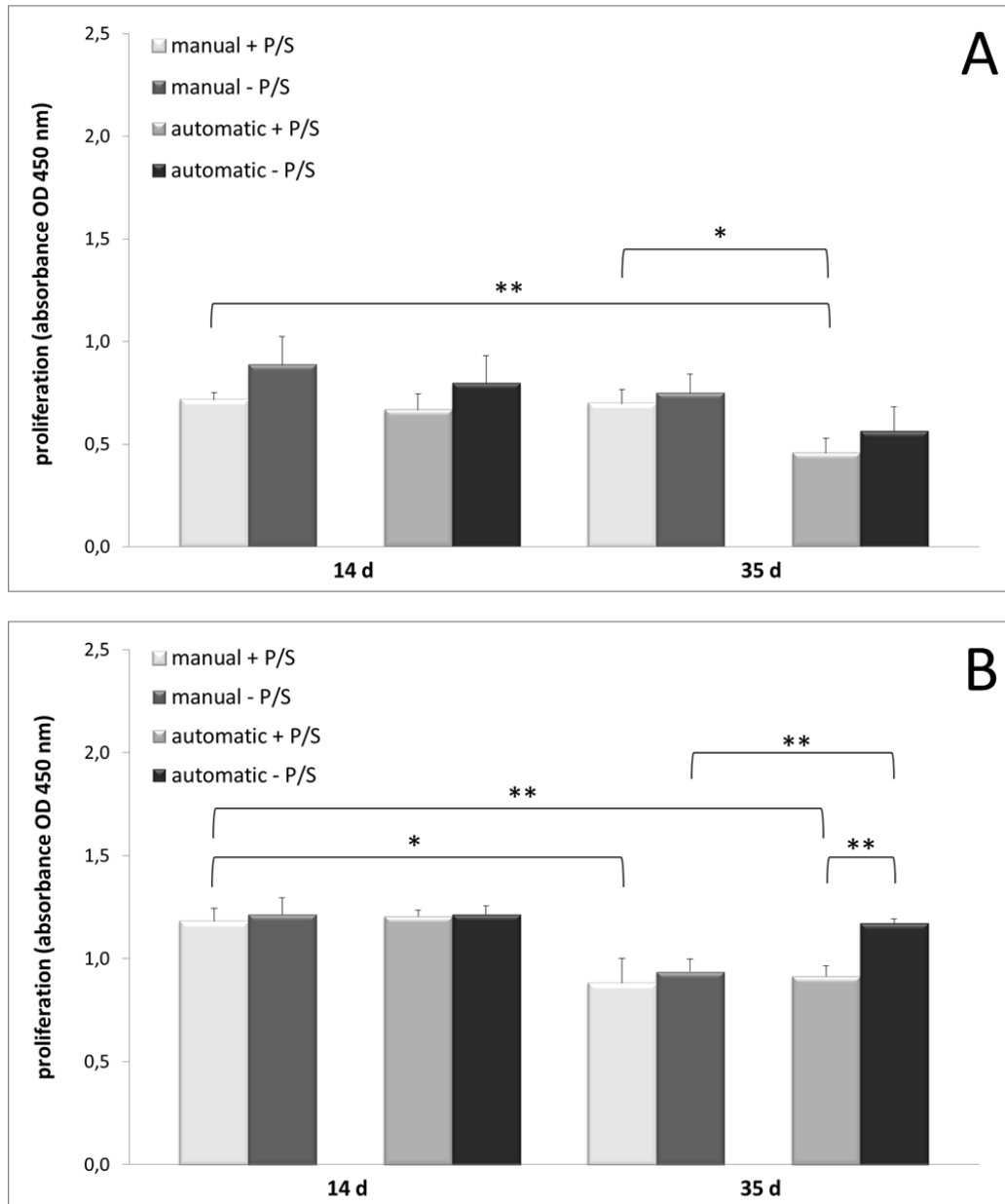
Figure 5-14 Proliferation rate of alginate-encapsulated HeLa cells detected by the EZ4U assay

Proliferation of automatically and manually produced HeLa cells embedded in alginate matrix cultivated in media with (+P/S) and without (-P/S) penicillin/streptomycin on day 14 and day 35 compared with manual (A; manual: n≥13; automatic: n≥12, in eight replicates) and automatic (B; manual: n≥6, automatic: n≥7; in eight replicates) bioscreening. Statistical analysis was done with student's t-test. Data are presented as mean value + standard error. Significances: *p<0.05, **p<0.01.

Spheroid Cultures

The manually and automatically manufactured spheroids have been quantified by manual and automatic bioscreening on day 14 and day 35 (see figure 5-15; manual bioscreening: n≥8 [manual], n≥6 [automatic], in eight replicates; automatic bioscreening: n≥6 [manual], n≥7 [automatic]; in six replicates). Manually produced spheroids cultivated in media with antibiotics on day 14 revealed as controls.

In general, a decreasing proliferation rate of automatically and manually produced spheroid cultures formed in hanging drops was determined from day 14 to day 35 (+P/S, -P/S) associated with a lower proliferation rate of spheroids cultivated in media without antibiotics.

In detail, both screening forms (manual, automatic) showed mostly a significantly decreased proliferation rate from day 14 until day 35 of spheroids cultivated in media with antibiotics compared to the control. The proliferation rates of manually manufactured spheroids were reduced about 35 % (+P/S, manual screening), 83 % (-P/S, $p < 0.001$, manual screening), 26 % (+P/S, $p < 0.05$, automatic screening) and 71 % (-P/S, significant: $p < 0.001$, automatic screening) on day 35. The proliferation of automatically produced spheroids decreased about 52 % (+P/S, $p < 0.05$, manual screening), 64 % (-P/S, $p < 0.05$, manual screening), 35 % (+P/S, automatic screening) and 60 % (-P/S, $p < 0.05$, automatic screening) on day 35.

The comparison of spheroids cultivated in different media (with antibiotics [+P/S], without antibiotics [-P/S]) showed always a reduced proliferation rate of spheroids in media without antibiotics with the manual and automatic bioscreening procedures on day 14 and day 35, whereby the differences of automatic handled spheroids were smaller compared to the manual manufactured spheroids. On day 14, the comparison of manually screened spheroids cultivated in different media (+P/S, -P/S) visualized an increased proliferation of cells in media with antibiotics (+P/S) about 44 % (manually produced, $p < 0.01$) and 16 % (automatically produced) related to cells in media without antibiotics. The automatically screened beads (+P/S) displayed an increased proliferation about 45 % (manual production, $p < 0.05$) and 16 % (automatic production) compared to spheroids in media without antibiotics on day 14. On day 35 the proliferation of manually produced beads (+P/S) was enhanced about 82 % (manual screening, $p < 0.01$) and 61 % (automatic screening, $p < 0.05$) similar to spheroids in media without antibiotics (-P/S). The proliferation rate of the automatically produced beads (+P/S) was increased about 24 % (manual screening) and 39 % (automatic screening) instead of spheroids in media without antibiotics.

The evaluation of the different manufacturing procedures of spheroid cultures presented an advanced proliferation of automatically produced spheroids in media without antibiotics (-P/S) compared to manually manufactured cultures about 36 % (day 14, manual screening), 67 % (day 35, $p < 0.05$, manual screening), 33 % (day 14, automatic screening) and 27 % (day 35, automatic screening). The proliferation rate of automatically produced spheroids cultivated in media with antibiotics (+P/S) was slightly increased compared to manually manufactured cultures about 6 % (day 14, manual screening) and 27 % (day 35, automatic screening). Instead, the proliferation of automatically processed spheroids with antibiotics was decreased about 27 % (day 35, manual screening) and 12 % (day 35, automatic screening) compared to the manually manufactured pellets.

The comparison of manual and automatic bioscreenings visualized analogous signal strengths and results. On day 14, the absorbance units of the automatic bioscreening were increased about 12 % (manual, +P/S), 9 % (manual,-P/S) and 5 % (automatic; +P/S, -P/S). On day 35 the cell proliferation determined by the automatic bioscreening was increased about 22 % (manual, +P/S), 64 % (manual,-P/S), 35 % (automatic; +P/S) and 19 % (automatic, -P/S) [52].
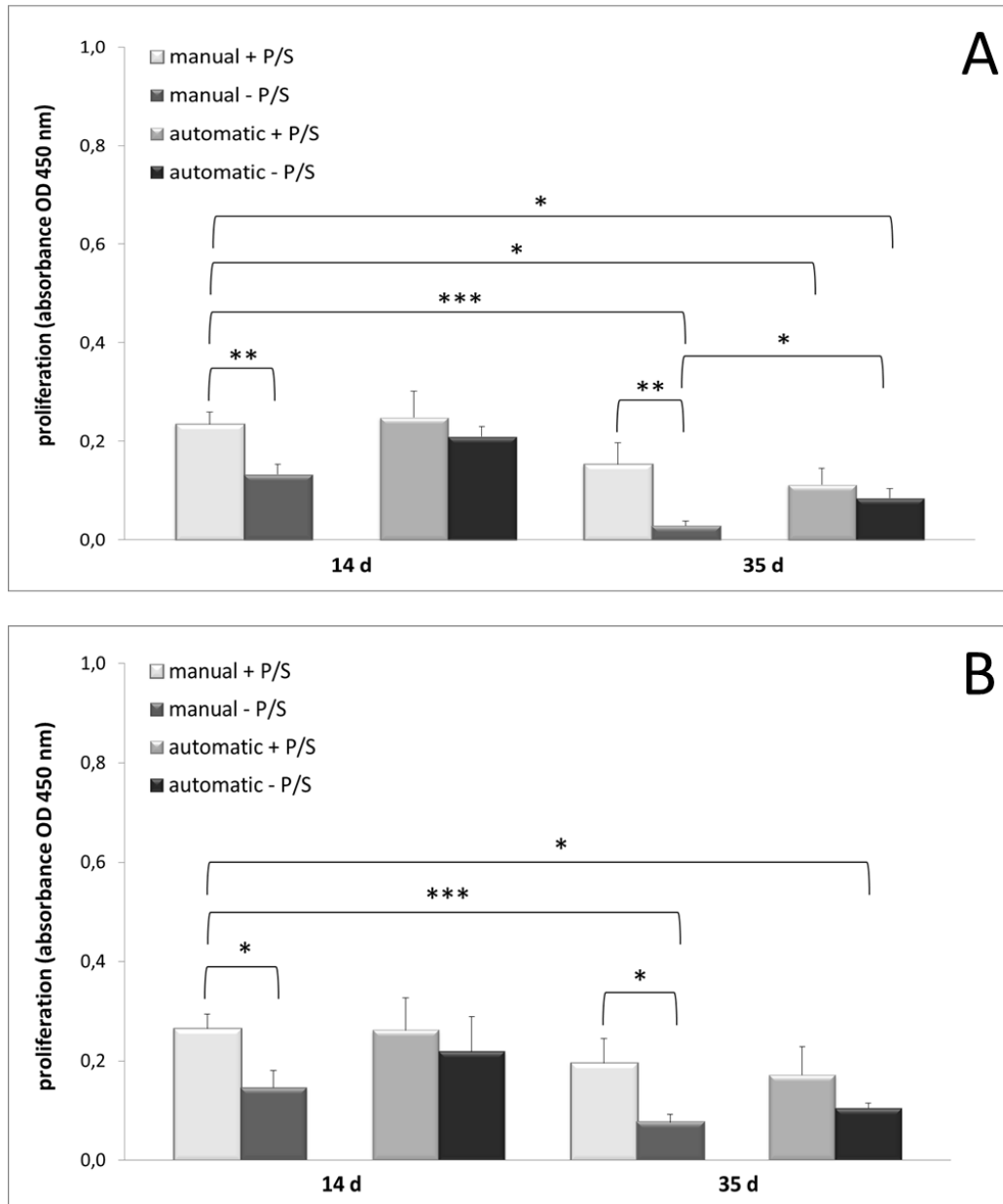
Figure 5-15 Proliferation of spheroid cultures formed in hanging drops detected by the EZ4U assay

Proliferation of automatically and manually produced spheroid cultures formed in hanging drops in media with (+P/S) and without (-P/S) penicillin/streptomycin on day 14 and day 35 compared with manual (A; manual: n≥8; automatic: n≥6, in eight replicates) and automatic (B; manual: n≥6, automatic: n≥7; in six replicates) bioscreening procedures. Statistical analysis was done with student's t-test. Data are presented as mean value ± standard error. Significances: ∗p<0.05, ∗∗p<0.01 and ∗∗∗p<0.001.

Pellets Cultures

The quantifications of manually and automatically manufactured pellet cultures were performed using manual and automatic bioscreenings of the EZ4U assay on day 14 and day 35 (see figure 5-16; manual bioscreening: n≥7 [manual], n≥8 [automatic]; automatic bioscreening: n≥6 [manual], n≥7 [automatic]; in eight replicates). Control were the manually produced spheroids cultivated in media with antibiotics on day 14.

In general, the proliferation rate of pellets in medium with antibiotics (+P/S) decreased from day 14 to day 35 contrary to pellets cultivated in media without antibiotics, where the proliferation increased from day 14 to day 35. Comparing the screening and manufacturing procedures, a decreased proliferation of pellet cultures in media without antibiotics compared to media with antibiotics on day 14 was shown. Instead, on day 35, the proliferation rate of pellets in media without antibiotics was increased compared to cultures with antibiotics. The automatically produced pellet cultures showed a higher proliferation compared to manually manufactured cells.

In detail, the manually produced pellet cultures displayed a decreased proliferation from day 14 to day 35 with both screening forms (manual and automatic) compared to the control (manually produced, +P/S, day 14). The proliferation of manually manufactured pellets significantly decreased about 37 % (+P/S, $p<0.05$, manual screening), 8 % (-P/S, $p<0.05$, manual screening), 22 % (+P/S, $p<0.05$, automatic screening) but increased about 14 % (-P/S, automatic screening) on day 35. Compared to the control, the proliferation of automatically created pellet cultures in medium without antibiotics decreased about 32 % (manual screening) and 8 % (-P/S, automatic screening) on day 35. In contrast, the proliferation of automatically produced pellets cultivated in media with antibiotic increased about 12 % (manual screening) and 7 % (automatic screening).

The comparison of manually and automatically manufactured pellet cultures showed mostly a higher proliferation rate of automatically produced pellet cultures in media with and without antibiotics. The manual screening displayed an increased proliferation of automatically produced pellets about 12 % (day 14, +P/S), 18 % (day 14, -P/S), 8 % (day 35, +P/S) and 19 % (day 35, -P/S). The automatic screening showed an increased proliferation of automatically manufactured pellets about 27 % (day 14, +P/S), 22 % (day 14, -P/S) and 4 % (day 35, +P/S). Instead, the proliferation of automatically produced pellets was decreased about 7 % (day 35, -P/S) compared to the manually manufactured pellets.

On day 14 the proliferation (manual and automatic bioscreening) of pellet cultures in media without antibiotics (-P/S, manual and automatic manufacturing) was decreased compared to pellet cultures in media with antibiotics (+P/S), contrary to day 35. Here, the proliferation rate of pellets (-P/S) was decreased about 18 % (manually manufactured, manual screening), 12 % (automatically manufactured, manual screening), 22 % (manual manufacturing, automatic screening) and 27 % (automatically manufactured, automatic screening) on day 14. Instead of day 35, the proliferation of pellet cultures (-P/S) was increased about 31 % (manually manufactured, manual screening), 40 % (automatically manufactured, $p<0.05$, manual screening), 24 % (manually manufactured, automatic screening) and 15n% (automatically manufactured, automatic screening).

All together, the signal strength of the detected absorbance of automatically screened pellet cultures is lower compared to the manually screened proliferation (average: 30 % [day 14] and 19 % [day 35]) [54].
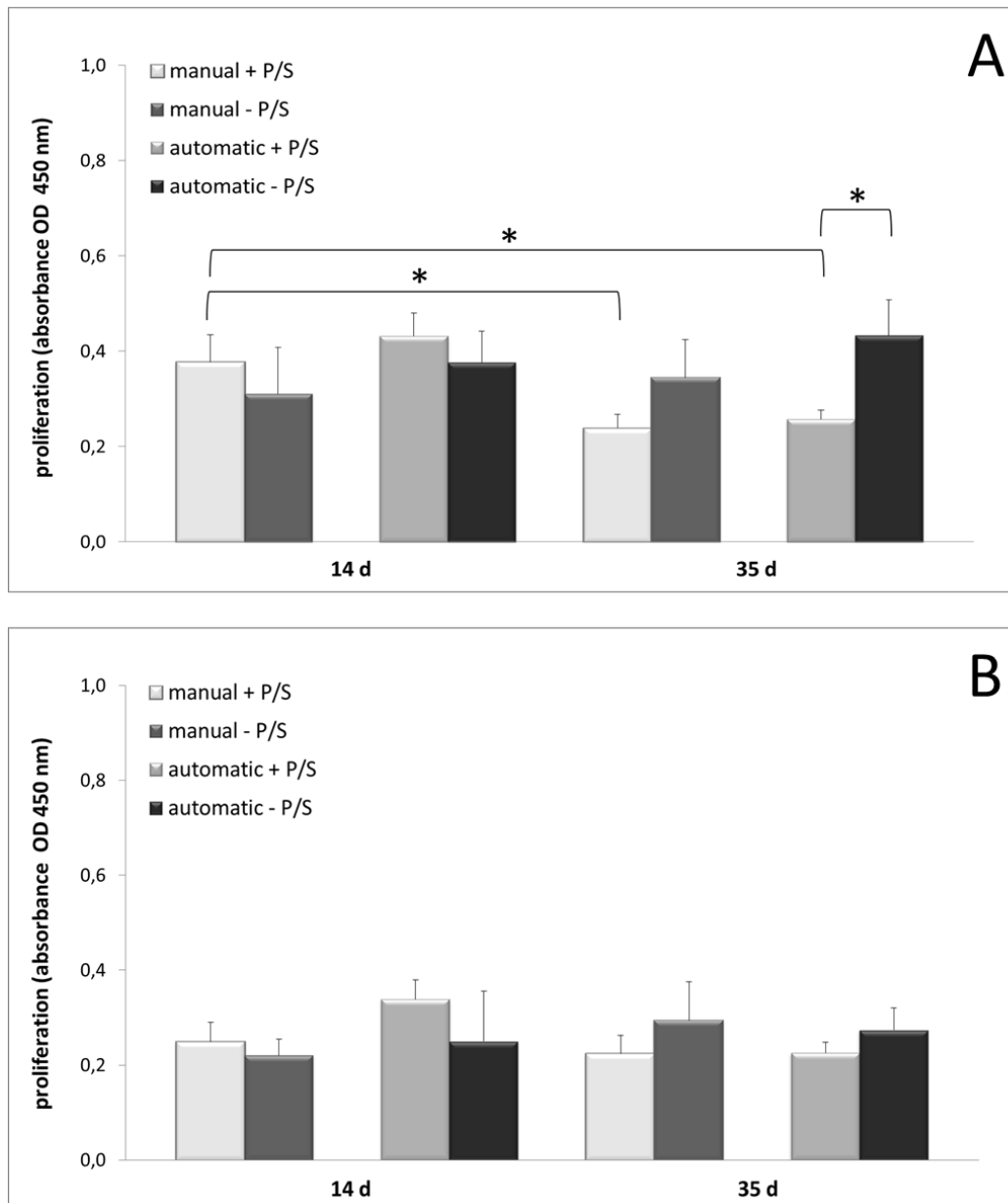
Figure 5-16 Proliferation of human chondrocytes in 3D pellet cultures detected by the EZ4U assay

Proliferation of automatically and manually produced pellet cultures derived from human chondrocytes in media with (+P/S) and without (-P/S) penicillin/streptomycin on day 14 and day 35 compared with manual (A; manual: n≥7; automatic: n≥8, in eight replicates) and automatic (B; manual: n≥6, automatic: n≥7; in eight replicates) bioscreening. Statistical analysis was done with student's t-test. Data are presented as mean value ± standard error. Significances: ∗p<0.05.

Summary – EZ4U Assay

The proliferation of manually and automatically produced 3D constructs (alginate beads, spheroid cultures, pellet cultures) was evaluated by the EZ4U assay on day 14 and day 35. This proliferation screening based on the reduction of tetrazolium salt to colored formazan and was quantified by the detection of absorbance. The results are summarized in the following table:

Table 5-9 Summary of the EZ4U assay

Feature	Alginate beads	Spheroid cultures	Pellet cultures
Proliferation by EZ4U assay	• mainly decreased proliferation rates from day 14 to day 35 (+P/S, -P/S) • higher proliferation of cells in media without P/S (manually and automatically produced) • lower proliferation of automatically produced beads (manual screening) • mainly higher proliferation of automatically produced beads (automatic screening)	• decreased proliferation from day 14 to day 35 (+P/S, -P/S) • lower proliferation of cells in media -P/S (manually and automatically produced) • mainly higher proliferation of automatically produced spheroids	• decreased proliferation from day 14 to day 35 (+P/S, manually and automatically produced) • increased proliferation from day 14 to day 35 (-P/S, manually and automatically produced) • day 14: lower proliferation of cells in media without P/S (manually and automatically produced) • day 35: higher proliferation of cells in media without P/S (manually and automatically produced) • mainly lower proliferation of manually produced pellets
Signal strength	• automatic screening: increased signal strength/absorbance units compared to manual screening	• automatic screening: increased signal strength/absorbance units compared to manual screening	• automatic screening: decreased signal strength/absorbance units compared to manual screening

DNA Quantification

After isolation of DNA, the DNA amounts of all 3D cultures were evaluated by the Quant-iT™PicoGreen®dsDNA Kit (Life Technologies).

Alginate Beads

The manual and automatic detection of the DNA amounts of manually and automatically formed alginate beads were performed on day 14 and day 35 (see figure 5-17 manual and automatic bioscreening: n≥6). The control and standard displayed manually manufactured pellet cultures cultivated in media with antibiotic on day 14.

In general, the DNA amount of cells embedded in alginate beads mainly decreased from day 14 to day 35. On day 14, the DNA amount of cells in alginate cultivated in media without antibiotics (-P/S) was higher in media with antibiotics (+P/S) which was in contrast to day 35. Nevertheless, the DNA amount of automatically produced pellet cultures was lower than manually manufactured beads on day 14.

In detail, the bioscreening (manual, automatic) showed a reduced DNA amount from day 14 to day 35 compared to the control (manually produced, +P/S, day 14). The DNA amount of manually manufactured alginate beads decreased about 669 ng/ml (+P/S, manual screening), 1,232.9 ng/ml (-

P/S, manual screening), 254.1 ng/ml (+P/S, automatic screening) and 986.9 ng/ml (-P/S, automatic screening). The DNA amount of automatically manufactured alginate beads reduced about 355.6 ng/ml (+P/S, manual screening), 706.5 ng/ml (-P/S, manual screening), 182.1 ng/ml (+P/S, automatic screening) and 564.5 ng/ml (-P/S, automatic screening).

Moreover, on day 14 the manual and automatic quantification displayed a higher DNA amount of cells in alginate matrix cultivated in media without antibiotics (-P/S) contrary to beads in media with antibiotics about 129.1 ng/ml (manual screening, manual manufacturing), 270.3 ng/ml (manual screening, automatic manufacturing), 427.6 ng/ml (automatic screening, manual manufacturing) and 71.4 ng/ml (automatic screening, automatic manufacturing). On day 35, the bioscreenings showed the contrary effect, whereby the DNA amount of beads in media without antibiotics (-P/S) decreased related to beads in media with antibiotics (+P/S) about 563.9 ng/ml (manual screening, manual manufacturing), 351 ng/ml (manual screening, automatic manufacturing), 732.8 ng/ml (automatic screening, manual manufacturing) and 382.4 ng/ml (automatic screening, automatic manufacturing). Moreover, on day 14 the DNA amount of automatically formed alginate beads were lower compared with manually manufactured beads (+P/S, -P/S) about 534.4 ng/ml (+P/S, manual screening), 393.2 ng/ml (-P/S, manual screening), 384.2 ng/ml (+P/S, automatic screening) and 740.4 ng/ml (-P/S, automatic screening). On day 35, an increased DNA amount of automatically produced beads compared with manually manufactured beads about 313.4 ng/ml (+P/S, manual screening) and 526.3 ng/ml (-P/S, manual screening), 72 ng/ml (+P/S, automatic screening) and 422.4 ng/ml (-P/S, automatic screening) was shown.

On day 14 the signal strength and DNA amount of automatically screened beads was mainly increased about 116.1 ng/ml (-P/S, manually produced), 232.5 ng/ml (+P/S, automatically produced) and 63.6 ng/ml (-P/S, automatically produced) compared to the manual bioscreening. But the signal strength of automatically screened beads showed a decreased DNA amount compared with the manually screening about 182.4 ng/ml (day 14, +P/S, manually produced), 32.2 ng/ml (day 35, +P/S, manually produced), 231.1 ng/ml (day 35, -P/S, manually produced), 8.9 ng/ml (day 35, +P/S, automatically produced) and 40.3 ng/ml (day 35, -P/S, automatically produced).
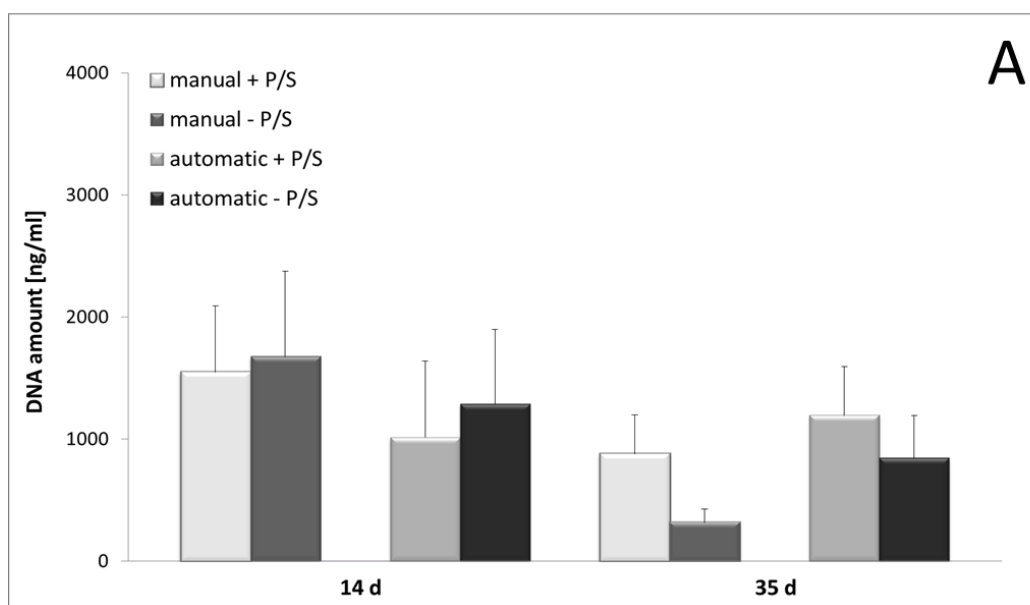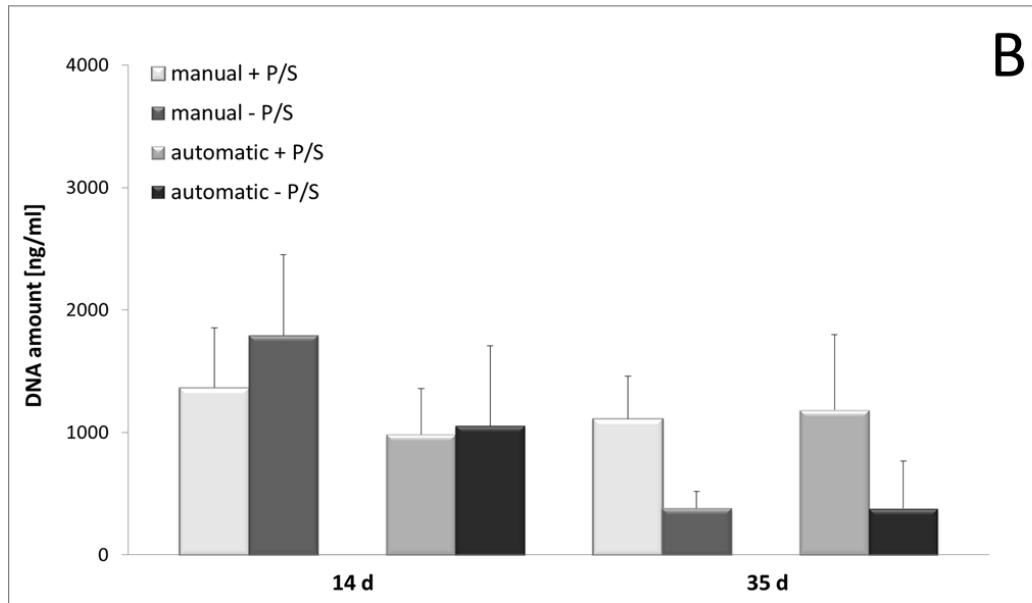
Figure 5-17 DNA amount of HeLa cells embedded in alginate matrix

DNA amount of automatically and manually produced alginate beads composed of cervix carcinoma cells in media with (+P/S) and without (-P/S) antibiotics (penicillin/streptomycin) on day 14 and day 35 compared with manual (A; manual: n≥6; automatic: n≥6) and automatic (B; manual: n≥6, automatic: n≥6) bioscreening. Statistical analysis was done with student's t-test. Data are presented as mean value ± standard error.

Spheroid Cultures

The manual and automatic DNA quantification of spheroid cultures was performed on day 14 and day 35 (see figure 5-18; manual bioscreening: n≥7 [manual], n≥6 [automatic]; automatic bioscreening: n≥6 [manual], n≥7 [automatic]). The control displayed the manual manufactured spheroids cultivated in media with antibiotics on day 14.

In general, cell proliferation and DNA amount was decreased from day 14 to day 35. The DNA amount of spheroids cultivated in media without antibiotics was lower compared to 3D cultures in media with antibiotics. The signal strength (DNA amount) of automatically screened spheroids is increased compared to manually screened spheroid cultures except of automatically produced spheroids cultivated in media without antibiotic (-P/S).

The bioscreening (manual, automatic) visualized a decreased DNA amount from day 14 to day 35 related to the control (manually produced, +P/S, day 14). The DNA amount of manually manufactured spheroids decreased about 1,926.8 ng/ml (+P/S, manual screening), 2,072.4 ng/ml (-P/S, significant: $p<0.05$, manual screening), 2,328.6 ng/ml (+P/S, automatic screening) and 2543.5 ng/ml (-P/S, significant: $p<0.05$, automatic screening). The DNA amount of automatically manufactured spheroids decreased about 1,642.9 ng/ml (+P/S, manual screening), 1,964.6 ng/ml (-P/S, significant: $p<0.05$, manual screening), 1,627.2 ng/ml (+P/S, automatic screening) and 2,498.5 ng/ml (-P/S, $p<0.05$, automatic screening).

The bioscreenings (manual, automatic) visualized always a decreased DNA amount of spheroids cultivated in media without antibiotics (-P/S) compared to the DNA amount of spheroids in media with antibiotics (+P/S) on day 14 and day 35. Herein, the decreased DNA amount of automatically manufactured spheroids in media without antibiotics (-P/S) was more marked. The DNA amount of

manually manufactured spheroids (-P/S) was reduced about 132.8 ng/ml (day 14, manual screening), 145.6 ng/ml (day 35, manual screening), 460.7 ng/ml (day 14, automatic screening) and 214.9 ng/ml (day 35, automatic screening). The DNA amount of automatically produced spheroids was decreased about 1248.2 (day 14, manual screening), 321.7 ng/ml (day 35, manual screening), 1939.7 ng/ml (day 14, automatic screening) and 871.3 ng/ml (day 35, automatic screening). Subsequently, on day 35 the DNA amount of automatically produced spheroids was higher than manually manufactured spheroid cultures (+P/S, -P/S) about 283.9 ng/ml (+P/S, manual screening), 107.8 ng/ml (-P/S, manual screening), 701.4 ng/ml (+P/S, automatic screening) and 45 ng/ml (-P/S, automatic screening). On day 14 the DNA amounts by manual and automatic bioscreening processes showed contrary results. The DNA amounts of automatically produced cultures were decreased related to manually manufactured speroids about 0.5 ng/ml (+P/S, manual screening) and 1339.1 ng/ml (-P/S, automatic screening). Instead, DNA amounts of automatically produced spheroids were higher compared with manually manufactured beads about 1,115.9 ng/ml (-P/S, manual screening) and 139.9 ng/ml (+P/S, automatic screening).

The results of the manual and automatic bioscreening were similar. Herein, the DNA amount of automatically screened spheroids was mainly increased about 501 ng/ml (day 14, +P/S, manually produced), 173.1 ng/ml (day 14, -P/S, manually produced), 641.4 ng/ml (day 14, +P/S, automatically produced), 99.2 ng/ml (day 35, +P/S, manually produced), 29.9 ng/ml (day 35, -P/S, manually produced) and 516.7 ng/ml (day 35, +P/S, automatically produced) compared to the manually bioscreening. Certainly, the DNA amount of automatically screened and manufactured spheroids cultivated in media without antibiotics was decreased about 50.1 ng/ml (day 14) and 32.9 ng/ml (day 35) [52].
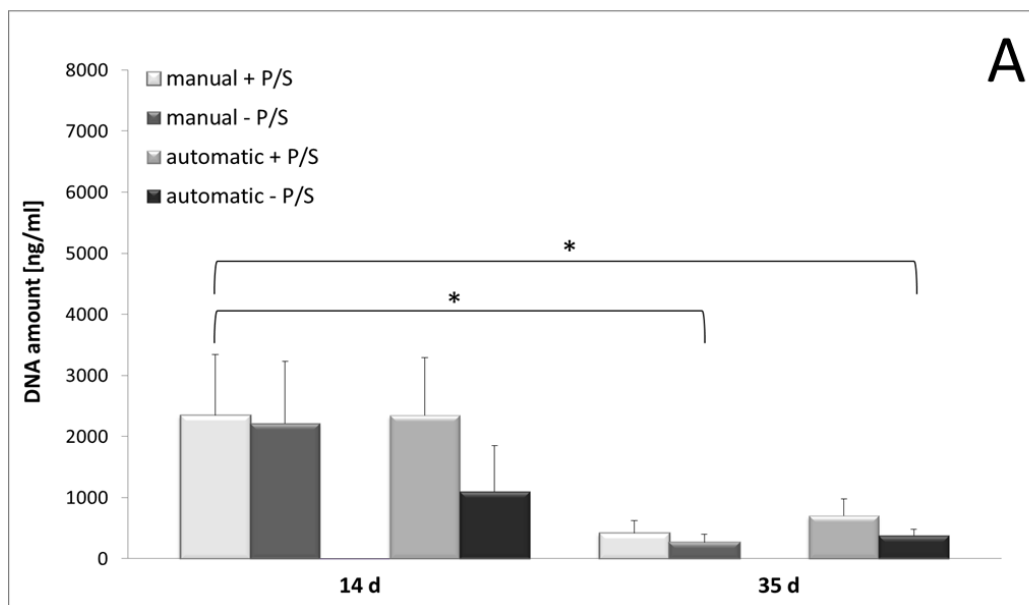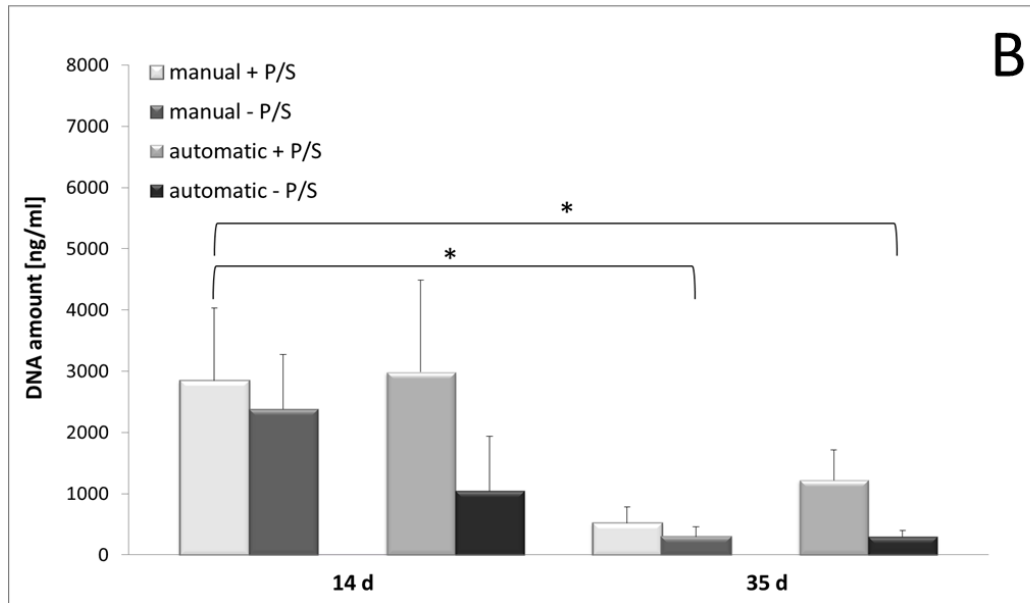
Figure 5-18 DNA amount of spheroid cultures (HeLa cells) formed in hanging drops

DNA amount of automatically and manually produced spheroid cultures composed of cervix carcinoma cells and formed in hanging drops in media with (+P/S) and without (-P/S) antibiotics (penicillin/streptomycin) on day 14 and day 35 compared with manual (A; manual: n≥7; automatic: n≥6) and automatic (B; manual: n≥6, automatic: n≥7) bioscreenings. Statistical analysis was done with student's t-test. Data are presented as mean value ± standard error. Significances of the comparison from day 14 until day 35 are based on the manually produced beads cultivated in media with antibiotic on day 14: *$p < 0.05$.

Pellets Cultures

The DNA quantification (manual and automatic) of manually and automatically produced pellet cultures was detected on day 14 and day 35 (see figure 5-19; manual bioscreening: n≥6 [manual], n≥7 [automatic]; automatic bioscreening: n≥6 [manual], n≥8 [automatic]). The manually manufactured pellet cultures cultivated in media with antibiotic (day 14) displayed the control.

In general, the DNA amount of pellet cultures increased from day 14 to day 35. The DNA amount of pellets cultivated in media without antibiotics (-P/S) was mainly higher than DNA amounts of pellets in media with antibiotics (+P/S). Subsequently, the DNA amount of automatically produced pellet cultures was higher than manually manufactured pellets.

In detail, the manual and automatic screening showed an increased DNA amount from day 14 to day 35 compared to the control (manually produced, +P/S, day 14). The DNA amount of manually manufactured pellets increased about 1,153.5 ng/ml (+P/S, manual screening), 719.8 ng/ml (-P/S, manual screening), 1,962.4 ng/ml (+P/S, automatic screening) and 1,017.7 ng/ml (-P/S, automatic screening). The DNA amount of automatically manufactured pellets enhanced about 2,821.3 ng/ml (+P/S, manual screening), 788.2 ng/ml (-P/S, manual screening), 2,369.3 ng/ml (+P/S, automatic screening) and 600.6 ng/ml (-P/S, automatic screening).

Additionally, the bioscreenings (manual, automatic) showed a lower DNA amount of pellets cultivated in media without antibiotics (-P/S) instead of pellets in media with antibiotics (+P/S) on day 14 and day 35. The decreased DNA amount of automatically manufactured pellets in media without antibiotic (-P/S) was higher. The DNA amount of manually manufactured pellets (-P/S) was reduced about 141.9 ng/ml (day 14, manual screening), 433.7 ng/ml (day 35, manual screening), 601.7 ng/ml (day 14, automatic screening) and 964.7 ng/ml (day 35, automatic screening). The DNA amount of

automatically formed pellets was reduced about 1,764.2 (day 14, manual screening), 2,033.1 ng/ml (day 35, manual screening), 2,203.9 ng/ml (day 14, automatic screening) and 1,768.2 ng/ml (day 35, automatic screening). On day 14, the DNA amount of automatically formed pellets were increased compared to manually manufactured pellets (+P/S, -P/S) about 2,340.9 ng/ml (+P/S, manual screening), 717.6 ng/ml (-P/S, manual screening), 2,165.5 ng/ml (+P/S, automatic screening) and 562.8 ng/ml (-P/S, automatic screening).

On day 35, the DNA amounts of automatically produced pellets were almost higher compared to manually manufactured beads about 1,667.6 ng/ml (+P/S, manual screening) and 68.4 ng/ml (-P/S, manual screening) and 497.4 ng/ml (+P/S, automatic screening). Instead, DNA amounts of automatically produced and screened pellets were reduced compared to manually manufactured pellets about 416.1 ng/ml (+P/S).

The signal strength and DNA amount of automatically screened cultures was slightly increased compared to the manual bioscreening about 555.1 ng/ml (day 14, +P/S, manually produced), 95.3 ng/ml (day 14, -P/S, manually produced), 380.2 ng/ml (day 14, +P/S, automatically produced), 1364 ng/ml (day 35, +P/S, manually produced), 853 ng/ml (day 35, -P/S, manually produced), 103 ng/ml (day 35, +P/S, automatically produced) and 368.5 ng/ml (day 35, -P/S, automatically produced). Instead, the DNA amount of automatically produced and screened pellets cultivated in media without antibiotics (-P/S) was reduced about 59.5 ng/ml compared to the manual screened pellets [54].
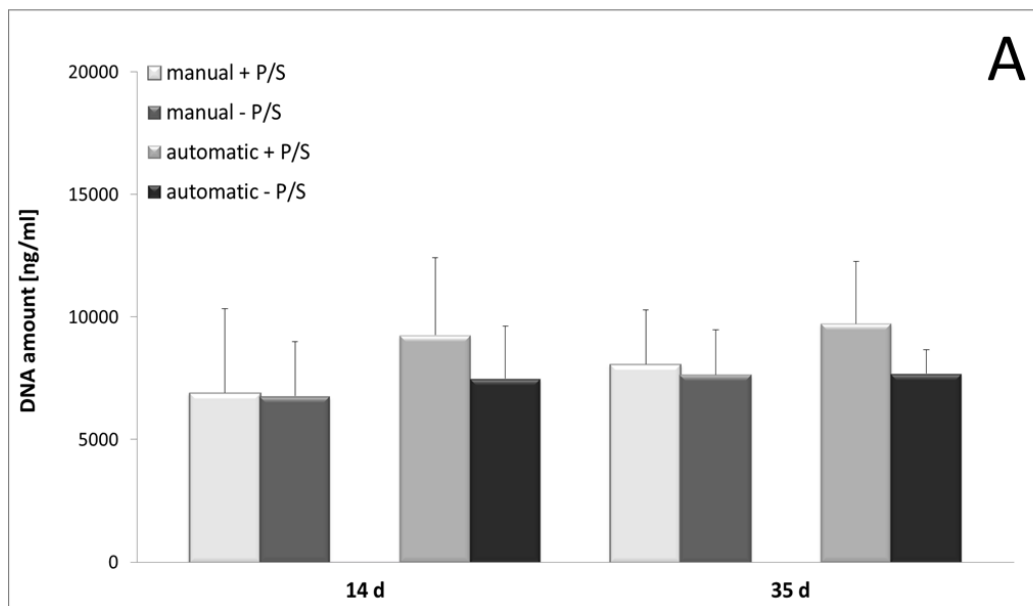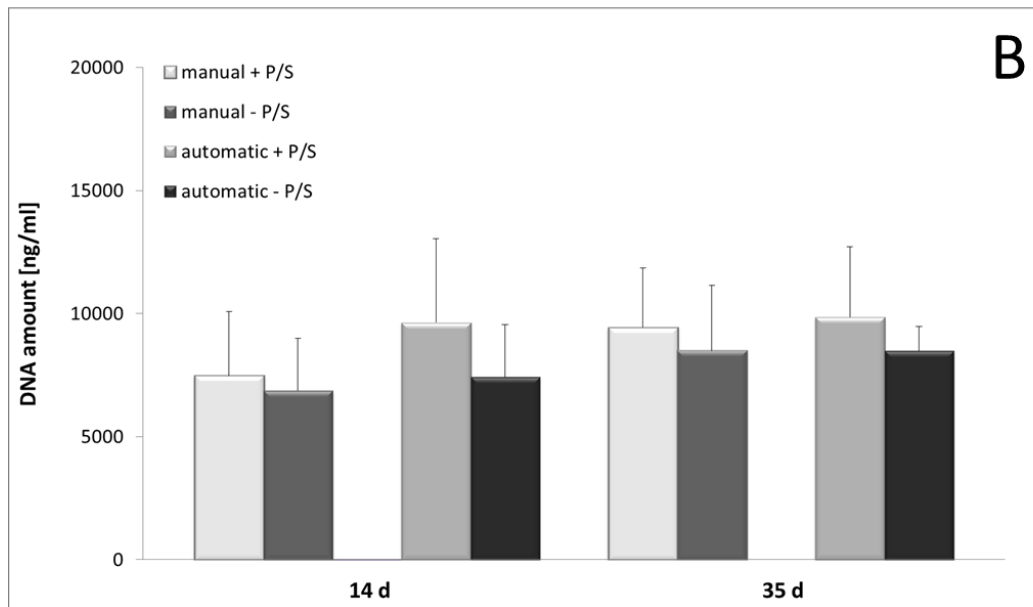
Figure 5-19 DNA amount of human chondrocytes in pellet cultures

The DNA amount of manually and automatically manufactured pellet cultures of human primary chondrocytes cultivated in media with (+P/S) and without (-P/S) antibiotics at two detection time points (day 14, day 35) compared with manual (A) (manually produced pellets: n≥6; automatically produced pellets: n≥7) and automatic (B) bioscreening procedures (manually produced pellets: n=6; automatically produced pellets: n=8). Statistical analysis was done with student's t-test. Data are presented as mean value ± standard error.

Summary – DNA Quantification

The proliferation of manually and automatically produced 3D constructs (alginate beads, spheroid cultures, pellet cultures) was also evaluated by DNA quantification on day 14 and day 35. The results are summarized in the following table:

Table 5-10 Summery of the DNA quantification

Feature	Alginate beads	Spheroid cultures	Pellet cultures
Proliferation by DNA quantification	• mainly decreased DNA amounts from day 14 to day 35 (+P/S,-P/S) • day 14: higher DNA amounts (-P/S); day 35: contrary effect • automatically screened beads: mainly increased DNA amount (day 14); day 35: contrary effect	• decreased DNA amount from day 14 to day 35 • higher DNA amounts (+P/S) on day 14 and 35 • mainly higher DNA amount of automatically produced spheroids on day 14 and 35 • automatically screened spheroids: mainly higher DNA amounts	• increased DNA amount from day 14 to day 35 • mainly higher DNA amounts (+P/S) on day 14 and 35 • automatically screened pellets: higher DNA amounts

5.3.2.2 Cytotoxicity

Adenylate Kinases Assay

The toxicity was directly evaluated by the determination of the adenylate kinase (AK) that is released through the plasma membrane of damaged cells in the supernatant. This detection of the screenings was manually and automatically evaluated on day 14 and day 35.

Alginate Beads

The cytotoxicity bioscreening (manual and automatic) of manually and automatically performed cells embedded in alginate matrix was discovered on day 14 and day 35 (see figure 5-20; manual bioscreening: $n\geq16$ [manual], $n\geq19$ [automatic]; automatic bioscreening: $n\geq9$ [manual], $n\geq6$ [automatic]; in eight replicates). Manually manufactured alginate beads cultivated in media with antibiotics on day 14 have been used as control.

Generally, the toxicity decreased from day 14 to day 35. The release of AK by cells encapsulated in alginate and cultivated in media with antibiotics (+P/S) was higher compared to cells in alginate beads in media without antibiotics (-P/S). The automatic bioscreening showed an increased cytotoxicity compared with the manual screening.

On day 35, both screening forms (manual, automatic) visualized mainly significant decreased cell cytotoxicity from HeLa-alginate beads cultivated in media without antibiotics compared to the control on day 14 (manual, + P/S) plus automatic screened beads with antibiotics. The HeLa-alginate beads cultivated in media without antibiotic (-P/S) showed a significantly reduced cytotoxicity about 36 % (manually manufactured, $p<0.01$, manual screening), 55 % (automatic manufactured, $p<0.001$, manually screening), 55 % (manual manufactured, $p<0.001$, automatic screening) and 34 % (automatic manufactured, $p<0.05$, automatic screening). The AK release of HeLa-alginate beads cultivated in media with antibiotics (+P/S) decreased rarely about 3 % (manual manufactured, manually screening) and 4 % (automatic manufactured, manually screening) whereas the automatic screening showed significant differences about 39 % (manual manufactured, $p<0.01$) and 33 % (automatic manufactured, $p<0.05$).

The AK release of alginate-embedded cells in media without antibiotic (-P/S) was lesser reduced to beads cultivated with antibiotics (+P/S). At the manually screening processes, cytotoxicity of beads without antibiotics (-P/S) was decreased about 10 % (day 14, manual produced), 18 % (day 14, automatic produced), 34 % (day 35, $p<0.05$, manual produced) and 53 % (day 35, $p<0.05$, automatic produced). The automatic bioscreening revealed a reduced cytotoxicity of beads without antibiotics (-P/S) about 12 % (day 14, manual produced), 8 % (day 14, automatic produced), 25 % (day 35, manual produced) and 2 % (day 14, automatic produced). The comparison of the manually and automatically manufactured beads showed almost comparable released AK levels of alginate beads in media with (+P/S) and without (-P/S) antibiotics on day 14 as well as for beads cultivated in media with antibiotics (+ P/S) on day 35 (manually and automatically screening). The toxicity of automatically produced beads performed at manual bioscreening processes was mainly lower than manual manufactured beads about 2 % (-P/S, day 14), 1 % (+P/S, day 14) and 30 % (-P/S, day 35) contrary to beads cultivated with P/S on day 14. The toxicity (automatic screening) of automatically produced beads was mainly higher than for manual manufactured beads about 2 % (-P/S, day 14), 1

% (+P/S, day 14) and 45 % (-P/S, day 35). However, the toxicity (automatic screening) of automatically produced beads was decreased about 2 % (+P/S, day 14).

The signal strength of automatically screened toxicity was always higher compared to the manual screening about more than one third (day 14) and one seventh (day 35). The toxicity of automatically screened beads increased about 44 % (+P/S, manual production, day 14), 43 % (-P/S, manual production, day 14), 38 % (+P/S, automatic production, day 14), 45 % (-P/S, automatic production, day 14), 11 % (+P/S, manual production, day 35), 21 % (-P/S, manual production, day 14), 20 % (+P/S, automatic production, day 35) and 62 % (-P/S, automatic production, day 35) [73].
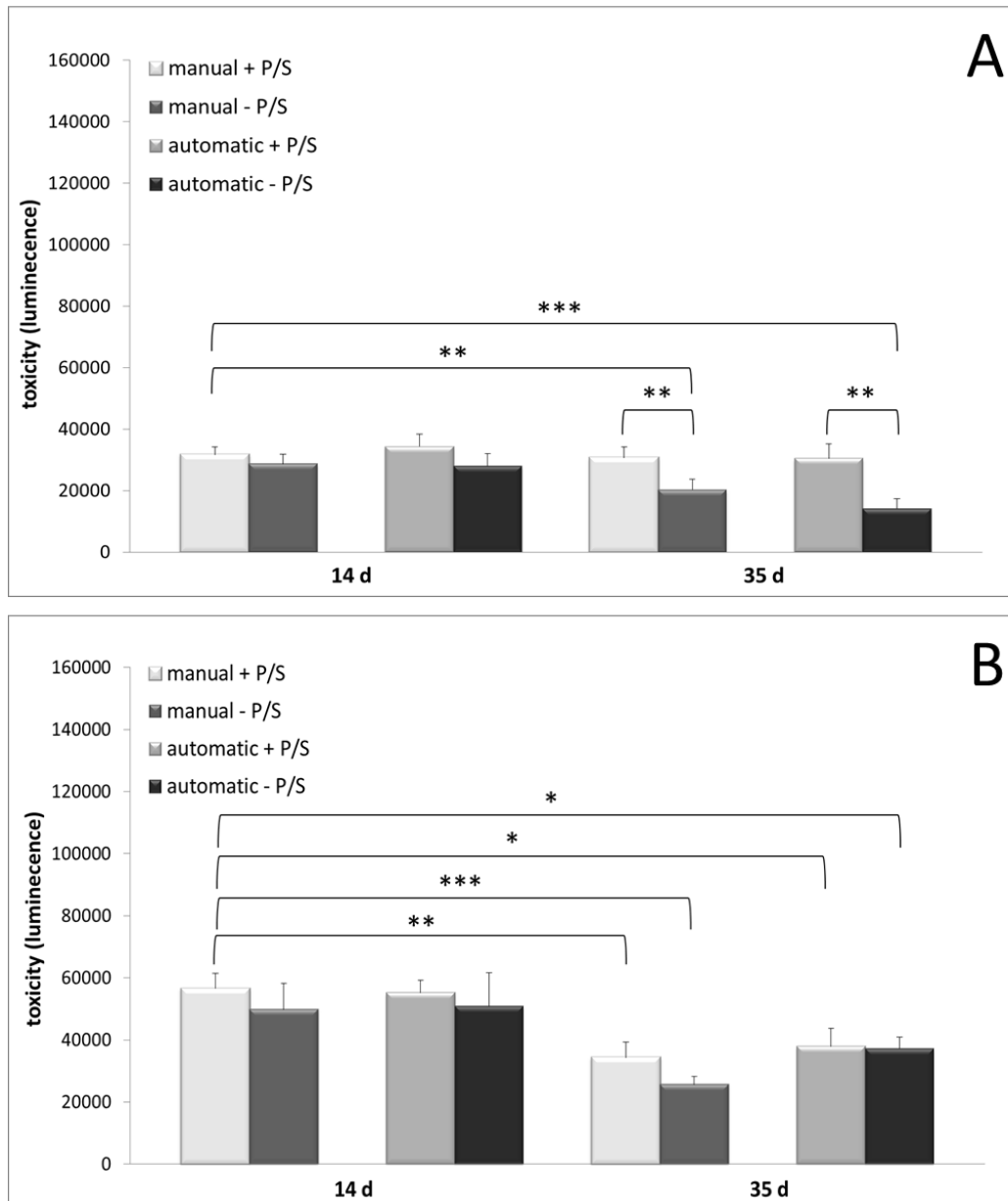
Figure 5-20 Cytotoxicity of HeLa cells encapsulated in alginate matrix detected by the AK assay

Release of adenylate kinases (AK) of automatically and manually produced HeLa cells encapsulated in alginate matrix cultivated in media with (+P/S) and without (-P/S) penicillin/streptomycin on day 14 and day 35 combined with manual (A; manual: n≥16; automatic: n≥19, in eight replicates) and automatic bioscreening (B; manual: n≥9, automatic: n≥6; in eight replicates). Statistical analysis was done with student's t-test. Data are presented as mean value ± standard error. Significances are based on the manually produced beads cultivated in media with antibiotic on day 14: ∗p<0.05, ∗∗p<0.01, ∗∗∗p<0.001 [49].

Spheroid Cultures

The toxicity of spheroids (HeLa cells) formed in hanging drops and cultivated in media with (+P/S) and without antibiotic (-P/S) were evaluated by quantification of adenylate kinases on two detection dates (day 14, day 35; see figure 5-21; manual bioscreening: n≥8 [manual], n≥6 [automatic]; automatic bioscreening: n≥9 [manual], n≥8 [automatic]; in six replicates). The manual manufactured spheroids in hanging drop cultures cultivated in media with antibiotic (+P/S) on day 14 have been used as control.

Generally, the release of AK decreased from day 14 until day 35 as well as of beads cultivated in media without antibiotics (-P/S). The automatic bioscreening showed a higher signal strength compared with manually screened spheroids.

In detail, the manual and automatic bioscreening showed an overall significantly decreased toxicity from day 14 to day 35 compared to the control. The toxicity of manually created spheroid cultures was reduced about 70 % (+P/S, p<0.01, manual screening), 74 % (-P/S, p<0.01, manual screening), 65 % (+P/S, p<0.05, automatic screening) and 75 % (-P/S, p<0.01, automatically screening). The toxicity of automatically produced spheroids was significantlydecreased about 69 % (+P/S, p<0.01, manual screening), 78 % (-P/S, p<0.001, manual screening), 64 % (+P/S, p<0.05, automatic screening) and 77 % (-P/S, p<0.01, automatic screening).

The comparison of manually and automatically produced spheroid cultures cultivated in different media revealed a reduced toxicity of spheroids in media without antibiotic (-P/S). On day 14 the toxicity of spheroids (-P/S) was slightly reduced about 11 % (manual production, manual screening), 19 % (automatic production, manual screening), 13 % (manual production, automatic screening) and 30 % (automatic production, automatic screening). On day 35 the toxicity of spheroids (-P/S) was also decreased about 13 % (manual production, manual screening), 28 % (automatic production, manual screening), 30 % (manual production, automatic screening) and 37 % (automatic production, automatic screening). The comparison of manually and automatically produced spheroid cultures in media without antibiotic (-P/S) showed decreased toxicity effects in contrast to spheroids in media with antibiotics (+P/S). The toxicity of automatically produced spheroids (-P/S) was decreased compared to the manually formed spheroids about 12 % (day 14, manual screening), 13 % (day 35, manual screening), 24 % (day 14, automatic screening) and 10 % (day 35, automatic screening). The AK rrelease of automatically formed spheroids (+P/S) were slightly increased compared with the manually designed spheroids about 3 % (day 14, manual screening), 6 % (day 35, manual screening) and 2 % (day 35, automatic screening) in contrast to automatically screened spheroids on day 14 which decreased about 5 %.

Commonly, both screening forms (manual, automatic) displayed analogous effects concerning the detection of toxicity. However, the luminescence and signal strength of automatically screened spheroids was enhanced associated with the higher toxicity of spheroids in media with antibiotics (+P/S) at both detection time points (day 14, day 35). On day 14, the cytotoxicity of automatically screened spheroids was increased about 17 % (+P/S, manual produced), 14 % (-P/S, manual produced), 15 % (+P/S, automatic produced) and 1 % (-P/S, automatic produced). On day 35, the cytotoxicity of automatically screened spheroids was enhanced about 31 % (+P/S, manual produced), 14 % (-P/S, manual produced), 27 % (+P/S, automatic produced) and 17 % (-P/S, automatic produced) [52].
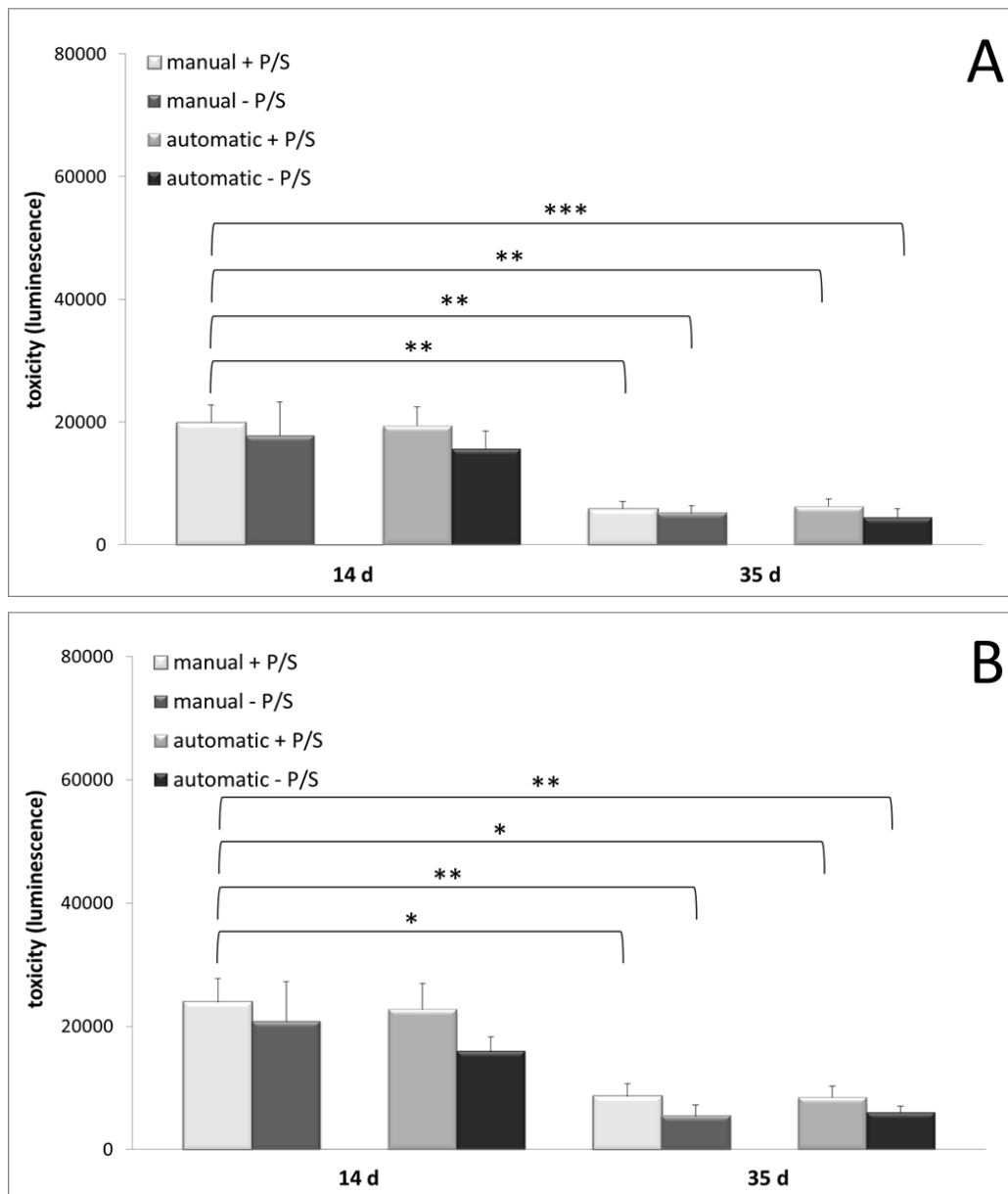
Figure 5-21 The cytotoxicity of spheroids in handing drops detected by the AK assay [52, 73]

Release of adenylate kinases (AK) of automatically and manually produced spheroid cultures formed in hanging drops cultivated in media with (+P/S) and without (-P/S) penicillin/streptomycin on day 14 and day 35 compared with manual (A; manual: n≥8, automatic: n≥6, in 6 replicates) and automatic (B; manual: n≥9, automatic: n≥8, in 6 replicates) bioscreening. Statistical analysis was done with student's t-test. Data are presented as mean value ± standard error. Significances of the comparison from day 14 until day 35 are based on the manually produced beads cultivated in media with antibiotic on day 14: *p<0.05, **p<0.01 and ***p<0.001 [52].

Pellets Cultures

The quantification of the cytotoxicity was manually and automatically performed on two detection time points (day 14, day 35; see figure 5-22; manual bioscreening: n≥6 [manual, automatic]; automatic bioscreening: n≥8 [manual, automatic]; in eight replicates). The manually manufactured pellet cultures cultivated in media with antibiotics on day 14 have been used as control.

In general, the cytotoxicity of manually and automatically produced pellets cultivated in different media (+P/S, -P/S) mainly decreased from day 14 to day 35 whereby the pellets in media without antibiotic showed enhanced toxicity levels compared to media with antibiotics. The signal strength of automatically screened beads was slightly higher compared to the manual performed screening.

In detail, the bioscreenings showed mainly a decreased cytotoxicity of pellet cultures related to the control from day 14 to day 35. The toxicities of pellet cultures in media with antibiotics (+P/S) were generally reduced about 21 % (manual production, manual screening), 23 % (automatic production, manual screening), 18 % (manual production, automatic screening) and 16 % (automatic production, automatical screening) on day 35. The toxicities (day 35) of pellet cultures in media without antibiotics (-P/S) were decreased about 7 % (manual production, manual screening) and 2 % (automatic production, automatic screening) contrary to manually produced pellets which were automatically screened. On day 35, the AK release toxicity of pellets (-P/S, automatic production, manual screening) was similar compared with the control.

The comparison of manually and automatically produced pellets showed an increased toxicity of pellets cultivated in media without antibiotics (-P/S) compared with pellets in media with antibiotics (+P/S) whereby the difference was stronger on day 35 of manually produced pellets. On day 14, the toxicity of pellets in media without antibiotics is slightly increased about 5 % (manual production, manual screening) and 12 % (manual production, automatic screening) and 1 % (automatic production, automatic screening). However, the automatically produced and manual screened pellets cultivated in media without antibiotic (-P/S) were similar to pellets in media with antibiotic (+P/S) on day 14. On day 35 the toxicity of pellets in media without antibiotics is increased about 18 % (manual production, manual screening), 32 % (automatic production, manual screening), 32 % (manual production, automatic screening) and 16 % (automatic production, automatic screening). Moreover, the comparison of manually and automatically formed pellet cultures displayed an increased cytotoxicity of automatically manufactured ones on day 14 about 11 % (+P/S, manual screening), 6 % (-P/S, manual screening) and 11 % (+P/S, automatic screening). Nevertheless, the automatically screened pellets (-P/S) showed the similar toxicity results. On day 35 the cytotoxicity of automatically produced pellets also increased about 9 % (-P/S, manual screening) and 2 % (+P/S, automatic screening). On day 35 the cytotoxicity of automatically produced pellets decreased about 2 % (+P/S, manual screening) and 12 % (-P/S, automatic screening).

The comparison of the screening forms (manual, automatic) showed slightly differences concerning the screening results of toxicity. On day 14, the automatically screening visualized a decreased toxicity and signal strength of pellets in media with antibiotics (+P/S) about 4 % (manual production) and 3 % (automatic production) in contrast to pellets in media without antibiotics. Herein, the toxicity increased about 4 % (manual production) and 2 % (automatic production). On day 35 the cytotoxicity and signal strength of automatically produced pellets in media with antibiotics (+P/S) was increased about 6 % (automatic production). The toxicity of automatically produced and screened pellets in media without antibiotics (-P/S) was decreased about 7 %.
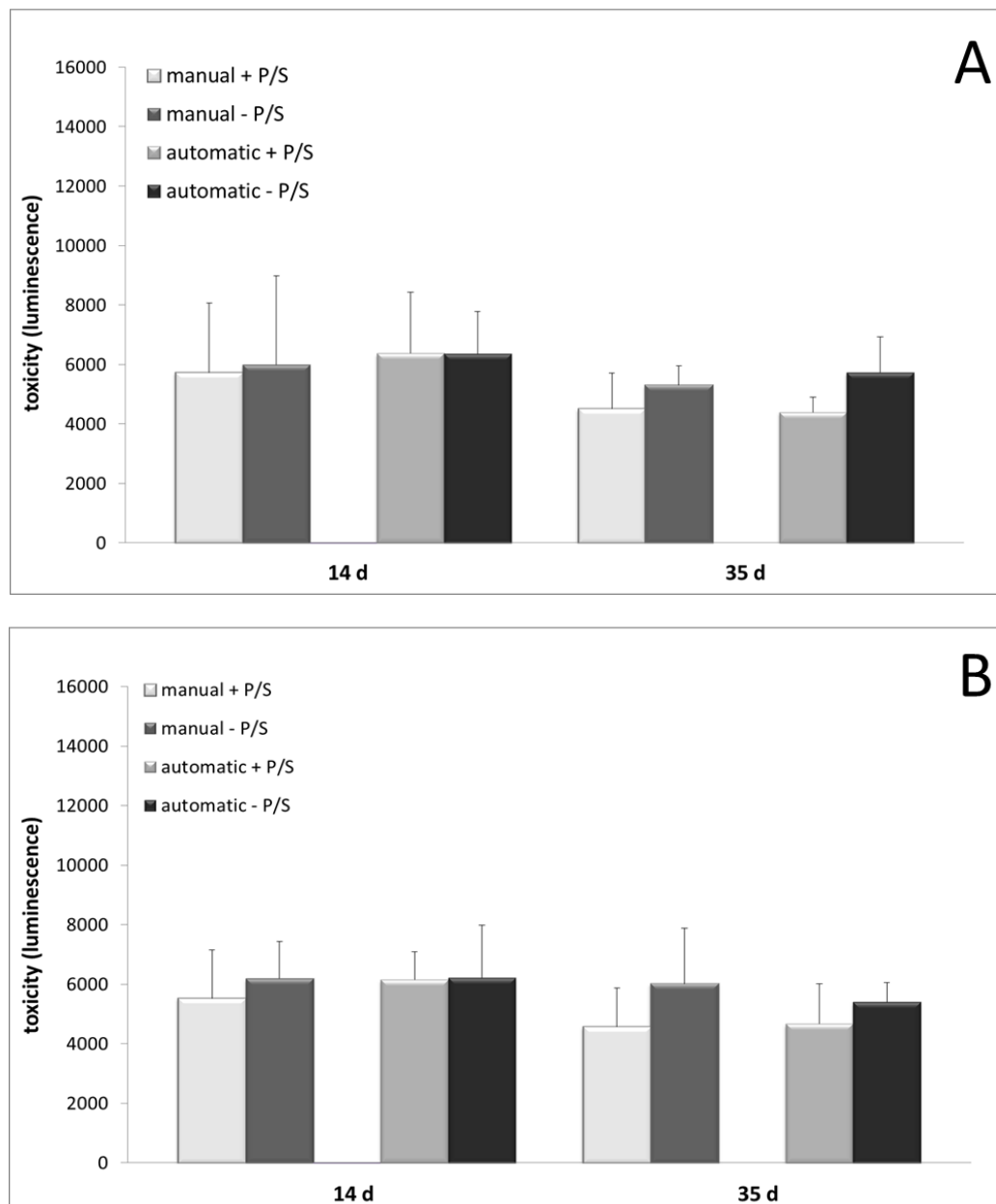
Figure 5-22 The cytotoxicity of primary chondrocytes cultivated as pellet cultures detected by the AK assay [54]

Release of adenylate kinases (AK) of automatically and manually produced pellet cultures of human primary chondrocytes in media with (+P/S) and without (-P/S) penicillin/streptomycin on day 14 and day 35 combined with manual (A) (manually produced pellets: n=6; automatically produced pellets: n≥6; in eight replicates) and automatic (B) bioscreening (manually produced pellets: n=8; automatically produced pellets: n≥8; in eight replicates). Statistical analysis was done with student's t-test. Data are presented as mean value ± standard error [54].

Summary – Adenylate Kinases Assay

The toxicity of manually and automatically produced 3D constructs (alginate beads, spheroid cultures, pellet cultures) was evaluated by the adenylate kinases assay on day 14 and day 35. The results are summarized in the following table:

Table 5-11 Summary of the cytotoxicity

Feature	Alginate beads	Spheroid cultures	Pellet cultures
Toxicity	• decreased toxicity from day 14 to day 35 • higher toxicity of cells in media + P/S • manually/automatically produced beads: comparable results • automatically screened beads: increased signal strength	• decreased toxicity from day 14 to day 35 • higher toxicity of cells in media + P/S • manually/automatically produced spheroids: comparable effects • automatic screening: increased signal strength	• decreased toxicity from day 14 to day 35 • higher toxicity of cells in media - P/S • increased cytotoxicity of automatically manufactured pellets on day 14 • manual and automatic screening: strength and cytotoxicity showed slightly differences

6 Performance

The performance compared the traditional manually processes with the automatically performed methods. The manual methods are completed by skilled laboratory staff. The automatic cell cultivations are realized using the Biomek® Cell Workstation. The automatic bioscreening was performed using a High Throughput Screening System.

The performance can be gained by looking at the throughput as well as efficiency, accuracy and standard error. These facts can be used to compare the quality of automatically produced 3D cell cultures compared with the traditional manual processes. The accuracy (a) shows possible variations of automatic produced 3D cultures related on the sizes and is calculation from the ratio of the arithmetic averages of automatic manufactured 3D cultures to manual produced cultures. The following formula visualized the evaluation of the accuracy:

$$a\ (\%) = \frac{x_2 * 100}{x_1}$$

Legend:

x_2 = arithmetic average of the sizes from manual manufactured 3D cultures

x_1 = arithmetic average of the sizes from automatic manufactured 3D cultures

The standard error of the mean (SEM) calculates the precision of the mean. The following formula imagined the evaluation of the standard error [143]:

$$SEM = \frac{s}{\overline{n}}$$

Legend:

s = variance of the probes

n = sample size

The throughput of cell culture processes is an important aspect for automatic processes. It covers the sample throughput and thus the efficiency of the process. Regularly, automation is connected with an increased throughput e.g. of cell cultures.

6.1 Cell Culture Processes

6.1.1 Throughput and Efficiency

The table 6-1 (n=3) visualizes the throughput and efficiency of different cell culture processes. The automatic processes can be performed in a 24 h mode. The automatic cell culture methods were completed by the Biomek® Cell Workstation. An operation mode of 23 hours (1,360 min.) can be realized with a loss of 1 hour in cause of cleaning steps and the addition of solutions. The manual

processes can be finished in a 6-hour (360 min.) operation shift. Herein, an 8-hour workday includes a loss of 2 hours in cause of breaks, concentration problems and other tasks.

The performance per workday is calculated with 100 %, which mean the handling of one cell culture flask or well plate (WP).

In general, all cell culture processes showed a slower cell culture process at an automatic operation mode for one process resulting from initializing processes of the devices, longer transport ways of the labware, disinfection and rinsing steps during the methods as well as slower liquid handling to prevent cell loss or separation in more steps because of a higher volume at the automatic procedures. But the projected time to disseminate and produce cell cultures at one workday in the automatic way (1,360 min.) is increased about 26 % compared with manual methods because the workday of laboratory staff is projected with 360 min. The automatic procedures show an increased throughput of plates for the production of 3D cell cultures and the seeding of suspension cells.

In detail, the basic process to detach adherent cell lines needs 34 min. (automatic) and 8 min. (manual) for one process and the projection shows the handling of 40 AutoFlasks™ (automatic) and 45 x T75 flasks (manual) performance per workday. The main reason for a longer automatic process is the liquid handling partly in stage besides initializing processes, disinfection steps and the longer transport ways. In detail, the cell culture flasks contain 15 ml medium and the removing of the supernatant needs four steps in contrast at the manual process where only one step is needed. The following automatic seeding process of adherent cells require 56 min. (one processes) and produce 15 x 96 well plates. However, the manual process needs only 6 min. and enables the production of 25 well plates. Primarily, the automatic process requires longer time since the cell seeding is not performed using eight channels like in the manual method. The automatic cell seeding by the liquid handler was carried out using three channels (5, 6, 8). Moreover, the mixing steps in the automatic process needed a longer time period. Rinsing steps of the steel cannulas are necessary to support the cell seeding of the exact cell number. The manual parallel cell seeding of four suspension cell lines is performed in 18 min. resulting in 20 well plates per workday. However, the automatic cell seeding lasts 76 min. for 18 well plates/shift. This is mainly due to the disinfections steps, the cell seeding by one channel to prevent cross contaminations and the rinsing steps to support the seeding of the exact cell number at the automatic procedure. The manual process enables the cell seeding by an eight channel pipette with the changing of the disposables after every pipetting step.

The production of 3D cell constructs consists of two sub-processes. Firstly, the adherent cell cultures have to be detached and consequently followed the 3D formation. The different 3D constructs required different amounts of cell culture flasks (alginate beads: 1 flask; spheroid cultures: 2 flasks; pellet cultures: 8 flasks) to obtain the required number of cells for one 96 well plate.

The duration of automatic alginate bead production (187 min.) for one plate is more than threefold increase compared with the manual process (56 min.) because the longer time period for the liquid handling, disinfections and rinsing steps. The main limiting steps are the sensitive removing of the supernatant from the pellets and the resuspending in alginate. But the projection shows the production of 7 plates (automatic) and 6 x 96 well plates (manual) per workday [123].

The automatic formation (98 min.) of hanging drops to produce spheroid cultures needs more than two fold more time compared with the manually method (42 min.) for one process. However, in summary, the automatic method enables the handling of 14 x 96 well plates in contrast to 8 x 96 well plates compared to the manual procedure per workday. The simple manually and automatically performed manufacturing of hanging drops is equal whereas the automatic detachment process

takes longer time because of the initializing steps like disinfection and rinsing of cannulas as well as the liquid handling in stages [123].

The automatic seeding of chondrocytes (460 min.) to form pellet cultures over the time needs also more than two fold more time related to the manually method (177 min.). But the projected performance visualizes the manufacturing of 3 x 96 well plates (automatic) and 2 x 96 well plates (manual) at one workday. The automatically performed pellet formation takes longer because of all disinfection steps. Especially, the automatic removing of supernatant is performed by using only one channel in a slow aspirating step to prevent cell loss. Moreover, the rinsing of the steel cannulas is important to seed the same cell number per well. Disposables of the manual pellet formation are changed, which saves time. Contrarily, the supernatant of the automatic process is changed at the specific wash station, which could not positioned next to the ALP at the liquid handler deck and is therefore time-consuming [123].

Table 6-1 Overview about the throughput and efficiency of cell culture processes

Feature	Method	Operation	Total duration of one process [min]	Production time per workday/ shift [min]	Projected performance per workday/shift (labware)
Throughput and efficiency	detachment process of adherent cells (4 min. incubation period)	automatic	34	1,380	40xAutoFlasks™
		manual	8	360	45xT75
	cell seeding of adherent cells	automatic	90 (34 min. cell detachment + 56 min. seeding)	1,380	15 WPs[1]
		manual	6 (8 min. cell detachment + 6 min. seeding)	360	25 WPs[1]
	cell seeding of suspension cells (4 cell lines)	automatic	76	1,380	18 WPs[1]
		manual	18	360	20 WPs[1]
Continued on next page					

Feature	Method	Operation	Total duration of one process	Production time per	Projected performance per

			[min]	workday/ shift [min]	workday/shift (labware)
Throughput and efficiency	alginate bead production	automatic	187 (34 min. cell detachment + 153 min. formation)	1,380	7 WPs[1]
		manual	56 (8 min. cell detachment + 48 min. formation)	360	6 WPs[1]
	spheroid production formed in hanging drops	automatic	98 (68 min. cell detachment + 30 min. formation)	1,380	14 HD-plates[2]
		manual	42 (16 min. cell detachment + 26 min. formation)	360	8 HD-plates[2]
	pellet culture production	automatic	460 (320 min. cell detachment + 140 min. formation)	1,380	3 WPs[1]
		manual	177 (112 min. cell detachment + 65 min. formation)	360	2 WPs[1]

Notes:

[1]WPs: 96 well plates

[2]HD-plates: 384 Perfect® Hanging Drop Well Plates

6.1.2 Accuracy - Size

The alginate beads and spheroid cultures enabled the size detection because of their shape. This permits the view to accuracy and standard error of the production process. Table 6-2 displays the accuracy related to the sizes (diameter) of alginate beads (automatic: n=10 [+P/S], n=6 [-P/S]; manual: n=8 [+P/S], n=7 [-P/S] and spheroid cultures (automatic: n=6 [+P/S, 14d], n=11 [+P/S, 35d], n=6 [-P/S, 14d], n=8 [-P/S, 35d]; manual: n=7 [+P/S, 14d], n=11 [+P/S, 35d], n=6 [-P/S, 14d], n=10 [-P/S, 35d]).

The comparison of the manual and automatic production of alginate beads showed an increase of diameter of automatic performed beads about 111.8 µm (+P/S) and 14 µm (-P/S). The automatic method produced less than 3.5 % bigger alginate beads compared to the standard method (manual process).

The evaluation of the spheroid cultures after manually and automatically manufacturing showed a decreased diameter of automatically performed spheroids about 28.5 µm (+P/S, day 14), 24.7 µm (-P/S, day 14) and 19.8 µm (+P/S, day 35). Only the diameter of automatically produced beads cultivated in media with antibiotics (+P/S) was increased about 24.4 µm on day 35. Thus, the automatic method showed differences of less than 7.2 % compared to the standard method (manual manufacturing).

Table 6-2 Overview about the accuracy in terms of the size (alginate beads, spheroid cultures; automatic: n≥6, manual: n≥7)

Feature	Method	Operation	Diameter
Accuracy (size)	alginate beads production	automatic	3,195.6 µm (+P/S), 3,185.4 µm (-P/S)
		manual	3,083.8 µm (+P/S), 3,171.4 µm (-P/S)
	spheroid production	automatic	335.4 µm (+P/S, day 14), 350.2 µm (-P/S, day 14); 269.7 µm (+P/S, day 35), 334.8 µm (-P/S, day 35)
		manual	363.9 µm (+P/S, day 14), 374.9 µm (-P/S, day 14); 289.5 µm (+P/S, day 35), 310.4 µm (-P/S, day 35)

6.1.3 Standard Error - Size

Table 6-3 displays the standard errors in terms of the sizes of alginate beads (automatic: n=10 [+P/S], n=6 [-P/S]; manual: n=8 [+P/S], n=7 [-P/S] and spheroid cultures (automatic: n=6 [+P/S, 14d], n=11 [+P/S, 35d], n=6 [-P/S, 14d], n=8 [-P/S, 35d]; manual: n=7 [+P/S, 14d], n=11 [+P/S, 35d], n=6 [-P/S, 14d], n=10 [-P/S, 35d]). The standard errors of the new adapted automatic processes were compared with the commonly manual methods.

The automatically manufactured alginate beads visualized a decreased standard error of beads cultivated in media with antibiotics (+P/S) about 80 % (41.1 µm) but an increased standard error in media without antibiotics about 20.9 % (12.2 µm) compared to manually performed spheroid cultures (standard) at the different media (±P/S).

On day 14, the standard errors of automatically performed spheroid cultures were enhanced about 15.9 µm (+P/S) and 0.3 µm (-P/S). The standard error of automatically produced spheroids was almost similar compared to manually performed spheroids (standard) on day 14. However, on day 35, the standard error of automatically manufactured spheroids was decreased about 1.7 µm (+P/S) and 34.6 µm (-P/S) resulting in a reduced standard error of the automatic method about 6 % (+P/S) and even 79.9 % (-P/S).

Table 6-3 Overview about the standard error in terms of the size (alginate beads, spheroid cultures; automatic: n≥6, manual: n≥7)

Feature	Method	Operation	Results [µm]
Standard error (size)	alginate beads	automatic	10.1 (+P/S), 58.4 (-P/S)

	production	manual	51.2 (+P/S), 46.2 (-P/S)
	spheroid production formed in hanging drops	automatic	47.0 (+P/S, day 14), 29.6 (-P/S, day 14); 26.8 (+P/S, day 35), 8.7 (-P/S, day 35)
		manual	32.1 (+P/S, day 14), 29.3 (-P/S, day 14); 28.5 (+P/S, day 35), 43.3 (-P/S, day 35)

6.2 High Throughput Screening (HTS) System

6.2.1 Throughput – HTS System

The following table 6-4 visualizes the throughput and efficiency of different bioscreening processes (n=3) in case of the duration of one screening process and its performance per workday of the serial process (manual: 360 min.; automatic: 1,380 min.). One process means the seeding of one well plate (WP).

In general, looking at the duration of one processes the automatic processes required longer times compared with the manual screening. But in consideration of the projected performance per workday of the serial procedures of a single processes, the throughput can be increased due to the longer production time per workday.

In detail, the combination of automatically performed EZ4U- and AK assay (199 min.) needed 9 min. longer than the traditional manual processes (190 min.). But the automatic methods would enable the 6 fold more processing procedures of bioscreenings and plates (6 WP) at the projected performance per workday for a serial procedure of a single process instead of the manually method (1 WP). The automatic DNA quantification (12 min.) required 4 min. more time for one processes (plate) compared to the manual screening (8 min.). However, the projected performance for the serial procedure visualize a 2.5-fold increase of plates processing and quantifications of automatic procedures (115 WP) compared with the manual quantification (45 WP). The duration of one process needed 6 minutes longer at the automatically performed screening (251 min.) compared with the manual screening (245 min.) in case of the WST assay. Nevertheless, the projected automatic bioscreening (WST assay) to detect the proliferation would enable a fivefold increase of plate processing at the automatic screening (5 WP) compared with the manual method (1 WP).

Table 6-4 Overview about the throughput in terms of bioscreening processes (n=3)

Feature	Method	Operation	Total duration of one process (plate) [min]	Production time per workday/	Projected performance per workday (labware)

				shift [min]	serial procedures of the single processes
Throughput and efficiency	EZ4U assay + AK assay	automatic	199	1380	6 WP
		manual	190	360	1 WP
	DNA quantification	automatic	12	1380	115 WP
		manual	8	360	45 WP
	WST assay	automatic	251	1380	5 WP
		manual	245	360	1 WP

6.2.2 Standard errors – HTS System

6.2.2.1 2D Cell Culture

WST Assay

The WST-proliferation assay is a traditional screening method to evaluate the proliferation of suspension cells by manual process steps. The WST assay was adapted at the HTS system to quantify the proliferation automatically. The different cell lines were cultivated in media with antibiotics (+P/S). The cell seeding was performed using the both processes (automatic [SEM: n=6; Jurkat, RS4, Molt4: n=7], manual [Jurkat: n=6; SEM, Molt4: n=8; RS4=9) with consequently automatic bioscreening. The standard errors are visualized in table 6-5.

In general, the automatic cell seeding enabled a reduction of standard errors during the automatic bioscreening compared to the traditional manual cell handling. The automatic cell seeding presented resulted in decreased standard errors about 19.4 % (Jurkat), 13.3 % (Molt4) and 12.5 % (RS4). However, the standard errors of automatically and manually performed SEM cells were similar.

Table 6-5 Overview about the standard error of suspension cell lines in terms of the WST assay (n≥6)

Feature	Method	Operation	Screening	Results [μm]
Standard error	WST assay (Jurkat cells)	automatic	automatic	0.29 (+P/S)
		manual		0.36 (+P/S)
Continued on next page				

Feature	Method	Operation	Screening	Results [μm]
	WST assay (SEM cells)	automatic	automatic	0.30 (+P/S)
		manual		0.30 (+P/S)
	WST assay	automatic	automatic	0.13 (+P/S)

	(Molt-4 cells)			
		manual		0.15 (+P/S)
	WST assay (RS-4 cells)	automatic	automatic	0.07 (+P/S)
		manual		0.08 (+P/S)

6.2.2.2 3D Cell Cultures

The following tables (6-6, 6-7, 6-8) give an overview about the standard errors of the bioscreening processes to investigate the different 3D cell culture systems (alginate beads, spheroid cultures, pellet cultures). The limiting factor was the number of produced 3D cell cultures and not the throughput. The quality of 3D cell cultures can be shown by visualization of standard errors of the bioscreening processes.

EZ4U Assay

The table 6-6 displays an overview about the standard errors of the manually and automatically produced 3D cell cultures in terms of the EZ4U assay. In general, the EZ4U proliferation bioscreenings (manual and automatic) demonstrated oftenly a lower standard error of automatically produced 3D cell cultures (alginate beads, spheroid cultures, pellet cultures) compared to the manually produced 3D constructs. The standard error of automatically screened beads was mainly lower compared to the manual handling in contrast to spheroid cultures. The pellet cultures visualized contrary standard errors.

Alginate Beads

The comparison of the both production processes (manual, automatic) presented mostly lower standard errors of automatically manufactured beads (±P/S) of the bioscreening methods (manual, automatic) compared with manually formed beads. The manual screening of automatically produced alginate beads showed an increased standard error about 53.2 % (day 14, +P/S), 6.9 % (day 35, +P/S) and 19.7 % (day 35, -P/S) contrary to day 14 where beads cultivated without antibiotics showed a decreased standard error about 2.2 %. However, the automatically screened and produced alginate beads displayed a decreased standard error about 50.8 % (day 14, +P/S), 47.9 % (day 14, -P/S), 57.1 % (day 35, +P/S) and 66.1 % (day 35, -P/S) compared to the manually manufactured beads.

The comparison of different bioscreening forms (manual, automatic) visualized mostly lower standard errors at the automatic screening compared with the manual proliferation screening processes. The standard errors of automatically produced and screened beads were reduced about 59.8 % (day 14, +P/S), 68.7 % (day 14, -P/S), 29.2 % (day 35, +P/S) and 82.1 % (day 35, -P/S) compared to the manually screened beads. The manually produced beads showed higher standard errors at the automatic bioscreening in media with antibiotics about 42.9 % (day 14) and 43.7 % (day 35). Instead, manually formed beads in media without antibiotics displayed a lower standard error about 41.6 % (day 14) and 33 % (day 35) for the automatic screening processes compared to the manual screening.

The standard errors of alginate beads (-P/S; manual, automatic) decreased at the bioscreening process (EZ4U assay) in contrast to beads in media with antibiotic (+P/S) with increasing standard errors from day 14 to day 35. The standard errors of cells embedded in alginate were reduced about 12.7 % (-P/S, manual screening, automatic production), 31.4 % (-P/S, manual screening, manual production), 50 % (-P/S, automatic screening, automatic production) and 22.5 % (-P/S, automatic screening, manual production) over the time. But the standard errors of cells in alginate matrix (+P/S) were mostly enhanced about 46.3 % (+P/S, manual screening, manual production), 39.2 % (+P/S, automatic screening, automatic production) and 47.1 % (+P/S, automatic screening, manual production) in contrast to beads (+P/S, manual screening, automatic production) with reduced standard errors about 6.5 % from day 14 to day 35.

The sample amounts of the manual screening, which were used to calculate the standard errors, are: n=18 (+P/S, manual production, 14d), n=19 (+P/S, manual production, 35d), n=20 (-P/S, manual production, 14d), n=13 (-P/S, manual production, 35d), n=26 (+P/S, automatic production, 14d), n=15 (+P/S, automatic production, 35d), n=27 (-P/S, automatic production, 14d) and n=12 (-P/S, automatic production, 35d). The sample totals of the automatic screening, which were used to estimate the standard errors, are: n=10 (+P/S, manual production, 14d), n=8 (+P/S, manual production, 35d), n=10 (-P/S, manual production, 14d), n=6 (-P/S, manual production, 35d), n=10 (+P/S, automatic production, 14d), n=11 (+P/S, automatic production, 35d), n=8 (-P/S, automatic production, 14d) and n=7 (-P/S, automatic production, 35d).

Spheroid Cultures

The comparison of the different production processes (manual, automatic) presented mostly higher standard errors of automatically manufactured spheroids compared with the traditional manual formed spheroids in the proliferation bioscreening. The screening (manual) of automatically produced spheroid cultures displayed mainly an enlarged standard error about 52.8 % (day 14, +P/S), 73.1 % (day 14, -P/S) and 47.4 % (day 35, -P/S) compared with manually shaped spheroids. This was contrary to spheroids in media with antibiotic on day 35 with a reduced standard error about 23.3 %. The automatically screened and produced alginate beads showed an increased standard error about 51.7 % (day 14, +P/S), 50 % (day 14, -P/S) and 12.3 % (day 35, +P/S) compared with the manually made beads. Instead, the automatically produced and screened spheroids in media without antibiotic displayed a lower standard error about 31.2 %.

The comparison of the both bioscreening processes (manual, automatic) displayed mainly increased standard errors of automatically screened spheroids (±P/S) compared with manual bioscreening which was in contrast to standard errors of manually produced spheroid cultures in media without antibiotic (-P/S). The manual and automatic bioscreenings showed higher standard errors of automatically produced and screened spheroids in media with antibiotics about 59.7 % (day 14) and 82.1 % (day 35) compared to manually formed spheroids. Instead, the automatically handled spheroids in media without antibiotics showed lower standard errors of about 59.7 % (day 14) and 82.1 % (day 35). The manually produced spheroids showed higher standard errors for the automatic bioscreening of spheroids cultivated in media with antibiotics about 19.7 % (day 14) and 42.1 % (day 35). Instead, manually formed spheroids in media without antibiotics display a lower standard error of about 10.3 % (day 14) and 42.1 % (day 35) for the automatic screening processes compared with the manual screening.

The standard errors of spheroid cultures (±P/S; manual, automatic) were mostly reduced at the bioscreening procedures (EZ4U assay) to detect the proliferation standard errors of manually produced spheroids in media with antibiotics from day 14 to day 35. The standard errors decreased about 37.7 % (+P/S, manual screening, automatic production), 75.6 % (-P/S, manual screening, automatic production), 52.4 % (-P/S, manual screening, manual production), 13.6 % (+P/S, automatic screening, manual production), 84.3 % (-P/S, automatic screening, automatic production) and 54.3 % (-P/S, automatic screening, manual production). However, on day 35 the standard errors of manually produced spheroids in media with antibiotics (+P/S) increased about 41.9 % (manual screening) and 42 % (automatic screening).

The sample sums of the manual screening, which were used to estimate the standard errors, are: n=13 (+P/S, manual production, 14d), n=8 (+P/S, manual production, 35d), n=9 (-P/S, manual production, 14d), n=9 (-P/S, manual production, 35d), n=12 (+P/S, automatic production, 14d), n=6 (+P/S, automatic production, 35d), n=11 (-P/S, automatic production, 14d) and n=7 (-P/S, automatic production, 35d). The sample sums of the automatic screening, which were used to calculate the standard errors, are: n=15 (+P/S, manual production, 14d), n=9 (+P/S, manual production, 35d), n=10 (-P/S, manual production, 14d), n=8 (-P/S, manual production, 35d), n=12 (+P/S, automatic production, 14d), n=8 (+P/S, automatic production, 35d), n=13 (-P/S, automatic production, 14d) and n=8 (-P/S, automatic production, 35d).

Pellet Cultures

Additionally, the comparison of the different production processes (manual, automatic) presented mainly lower standard errors of automatically produced pellet cultures compared with the manual manufacturing method. The standard errors were decreased about 17.2 % (day 14, +P/S), 33 % (day 14, -P/S) and 34.5 % (day 35, +P/S) compared with manually formed pellets contrary to pellets in media without antibiotics (-P/S) on day 35 which showed increased standard error about 5.1 % at manually bioscreening processes. The automatically handled pellet cultures (production, screening) displayed increased standard errors about 2.5 % (+P/S) and 67.9 % (-P/S) compared with the manually produced pellets on day 14. However, the automatically handled pellet cultures (production, screening) showed lower standard errors about 43.2 % (±P/S) similar to manually produced pellets on day 35.

The comparison of different screening forms (manual, automatic) presented mostly decreased standard errors of automatically evaluated pellets compared to the manually screened pellets on day 14 which was in contrast to day 35. The standard errors of automatically produced and screened pellets were decreased about 14.6 % (day 14, +P/S) and 38.7 % (day 35, -P/S). Instead, automatically produced and screened pellet cultures displayed higher standard errors about 14.6 % (day 14, +P/S) and 38.7 % (day 35, -P/S). The automatically screened and manually produced pellet cultures showed decreased standard errors about 31 % (+P/S) and 64.9 % (-P/S) on day 14 compared to the manual bioscreening. However, on day 35 the manually formed pellet cultures (automatic screening) showed higher standard errors about 21.6 % (+P/S) and 2.5 % (-P/S).

The proliferation bioscreening by the EZ4U assay revealed mostly decreased standard errors of pellet cultures (±P/S; manual, automatic) from day 14 to day 35. The standard errors of pellet cultures were reduced about 60.4 % (+P/S, manual screening, automatic production), 50 % (+P/S, manual screening, manual production), 18.6 % (-P/S, manual screening, manual production), 48.8 % (+P/S,

automatic screening, automatic production), 56.6 % (-P/S, automatic screening, automatic production) and 7.5 % (+P/S, automatic screening, manual production) over the time. Instead, the standard errors of spheroids in media without antibiotics (-P/S) were increased about 22 % (manual screening, automatic production) and 58 % (automatic screening, manual production).

The sample amounts of the manual screening, which were used to evaluate the standard errors, are: n=7 (+P/S, manual production, 14d), n=8 (+P/S, manual production, 35d), n=7 (-P/S, manual production, 14d), n=6 (-P/S, manual production, 35d), n=9 (+P/S, automatic production, 14d), n=11 (+P/S, automatic production, 35d), n=8 (-P/S, automatic production, 14d) and n=9 (-P/S, automatic production, 35d). The sample quantities of the automatic screening, which were used to estimate the standard errors, are: n=6 (+P/S, manual production, 14d), n=6 (+P/S, manual production, 35d), n=6 (-P/S, manual production, 14d), n=7 (-P/S, manual production, 35d), n=12 (+P/S, automatic production, 14d), n=11 (+P/S, automatic production, 35d), n=7 (-P/S, automatic production, 14d) and n=6 (-P/S, automatic production, 35d).

Table 6-6 Overview about the standard error of 3D cell cultures (n≥6) in terms of the EZ4U assay

Feature	Method	Operation	Screening	Results [µm]
Standard error	EZ4U assay (alginate beads)	automatic	manual	day 14: 0.077 (+P/S), 0,134 (-P/S)
				day 35: 0.072 (+P/S), 0.117 (-P/S)
		manual		day 14: 0.036 (+P/S), 0.137 (-P/S)
				day 35: 0.067 (+P/S), 0.094 (-P/S)
		automatic	automatic	day 14: 0.031 (+P/S), 0.042 (-P/S)
				day 35: 0.051 (+P/S), 0.021 (-P/S)
		manual		day 14: 0.063 (+P/S), 0.080 (-P/S)
				day 35: 0.119 (+P/S), 0.062 (-P/S)
	EZ4U assay (spheroid cultures)	automatic	manual	day 14: 0.053 (+P/S), 0.078 (-P/S)
				day 35: 0.033 (+P/S), 0.019 (-P/S)
		manual		day 14: 0.025 (+P/S), 0.021 (-P/S)
				day 35: 0.043 (+P/S), 0.010 (-P/S)
Continued on next page				

Feature	Method	Operation	Screening	Results [µm]
Standard error	EZ4U assay (spheroid cultures)	automatic	automatic	day 14: 0.066 (+P/S), 0.070 (-P/S)
				day 35: 0.057 (+P/S), 0.011 (-P/S)
		manual		day 14: 0.029 (+P/S), 0.035 (-P/S)
				day 35: 0.050 (+P/S), 0.016 (-P/S)
	EZ4U assay (pellet cultures)	automatic	manual	day 14: 0.048 (+P/S), 0.066 (-P/S)
				day 35: 0.019 (+P/S), 0.075 (-P/S)
		manual		day 14: 0.058 (+P/S), 0.097 (-P/S)
				day 35: 0.029 (+P/S), 0.079 (-P/S)

		automatic	automatic	day 14: 0.041 (+P/S), 0.106 (-P/S)
				day 35: 0.021 (+P/S), 0.046 (-P/S)
		manual		day 14: 0.040 (+P/S), 0.034 (-P/S)
				day 35: 0.037 (+P/S), 0.081 (-P/S)

DNA Quantification

The table 6-7 gives an overview about the standard errors of DNA quantifications of manually and automatically produced 3D cell cultures.

In general, the bioscreenings (manual, automatic) to evaluate the DNA quantification showed mostly lower standard errors of automatically produced 3D cell cultures compared to the manually produced 3D constructs. The alginate beads displayed decreased standard errors of the automatic quantification compared to the manual screening on day 14. The spheroid cultures in media with antibiotics (+P/S) showed mainly increased standard errors (day 14, day 35) for the automatic screening which was in contrast to spheroids in media without antibiotics (-P/S). The pellet cultures (production: manual, automatic) visualized mainly increased standard errors for the automatic quantification (±P/S). The standard errors of 3D cell cultures (alginate beads, spheroid cultures, pellet cultures) cultivated in different media (±P/S) were mainly decreased from day 14 to day 35.

Alginate Beads

In detail, the comparison of the different manufacturing forms (manual, automatic) displayed mainly enhanced standard errors of automatically produced alginate beads for the manual DNA quantification and automatic screening on day 35 which was in contrast to the automatic quantification on day 14. The automatically produced alginate beads showed increased standard errors for the bioscreening (manual) compared to manually formed beads of about 13.1 % (day 14, +P/S), 21.1 % (day 35, +P/S) and 68.1 % (day 35, -P/S). On day 14, beads without antibiotic presented a reduced standard error about 11.9 %. Furthermore, the automatically screened and produced alginate beads displayed reduced standard errors of about 23 % (+P/S) and 34.3 % (-P/S) compared to the manually formed beads. On day 35, the automatically formed beads showed increased standard errors compared to manually produced beads for the automatic bioscreening about 43.9 % (+P/S) and 63.9 % (-P/S).

The comparison of the bioscreening processes (manual, automatic) of alginate-embedded cells (manual and automatic production) presented mostly decreased standard errors at the automatically performed DNA quantification compared to the manually performed quantification. The automatically formed beads showed decreased standard errors at the automatically performed bioscreening compared with manually screened beads about 39.9 % (+P/S) and 29.8 % (-P/S) on day 14 whereas an opposite effect on day 35 was visible. Herein, automatically formed beads displayed increased standard errors at the automatically performed bioscreening related to the manually screened beads about 34.7 % (+P/S) and 8.2 % (-P/S). The manually produced beads presented lower standard errors for the automatic bioscreening about 10.1 % (+P/S) and 5.8 % (-P/S) on day 14. Instead, on day 35 the automatic bioscreening of manually formed beads showed higher standard errors compared to the manual screening about 8.2 % (+P/S) and 18.8 % (-P/S).

The standard errors of the DNA screening (manual, automatic) in different media (±P/S) were mainly decreased from day 14 to day 35. The standard errors were reduced about 35.5 % (+P/S, manual screening, automatic production), 42.3 % (-P/S, manual screening, automatic production), 41.5 % (+P/S, manual screening, manual production), 83.8 % (-P/S, manual screening, manual production), 29.1 % (+P/S, automatic screening, manual production), 10.4 % (-P/S, automatic screening, automatic production) and 78.3 % (-P/S, automatic screening, manual production). However, the standard errors of automatically produced beads in media with antibiotics (production, screening) increased about 39.1 % (+P/S) from day 14 to day 35.

The sample amounts of the manual screening, which were applied to estimate the standard errors, are: n=8 (+P/S, manual production, 14d), n=8 (+P/S, manual production, 35d), n=9 (-P/S, manual production, 14d), n=6 (-P/S, manual production, 35d), n=8 (+P/S, automatic production, 14d), n=6 (+P/S, automatic production, 35d), n=6 (-P/S, automatic production, 14d) and n=7 (-P/S, automatic production, 35d). The sample numbers of the automatic screening, which were used to calculate the standard errors, are: n=9 (+P/S, manual production, 14d), n=8 (+P/S, manual production, 35d), n=12 (-P/S, manual production, 14d), n=6 (-P/S, manual production, 35d), n=13 (+P/S, automatic production, 14d), n=9 (+P/S, automatic production, 35d), n=9 (-P/S, automatic production, 14d) and n=6 (-P/S, automatic production, 35d).

Spheroid Cultures

The comparison of the different manufacturing forms (manual, automatic) presented mainly lower standard errors of spheroid cultures (±P/S; automatic production) at the manual DNA quantification as well as for automatically screened spheroids cultivated without antibiotics. The opposite effect was shown for automatically performed (screening, production) spheroids (+P/S). The manually performed bioscreening of spheroid cultures (automatic production) displayed a decreased standard error related to manually formed cultures about 5.4 % (+P/S) and 25.9 % (-P/S) on day 14. At day 35, the standard errors of automatically formed spheroid cultures were increased about 26.3 % (+P/S) and 21 % (-P/S). The automatic bioscreening visualized higher standard errors of automatically formed spheroid cultures in media with antibiotics (+P/S) compared to manually produced spheroids about 20.7 % (day 14) and 45.2 % (day 35). However, the spheroid cultures (automatic production) in media without antibiotics showed lower standard errors for the automatic bioscreening and manually formed spheroids about 40.3 % (day 14) and 42.1 % (day 35).

The comparison of the screening processes showed increased standard errors of spheroids cultivated in media with antibiotics (+P/S, manual and automatic production) at the automatically performed DNA quantification contrary to standard errors of spheroids (-P/S, manual and automatic production) at the manual bioscreening. The standard errors of automatically manufactured spheroids (+P/S) increased at the automatic performed bioscreening compared to the manually screening about 36.8 % (day 14) and 43.8 % (day 35). The automatically produced spheroids in media without antibiotics (-P/S) showed decreased standard errors at the automatic bioscreening about 29.6 % (day 14) and 8.5 % (day 35). The manually produced spheroid cultures visualized mainly an increased standard error of automatically screened spheroid cultures compared to the manual bioscreening about 15.7 % (day 14, +P/S), 24.3 % (day 35, +P/S) and 20 % (day 35, -P/S). Nevertheless, the manually shaped spheroids (-P/S) showed a decreased standard error at the automatic bioscreening compared with the manually screening about 12.6 % on day 14.

The standard errors of manually and automatically performed (±P/S, production, screening) spheroids DNA quantifications were completely reduced from day 14 to day 35 about 70.6 % (+P/S, manual screening, automatic production), 86.6 % (-P/S, manual screening, automatic production), 79.5 % (+P/S, manual screening, manual production), 87.4 % (-P/S, manual screening, manual production), 66.9 % (+P/S, automatic screening, automatic production), 82.6 % (-P/S, automatic screening, automatic production), 77.1 % (+P/S, automatic screening, manual production) and 82 % (-P/S, automatic screening, manual production).

The sample numbers of the manual screening, which were applied to calculate the standard errors, are: n=7 (+P/S, manual production, 14d, 35d), n=7 (-P/S, manual production, 14d), n=9 (-P/S, manual production, 35d), n=7 (+P/S, automatic production, 14d, 35d), n=6 (-P/S, automatic production, 14d) and n=11 (-P/S, automatic production, 35d). The sample quantities of the automatic screening, which were used to evaluate the standard errors, are: n=6 (+P/S, manual production, 14d, 35d), n=6 (-P/S, manual production, 14d, 35d), n=7 (+P/S, automatic production, 14d, 35d), n=9 (-P/S, automatic production, 14d, 35d) and n=6 (-P/S, automatic production, 35d).

Pellet cultures

The comparison of the manufacturing methods (manual, automatic) presented mainly decreased standard errors of pellets (automatic production) of the manual DNA quantification (±P/S) and automatically performed quantification (-P/S) compared to standard errors of manually produced pellets. This was in contrast to standard errors of pellets in media with antibiotic (+P/S, automatic screening). The manual DNA quantification of the manufacturing forms (automatic, manual) showed lower standard errors of automatically produced pellets compared to manually manufactured pellets about 7.3 % (+P/S) and 4.1 % (-P/S) on day 14. However, on day 35 the standard errors of automatically manufactured pellets were increased about 12 % (+P/S) and 47.5 % (-P/S) for the manual bioscreening. The automatic DNA quantification of pellet cultures (+P/S, automatic production) showed increased standard errors compared to manually produced pellets about 23.6 % (day 14) and 15.5 % (day 35). The standard errors of pellet cultures (-P/S, automatic production) were reduced compared to manually produced pellets about 1 % (day 14) and 62.9 % (day 35) for the automatic screening.

The comparison of the screening methods showed mainly enhanced standard errors of pellets (manual production, automatic production) cultivated in media with antibiotics (+P/S) in contrast to standard errors of pellets in media without antibiotics (-P/S). Here, mainly reduced standard errors at the automatically performed DNA quantification compared to the manually screening were determined. The manually and automatically produced pellets displayed mainly increased standard errors of automatically screened and produced pellet cultures compared to the manually quantification about 7.2 % (day 14, +P/S), 12.1 % (day 35, +P/S) and 1.8 % (day 35, -P/S). In contrast, the standard error of automatically handled pellets (screening, production) in media without antibiotics (-P/S) was decreased about 1.1 % on day 14. The manually produced pellet cultures (automatic screening) displayed lower standard errors on day 14 compared to the manually screening about 23.7 % (+P/S) and 4.3 % (-P/S). However, the automatic screening showed increased standard errors of manually formed pellets on day 35 about 8.4 % (+P/S) and 30.5 % (-P/S).

The standard errors of quantified DNA of manually and automatically produced (±P/S, screening,) pellets showed mainly decreased values from day 14 to day 35 instead of automatically screened

pellets (manual production) cultivated in media without antibiotics. The standard errors were decreased about 20.3 % (+P/S, manual screening, automatic production), 54.5 % (-P/S, manual screening, automatic production), 34.9 % (+P/S, manual screening, manual production), 16.8 % (-P/S, manual screening, manual production), 15.8 % (+P/S, automatic screening, automatic production), 53.5 % (-P/S, automatic screening, automatic production) and 6.9 % (+P/S, automatic screening, manual production). The standard error of pellets was increased about 20 % (-P/S, automatic screening, manual production) from day 14 to day 35.

The sample sums of the manual screening, which were used to calculate the standard errors, are: n=7 (+P/S, manual production, 14d), n=6 (+P/S, manual production, 35d), n=6 (-P/S, manual production, 14d, 35d), n=7 (+P/S, automatic production, 14d), n=8 (+P/S, automatic production, 35d), n=8 (-P/S, automatic production, 14d) and n=7 (-P/S, automatic production, 35d). The sample amounts of the automatic screening, which were used to evaluate the standard errors, are: n=8 (+P/S, manual production, 14d), n=7 (+P/S, manual production, 35d), n=6 (-P/S, manual production, 14d), n=7 (-P/S, manual production, 35d), n=8 (+P/S, automatic production, 14d, 35d) and n=7 (-P/S, automatic production, 14d, 35d).

Table 6-7 Overview about the standard errors of 3D cell cultures (n≥6) in terms of the DNA quantification

Feature	Method	Operation	Screening	Results [µm]
Standard error	DNA quantification (alginate beads)	automatic	manual	day 14: 624.8 (+P/S), 613.2 (-P/S) day 35: 402.9 (+P/S), 354.1 (-P/S)
		manual	manual	day 14: 542.9 (+P/S), 696.0 (-P/S) day 35: 317.8 (+P/S), 113.1 (-P/S)
		automatic	automatic	day 14: 375.6 (+P/S), 430.6 (-P/S) day 35: 616.6 (+P/S), 385.7(-P/S)
		manual	automatic	day 14: 488.1 (+P/S), 655.5 (-P/S) day 35: 346.1 (+P/S), 139.3 (-P/S)
Continued on next page				

Feature	Method	Operation	Screening	Results [µm]
Standard error	DNA quantification (spheroid cultures)	automatic	manual	day 14: 946.7 (+P/S), 757.3 (-P/S) day 35: 278.8 (+P/S), 101.4 (-P/S)
		manual	manual	day 14: 1,001.2 (+P/S), 1,021.5 (-P/S) day 35: 205.6 (+P/S), 128.4 (-P/S)
		automatic	automatic	day 14: 1,497.3 (+P/S), 532.8 (-P/S) day 35: 495.8 (+P/S), 92.8 (-P/S)
		manual	automatic	day 14: 1,187.3 (+P/S), 892.6 (-P/S) day 35: 271.6 (+P/S), 160.4 (-P/S)
	DNA quantification (pellet cultures)	automatic	manual	day 14: 3,159.4 (+P/S), 2,119.8 (-P/S) day 35: 2,518.8 (+P/S), 964.2 (-P/S)
		manual	manual	day 14: 3,406.6 (+P/S), 2,210.1 (-P/S) day 35: 2,216.6 (+P/S), 1,838.1 (-P/S)
		automatic	automatic	day 14: 3,402.8 (+P/S), 2,095.6 (-P/S) day 35: 2,865.0 (+P/S), 982.1 (-P/S)

| | | manual | | day 14: 2,599.2 (+P/S), 2,116.0 (-P/S) |
| | | | | day 35: 2,420.6 (+P/S), 2,644.3 (-P/S) |

Adenylate Kinases Assay

Table 6-8 visualizes the standard errors of the manually and automatically produced 3D cell cultures in case of detection the cytotoxicity (manual and automatic) by the AK assay.

In general, the bioscreenings (manual, automatic) to evaluate the cytotoxicity displayed mostly a higher standard error of automatically produced alginate beads and spheroid cultures compared to manually produced 3D constructs except of pellet cultures. The automatically and manually produced 3D cell cultures showed mainly increased standard errors for the automatic toxicity screening compared to the manual bioscreening (day 14, 35). The standard errors of alginate beads cultivated in media without antibiotic (-P/S) were reduced from day 14 to day 35 contrary to beads in media with antibiotic (+P/S). The spheroid cultures (±P/S) showed a decreased standard error from day 14 to day 35.

Alginate Beads

The comparison of the different manufacturing forms (manual, automatic) presented mainly increased standard errors of automatically produced beads compared to the manually manufactured beads (±P/S) for both bioscreening processes (manual, automatic). The standard errors of automatically formed beads were higher about 35.8 % (day 14, +P/S), 22.3 % (day 14, -P/S) and 26.5 % (day 35, +P/S) at the manual performed bioscreening. However, the standard error of automatically formed beads (-P/S) was lower about 5.9 %. For the automatically performed toxicity screening, the standard errors of beads (automatic production) were increased compared to manually formed beads about 22.7 % (day 14, -P/S), 13 % (day 35, +P/S) and 32.7 % (day 35, -P/S) contrary to beads on day 14 (-P/S) which showed a reduced standard error (15.3 %).

The comparison of the bioscreening processes (manual, automatic) of manually and automatically manufactured beads showed mainly increased standard errors at the automatically performed toxicity screening compared with the manually bioscreening. The standard errors of automatically handled beads (screening, production) were higher about 1 % (day 14, +P/S), 62.5 % (day 14, -P/S), 16.7 % (day 35, +P/S) and 11.6 % (day 35, -P/S). For the automatic bioscreening, the standard errors of manually formed beads were higher about 48.8 % (day 14, +P/S), 62.3 % (day 14, -P/S) and 29.6 % (day 35, +P/S) compared to the manually screened beads (day 35, -P/S) which had a reduced standard error about 28.4 %.

The standard errors of the bioscreenings were increased from beads with antibiotics (+P/S) in contrast to beads in media without antibiotics (-P/S) from day 14 to day 35. The standard errors of automatically and manually produced beads in media with antibiotic (+P/S) were increased at both bioscreening processes on both detection time points. At the manually bioscreening, the standard errors were enhanced about 17.9 % (automatic production) and 28.4 % (manual production). For the automatic bioscreening the standard errors were also higher about 31 % (automatic production) and 2.2 % (manual production). The standard errors of beads (manual production, automatic production) in media without antibiotic (-P/S) were mainly decreased from day 14 to day 35 forr both

bioscreening processes. At the manually bioscreening, the standard errors were reduced about 18.9 % (automatic production) and enhanced about 9.8 % (manual production). For the automatic bioscreenings, the standard errors were also reduced about 65.6 % (automatic production) and 70.1 % (manual production).

The sample amounts of the manual screening, which were applied to calculate the standard errors, are: n=22 (+P/S, manual production, 14d, 35d), n=20 (-P/S, manual production, 14d), n=16 (-P/S, manual production, 35d), n=24 (+P/S, automatic production, 14d), n=22 (+P/S, automatic production, 35d), n=25 (-P/S, automatic production, 14d) and n=17 (-P/S, automatic production, 35d). The sample sums of the automatic screening, which were used to estimate the standard errors, are: n=9 (+P/S, manual production, 14d), n=10 (+P/S, manual production, 35d), n=13 (-P/S, manual production, 14d), n=9 (-P/S, manual production, 35d), n=9 (+P/S, automatic production, 14d), n=10 (+P/S, automatic production, 35d), n=10 (-P/S, automatic production, 14d) and n=6 (-P/S, automatic production, 35d).

Spheroid Cultures

The evaluation (manual and automatic bioscreening) of the manual and automatic hanging drop production to create spheroid cultures over the time showed mainly enhanced standard errors of automatically produced spheroids compared to manually formed spheroid cultures (±P/S). For the manually performed bioscreening, the standard errors of automatically manufactured spheroids were mostly increased about 6.4 % (day 14, +P/S), 3.1 % (day 35, +P/S) and 12.5 % (day 35, -P/S) in contrast to spheroids (day 14, -P/S) which displayed a reduced standard error (47.7 %). For the automatic bioscreening the standard errors of spheroids (production: automatic) were also increased compared to manually formed spheroids about 8.6 % (day 14, +P/S), 10.6 % (day 35, +P/S) and 37.4 % (day 35, -P/S). However, the standard error of automatically formed spheroid cultures (day 14, -P/S) was lower about 63.1 %.

The comparison of the bioscreening processes (manual, automatic) of manually and automatically produced spheroid cultures displayed mainly enhanced standard errors for the automatic toxicity bioscreening compared to the screening processes performed in the manual way. The standard errors of automatically performed spheroid cultures (production, screening) were mainly increased about 1 % (day 14, +P/S), 62.5 % (day 14, -P/S) and 16.7 % (day 35, +P/S) in contrast to spheroids on day 14 (-P/S) with a lower standard error (47.7 %). For the automatic bioscreening, the standard errors of manually formed beads were higher about 27.7 % (day 14, +P/S), 15.4 % (day 14, -P/S) and 33 % (day 35, +P/S) compared to the manually screened spheroids on day 35 (-P/S) which had a reduced standard error about 9.7 %.

The standard errors of the spheroid-toxicity screening (±P/S) were overall decreased from day 14 to day 35 about 59 % (+P/S, manual screening, automatic production), 50.1 % (-P/S, manual screening, automatic production), 57.6 % (+P/S, manual screening, manual production), 77.1 % (-P/S, manual screening, manual production), 52.6 % (+P/S, automatic screening, automatic production), 25.4 % (-P/S, automatic screening, automatic production), 53.6 % (+P/S, automatic screening, manual production) and 82.5% (-P/S, automatic screening, manual production).

The sample sums of the manual screening, which were used to calculate the standard errors, are: n=15 (+P/S, manual production, 14d), n=8 (+P/S, manual production, 35d), n=11 (-P/S, manual production, 14d), n=9 (-P/S, manual production, 35d), n=11 (+P/S, automatic production, 14d), n=7 (+P/S, automatic production, 35d), n=11 (-P/S, automatic production, 14d) and n=6 (-P/S, automatic

production, 35d). The sample amounts of the automatic screening, which were applied to estimate the standard errors, are: n=15 (+P/S, manual production, 14d), n=8 (+P/S, manual production, 35d), n=11 (-P/S, manual production, 14d), n=10 (-P/S, manual production, 35d), n=10 (+P/S, automatic production, 14d), n=7 (+P/S, automatic production, 35d), n=10 (-P/S, automatic production, 14d) and n=6 (-P/S, automatic production, 35d).

Pellet cultures

The evaluation of the manually and automatically produced pellet cultures displayed mainly decreased standard errors of automatically produced pellets compared to manually formed pellet cultures (±P/S) for the bioscreening processes (manual, automatic). For the manual toxicity bioscreening, the standard errors of automatically manufactured pellets were mainly decreased about 12.1 % (day 14, +P/S), 52.8 % (day 14, -P/S) and 58.1 % (day 35, +P/S) compared to manually produced pellets. However, the automatically produced pellet (-P/S) showed an increased standard error on day 35 (45.8 %). For the automatic bioscreening, the standard errors of pellet cultures (production: automatic) were decreased compared to standard errors of manually formed pellet cultures. The standard errors of automatically produced pellets were decreased about 41.5 % (day 14, +P/S) and 65 % (day 35, -P/S). However, the standard errors of pellets (automatic production) were increased about 30.3 % (day 14, -P/S) and 2.3 % (day 35, +P/S).

The comparison of the bioscreening processes (manual, automatic) of manually and automatically produced pellet cultures showed that 50 % of the probes had decreased standard errors for the automatic toxicity bioscreening compared to the manually performed screening processes.

The standard errors of automatically handled pellet cultures (production, screening) were partly increased about 20.5 % (day 14, -P/S) and 62.2 % (day 35, +P/S). The other standard errors of automatically screened and produced pellets were decreased about 54.4 % (day 14, +P/S) and 45.1 % (day 35, -P/S). For the automatic bioscreening, the standard errors of manually produced pellets were decreased about 31.3 % (+P/S) and 58.6 % (-P/S) compared with manually screened pellets on day 14. But on day 35, standard errors of manually produced pellet cultures were increased about 7.6 % (+P/S) and 65.5 % (-P/S).

The toxicity screening (manual, automatic) of automatically produced pellet cultures (±P/S) presented mainly decreased standard errors from day 14 to day 35. The manually and automatically produced pellets presented decreased standard errors at the manually bioscreening from day 14 to day 35 about 75.4 % (+P/S, automatic production), 16.2 % (-P/S, automatic production), 48.4 % (+P/S, manual production) and 78.5 % (-P/S, manual production). For the automatic bioscreening, the standard errors were decreased about 63.4 % (-P/S, automatic production) and 18.7 % (+P/S, manual production) from day 14 to day 35 whereas the other half of standard errors were increased about 29.7 % (+P/S, automatic production) and 33.4 % (-P/S, manual production).

The sample amounts of the manual screening, which were used to evaluate the standard errors, are: n=6 (±P/S, manual production, 14d), n=8 (±P/S, manual production, 35d), n=7 (+P/S, automatic production, 14d), n=10 (+P/S, automatic production, 35d), n=6 (-P/S, automatic production, 14d) and n=8 (-P/S, automatic production, 35d). The sample sums of the automatic screening, which were applied to evaluate the standard errors, are: n=8 (±P/S, manual production, 14d), n=6 (±P/S, manual production, 35d), n=12 (+P/S, automatic production, 14d), n=10 (+P/S, automatic production, 35d), n=10 (-P/S, automatic production, 14d) and n=6 (-P/S, automatic production, 35d).

Table 6-8 Overview about the standard errors of 3D cell cultures (n≥6) in terms of the AK assay

Feature	Method	Operation	Screening	Results [μm]
Standard error	AK assay (alginate beads)	automatic	manual	day 14: 3,911.3 (+P/S), 4,031.8 (-P/S) day 35: 4,766.6 (+P/S), 3,270.8 (-P/S)
		manual		day 14: 2,509.4 (+P/S), 3,133.5 (-P/S) day 35: 3,502.7 (+P/S), 3,475.7 (-P/S)
		automatic	automatic	day 14: 3,950.0 (+P/S), 10,763.3 (-P/S) day 35: 5,722.1 (+P/S), 3,698.6(-P/S)
		manual		day 14: 4,870.0 (+P/S), 8,320.1 (-P/S) day 35: 4,977.8 (+P/S), 2,488.7 (-P/S)
	AK assay (spheroid cultures)	automatic	manual	day 14: 3,055.0 (+P/S), 2,874.4 (-P/S) day 35: 1,252.1 (+P/S), 1,435.1 (-P/S)
		manual		day 14: 2,860.1 (+P/S), 5,495.4 (-P/S) day 35: 1,213.8 (+P/S), 1,255.7 (-P/S)
		automatic	automatic	day 14: 4,273.6 (+P/S), 2,393.6 (-P/S) day 35: 2,025.8 (+P/S), 1,810.4 (-P/S)
		manual		day 14: 3,905.4 (+P/S), 6,492.4 (-P/S) day 35: 1,811.4 (+P/S), 1,133.7 (-P/S)
Continued on next page				

Feature	Method	Operation	Screening	Results [μm]
Standard error	AK assay (pellet cultures)	automatic	manual	day 14: 2,060.8 (+P/S), 1,410.7 (-P/S) day 35: 507.0 (+P/S), 1,182.5 (-P/S)
		manual		day 14: 2,345.4 (+P/S), 2,985.9 (-P/S) day 35: 1,210.2 (+P/S), 640.8 (-P/S)
		automatic	automatic	day 14: 942.3 (+P/S), 1,773.4 (-P/S) day 35: 1,341.3 (+P/S), 649.4 (-P/S)
		manual		day 14: 1,612.0 (+P/S), 1,235.4 (-P/S) day 35: 1,310.2 (+P/S), 1,856.0 (-P/S)

6.3 Summary

This chapter includes overviews to summarize the new integrated methods in the case of the performance (throughput, efficiency, accuracy, standard errors). The new adapted methods are the automatic handlings of 3D cell cultures (alginate beads, spheroid cultures, pellet cultures).

6.3.1 3D cell cultures

The following table (see figure 6-9) summarizes the performance results of 3D cell cultures (alginate beads, spheroid cultures, pellet cultures). The automatically performed methods (production, screening) are compared to standard manual handling processes.

Table 6-9 Summery of the performance of 3D cell cultures

Feature	Alginate beads	Spheroid cultures	Pellet cultures
Throughput and efficiency	• process times: 187 min. (auto) 56 min. (manual) • projected plates/day: 7 well plates (automatic) 6 well plates (manual)	• process times: 98 min. (auto) 42 min. (manual) • projected plates/day: 14 well plates (automatic) 8 well plates (manual)	• process times: 460 min. (automatic) 177 min. (manual) • projected plates/day: 3 well plates (automatic) 2 well plates (manual)
Accuracy (diameter)	• minor increased (3.5%) of automatically produced beads	• differences of manual and automatic produced spheroids: 7.2 % • mainly decreased diameter of automatically produced spheroids	-
Continued on next page			

Feature	Alginate beads	Spheroid cultures	Pellet cultures
Standard error (size)	• size detection on day 1 • +P/S: decreased standard error of automatically produced beads • -P/S: increased standard error of automatically produced beads	• size detection on day 14 and 35 • day 14: similar standard error of automatically produced spheroids (±P/S) • day 35: decreased standard error of automatically produced spheroids (±P/S)	-
Standard error (EZ4U Assay)	• **comparison-production:** mostly lower of automatically manufactured beads (±P/S) • **comparison-bioscreening:** mostly lower for automatically screened beads (day 14, 35) • **evaluation day 14 to day 35:** decreased -P/S in	• **comparison-production:** mostly higher of automatically manufactured spheroids (±P/S) • **comparison-bioscreening:** mainly higher of automatically screened spheroids (day 14, 35) • **evaluation day 14 to day 35:** mostly reduced	• **comparison-production:** mainly lower of automatically produced pellet cultures (±P/S) • **comparison-bioscreening:** mostly lower of automatically screened pellets on day 14 in contrast to day 35 • **evaluation day 14 to day 35:** mostly decreased

	contrast to beads +P/S		
Standard error (DNA Quantification)	• **comparison-production:** mainly enhanced of automatically produced beads at the manual quantification and automatic screening on day 35; decreased for the automatically quantification on day 14 • **comparison-bioscreening:** mostly decreased at the automatically performed DNA quantification	• **comparison-production:** mainly lower of spheroids (±P/S; production: automatic) at the manual DNA quantification and at automatically screened spheroids -P/S; opposite effect for automatically performed (production, screening) spheroids (+P/S) • **comparison-bioscreening:** increased of spheroids cultivated +P/S for the automatically performed DNA quantification contrary to standard errors of spheroids P/S	• **comparison-production:** mainly decreased of pellets (production: automatic) at the manually DNA quantification (±P/S) and automatically performed quantification -P/S in contrast to standard errors of pellets +P/S • **comparison-bioscreening:** mainly increased of pellets +P/S at the automatically performed DNA quantification in contrast to standard errors of pellets -P/S
Continued on next page			

Feature	Alginate beads	Spheroid cultures	Pellet cultures
Standard error (DNA Quantification)	• **evaluation day 14 to day 35:** mainly decreased	• **evaluation day 14 to day 35:** completely decreased	• **evaluation day 14 to day 35:** mainly decreased
Standard error (AK assay)	• **comparison-production:** mainly enhanced standard errors of automatically produced beads (±P/S) for both bioscreenings (manual, automatic) • **comparison-bioscreening:** mainly increased for the automatically toxicity screening • **evaluation day 14 to day 35:** increased for +PS, mainly decreased for -P/S	• **comparison-production:** mainly enhanced of automatically produced spheroids (±P/S) for the bioscreening (manual, automatic) • **comparison-bioscreening:** mainly increased for the automatically toxicity bioscreening • **evaluation day 14 to day 35:** decreased (±P/S)	• **comparison-production:** mainly decreased of automatically shaped pellets (±P/S) for the bioscreening processes (manual, automatic) • **comparison-bioscreening:** automatically handled pellet cultures (production, screening) only consolidated to half increased • **evaluation day 14 until day 35:** mainly decreased from (±P/S)

7 Discussion

The automatic cell cultivation is an important tool for simplifying routine laboratory work. The automatic methods are independent of skill levels and daily constitution of laboratory staff in combination with a constant quality and performance of the methods. The Biomek® Cell Workstation was configured as a flexible and compatible system for automatic cell cultivation processes [110]. The automatic processes were compared with the regularly manual methods to visualize the substitutability of the manual handling [72].

7.1 Cell Culture Systems

The cell culture systems are used for automatic cell culture processes under sterile conditions. There are different cell culture systems published and available on the market. However, the Biomek® Cell Workstation displays a unique system with a wide broad of cell culture processes. Commercially available cell culture systems are mainly horizontal arranged flexible systems (CompaTSelecT, AI CELLHOST, Cellerity™, BioCel Systems) with different footprints and labware transport as well as connection of the devices by a robotic manipulator arm. However, the Biomek® Cell Workstation is vertical arranged to reduce the required space but includes high flexibility. The labware is transported by a lift and the liquid handler on the deck, which is associated with the needed devices [110, 111, 144, 145]. Nevertheless, the cell culture system of Kato et al. shows the smallest footprint (70 cm x 60 cm x 86 cm) but loss of flexibility with a low number of integrated devices, e.g. no cell counter [146]. The liquid transfers found diverse solutions at the cell culture systems. Basically, a tubing system with valves was used. The cell culture systems used re-useable tips (Cellerity™, AI.CELLHOST) [111, 144, 145] or serological pipettes with one dispensing channel (CompacTSelecT) [111]. Conversely, the Biomek® Cell Workstation used eight steel cannulas for the liquid transfer during the cell processes to support the flexible cultivation methods and labware [110]. The different modified pipetting templates supported the manifold cultivation processes. Cell culture flasks are required to cultivate the cell lines. Different flasks and labware are used in automatic cultivation processes, e.g. RoboFlask™ (Cellerity™) for automatic cell culture processes in a 96 well format [144], regular T75 flask (CompacTSelecT, system reported by Kino-oka et al.) [111, 147] or one cell culture dish (system reported by Kato et al.) [146]. The Biomek® Cell Workstation also supports the management of flask and labware in a 96 well format by using the CellStar AutoFlasks™. The main benefit is the horizontal cell handling during the processes because this prevents the cells from dying by drying out and shear stress [110]. The published cell culture systems are used for cell expansion of adherent cell lines. However, the Biomek® Cell Workstation enables the manifolds cell processing by cultivation of adherent cells, suspension cells and the manufacturing of 3D constructs (alginate beads, spheroid cultures, pellet cultures).

7.2 2D Cell Cultures

The evaluation and exclusion of contaminations are very important aspects at the automatic cell cultivation. The contaminations can be caused by microorganism (spores, fungi, bacteria) or other cell lines. This is called cross-contamination. The presented data are unique and represent a new

method to check cross contaminations. It is well known that contamination and especially cross contamination represent always a set-back in automation processes. Well published is the trouble with cross contamination for the Polymerase Chain Reaction (PCR). Manual procedures have a greater possibility of cross contaminating the sample than robots that handle DNA or RNA [148, 149]. Even metal contamination has been discussed and published in automatic processes [150]. We are still in the pipeline talking about cross contamination in cell culturing with more than one cell line. Disinfection cycles are fundamental for automatic systems with eight pipes or less. Otherwise it would be possible to run each and every cell line separately in pipes and needles. The disinfection program offers a low-cost and is a still safe and efficient way to use several cell lines in one test run and even co-cultivate cell lines without concern about the contamination [134]. The new ways to investigate cross contaminations can be presented by microscopic analysis or quantified by FACS analysis.

Primarily, the screening of 2D cell cultures is focused on the detection of the proliferation. This assays base on the conversation of tetrazolium salt to colored formazan and detection of the absorbance.

The basically examination of manually and automatically performed 2D cell cultures (HeLa cells) presented the evaluation of cell proliferation by the EZ4U assay. Three test groups were investigated: manual/sterile, automatic/sterile and manual/unsterile. Altogether, the adherent cells were manually and automatically seeded from 1,000 cells/well up to 8,000 cells/well associated with an increased proliferation at amplified cell number per well. However, the proliferation of automatically seeded cells (automatic/sterile) was minor lower for all cell numbers compared with manually performed cells (manual/sterile). This is warranted by the low cell loss at automatic cell processes.

Proliferation assays can be used for the investigation of compounds to detect effects of compounds on cell cultures. The detection of the proliferation serves to evaluate indirectly the toxicity of compounds. Hereof, the cells were seeded (manual, automatic) and treated with cyclophosphamide. The consequently EZ4U-assay was used to evaluate the growth curves. These served to determine the EC_{50} (half maximal effective dose) values in the range from 17.5 mmol/l to 8.75 mmol/l. Normally, the cytotoxicity and half-maximal concentrations are regularly quantified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tests in media with antibiotics. Hereof, EC_{50} values of different cancer cell types are 28 µmol/l for 9L glioblastoma cells, 22.5 µg/ml for A431 skin cancer cells and 28 µg/ml MCF 7 for breast cancer cells [3, 151]. These EC_{50} values are essential lower than of quantified cervix carcinoma cells.

The published papers with automatic systems for cell cultivation address so far exclusively monolayer cultures. The suspension cell lines are another cell type, which are essential for the research, especially for the investigation of leukemia [110]. Regularly, the proliferation of suspension cells is evaluated by the WST-1 assay (Roche) [152]. The proliferation of automatically processed cells is non-significant reduced compared to the manual seeded cells. A Non-significant decrease of the proliferation might be associated with stress on the cells caused by sheer forces and consequently increased cell death and cell loss during the automatic transfer steps [110].

7.3   3D Cell Cultures

The increased aging population mainly causes the rise of diseases like cancer and cartilage defects. Different forms of cancer are widespread diseases in the worldwide population [51, 52]. Cancer diseases are combined with high therapeutic costs without warranties of healing [73, 153]. These diseases still have a great importance in research to investigate the processes of cancer and evaluate new drugs by compound screenings [46, 52].

The rheumatoid arthritis, deformation and traumata require new methods for the investigation of cartilage tissue and consequently cartilage regeneration [54, 154].

In this respect, three dimensional cell cultures are an important strategy for compound screenings [20], tissue engineering [74] and investigation of tumors [76]. Three dimensional cell cultures enable the mimic of *in vivo* conditions [73]. The replacement of 2D cell cultures by 3D cell cultures is required for a better simulation of *in vivo* conditions [19] as well as for increasing the efficiency and cost-effectivity of drugs in preclinical studies [52].

Popular arrangements to cultivate cells in three dimensional forms are bead cultures, spheroid cultures and pellet cultures. These 3D cell cultures are useful for different cell lines and applications. The cells embedded in sodium alginate display a suitable source for the investigation of cancer diseases [40, 72, 73, 78]. The manual manufacturing is an established process in research [31, 155]. The formation of spheroid cultures in hanging drops is a popular method to produce homogenous 3D constructs in a short time period. This manual method is a suitable method to form 3D cell cultures in an automatic way [46, 52, 73]. The common *in vitro* forms to cultivate chondrocytes in 3D constructs are pellet cultures. But the production and analysis of pellet cultures are time consuming processes. At present, the different cell culture platforms and screening systems do not realize these challenges for human primary chondrocytes in an automatic way [54, 57, 111].

Novel is the combination of cell detachment and 3D formation of alginate beads, spheroid cultures and pellet cultures with consequently bioscreening processes. The integrated alginate bead production is close to the manual manufacturing method and new for automatic processes. In case of spheroid cultures the bioscreenings procedures (proliferation, cytotoxicity) performed are already non-published assays [72]. Concerning pellet cultures the automatic handling of human chondrocytes is new associated with fully automatic manufacturing and screening [54].

The automatic manufacturing partly required other devices and every 3D cell culture requires specific production methods.

The cervix carcinoma cells were manually and automatically embedded in 1.2 % sodium alginate matrix to produce beads. These cultures were compared by manual and automatic proliferation and toxicity screening methods [73]. The established method is a down-screened process of the manual alginate bead production. The cells are likewise embedded in 1.2 % sodium alginate ($2x10^6$ cells/ml) and dropped in 100 mM CaCl$_2$ solution for bead formation. Jonitz *et al.* pooled 10-15 beads into a 48 well plate within the manual procedure [31]. Instead of this, one bead/well is produced in the automatic process for further individual tests. This down-screening process enables the reduction of required solutions and media. The comparison of the manual and automatic manufacturing process visualized equivalent results related on the size, proliferation and toxicity of HeLa cells encapsulated in alginate matrix [54].

In accordance to Tung *et al.* [48], the formation of spheroid cultures (HeLa cells) was performed in 384 hanging drop well plates to investigate the long-term stability and cell behavior of this 3D constructs. We also produced 192 spheroids in a 384 hanging drop well plate with a water reservoir which protects against evaporation. The cell suspension was seeded in 24 columns and 16 rows,

arranged in an offset manner. The Perfecta®3D Hanging Drop Plates were sandwiched with a lid and a tray with a further reservoir for water. The 50 % media change two times the week supported the cell proliferation and formation of spheroids, like the daily 30 % media change [48, 54, 156].

The pellet formation was performed in 96 well plates in high-density cell cultures. Penick *et al.* investigated the chondrogenic potential of bone marrow-derived mesenchymal stem cells (hMSC) by the translation of the regular formation of aggregates within 15 ml tubes to 96 well plates, a high throughput aggregate system with positive effects for the 3D cell cultures [55]. Schon *et al.* propagated the manual pellet production in a 96 well format, a high throughput production process instead of pellet production in tubes. The reasons are the saving of time (capping, uncapping cell culture vessels) and medium (reduced volume). Additionally, Schon *et al.* produced pellet cultures in a large scale 96 well format with an increased amount of pellet cultures associated with reduced costs [57]. We strongly support these views. The 96 well formats for manufacturing 3D cell cultures support the automation of these methods. Especially, the stable automatic manufacturing process has the potential for further reduction of time and costs. Ibold *et al.* compared the manual and automatic cell seeding of the 3-Dimensional Pannus Model in a 96-Well format from chondrocytes of porcine femur bones. The automatic cell seeding was realized with the CyBi™-Disk workstation (CyBio AG, Jena, Germany). The automatic process was associated with a reduced accuracy, cell count, pipetting time/plate, failure rate and higher reproducibility compared to the manual process [84]. However, the pellet formation using the Biomek® Cell Workstation is characterized by mainly similar screening results which are associated with a high accuracy and cell count [54].

The microscopic analysis enabled the investigation of 3D cell cultures in the cases of evaluation over the time, histological staining and detection of the sizes. The evaluation over the times showed the changes of the 3D constructs from week one until week five.

Fischbach *et al.* and Xu *et al.* displayed increased cell accumulations over the time in cell-alginate beads [78, 157]. These present necrotic and apoptotic niches are located in the center of tumors [158]. In our studies, the development of cell-aggregates within the alginate beads was confirmed from day 14 [73].

Tung *et al.* reported the formation and long-term cultivation of spheroids in hanging drops about 12 days. Even after a short period of one week stable spheroids were formed. This 3D constructs increased size until day 12 [48]. Amann *et al.* also showed an increased volume of Colo699 cancer cells until day 10 [159]. Kelm *et al.* showed in increased volume of HepG2 spheroids until day 15 and following decreased volume [160]. We support these results with an increased size until day 14. Subsequently, a spheroid with loose aggregation in the environment was shown from day 21 until day 35 [52].

Schon *et al*. investigated shape and size of manually produced pellet cultures in tubes and well plates. They detected an increased spherical over time and consistent roundness of the pellets cultures [78]. We support these observations. In our study, the pellet formation in both media (+P/S, -P/S) lasted up to day 21 including an increase in size [54].

The alginate beads and pellet cultures enabled additionally histological investigations.

The alginate beads were marked with Papanicolaous staining. The increased growth of the cell aggregates were shown from day 14 [73].

We imagined the newly synthesized matrix by Safranin-O- and Alcian blue staining of sectioned pellet cultures. We could not show new matrix on day 14, whereby, on day 35, an increase of Safranin-O-

and Alcian blue stained matrix was detectable. Schon *et al.* were also able to visualize new matrix in pellet cultures already from day 21 [57]. However, Jonitz *et al.* showed new matrix only on day 35 [31, 54].

Specific software (Image pro plus software) enabled the detection of the sizes. The alginate beads and spheroid cultures allowed these investigations in cause of their regular conformation.

The sizes (diameter) of HeLa-cell alginate beads amount 3,128 µm (manual production) and 3,191 µm (automatic production). These sizes showed the successful translation of the manual production into automatic procedures [49]. Certainly, automatically manufactured alginate beads are µ-constructs with a more than threefold decreased size. Moshaverinia *et al.* encapsulated dental derived mesenchymal stem cells and automatically generated scaffolds with a size of 1,000 µm ± 100 µm [33]. Chen *et al.* and Kim *et al.* automatically produced alginate beads with a size of 500 µm [77, 161]. Tagler *et al.* reduced the size of alginate constructs to 80 µm [32]. We suggest that an increased size of the cancer constructs better simulates in vivo conditions and cancer tissues for further investigations by the cell density and the environmental conditions [49].

The diameter (µm) of the manually and automatically produced spheroids (+P/S; -P/S) reached in the average 356.1 µm (day 14) and 301.1 µm (day 35). But the literature propagated the increased size until less than 15 days [48, 159, 160]. Kelm *et al.* reported that spheroid cultures of HeLa cells showed a brittle constitution at day 5 [156]. However long-term cultivation could enable the development of more stable and *in vivo* similar tumor constructs. Kelm *et al.* showed the development of low density (250 cells/well) spheroid cultures in hanging drops with sizes of 250 µm (HepG2 cells) and 308 µm (MCF-7 cells) at day 5 formed in hanging drops in a MicroWell MiniTray (Nunc). These enabled the production of 60 hanging drops per time. Our high-density spheroid constructs (40,000 cells/well) showed merely minor increased sizes on day 14. But we produced 196 hanging drops in the Perfecta® 3D Hanging Drop Plate per time [52].

In order to investigate the cell behavior within 3D constructs (alginate beads, spheroid cultures, pellet cultures) bioscreening by different assays were done after incubation periods of day 14 and day 35. These screenings should be the basis for advanced investigations e.g. compound tests. Nevertheless, the 3D constructs include different characteristics and the right bioscreening process should have been detected. The proliferation was evaluated by the EZ4U assay. The other method to evaluate the cell proliferation in 3D constructs is the quantification of DNA. This method is useable for long term cultivation of 3D cell constructs because the compaction of the constructs might reduce the conversion of the reagent inside the 3D cell culture by living cells [52]. The cytotoxicity was detected by the adenylate kinases released by the damaged plasma membrane of dead cells [73]. Regularly, the toxicity is evaluated by fluorescence microscopy in combination with the Live/Dead assay or indirect quantified by detection of the proliferation. Hereof, researchers assume that a low proliferation is combined with a high toxicity and vice versa after a specific incubation period. This has to be investigated for 3D constructs. The bioscreenings were performed in manual and automatic processes.

Coward *et al.* published an increased proliferation till day ten of low density alginate beads (0.5×10^6 cells/ml alginate) and higher proliferation rates of cells in three dimensional cell culture systems caused by lower shear stress [74]. However, Moshaverinia *et al.* showed a decreased proliferation of a high density alginate bead cultures (2×10^6 cells/ml alginate) till day 14 [79]. Our bioscreening

confirmed these results. The EZ4U assay and DNA quantification (manual, automatic) also visualized the partly significant reduction of the proliferation of high-density alginate beads from day 14 till day 35. The reduction of the proliferation rates can be explained by the development of cell-aggregates and proliferation resistance caused by limited space in the beads [78]. The reduced cell growth is also combined with the accumulation of metabolism end products and hypoxia within three dimensional cell culture systems [73, 162, 163].

The investigation of the proliferation of spheroids in hanging drop cultures is manifold. The bioscreenings were evaluated by Alamar blue [48], staining with KI-67 [159, 164], a BrdU incorporation assay [164] and FACS analysis [159]. Analogous to Jogensen *et al.* [164], the proliferation of spheroid cultures over the time was decreased. However, the CIS cells showed a partly significant decreased proliferation until day 14 [164]. We detected the proliferation of cervix carcinoma cells-spheroids by the EZ4U proliferation assay until day 35 with the same consequence, the significantly reduced proliferation from day 14 to day 35. Instead, Amann *et al.* showed an increased cancer cell proliferation (A549 and Colo699) until day 10 [159]. However, the detection of the DNA amount visualized the similar results related on the proliferation like the EZ4U proliferation bioscreening. Nevertheless, the evaluation of the DNA required the prior isolation but enabled the direct quantification of the DNA amount (ng/ml). The spheroids were mainly long-term cultivated until 15 days. Consequently, the compaction was not to strong that reagents are unable to reach all cells in the construct [52].

Regularly, cell proliferation of chondrocytes in 3D constructs was evaluated by DNA analysis [31, 57, 84]. Schon *et al.* published a decreased DNA amount of pellet cultures over the time of 28 days because of predominantly re-differentiation processes [57]. Instead, Jonitz *et al.* embedded human chondrocytes in alginate matrix. This 3D construct supported an significantly increased DNA amount from day 14 to day 35 by the addition of chondrogenic growth factors (IGF-1 and TGF-β1) [31]. We detected a minor increased DNA amount from day 14 to day 35 in pellet cultures, which seems to be associated with an increased proliferation of the chondrocytes over the time. On day 14 and day 35, the detection of the proliferation by the EZ4U is comparable with the DNA quantification and displays predominantly similar results. In detail, the proliferation of automatically produced pellet cultures is mainly increased compared to the manually produced pellets (±P/S). The increased compactness and formation of new matrix might affect the screening by the EZ4U assay on day 35 so that the reagent could not be reduced by every vital cell in the 3D cell culture [54].

Usually, the Live/Dead assay is used for the differentiation of living and death cells embedded in alginate by microscopic (fluorescence) evaluation, which do not allow quantification processes. Certainly, cytotoxicity bioscreening of cells in alginate beads was not a common screening form. Xu *et al.* published that the reduction of the toxicity in alginate beads over the time is connected with the reduced proliferation rate caused by proliferation resistance [78]. Vemmer *et al.* supported this by the meaning that the embedded cells are protected against biotic and abiotic factors resulting in a longer persistence [28]. Our screening methods confirmed the quantified reduction of the toxicity. The results showed a decreased cytotoxitiy of alginate beads from day 14 to day 35 [73].

The evaluation of the cytotoxicity in spheroid cultures formed in hanging drops is a rather neglected issue. Merely, Kelm *et al.* detected death cells of HepG2 spheroids by Live/Dead assay and consequently microscopic imaging with a low concentration of death cells [160]. In contrast, we directly detected the cytotoxicity by the quantification of adenylate kinases, which decreased from day 14 to day 35. This could be explained by the increased concentration of metabolic products by

the media change and hypoxic conditions inside the spheroid [81] by more compaction of 3D constructs over the time resulting in a reduced amount of living cells over the time [52].

We investigated a new cytotoxicity and non-published bioscreening for pellet cultures. The toxicity of pellet cultures (manual and automatic production) decreased from day 14 to day 35. The cytotoxicity had to be detectable by diffusion and exchange of substances. This toxicological bioscreening might be an indicator for the quality and survival of chondrocytes within pellet cultures [54].

In general, antibiotics for cell cultures such as penicillin and streptomycin (P/S) are routinely used because they are low-dose, nontoxic, and stable under different temperature conditions and pH values [142, 165]. But the investigation of the possible influence of antibiotics is a neglected topic in the cell culture research, especially for 3D cell cultures.

Duewelkhenke *et al.* could not substantiate any effect of penicillin and streptomycin on proliferation, metabolic activity, and production of lactate in 2D HeLa cell cultures [166]. Instead, Cohen *et al.* published the negative proliferation (mRNA expression) and differentiation effect of P/S compared to cells without antibiotic [120, 142]. The basic investigations of different 2D test groups (manual/sterile, automatic/sterile, manual/unsterile) showed opposite results. The adherent HeLa cells cultivated under sterile conditions (manual, automatic) support the results of Cohen *et al.*. Herein, the cells cultivated in media without antibiotics showed pro-proliferative behavior compared with the lower proliferation of cells in media without antibiotics. Contrary, the manually seeded HeLa cells (test group: manual/unsterile) in media without antibiotics (-P/S) presented mainly significant (p<0.01, p<0.001) decreased proliferation rates under unsterile conditions. Thus, the culture conditions (sterile, unsterile) seem to have strong influence of the cell proliferation. Generally, the EC_{50} concentrations and effect of cyclophosphamide of cells with antibiotics (+P/S) are higher than of cells without P/S. The half-maximal concentrations of cells without antibiotic were identified in the range of 15-16 mmol/l and of cells with antibiotic from 12-15 mmol/l. Obviously higher concentrations of cytostatic agent had to be used to kill carcinoma cells in media with antibiotics [143]. Higher concentrations of the cytotoxic agent are needed to reduce the proliferation of cervix carcinoma cells in media with antibiotics (+P/S) to an effect of 50 % although cells in media without antibiotic (-P/S) show the pro-proliferative characteristics (sterile).

The differences of 3D cell cultures in different media (± antibiotics) were mainly non-significant at the bioscreening processes. The alginate beads showed higher cell proliferation in media without antibiotics (-P/S) quantified by the EZ4U assay (day 14, day 35). Merely, this was confirmed by the DNA quantification on day 14. On day 35 the opposite effect with lower DNA amount of beads in media without antibiotics (-P/S) was shown. The mainly higher proliferation of cells embedded in alginate matrix cultivated in media without antibiotics (-P/S) was associated with lower toxicity of beads in media without antibiotics (-P/S) on day 14 and day 35. The spheroids formed in hanging drops cultivated in media without antibiotics (-P/S) showed lower cell proliferation at both bioscreening processes (EZ4U assay, DNA quantification) at both detection dates (day 14, day 35). However, the cytotoxicity of spheroids in media without antibiotics (-P/S) was lower, too. The investigations of the cell proliferation showed mainly lower DNA amounts of pellets in media without antibiotics (-P/S; day 14, day 35). This was also detectable by the EZ4U assay on day 14. Contrary, on day 35 the cell proliferation was higher of pellets in media without antibiotics (-P/S). Nevertheless, the AK assay displayed a higher toxicity of pellets in media without antibiotics (-P/S).

The antibiotics seem to promote the proliferation of cervix carcinoma 3D spheroid cultures formed in hanging drops [72] in contrast to the cultivation of cervix carcinoma cells within alginate matrix. But

the cytotoxicity of cervix carcinoma cells in media without antibiotics (-P/S) presented lower toxicities. However, the cultivation of pellet cultures in media without antibiotic (-P/S) showed mainly lower proliferations associated with increased cytotoxicity.

The automation of processes propagated a reduction of the automatically performed procedures compared with the manual methods. Indeed, this has been only partially realized in the cell culture processes. The automatic production of 3D cell cultures showed mainly lower standard errors by evaluation of the proliferations compared to standard errors of the manually performed handling. However, the toxicity screening was combined with a higher standard error of the automatically produced 3D cultures consisting of cervix carcinoma cells (alginate beads, spheroid cultures). Contrary, the automatically produced pellet cultures showed reduced standard errors for the toxicity bioscreening. Reasons for this are the variability of biological processes during the work with cell cultures or a higher cell loss in the automatic processes. Nevertheless, the automatic processes were only minor different compared with the traditional manual methods, which support the possible replacement of the common procedures by automatic procedures.

8 Conclusion

In this work, the Biomek® Cell Workstation is presented as a flexible system for the automatic cell cultivation. After modifications, this system enables the cultivation of different 2D cell types (adherent cells, suspension cells). Additionally, the Biomek® Cell Workstation enables the passaging of cells, the expansion, media change and dissemination of exact cell numbers in well plates for further bioscreenings. In the last years, 3D cell constructs replace 2D cell cultures in case of better simulation of *in vivo* condition and physiological tissue. The manufacturing of different 3D cell cultures (alginate beads, spheroid cultures, and pellet cultures) could be realized using the Biomek® Cell Workstation.

This complex, moveable and flexible system is vertical arranged with a small footprint. The integrated devices are not producer-bound. Furthermore, the used AutoFlasks™ have almost the similar growth area like commonly used T75cm^2 cell culture vessels. The central component is the liquid handler, which is connected with the integrated devices. There is no robot required to connect the devices that may exceed the costs. The sterile cell handling is guaranteed by the housing with the HEPA-Filter, the UV-light, the flushing steps for cleaning and decontamination of the tube system for contamination free cell cultivation. The benefits of the software are the simple optimization of liquid handler steps (Biomek® software), the intuitive programming and scheduling of the methods as well as the graphical overview and monitoring of the running processes (SAMI®Proccess Definiton Editor) [110].

The basis for successful automatic cell cultivation should be the prevention and exclusion of contaminations in any form. Standardized rinsing steps with water and disinfection solutions during the processes as well as the flushing of the tubing systems are fundamental for the automatic cell cultivation. The exclusion of cross contamination is an important point at automatic systems for the cultivation of different cell lines. The new methods to evaluate this can be fluorescence labeling of cell lines with PKH stains and consequently microscopic analysis or quantifications by FACS analysis after automatic cell cultivation processes.

The cervix carcinoma cell cultures in three different test groups (manual/sterile, manual/unsterile, and automatic/sterile) were investigated for the basically investigation of adherent 2D cell cultures. The cells were disseminated manually and automatically with and without antibiotics under sterile and unsterile conditions. Growth curves were recorded to find EC$_{50}$ values for cells treated with the chemosensitivity agent cyclophosphamide. Similar performance of automatic and manual dissemination was indicated by proliferation rates, growth curves, and half-maximal effective dose (EC$_{50}$). The EZ4U proliferation test was used to evaluate the proliferation and toxicity of cells in automatic bioscreening [142].

The automatic cultivation and dissemination of suspension cells has not been published before and was realized with the Biomek® Cell Workstation. The proliferation, detected by the WST-1 assay, showed non-significant differences between manual and automatic processes combined with a lower standard error of the automatic method.

The cultivation of high density alginate beads over 35 days is connected with the development of constructs simililar to cancer tissue by the aggregates. This is associated with a decreased proliferation and toxicity due to the limited space in alginate beads, the arrest of the cells and the cell growth. In summary, we successfully established a new method for the automatic alginate bead production. The automatically produced beads are comparable with the manual produced beads based on the size (average 3 mm) and the decrease of the proliferation as well as the cytotoxicity

from day 14 to day 35. This new automatic manufacturing method is close to the manual alginate bead production. Non-published fully automatic processes were shown, which includes the manufacturing, cultivation and bioscreening of three dimensional alginate bead cultures. We recommend the cultivation without antibiotics (-P/S) for a better simulation of *in vivo* conditions. The proliferation of beads in media without antibiotics (-P/S) is higher and the toxicity is lower compared to the beads with antibiotics [73]. The proliferation screening is possible with the EZ4Uassay and the DNA quantification. The proliferation screening of long-term cultivated 3D constructs should be realized using the DNA quantification because the reagent possibly not reaches the center of the cell aggregates.

The production of spheroids in hanging drops is a fast method to form 3D cell cultures. Tumor tissue mimicking spheroids can be formed in less than two weeks. This manufacturing form associated with 384 well plates is predestined for the automatic production an industrial scale format and the subsequently high throughput bioscreening. The adapted bioscreening assays to evaluate the proliferation for spheroids are manifold. The automatic manufacturing of spheroid cultures by the Biomek® Cell Workstation with the consequently non-published screenings to detect the cytotoxicity (adenylate kinase assay) and cell proliferation (EZ4U assay, DNA isolation and quantification) was successfully adapted. The toxicities and proliferations of spheroids in media without antibiotics (-P/S) were increased but this cultivation form is a better mimicking of *in vivo* conditions and prevents possible interactions between compound and antibiotics at the compound screening. The automatic processes enable the replacement of the manual methods shown by the similar results. The long-term formation of spheroids in hanging drops should not last longer than 14 days because the proliferations significantly decreased to day 35 [52].

Pellet cultures were successfully established as an automatic solution for the manufacturing and screening of pellet cultures with human chondrocytes. The pellet manufacturing by the Biomek® Cell Workstation is followed by a consequently bioscreening using the high throughput screening system and these combined processes are non-published. The comparative analyses of manually and automatically produced pellet cultures show similar results. The pellet formation, the content of newly synthesized matrix as well as the proliferation and toxicity are comparable from day 14 until day 35. The proliferation increased from day 14 to day 35 in contrast to the cytotoxicity. The process automation is advantageous for the therapy of variety diseases. The proliferation screening by the EZ4U assay shows predominantly similar results compared with the regular detection of the DNA amount. Furthermore, we advise the cultivation of pellets in media without antibiotics (-P/S) resulting from the analysis as well as an improved simulation of *in vivo* conditions [54].

The manual methods are replaceable by the automatic techniques. Furthermore, it can be concluded that the antibiotic-free cultivation method is optimal; especially since the dissemination of cells without antibiotics using the automatic cell cultivation system is stable. Chemosensitivity of agents should be measured in antibiotic-free media to exclude any influence of the antibiotics on the cells [142]. The same applies for the 3D cell constructs.

9 Outlook

In the future, a further development of the Biomek® Cell Workstation might improve the processes and would enable the integration of new methods in the field of cell culture research.

The exclusion of cross contamination can be improved by cultivation of more than two cell lines as well as the influence of long-term cultivation, more than two weeks in the Biomek® Cell Workstation, in order to evaluate possible growing of one cell in the pipes over the detection limit. Another possibility would be the cultivation of suspension cells, which has a total different dynamic in cultivating and is probably more difficult to eliminate out of the pipes which can lead to higher risks of contaminations. Furthermore, the consequently FACS analysis could distinguish the different cell lines in a simple way in case of their sizes. Subsequently, the evaluation of variations for disinfection processes to maximize the time saving [134].

The automatic investigation of suspension cells can be extended by treatment of the cells with compounds for subsequently screening of the proliferation by the high throughput screening system [110].

The alginate beads display a great potential for further investigations in the field of compound screenings and biomaterial research. Next to the cells biomaterials and drug depots can be embedded into the alginate matrix in an automatic way to evaluate the cell behavior by consequently bioscreening processes. Furthermore, the DNA quantification of long term cultures can be improved by integration of automatically cell isolation and a reduction of cell loss at this process.

The spheroid cultures processes can be extended by co-cultivation of different cell lines. Hereof, for instance, human osteoblasts can be cultivated with bone cancer cells to investigate the behavior of cancer cells toward the healthy cells. The labeling with GFP (green fluorescent protein) would improve the microscopic analysis. Furthermore, the consequently compound screening of spheroids formed in hanging drops should be adapted [52].

The investigation of pellet cultures can be improved by automatic DNA isolation [54]. Additionally, the reduction of cell loss should be improved. Regarding this, the most sensitive step is removing of the supernatant after the centrifugation step. A method or system should be capable to detect the pellet for removing the supernatant from the opposite site and diminish cell loss. The recognition of the pellet could be performed using a camera system at the syringe or a scanning procedure of the bottom from well plates.

The further equipping of the Biomek® Cell Workstation should be the integration of a device to investigate the confluence of the monolayer before the cell culture processes. Additionally, the integration of another liquid handler could improve the throughput of the production of 3D cell cultures. A modified Biomek® NX MC with a steel cannula displays an adequate solution. The steel cannula enables the regular cell culture processes e.g. solution transfer within the AutoFlask™ and the 96 well pipetting head permit the fast production of the 3D cell cultures.

Subsequently, new methods can be integrated at the automatic systems. Suitable 3D cell cultures constructs for automatic processing are skin models. Skin cells can embedded in hydrogel and cultivated within inserts. Furthermore, the automatic histological staining would improve the cell culture research performed using the High Throughput Screening System.

10.1 Summary (English)

In this work the modification of the Biomek® Cell Workstation was improved. Next to the basically adherent cell cultivation the new integrated devices, ALPs and processes allowed the further cultivation of suspension cells and the manufacturing of 3D cell constructs (alginate beads, spheroid cultures, pellet cultures). The processes were manually and automatically performed to evaluate the automatic translation. The consequently bioscreenings (proliferation: EZ4U assay, DNA quantification; cytotoxicity: AK-assay) by the High Throuput Screening System enabled the quality control and display the basis for further investigations.

The combination of integrated and new adapted standardized disinfection and decontamination procedures guaranteed the contamination free automatic cell cultivation. The investigation of 2D cell cultures (adherent cells, suspension cells) showed minor lower proliferation of automatically processed cells (EZ4U assay, WST assay). The cells (adherent cells) under sterile conditions in media without antibiotic (-P/S) showed higher cell proliferation in contrast to unsterile handled cells. The half maximal effective dose (EC_{50}) of cells in media with antibiotics (+P/S) was higher after cyclophosphamide treatment. The 3D cell constructs containing cervix carcinoma cells (alginate beads, spheroid cultures) and human chondrocytes (pellet cultures). The microscopic analysis and Papanicolaous staining of alginate beads with embedded cells showed an average size of 3.19 mm since the manufacturing (day 1) associated with a formation of cell aggregates over the time. The spheroids in hanging drops already developed within two weeks and reached a maximum size of 0.36 mm. The proliferation and cytotoxicity decreased from day 14 to day 35. The development of pellet cultures with new synthesized matrix (Safranin-O-, Alcian blue staining) needed 35 days. The proliferation was mainly increased instead of the toxicity from day 14 to day 35. The manually and automatically evaluated results of the manufacturing processes and the bioscreening showed similar results compared to the automatic processes. Furthermore, the cultivation and detection of cell behavior in media without antibiotics (-P/S) was successfully implemented for the automatic cell handling and better mimicking of physiological conditions. The automatic methods enable the replacement of the traditional manual handling. The influence of antibiotics on the cell cultures is quite different. The proliferation of the cervix carcinoma (HeLa) cells cultivated in media without antibiotics (-P/S) in the 2D cell culture and the alginate beads showed an increased cell growth compared to cells in media with antibiotics (+P/S), instead of HeLa cell-spheroids formed in hanging drops and pellet cultures. The cytotoxicity of 3D cancer cultures (hanging drops, alginate beads) in media without antibiotics (-P/S) was decreased compared to 3D cultures in media with antibiotics (+P/S), contrary to pellet cultures with an increased cytotoxicity. The reduced standard errors of automatically processed cells could be partly achieved (2D cell cultures, 3D cell cultures-EZ4U assay). The Biomek® Cell Workstation is a flexible cell culture system to process different cell lines and 3D constructs in the automatic way.

10.2 Summary (German)

Die zunehmende alternde Bevölkerung ist verbunden mit verschiedenen Erkrankungen (z.B. Krebs, defekte des Gelenkapparates), welches neue therapeutische Strategien benötigt. In den letzten 30 Jahre ersetzen 3D Zellkulturen die regulären 2D Kulturen aufgrund der besseren Simulation von in vivo Bedingungen und dem Nachahmen von physiologischen Gewebe. Diese manuellen Methoden

benötigen viel Zeit und sind mit hohen Kosten verbunden. Deswegen können die automatisierte Herstellung und die biologischen Untersuchungen diese Prozesse sowie die Zellkulturforschung verbessern. In dieser Arbeit erfolgt die Modifikation der Biomek® Cell Workstation. Neben der grundlegenden Kultivierung von adhärenten Zellen erlauben neu integrierte Geräte, ALPs und Prozesse die Kultivierung von Suspensionszellen und die Herstellung von 3D Zellkulturen (Alginatbeads, Sphäroid Kulturen, Pellet Kulturen). Diese Prozesse wurden manuell und automatisiert durchgeführt, um die automatisierte Übersetzung zu untersuchen. Die anschließende mikroskopische Untersuchung und das Bioscreening (Proliferation: EZ4U-Assay, DNA Quantifizierung; Zytotoxität: AK-Assay) wurden mit der Hochdurchsatzuntersuchungsanlage durchgeführt, welche die Qualitätskontrolle ermöglicht und Basis für weitere Untersuchungen ist z.B. Untersuchung von Präparaten. Zudem wurden die Notwendigkeit und der mögliche Einfluss von Antibiotika auf Zelllinien, Untersuchungen, Wirkstoffen sowie auf den automatisierten Prozess geprüft.

Die Kombination von integrierten und neu adaptieren standarisierten Desinfektions- und Dekontaminationsprozessen garantierte die kontaminationsfreie Zellkultivierung. Die Untersuchung von 2D Kulturen (adhärente Zellen, Suspensionszellen) zeigte geringfügig niedrigere Proliferation von automatisiert prozessierten Zellen (EZ4U assay, WST assay). Zellen (adhärente Zellen), die unter sterilen Bedingungen sowie in Medium ohne Antibiotika (-P/S) kultiviert wurden, zeigten eine höhere Zellproliferation im Gegensatz zu unsteril kultivierten Zellen. Die halbmaximal effektive Dosis (EC50) von Zellen in Medium mit Antibiotika (+P/S) war höher nach der Behandlung mit Cyclophosphamid. Die 3D Zell-Konstrukte enthielten zervikale Krebszellen (Alginatbeads, Sphäroid Kulturen) und menschliche Knorpelzellen (Pellet Kulturen). Die mikroskopischen Untersuchungen und Papanicolaous-Färbungen der Alginatbeads mit eingebetteten Zellen zeigten im Durchschnitt eine Größe von 3,19 mm seit dem Tag der Herstellung (Tag 1) verbunden mit der Bildung von Zellaggregaten über die Zeit. Die Sphäroide in hängenden Tropfen entwickelten sich innerhalb von zwei Wochen und erreichten eine maximale Größe von 0,36 mm. Die Proliferation und Zytotoxität nahmen von Tag 14 bis Tag 35 ab. Die Entstehung von Pellet Kulturen mit neu synthetisierter Matrix (Safranin-O-, Alcian blau Färbungen) benötigte 35 Tage. Die manuell und automatisiert untersuchten Ergebnissen der Herstellungsprozesse und der biologischen Untersuchungen zeigten gleiche Resultate verglichen mit den automatisierten Prozessen. Zudem wurde die Kultivierung und Untersuchung vom Zellverhalten in Medium ohne Antibiotika für die automatisierten Zellkultivierung und der besseren Nachahmung von physiologischen Bedingungen erfolgreich implementiert. Die automatisierten Methoden ermöglichen das Ersetzen der traditionellen manuellen Handhabung. Der Einfluss von Antibiotika auf Zellkulturen ist recht unterschiedlich. Die Proliferation zervikaler Krebszellen (HeLa) kultiviert in Medium ohne Antibiotika (-P/S) in 2D Zellkulturen und Alginatbeads zeigte einen gesteigerten Zellwachstum verglichen mit Zellen kultiviert in Medium mit Antibiotika (+P/S), im Gegensatz zu HeLa-Sphäroiden geformt in hängenden Tropfen und Pellet Kulturen. Die Zytotoxität von 3D Krebskulturen (hängende Tropen, Alginatbeads) in Medium ohne Antibiotika (-P/S) war reduziert verglichen zu 3D Kulturen in Medium mit Antibiotika (+P/S), im Gegensatz zu Pellet Kulturen mit einer höheren Zytotoxität. Die reduzierten Standardfehler von automatisiert prozessierten Zellen konnte teilweise erreicht werden (2D Zellkulturen, 3D Zellkulturen-EZ4U Assay). Die Biomek® Cell Workstation ist ein flexibles Zellkultivierungssystem um verschiedene Zelllinien und 3D Konstrukte automatisiert zu prozessieren.

11 References

[1] Tomasetti, C., Vogelstein, B., Cancer etiology. Variation in cancer risk among tissues can be explained by the number of stem cell divisions. *Science (New York, N.Y.)* 2015, *347*, 78–81.

[2] Robert Koch-Instituts und der Gesellschaft epidemiolische Krebsregister in Deutschland e.V, Krebs in Deutschland 2007/2008 [Internet], 8th Ed. [cited 2013 Jul 10]. Available from: http://www.gekid.de/Doc/krebs_in_deutschland_2012.pdf.

[3] Awasare, S., Bhujbal, S., Nanda, R., In vitro cytotoxic activity of novel oleanane type of triterpenoid saponin from stem bark of Manilkara zapota Linn. *Asian Journal of Pharmaceutical and Clinical Research* 2012, *5*, 183–188.

[4] Mayr, L. M., Bojanic, D., Novel trends in high-throughput screening. *Current Opinion in Pharmacology* 2009, *9*, 580–588.

[5] Kolukisaoglu, Ü., Thurow, K., Future and frontiers of automatic screening in plant sciences. *Plant Science* 2010, *178*, 476–484.

[6] Markin, R. S., Whalen, S. A., Laboratory automation: trajectory, technology, and tactics. *Clinical chemistry* 2000, *46*, 764–771.

[7] Blow, N., Lab automation: tales along the road to automation. *Nature Methods* 2008, *5*, 109–112.

[8] Chapman, T., Lab automation and robotics: Automation on the move. *Nature* 2003, *421*, 661, 663, 665-6.

[9] Carere, A., Stammati, A., Zucco, F., In vitro toxicology methods: impact on regulation from technical and scientific advancements. *Toxicology Letters* 2002, *127*, 153–160.

[10] Junginger, S., System for automatic cell culture [Internet], Fakultät für Informatik und Elektrotechnik (IEF), 2011 [cited 2013 Jul 25]; [Dissertation]. Available from: http://rosdok.uni-rostock.de/file/rosdok_derivate_000000004620/Dissertation_Junginger_2011.pdf.

[11] Thomas, R., Ratcliffe, E., Automatic Adherent Human Cell Culture (Mesenchymal Stem Cells), in: Mitry, R. R., Hughes, R. D. (Eds.), *Human Cell Culture Protocols*. *Methods in Molecular Biology*, Humana Press, Totowa, NJ 2012, pp. 393–406.

[12] Lindl, T., Gstraunthaler, G., *Zell- und Gewebekultur: Von den Grundlagen zur Laborbank*, 6th Ed., Spektrum Akademischer Verlag, Heidelberg, Neckar 2008.

[13] Pampaloni, F., Stelzer, E., Masotti, A., Three-Dimensional Tissue Models for Drug Discovery and Toxicology. *Recent Patents on Biotechnology* 2009, *3*, 103–117.

[14] Manpadi, M., Uglinskii, P. Y., Rastogi, S. K., Cotter, K. M. et al., Three-component synthesis and anticancer evaluation of polycyclic indenopyridines lead to the discovery of a novel

indenoheterocycle with potent apoptosis inducing properties. *Organic & biomolecular chemistry* 2007, *5*, 3865–3872.

[15] Bartis D., Pongrácz J., *Three dimensional tissue cultures and tissue engineering*, 2011.

[16] Cabral, J., Ex vivo expansion of hematopoietic stem cells in bioreactors. *Biotechnology Letters* 2001, *23*, 741–751.

[17] Tang, Y.-J., Li, H.-M., Hamel, J.-F., Suspension Culture Process of MethA Tumor Cell for the Production of Heat-Shock Protein Glycoprotein 96: Process Optimization in Spinner Flasks. *Biotechnology Progress* 2007, *23*, 1363–1377.

[18] Beckman Coulter, Inc., Automating Cell Culture Processes with Integrated Biomek® Workstations [Internet], 2012. Available from: http://www.analis.be.

[19] Lehmann, R., Thurow, K., Weippert, M., Human vs. Machine in Life Science Automation: Comparing Effectiveness of Manual and Automatic 3-D Cell Culturing Processes. *Proceedings of the 5th International Conference on Applied Human Factors and Ergonomics AHFE 2014.* 2014.

[20] Rimann, M., Graf-Hausner, U., Synthetic 3D multicellular systems for drug development. *Current Opinion in Biotechnology* 2012, *23*, 803–809.

[21] Tebenkova, I., Vergleich zweier trägerfreier Kultursysteme bei der chondrogenen Differenzierung humaner mesenchymaler Stammzellen [Internet], Ludwig-Maximilians-Universität München, München. Orthopädische Klinik und Poliklinik Großhadern der LMU München, 2011 [cited 2013 Jun 30]. Available from: http://edoc.ub.uni-muenchen.de/13240/1/Tebenkova_Irina.pdf.

[22] reinnervate, alvetex 96 well scaffold: Instructions for use and example applications [Internet], reinnervate-The REAL 3D Cell Culture Company, 2014 [cited 2014 Oct 28]. Available from: http://reinnervate.com/using-alvetex/protocols/alvetex-scaffold-96-instructions-and-examples/.

[23] GE Healthcare, Cytodex 1, Cytodex 3: Instructions 18-1119-79 AD [Internet], GE Healthcare Bio-Sciences AB, 2006 [cited 2014 Oct 28]. Available from: https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1314716762536/litdoc18111979_201112141640 06.pdf.

[24] Orive, G., Maria Hernández, R., Rodríguez Gascón, A., Calafiore, R. et al., History, challenges and perspectives of cell microencapsulation. *Trends in Biotechnology* 2004, *22*, 87–92.

[25] Hunt, N. C., Grover, L. M., Cell encapsulation using biopolymer gels for regenerative medicine. *Biotechnology Letters* 2010, *32*, 733–742.

[26] Gibbs, B. F., Kermasha, S., Inteaz, A., Encapsulation in the food industry: a review. *International Journal of Food Sciences and Nutrition* 1999, *50*, 213–224.

[27] Onwulata, C. I., Encapsulation of new active ingredients. *Annual review of food science and technology* 2012, *3*, 183–202.

[28] Vemmer, M., Patel, A. V., Review of encapsulation methods suitable for microbial biological control agents. *Biological Control* 2013, *67*, 380–389.

[29] Wandrey, C., Bartkowiak, A., Harding, S. E. (Eds.), *Encapsulation Technologies for Active Food Ingredients and Food Processing: Materials for Encapsulation*, Springer New York, 2010.

[30] Obst, M., Steinbüchel, A., Microbial degradation of poly(amino acid)s. *Biomacromolecules* 2004, *5*, 1166–1176.

[31] Jonitz, A., Lochner, K., Peters, K., Salamon, A. et al., Differentiation Capacity of Human Chondrocytes Embedded in Alginate Matrix. *Connective Tissue Research* 2011, *52*, 503–511.

[32] Tagler, D., Makanji, Y., Anderson, N. R., Woodruff, T. K. et al., Supplemented αMEM/F12-based medium enables the survival and growth of primary ovarian follicles encapsulated in alginate hydrogels. *Biotechnology and Bioengineering* 2013, *110*, 3258–3268.

[33] Moshaverinia, A., Chen, C., Akiyama, K., Xu, X. et al., Encapsulated dental-derived mesenchymal stem cells in an injectable and biodegradable scaffold for applications in bone tissue engineering. *Journal of biomedical materials research. Part A* 2013, *101*, 3285–3294.

[34] Zähringer, H., Produktübersicht: 3D-Zellkultursysteme: Wirtschaft. *Laborjournal* 2014, 78–82.

[35] Caicedo-Carvajal, C. E., Liu, Q., Remache, Y., Goy, A. et al., Cancer Tissue Engineering: A Novel 3D Polystyrene Scaffold for In Vitro Isolation and Amplification of Lymphoma Cancer Cells from Heterogeneous Cell Mixtures. *Journal of tissue engineering* 2011, *2011*, 362326.

[36] Gesim, BioScaffolder: 3D Prototyping and More [Internet], Gesim, 2013 [cited 2014 Oct 28]. Available from: http://www.gesim.de/upload/PDFs/GESIM-BioScaffold_EN.pdf.

[37] Timm, D. M., Chen, J., Sing, D., Gage, J. A. et al., A high-throughput three-dimensional cell migration assay for toxicity screening with mobile device-based macroscopic image analysis. *Scientific reports* 2013, *3*, 3000.

[38] Haisler, W. L., Timm, D. M., Gage, J. A., Tseng, H. et al., Three-dimensional cell culturing by magnetic levitation. *Nature Protocols* 2013, *8*, 1940–1949.

[39] Tseng, H., Gage, J. A., Raphael, R. M., Moore, R. H. et al., Assembly of a three-dimensional multitype bronchiole coculture model using magnetic levitation. *Tissue engineering. Part C, Methods* 2013, *19*, 665–675.

[40] Pampaloni, F., Reynaud, E. G., Stelzer, E. H. K., The third dimension bridges the gap between cell culture and live tissue. *Nature Reviews Molecular Cell Biology* 2007, *8*, 839–845.

[41] van Opstal, D., Boter, M., Jong, D. de, van den Berg, C. et al., Rapid aneuploidy detection with multiplex ligation-dependent probe amplification: a prospective study of 4000 amniotic fluid samples. *European Journal of Human Genetics* 2008, *17*, 112–121.

[42] Shield, K., Ackland, M. L., Ahmed, N., Rice, G. E., Multicellular spheroids in ovarian cancer metastases: Biology and pathology. *Gynecologic Oncology* 2009, *113*, 143–148.

[43] Hirschhaeuser, F., Menne, H., Dittfeld, C., West, J. et al., Multicellular tumor spheroids: An underestimated tool is catching up again. *Journal of Biotechnology* 2010, *148*, 3–15.

[44] Lin, R.-Z., Chang, H.-Y., Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnology Journal* 2008, *3*, 1172–1184.

[45] Mehta, G., Hsiao, A. Y., Ingram, M., Luker, G. D. et al., Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy. *Journal of Controlled Release* 2012, *164*, 192–204.

[46] Breslin, S., O'Driscoll, L., Three-dimensional cell culture: the missing link in drug discovery. *Drug Discovery Today* 2013, *18*, 240–249.

[47] Torisawa, Y.-S., Takagi, A., Nashimoto, Y., Yasukawa, T. et al., A multicellular spheroid array to realize spheroid formation, culture, and viability assay on a chip. *Biomaterials* 2007, *28*, 559–566.

[48] Tung, Y.-C., Hsiao, A. Y., Allen, S. G., Torisawa, Y.-s. et al., High-throughput 3D spheroid culture and drug testing using a 384 hanging drop array. *The Analyst* 2011, *136*, 473–478.

[49] Torisawa, Y.-S., Chueh, B.-H., Huh, D., Ramamurthy, P. et al., Efficient formation of uniform-sized embryoid bodies using a compartmentalized microchannel device. *Lab on a chip* 2007, *7*, 770–776.

[50] Lee, W. G., Ortmann, D., Hancock, M. J., Bae, H. et al., A hollow sphere soft lithography approach for long-term hanging drop methods. *Tissue Engineering Part C: Methods* 2010, *16*, 249–259.

[51] Hanahan, D., Weinberg, R. A., The Hallmarks of Cancer. *Cell* 2000, *100*, 57–70.

[52] Lehmann, R., Roddelkopf, T., Junginger, S., Thurow, K., Comparative Investigations of Manually and Automatically Manufactured and Screened Cervix Carcinoma Spheroid Cultures Formed in Perfecta3D® Hanging Drop Plates. *Engineering in life sciences* 2014 (under review).

[53] Schulz, J. C., Stumpf, P. S., Katsen-Globa, A., Sachinidis, A. et al., First steps towards the successful surface-based cultivation of human embryonic stem cells in hanging drop systems. *Engineering in life sciences* 2012, *12*, 584–587.

[54] Lehmann, R., Gallert, C., Roddelkopf, T., Junginger, S. et al., Comparative Analysis of Manually and Automatically Produced Pellet Cultures of Human Primary Chondrocytes. *Engineering in Life Sciences* 2014 (under review).

[55] Penick, K., Solchaga, L., Welter, J., High-throughput aggregate culture system to assess the chondrogenic potential of mesenchymal stem cells. *BioTechniques* 2005, *39*, 687–691.

[56] Ong, S.-Y., Dai, H., Leong, K. W., Inducing hepatic differentiation of human mesenchymal stem cells in pellet culture. *Biomaterials* 2006, *27*, 4087–4097.

[57] Schon, B. S., Schrobback, K., Ven, M., Stroebel, S. et al., Validation of a high-throughput microtissue fabrication process for 3D assembly of tissue engineered cartilage constructs. *Cell and Tissue Research* 2012, *347*, 629–642.

[58] Huang, A. H., Motlekar, N. A., Stein, A., Diamond, S. L. et al., High-Throughput Screening for Modulators of Mesenchymal Stem Cell Chondrogenesis. *Annals of Biomedical Engineering* 2008, *36*, 1909–1921.

[59] Chang, C.-H., Lin, H.-Y., Fang, H.-W., Loo, S.-T. et al., Chondrogenesis from immortalized human mesenchymal stem cells: comparison between collagen gel and pellet culture methods. *Artificial Organs* 2008, *32*, 561–566.

[60] Markovitz-Bishitz, Y., Tauber, Y., Afrimzon, E., Zurgil, N. et al., A polymer microstructure array for the formation, culturing, and high throughput drug screening of breast cancer spheroids. *Biomaterials* 2010, *31*, 8436–8444.

[61] Yoshii, Y., Waki, A., Yoshida, K., Kakezuka, A. et al., The use of nanoimprinted scaffolds as 3D culture models to facilitate spontaneous tumor cell migration and well-regulated spheroid formation. *Biomaterials* 2011, *32*, 6052–6058.

[62] InSphero, 3D InSight™ Liver Microtissues: Organotypic 3D Liver Models for ADME-Tox & DMPK [Internet], InSphero AG, 2014 [cited 2014 Oct 28]. Available from: http://www.insphero.com/images/LiverMT_Product_Guide_FinalLR.PDF.

[63] Cervinkova, D., Babak, V., Marosevic, D., Kubikova, I. et al., The role of the qacA gene in mediating resistance to quaternary ammonium compounds. *Microbial drug resistance (Larchmont, N.Y.)* 2013, *19*, 160–167.

[64] GE Healthcare, ReadyToProcess WAVE™ 25 [Internet], GE Healthcare Life Sciences, 2013 [cited 2014 Oct 31]. Available from: http://www.gelifesciences.com/gehcls_images/GELS/ Related%20Content/Files/1372245760275/litdoc29056695_20131105235910.pdf.

[65] Shayan, N., Ebrahimi, M., Beiki, B., Janzamin, E., A non-rotational, computer-controlled suspension bioreactor for expansion of umbilical cord blood mononuclear cells. *Biotechnology Letters* 2012, *34*, 2125–2131.

[66] Ebers Medical Technology, teb 1000: Bioreactor designed for tissue engineering [Internet], Ebers Medical Technology [cited 2014 Oct 31]. Available from: http://www.meintrup-dws.de/assets/product-pdfs/TEB1000/teb1000_brochure.pdf.

[67] Merck Millipore, CellASIC® ONIX Live Cell Analysis: Platform [Internet], Merck Millipore, 2014 [cited 2014 Oct 31]. Available from: http://www.emdmillipore.com/DE/de/life-science-research/cell-culture-systems/cellASIC-live-cell-imaging/platform.

[68] Dunn Labortechnik GmbH, Overview of Applications of Flexcell Products [Internet], Dunn Labortechnik GmbH, 2009 [cited 2014 Oct 31]. Available from: http://www.dunnlab.de/pdf/Flexcell/Flex_application.pdf.

[69] Dunn Labortechnik GmbH, Flexcell Tissue Train: Culture System [Internet], Dunn Labortechnik GmbH, 2009 [cited 2014 Oct 31]. Available from: http://www.dunnlab.de/pdf/Flexcell/ Product%20Guide%202014_FX-5000T.pdf.

[70] Dunn Labortechnik GmbH, Flexcell F-5000TM: Compression System [Internet], Dunn Labortechnik GmbH, 2009 [cited 2014 Oct 31]. Available from: http://www.dunnlab.de/pdf/Flexcell/Product%20Guide%202014_FX-5000C.pdf.

[71] Dunn Labortechnik GmbH, Flexcell Tissue Train Culture System [Internet], Dunn Labortechnik GmbH, 2009 [cited 2015 Jan 14]. Available from: http://www.dunnlab.de/pdf/Flexcell/ Product%20Guide%202014_FX-5000TT.pdf.

[72] Kumachev, A., Greener, J., Tumarkin, E., Eiser, E. et al., High-throughput generation of hydrogel microbeads with varying elasticity for cell encapsulation. *Biomaterials* 2011, *32*, 1477–1483.

[73] Lehmann, R., Gallert, C., Roddelkopf, T., Junginger, S. et al., 3 Dimensional Cell Cultures: A Comparison Between Manually and Automatically Produced Alginate Beads. *Cytotechnology* 2015, DOI: 10.1007/s10616-015-9861-1.

[74] Coward, S. M., Legallais, C., David, B., Thomas, M. et al., Alginate-encapsulated HepG2 Cells in a Fluidized Bed Bioreactor Maintain Function in Human Liver Failure Plasma. *Artificial Organs* 2009, *33*, 1117–1126.

[75] Coward, S. M., Selden, C., Mantalaris, A., Hodgson, Humphrey J F, Proliferation rates of HepG2 cells encapsulated in alginate are increased in a microgravity environment compared with static cultures. *Artificial Organs* 2005, *29*, 152–158.

[76] Rokstad, A. M., Gustafsson, B. I., Espevik, T., Bakke, I. et al., Microencapsulation of small intestinal neuroendocrine neoplasm cells for tumor model studies. *Cancer Science* 2012, *103*, 1230–1237.

[77] Kim, J., Sachdev, P., Sidhu, K., Alginate microcapsule as a 3D platform for the efficient differentiation of human embryonic stem cells to dopamine neurons. *Stem cell research* 2013, *11*, 978–989.

[78] Xu, X.-x., Liu, C., Liu, Y., Li, N. et al., Encapsulated human hepatocellular carcinoma cells by alginate gel beads as an in vitro metastasis model. *Experimental Cell Research* 2013, *319*, 2135–2144.

[79] Moshaverinia, A., Xu, X., Chen, C., Akiyama, K. et al., Dental mesenchymal stem cells encapsulated in an alginate hydrogel co-delivery microencapsulation system for cartilage regeneration. *Acta Biomater* 2013, *9*, 9343–9350.

[80] Leslie, S. K., Cohen, D. J., Sedlaczek, J., Pinsker, E. J. et al., Controlled release of rat adipose-derived stem cells from alginate microbeads. *Biomaterials* 2013, *34*, 8172–8184.

[81] Cavnar, S. P., Salomonsson, E., Luker, K. E., Luker, G. D. et al., Transfer, imaging, and analysis plate for facile handling of 384 hanging drop 3D tissue spheroids. *Journal of laboratory automation* 2014, *19*, 208–214.

[82] Hsiao, A. Y., Tung, Y.-C., Qu, X., Patel, L. R. et al., 384 hanging drop arrays give excellent Z-factors and allow versatile formation of co-culture spheroids. *Biotechnology and Bioengineering* 2012, *109*, 1293–1304.

[83] Hsiao, A. Y., Tung, Y.-C., Kuo, C.-H., Mosadegh, B. et al., Micro-ring structures stabilize microdroplets to enable long term spheroid culture in 384 hanging drop array plates. *Biomedical Microdevices* 2012, *14*, 313–323.

[84] Ibold, Y., Frauenschuh, S., Kaps, C., Sittinger, M. et al., Development of a high-throughput screening assay based on the 3-dimensional pannus model for rheumatoid arthritis. *Journal of Biomolecular Screening* 2007, *12*, 956–965.

[85] co.don AG, Gelenkknorpeltransplantation: Ohne künstliche oder fremde Materialien [Internet], co.don AG, 2014 [cited 2014 Nov 5]. Available from: http://www.codon.de.

[86] Mountzios, G., Soultati, A., Pectasides, D., Pectasides, E. et al., Developments in the systemic treatment of metastatic cervical cancer. *Cancer Treatment Reviews* 2013, *39*, 430–443.

[87] Varma H., Lo D.C., Stockwell B.R., High-Throughput and High-Content Screening for Huntington's Disease Therapeutics. *Neurobiology of Huntington's Disease: Applications to Drug Discovery* 2011, *5*.

[88] Fernandes, T. G., Diogo, M. M., Clark, D. S., Dordick, J. S. et al., High-throughput cellular microarray platforms: applications in drug discovery, toxicology and stem cell research. *Trends in Biotechnology* 2009, *27*, 342–349.

[89] Ferreira, M. V., Jahnen-Dechent, W., Neuss, S., Standardization of Automatic Cell-Based Protocols for Toxicity Testing of Biomaterials. *Journal of Biomolecular Screening* 2011, *16*, 647–654.

[90] Zanella, F., Lorens, J. B., Link, W., High content screening: seeing is believing. *Trends in Biotechnology* 2010, *28*, 237–245.

[91] Bray, M.-A., Fraser, A. N., Hasaka, T. P., Carpenter, A. E., Workflow and Metrics for Image Quality Control in Large-Scale High-Content Screens. *Journal of Biomolecular Screening* 2012, *17*, 266–274.

[92] Sannes, L. J., HIGH-CONTENT SCREENING: Parallel Analysis Fuels Accelerated Discovery and Development. *CHI Reports* 2002.

[93] HighTech Business Descisions, High-Throughput Screening 2002: New Stategies and Technologies: Volume I: Report 2002.

[94] Dainiak, M. B., Savina, I. N., Musolino, I., Kumar, A. et al., Biomimetic macroporous hydrogel scaffolds in a high-throughput screening format for cell-based assays. *Biotechnology Progress* 2008, *24*, 1373–1383.

[95] Lang, P., Yeow, K., Nichols, A., Scheer, A., Cellular imaging in drug discovery. *Nature reviews. Drug discovery* 2006, *5*, 343–356.

[96] Dobson, C. M., Chemical space and biology. *Nature* 2004, *432*, 824–828.

[97] Amouretti, X., Impact of HTS on multidetection microplate readers and benefits for life science research laboratories. *American Biotechnology Laboratory* 2006, *24*, 16.

[98] Instrument Business Outlook, Multimode microplate reader market [Internet], 2003 [modified 2003 Apr 30; cited 2013 Jul 23]. Available from: http://www.thefreelibrary.com.

[99] TURNER designs, TrilogyTM Laboratory Absorbance: theory of absorbance [Internet], TURNER designs [cited 2014 Nov 10]. Available from: http://www.turnerdesigns.com/t2/doc/appnotes/S-0075.pdf.

[100] So, P., Dong, C.Y., Fluorescence Spectrophotometry [Internet], Massachusetts Institute of Technology, 2002 [cited 2014 Nov 10]. Available from: http://web.mit.edu/solab/Documents/Assets/So-Fluorescence%20spectrophotometry.pdf.

[101] Moerke, N. J., Fluorescence Polarization (FP) Assays for Monitoring Peptide-Protein or Nucleic Acid-Protein Binding. *Current protocols in chemical biology* 2009, *1*, 1–15.

[102] Diamandis, E. P., Christopoulos, T. K., Time-Resolved Fluorescence, in: Kessler, C. (Ed.), *Nonradioactive Analysis of Biomolecules*, Springer Berlin Heidelberg, Berlin, Heidelberg 2000, pp. 289–294.

[103] Lakowicz, J. R., *Topics in Fluorescence Spectroscopy: Probe Design and Chemical Sensing*. *Topics in Fluorescence Spectroscopy*, Vol. *4*, Springer US, Boston, MA 2002.

[104] Wang, Z., Li, P., Guo, Q., Yang, Z., Luminescence and energy transfer of tunable emission phosphor Ca2PO4Cl:Ce(3+), Mn(2+). *Spectrochimica acta. Part A, Molecular and biomolecular spectroscopy* 2014, *137C*, 871–876.

[105] Worzella, T., Larson B., Automatic multiplexing of PROMEGA'S* cell-based assays with the BECKMAN COULTER BIOMEK® FX AND DTX 880 multimode detector [Internet], Beckman Coulter, Inc., 2005 [cited 2014 Nov 10]. Available from: http://www.beckmancoulter.de/beckman2_media/Life+Sciences/Produkte/Instrumente/Laboraautomation/Produkte/Pipettierer/Biomek+FXp/Apps/Multiplexing+Promega+Cell+Assays.pdf.

[106]   BMG Labtech, PHERAstar FS: Der ultimative Mikroplatten-Reader für High-Throughput Screening [Internet], BMG Labtech, 2014. Available from: http://www.bmglabtech.com/de/produkte/mikroplatten-reader/pherastar-fs-obj-42-741.html.

[107]   Oksa, V.-V., EnVision® Multilabel Reader: EnVision ® Multilabel Plate Reader Combines Filters and Monochromators [Internet], PerkinElmer Inc., 2014 [cited 2014 Nov 10]. Available from: http://www.perkinelmer.com/catalog/product/id/2104-0010.

[108]   Molecular Devices Inc., Microplate Readers [Internet], Molecular Devices Inc., 2014 [cited 2014 Nov 10]. Available from: http://www.moleculardevices.com/systems/microplate-readers.

[109]   Koppalt, T., Cell Culture Automation [Internet], 2009. Available from: http://www.labmanager.com.

[110]   Lehmann, R., Severitt, J. C., Roddelkopf, T., Junginger, S. et al., Biomek® Cell Workstation: a variable system for automatic cell cultivation. *Journal of the Assiociation for Laboratory Automation* 2014 (under review).

[111]   Liu, Y., Hourd, P., Chandra, A., Williams, D. J., Human cell culture process capability: a comparison of manual and automatic production. *Journal of Tissue Engineering and Regenerative Medicine* 2010, *4*, 45–54.

[112]   Reichl, F.-X., Esters, M., Simon, S., Seiss, M. et al., Cell death effects of resin-based dental material compounds and mercurials in human gingival fibroblasts. *Archives of Toxicology* 2006, *80*, 370–377.

[113]   RETISOFT Inc., Genera Software [Internet], RETISOFT Inc., 2014 [cited 2014 Nov 10]. Available from: http://www.retisoft.ca/software/genera.php.

[114]   PAA, Overlord3, real-time static dynamic scheduling laboratory automation software. [Internet], 2004 [cited 2012 Apr 18]. Available from: http://www.paa.co.uk.

[115]   AGILENT TECHNOLOGIES, Agilent Technologies VWorks Automation Control-User Guide [Internet], 2009 [cited 2012 Apr 18]. Available from: http://www.chem.agilent.com.

[116]   Beckman Coulter, Inc., Cell Culture goes fully automatic Biomek® Cell Workstation. [Internet] [cited 2012 Apr 13]. Available from: http://www.beckmancoulter.de.

[117]   Beckman Coulter Inc., Beckman Coulter Integrated Solutions [Internet], 2011 [cited 2014 Nov 11]. Available from: https://www.beckmancoulter.com/wsrportal/bibliography?docname=BR-14746.pdf.

[118]   Lincoln, C. K., Gabridge, M. G., Cell culture contamination: Sources, consequences, prevention, and elimination. *Methods in Cell Biology* 1998, *57*, 49–65.

[119]   Kuhlmann, I., The prophylactic use of antibiotics in cell culture. *Cytotechnology* 1995, *19*, 95–105.

[120]   Cohen, S., Samadikuchaksaraei, A., Polak, J. M., Bishop, A. E., Antibiotics Reduce the Growth Rate and Differentiation of Embryonic Stem Cell Cultures. *Tissue Engineering* 2006, *12*, 2025–2030.

[121]   Tomasz, A., From penicillin-binding proteins to the lysis and death of bacteria: a 1979 view. *Reviews of infectious diseases* 1979, *1*, 434–467.

[122]   Francois, B., Crystal structures of complexes between aminoglycosides and decoding A site oligonucleotides: role of the number of rings and positive charges in the specific binding leading to miscoding. *Nucleic Acids Research* 2005, *33*, 5677–5690.

[123]   Lehmann, R., Gallert, C., Roddelkopf, T., Junginger, S. et al., Biomek® Cell Workstation: a flexible system for automatic 3D cell cultivation. *Journal of the Assiociation for Laboratory Automation* 2015 (under review).

[124]   Beckman Coulter, Inc., Biomek® NXP Laboratory Automation Workstation [Internet], 2006 [cited 2014 Jul 16]. Available from: www.beckmancoulter.com.

[125]   TRIAUD, F., CLENET, D., CARIOU, Y., Lenell, T. et al., Evaluation of automatic cell culture incubators. *Journal of the Association for Laboratory Automation* 2003, *8*, 82–86.

[126]   Beckman Coulter, Inc., Automatic Labware Positioners (ALPs): ALPs Instructions For Use [Internet], 2012 [cited 2014 Jul 10]. Available from: https://www.beckmancoulter.com.

[127]   Beckman Coulter, Inc., Static Peltier Device [Internet], 2010 [cited 2014 Jul 10]. Available from: https://www.beckmancoulter.com.

[128]   Velocity11, VSpin™ User Manual version 3.0 [Internet], 2002 [cited 2013 Jul 25]. Available from: http://www.velocity11.com.

[129]   BD Biosciences, BD Biosciences - Discovery Labware Supplement [Internet], 2008 [modified https://www.bd.com/fr/biosciences/pdfs/helping_all_people.pdf; cited 2014 Dec 1].

[130]   greiner bio one, CELLSTAR ® AutoFlask™ Cell culture flask for automatic systems: Instructions for Use [Internet] [cited 2013 Jul 25]. Available from: http://www.lambda.at/UserFiles/File/AutoFlask/CELLSTAR_AutoFlask_Instruction_for_Use.pdf.

[131]   Corning Incorporated, Corning® RoboFlask® Cell Culture Vessels [Internet], 2012 [cited 2014 Dec 2]. Available from: http://www.corning.com/uploadedFiles/Lifesciences/PDFs/ProductInformation/CellCultureandBioprocess/CLS-CC-019REV2.pdf.

[132]   greiner bio-one, CELLSTAR AUTOFLASK TM Cell culture flask for automatic systems: Instructions for use [Internet] [cited 2014 Jul 15]. Available from: www.gbo.com/bioscience.

[133]   Beckman Coulter, Inc., Reservoirs [Internet], Beckman Coulter, Inc., 2010-2014 [cited 2014 Jul 15]. Available from: www.beckmancoulter.com.

[134]   Lehmann, R., Severitt, J. C., Roolf C., Thurow K., Role and Avoidance of Cross Contaminations in Automation Cell Culture Processes. *Journal of the Assiociation for Laboratory Automation* 2014 (under review).

[135]   Gallert, C., Lehmann, R., Roddelkopf, T., Junginger, S. et al., High Throughput Screening System for Measuring the Effect of Drugs on 3D Cell Cultures. *IEEE conference* 2014.

[136]   Armitage, P., Berry, G., Matthews, J. N. S, *Statistical methods in medical research*, 4th Ed., Blackwell Scientific Publications, Oxford, Boston 2002.

[137]   Altman, D. G., *Practical statistics for medical research*, 1st Ed., Chapman and Hall, London, New York 1991.

[138]   Bland, J. M., Altman, D. G., Statistics Notes: Measurement error. *British Medical Journal* 1996, *313*, 744.

[139]   Chatterjee, R., CELL BIOLOGY: Cases of Mistaken Identity. *Science* 2007, *315*, 928–931.

[140]   Dirks, W. G., Faehnrich, S., Estella, Isabelle Annick Janine, Drexler, H. G., Short tandem repeat DNA typing provides an international reference standard for authentication of human cell lines. *ALTEX* 2005, *22*, 103–109.

[141]   Boutonnât, J., Barbier, M., Rousselle, C., Muirhead, K. A. et al., Usefulness of PKHs for studying cell proliferation. *Comptes Rendus de l'Académie des Sciences - Series III - Sciences de la Vie* 1998, *321*, 901–907.

[142]   Lehmann R., Vetter L., Person L., Thurow K., Evaluation of Antibiotic-Free Cell Cultures in Manual and Automatic Cell Handling [Internet], American Laboratory, 2013 [cited 2013 Nov 5]. Available from: http://www.americanlaboratory.com/914-Application-Notes/148434-Evaluation-of-Antibiotic-Free-Cell-Cultures-in-Manual-and-Automatic-Cell-Handling/.

[143]   Koschack, J., Standardabweichung und Standardfehler: der kleine, aber feine Unterschied. *ZFA - Zeitschrift für Allgemeinmedizin* 2008, *84*, 258–260.

[144]   TECAN, Cellerity™: The fully automatic solution for cell culture work [Internet], Tecan Trading AG, 2004 [cited 2014 Sep 10]. Available from: http://www.tecan.com/platform/apps/checkdoc/index.asp?doc_Id=157,81&prot=1,1.

[145]   Jain, S., Sondervan, D., Rizzu, P., Bochdanovits, Z. et al., The Complete Automation of Cell Culture: Improvements for High-Throughput and High-Content Screening. *Journal of Biomolecular Screening* 2011, *16*, 932–939.

[146]   Kato, R., Iejima, D., Agata, H., Asahina, I. et al., A Compact, Automatic Cell Culture System for Clinical Scale Cell Expansion from Primary Tissues. *Tissue Engineering Part C: Methods* 2010, *16*, 947–956.

[147]   Kino-oka, M., Chowdhury, S. R., Muneyuki, Y., Manabe, M. et al., Automating the Expansion Process of Human Skeletal Muscle Myoblasts with Suppression of Myotube Formation. *Tissue Engineering Part C: Methods* 2009, *15*, 717–728.

[148]   Mifflin, T. E., Estey, C. A., Felder, R. A., Robotic automation performs a nested RT-PCR analysis for HCV without introducing sample contamination. *Clinica Chimica Acta* 2000, *290*, 199–211.

[149]   Wilke, W. W., Jones, R. N., Sutton, L. D., Automation of polymerase chain reaction tests. *Diagnostic Microbiology and Infectious Disease* 1995, *21*, 181–185.

[150]   Beebe, M., Anderson, S., Monitoring wafer cleanliness and metal contamination via VPD ICP-MS: Case studies for next generation requirements. *Microelectronic Engineering* 2010, *87*, 1701–1705.

[151]   Jounaidi, Y., Enhanced antitumor activity of P450 prodrug-based gene therapy using the low Km cyclophosphamide 4-hydroxylase P450 2B11. *Molecular Cancer Therapeutics* 2006, *5*, 541–555.

[152]   Khalife, R., Stephany, E.-H., Tarras, O., Hodroj, M. H. et al., Antiproliferative and proapoptotic effects of topotecan in combination with thymoquinone on acute myelogenous leukemia. *Clinical lymphoma, myeloma & leukemia* 2014, *14 Suppl*, 46-55.

[153]   Bogenrieder, T., Herlyn, M., Axis of evil: molecular mechanisms of cancer metastasis. *Oncogene* 2003, *22*, 6524–6536.

[154]   Hunziker, E. B., Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2002, *10*, 432–463.

[155]   Fischbach, C., Kong, H. J., Hsiong, S. X., Evangelista, M. B. et al., Cancer cell angiogenic capability is regulated by 3D culture and integrin engagement. *Proceedings of the National Academy of Sciences* 2009, *106*, 399–404.

[156]   Takagi, M., Hayashi, H., Yoshida, T., The effect of osmolarity on metabolism and morphology in adhesion and suspension chinese hamster ovary cells producing tissue plasminogen activator. *Cytotechnology* 2000, *32*, 171–179.

[157]   Fischbach, C., Chen, R., Matsumoto, T., Schmelzle, T. et al., Engineering tumors with 3D scaffolds. *Nature Methods* 2007, *4*, 855–860.

[158]   Cassidy, M. B., Lee, H., Trevors, J. T., Environmental applications of immobilized microbial cells: A review. *Journal of Industrial Microbiology* 1996, *16*, 79–101.

[159]   Amann, A., Zwierzina, M., Gamerith, G., Bitsche, M. et al., Development of an innovative 3D cell culture system to study tumour--stroma interactions in non-small cell lung cancer cells. *PLoS ONE* 2014, *9*, e92511.

[160]   Kelm, J. M., Timmins, N. E., Brown, C. J., Fussenegger, M. et al., Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. *Biotechnology and Bioengineering* 2003, *83*, 173–180.

[161]   Chen, A. A., Underhill, G. H., Bhatia, S. N., Multiplexed, high-throughput analysis of 3D microtissue suspensions. *Integrative biology : quantitative biosciences from nano to macro* 2010, *2*, 517–527.

[162]   Malda, J., Klein, T. J., Upton, Z., The Roles of Hypoxia in the In Vitro Engineering of Tissues. *Tissue Engineering* 2007, *13*, 2153–2162.

[163]   Jonitz, A., Lochner, K., Lindner, T., Hansmann, D. et al., Oxygen consumption, acidification and migration capacity of human primary osteoblasts within a three-dimensional tantalum scaffold. *Journal of Materials Science: Materials in Medicine* 2011, *22*, 2089–2095.

[164]   Jørgensen, A., Young, J., Nielsen, J. E., Joensen, U. N. et al., Hanging drop cultures of human testis and testis cancer samples: a model used to investigate activin treatment effects in a preserved niche. *British journal of cancer* 2014, *110*, 2604–2614.

[165]   Bohannon, L. K., Owens, S. D., Walker, N. J., Carrade, D. D. et al., The effects of therapeutic concentrations of gentamicin, amikacin and hyaluronic acid on cultured bone marrow-derived equine mesenchymal stem cells. *Equine Veterinary Journal* 2013, *45*, 732-736.

[166]   Duewelhenke, N., Krut, O., Eysel, P., Influence on Mitochondria and Cytotoxicity of Different Antibiotics Administered in High Concentrations on Primary Human Osteoblasts and Cell Lines. *Antimicrobial Agents and Chemotherapy* 2006, *51*, 54–63.

[167]   Jonitz, A., Lochner, K., Tischer, T., Hansmann, D. et al., TGF-β1 and IGF-1 influence the re-differentiation capacity of human chondrocytes in 3D pellet cultures in relation to different oxygen concentrations. *International journal of molecular medicine* 2012, *30*, 666–672.

12 Supplements

12.1 Materials and Methods

12.1.1 Materials

12.1.1.1 Lists of Materials

The following tables summarize the used devices (see table 12-1), kits (12-2), chemicals (12-3), media and supplements (12-4), buffers (12-5) as well as consumables (12-6).

Table 12-1 Devices

Devices	Manufacturers	Device/serial numbers	Software
MiniSpin mini-centrifuge	Eppendorf, Hamburg	5452 13886	-
Thermomixer comfort	Eppendorf, Hamburg	5355 05734	-
ViCell- cell counter XR AK 03009	Beckman Coulter, Krefeld	AK 03009	ViCell-software
Pherastar microplate reader	BMG Labtech, Ortenberg	470-0054	SAMI®- / Biomek-software
Vacusafe Comfort	Heidolph, Schwabach	400429	-
Water bath VWB 18	VWR International GmbH, Darmstadt	1106414	-
Incubator Heracell 240	Kendro, Hanau	40474262	-
Microscope Leica DMIL	Leica, Wetzlar	090-135.002	Leica-software
Autoclave 2540 EL	Systec, Wettenberg	2205 073	-
Continued on next page			

Devices	Manufacturers	Device/serial numbers	Software
Water purification plant MilliQ®-Synthesis A10	Millipore, Darmstadt	F4PN78538B	-
Biomek® Cell Workstation	AIG, Rostock; Beckman Coulter, Krefeld	A318400154	SAMI®- / Biomek®-software
VSpin™ centrifuge	Velocity11, Palo Altro	14.00278.0000	SAMI®- / Biomek®-Software
Leica RM 225 rotary	Leica, Wetzlar	-	-

microtome			
Paraffin- drawing bath TFB 45	Medite, Burgdorf	-	-
heating-plate	Medax Nagel GmbH, Kiel	-	-

Table 12-2 Kits

Kits	Manufactures	Order numbers
EZ4U-Assay	Biozol, Eching	BI-5000
Adenylat-Kinase-Assay (AK-Assay)	Promocell, Heidelberg	PK-CA 577-K312
Precellys Steel Kit	PEQLAB Biotechnologie GmbH, Erlangen	91-PCS-MK 28
Quant-iT™PicoGreen®dsDNA kit	Life Technologies, Carlsbad	P 11496
Safranin-O/Fast green staining kit	Morphisto, Frankfurt am Main	12287.00500
Papanicolaous- staining kit	Morphisto, Frankfurt am Main	11919.00500
Alcian blue - fast red- staining kit	Morphisto, Frankfurt am Main	13416.00500

Table 12-3 Chemicals

Chemicals	Degree of purity	Manufactures
Phosphate buffered saline (PBS)	.	PAA, Cölbe
Trypsin/EDTA	-	Sigma Aldrich, Seelze
Alginic acid (alginate)	-	Sigma Aldrich, Seelze
Sodium chloride (NaCl)	Puriss. p. A.	Fluka, Dresden
Calcium chloride (CaCl$_2$)	97%	Sigma Aldrich, Seelze
Sodium citrate	pure (≥99%)	Roth, Karlsruhe
Cacodylic acid	98%	Merck, Darmstadt

Paraformaldehyde (PFA)	pure	Roth, Karlsruhe
Barium chloride (BaCl$_2$)	extra pure	Riedel-de-Haen, Seelze
Embedding medium DPX mountant	-	Sigma Aldrich, Seelze

Table 12-4 Media and supplements

Media and supplements	Manufactures	Order numbers
Dulbecco's modified eagles medium (DMEM)	Sigma Aldrich, Seelze, Seelze	D 5546
Dulbecco's modified eagles medium GlutaMax (DMEM)	Life Technologies, Darmstadt	31966021
L-glutamine	Biochrom, Berlin	K0283
Penicillin/ streptomycin	Sigma Aldrich, Seelze	P 4333
Fetal bovine serum (FBS)	Sigma Aldrich, Seelze	F 0804
Amphotericin B	Sigma Aldrich, Seelze	A 2942
Vitamin C	Sigma Aldrich, Seelze	A 4034
Continued on next page		

Media and supplements	Manufactures	Order numbers
RH IGF-1	RD Systems, Wiesbaden	291-G1-200
RH TGF-β1	Tebu-bio, Offenbach	100-21C-B
ITS Premix	BD Biosciences, Heidelberg	354352
Dexamethasone	Sigma Aldrich, Seelze	D 2915
Citric acid	Sigma Aldrich, Seelze	C 2404
Bovine serum albumin (BSA)	Roth, Karlsruhe	CP 84.2

Table 12-5 Buffers

Buffers	Order numbers	Composition
0.9% NaCl-solution	NaCl: S 7653	4.5 g NaCl in 50 ml *aqua dest.* solved
Alginate-solution	Alginic acid: A 2158 NaCl: S 7653	0.6 g alginate in 50 ml 0.9 % NaCl solved
100 mM CaCl$_2$-solution	CaCl$_2$: 38,315-5	5.54 g CaCl$_2$ in 500 ml *aqua dest.* solved
Lysis buffer for alginate beads	Sod. Citrate: HN13.1 NaCl: S 7653	55 mM sodium citrate (1,618 g) + 150 nM sodium chloride (0.878 g) in 100 ml *Aqua dest.* solved
Vitamin C-solution	vit. C: A 4034	1 g vitamin C in 100 ml *Aqua dest.* solved
2 mg BSA / ml for medium + growth factors	BSA: CP84.2 PBS: H15-011	14 mg BSA in 7 ml 1 x (sterile) PBS solved
Continued on next page		

Buffers	Order numbers	Composition
0,1 M cacodyl buffer, pH 7,4	Cacodylic acid: C-0125	4.28 g in 100 ml *aqua dest.* solved, adjusted to pH 7.4 with HCl
0,1 M cacodyl buffer + 4 % PFA + 10 mM CaCl$_2$	Cacodylic acid: C-0125 PFA: 0335.3 CaCl$_2$: 38,315-5	36.75 mg CaCl$_2$ in 25 ml 0.1 M cacodyl buffer solved + 1 g PFA
0,1 M cacodyl buffer + 50 mM BaCl$_2$	Cacodylic acid: C-0125 BaCl$_2$: 11411	305 mg BaCl$_2$ in 25 ml 0.1 M cacodyl buffer solved
RH IGF-1	rH IGF-1: 291-G1-200 BSA: CP84.2	200 µg lyophilized protein in 2000 µl 0.1 % BSA in 1 x (sterile)

	PBS: H15-011	PBS solved (2 ml BSA + 2000 µl PBS)
RH TGF β1	rH TGF β1: 100-21C-B citric acid: C 2404 BSA: CP84.2	5 µg lyophilized protein in 100 µl 10 mM citrate acid solved + 900 µl BSA (2 mg / ml)
10 mM citrate acid	citric acid: C 2404	0.192 g in 100 ml *aqua dest.* solved
10 mM dexamethasone	dexamethasone: D 2915	39.2 mg in 10 ml *aqua dest.* solved
Ethanol 96 % **(ethanol puritiy degree: HPLC-grade)**	EtOH: AP1612,2500	192 ml ethanol (100 %) + 8 ml *aqua dest.* diluted
Ethanol 80 % **(ethanol puritiy degree: HPLC-grade)**	EtOH: AP1612,2500	160 ml ethanol (100 %) + 40 ml *aqua dest.* diluted
Continued on next page		

Buffers	Order numbers	Composition
Ethanol 70 % **(ethanol puritiy degree: HPLC-grade)**	EtOH: AP1612,2500	140 ml ethanol (100 %) + 60 ml *aqua dest.* diluted
Ethanol 60 % **(ethanol puritiy degree: HPLC-grade)**	EtOH: AP1612,2500	120 ml ethanol (100 %) + 80 ml *aqua dest.* diluted
Ethanol 50 % **(ethanol puritiy degree: HPLC-grade)**	EtOH: AP1612,2500	100 ml ethanol (100 %) + 100 ml *aqua dest.* diluted

Table 12-6 Consumables

Consumables	Manufactures	Order numbers
T75-flasks	Sarstedt, Nümbrecht	83.1813.002
CELLSTAR® AutoFlask™	greiner, Frickenhausen	779 190 (Susp.cells) 779 160 (ad. Cells)
100 Sterican® single-use needles	Roth, Karlsruhe	0,60 x 30 mm: X 129.1 0,60 x 60 mm: C720.1 0,60 x 80 mm: C629.1
Injekt® Solo disposable syringes 20 ml	Roth, Karlsruhe	0059.1
96 well plate	greiner, Frickenhausen	655 180
96 well plate for suspension cells	greiner, Frickenhausen	655 185
Continued on next page		

Consumables	Manufactures	Order numbers
Disposable serological polystyrene pipette 5-25 ml	Sarstedt, Nümbrecht	5 ml: 86.1253.001 10 ml: 86.1254.001 25 ml: 86.1685.001
PP- centrifuge tubes 15, 50 ml	Roth, Karlsruhe	15 ml: N 459.1 50 ml: N 463.1
Closure foil, sterile	Roth, Karlsruhe	EN 85.1
96well deep well plates	greiner, Frickenhausen	1 ml U-Boden: 780 261 2ml V-Boden: 780 271
Lids for well plates and deep well plates	greiner, Frickenhausen	656 101

Glass bottles with threaded fittings and lid (Schott)	Roth, Karlsruhe	100 ml: HL99.1 250 ml: HT00.1 500 ml: HT01.1 1000 ml: HT02.1
1,5ml- reaction vessels	VWR International GmbH, Darmstadt	211-0007
Sterile filters 0,45µM	Sarstedt, Nümbrecht	syringe filter: P667.1 top filter: 83.1823.100
Eppendorf pipettes	Eppendorf, Hamburg	single-channel pipette 0.5–10 µL: 3120000020 single-channel pipette 2–20 µL: 3120000038 single-channel pipette 10–100 µL: 3120000046
Continued on next page		

Consumables	Manufactures	Order numbers
Eppendorf pipettes	Eppendorf, Hamburg	single-channel pipette 20–200 µL: 3120000054 single-channel pipette 30–300 µL: 3120000100 single-channel pipette 100–1000 µL: 3120000062 single-channel pipette 0.5–5 mL: 3120000070 eight-channel pipette 10–100 µL: 3122000035

		eight-channel pipette 30–300µL: 3122000051
Disposables for Eppendorf-pipettes	Eppendorf, Hamburg	epT.I.P.S.®-Reloads 0.1 – 10 µL: 0030073363 epT.I.P.S.®-Reloads 0.5 – 20 µL: 0030073401 epT.I.P.S.®-Reloads 2 – 200 µL: 0030073428 epT.I.P.S.®-Reloads 20 – 300 µL: 0030073444 epT.I.P.S.®-Reloads 50 – 1000 µL: 0030073460 epT.I.P.S.®- Standard 0.1 – 5 mL: 0030000978
Slides	Roth, Karlsruhe	H 867.1
Continued on next page		

Consumables	Manufactures	Order numbers
Brushes	Leica, Wetzlar	-
Scalpel	R. Wurach, Berlin	-
Microtome-knives N35	Feather®, Osaka	-

12.1.2 Methods

12.1.2.1 Cell Cultivation

Manual Cell Cultivation

Cervix Carcinoma Cells

HeLa cells were cultivated in 75 cm² cell culture flasks at 37 °C and 5 % CO_2. Dulbecco's modified eagle medium (DMEM) was supplemented with (1 %) and without penicillin/streptomycin (Sigma-Aldrich) and 10 % fetal bovine serum (FBS). For the splitting process the conditioned medium was removed, cells were washed in saline buffered with 5 ml phosphate (PBS; PAA, Cölbe, Germany) and were treated with 2 ml Trypsin/EDTA (Sigma-Aldrich, Seelze, Germany) for 4 minutes at 37 °C. Trypsin/EDTA was then neutralized with 5 ml DMEM. The cell suspension (1 ml) was seeded in new T75 flasks with 14 ml DMEM medium [73].

Primary Chondrocytes

Primary chondrocytes were isolated and cryoconservated from the department of Orthopaedics, Biomechanics and Implant Technology Research Laboratory of University Rostock [31, 167]. The cells (1ml) were defrosted in a water bath (30 seconds, 37 °C; Memmert, Schwabach, Germany) and resuspended in 14 ml Dulbeccos Modified Eagle-GlutaMax medium (DMEM; Life technologies, Darmstadt, Germany) supplemented with 10 % FBS (fetal bovine eagle medium), 1 % amphotericin B, with or without 1 % penicillin/streptomycin and 0.5 % ascorbic acid (all purchased from Sigma-Aldrich, Seelze, Germany). The cells were manually expanded in 75 cm² flasks in a clean room. In passage two, chondrocytes were splitted into 75 cm² flasks (manual process; greiner) and CELLSTAR® AutoFlasks™ (automatic process; greiner, Frickenhausen, Germany) [54].

Manual 3D Formation

Alginate Bead Manufacturing

HeLa cell monolayer was washed with 5 ml PBS (PAA) after removing the supernatant (15 ml). The cells were detached using trypsin for 4 minutes. Subsequently, the reaction was stopped with medium (4 ml) and cells were collected in a modular reservoir. The cells were detected by transfer of cell suspension (500 µl) into the ViCell cell counter and evaluation of the viability. Subsequently 1 x 10^6 cells were transferred per well into the deep well plate. The same volume and wells were equipped with sterile water in a second deep well plate as counter weight. The deep well plates were centrifuged by the ViCell™ centrifuge (1150 rpm, 5 min). After removing the supernatant, the pellet was resuspended in 500 µl alginate/well. The cell/alginate solution was absorbed into a syringe equipped with cannula. The cell/alginate suspension was added dropwise to $CaCl_2$ (Sigma Aldrich) with a disposable syringe. Alginate beads were washed one time in 200 µl NaCl (Fluka, Dresden, Germany) and two times in 130 µl NaCl with a eight cannel Eppendorf pipette. Than 200 µl fresh Dulbeccos Modified Eagles medium (DMEM; Sigma Aldrich) per well with 10 % fetal bovine serum (FBS), with 2 mM L-glutamine (sigma aldrich) and with/without 1 % penicillin-streptomycin (P/S; Sigma Aldrich) was added [73]. The media change was done two times per week by exchange of 200 µl medium/well.

Spheroid Culture Manufacturing

The supernatant (15 ml) of the monolayer was removed by a serological pipette and the cells were rinsed with 5 ml phosphate buffered saline (PBS; Sigma Aldrich). The cells were treated with 2 ml trypsin/EDTA (Sigma Aldrich) and incubated for 4 minutes. The detachment process was stopped by the addition of 4 ml medium (Sigma Aldrich). The cell suspension was transferred into a sterile 50 ml tube. Subsequently, the cells were counted and viability was tested using the ViCell™ XR (Beckman Coulter) with 500 µl cell suspension. The cells were diluted (40,000 cells/30 µl) with the specific medium (+P/S; -P/S) in a modular reservoir. The cell suspension (30 µl) was transferred into every second well of the Perfecta3D® Hanging Drop Plates (3D Biomatrix Inc., Huron Parkway, USA) using a one channel 200 µl Eppendorf-pipette (Eppendorf, Hamburg, Germany). The media change was performed two times per week by exchange of 15 µl medium [52].

Pellet Culture Manufacturing

Chondrocytes were cultivated in 15 mL DMEM medium with supplements in 75 cm² cell culture flasks (greiner) at 37 °C and 5 % CO_2. The supernatant was removed by serological pipettes. Than the chondrocytes were washed with 5 ml PBS (PAA, Cölbe, Germany). An addition of 2 ml Trypsin/EDTA (Sigma Aldrich) was followed by an incubation of the cells for 10 minutes at 37 °C and 5 % CO_2. Trypsin/EDTA (Sigma Aldrich) was neutralized with fresh media (3 ml) and the cell suspension was transferred into a 50 ml tube (Carl Roth, Karlsruhe, Germany). After cell count measurement by the Vi-CELL™XR (Beckman Coulter) the cells were aliquoted (0.5×10^6 cells/well) into a deep well plate (greiner). The cells were centrifuged by the Vspin™ at 2,250 rpm of minimum speed for 5 minutes. The conditioned media was removed and the cell pellets were resuspended in 200 µl fresh media with supplements/well transferred into a new 96 well plate for suspension cells where cells organized themselves to pellets. The supplements are dexamethasone (final concentration: 100 nM), ascorbic acid (final concentration: 50 µg/mL; both: Sigma Aldrich), rh IGF-1 (final concentration: 50 ng/mL; RD Systems, Wiesbaden, Germany), rh TGF-β1 (final concentration: 50 ng/mL, Tebu-bio, Offenbach, Germany) and ITS™ Premix (complete medium to ITS™ in a 100:1 ratio; BD Bioscience, Heidelberg, Germany). Then followed a shaking period (0.5 h) using the thermomixer (37 °C; 300 rpm) (Eppendorf, Hamburg, Germany) and the consequently incubation at the Cytomat (37 °C, 5 % CO_2; Thermo Fisher Scientific) [54].

Automatic cell cultivation

Disinfection

Naturally, before starting a new test run, the Biomek® Cell Workstation was disinfected over night with 70 % Isopropanol by purging and syringing the pipes and steel needles. If the workstation was not in use for more than two days, the pipes were filled with Korsolex® basic (Bode-Chemistry)

especially to minimized growth of bacteria or fungi. After every cycle the disinfection time was set on 15 minutes. Pipetting steps were attended with cleansing steps with autoclaved water.

The washing station on deck provided the purgation of the steel needles. The pipetting pump with steel needles drew back autoclaved water or isopropanol and dispensed it several times in order to clean the pipes. Steel needles were passively washed by dipping the tips of the needles into an isopropanol bath [134].

Microorganism - free Cultivation

Before starting the experiment, the Biomek® NX was set back, cleansed and disinfected. The liquid handler deck was decontaminated with UV-radiation (15 min.). The channel 5 pipetted exclusively for red-marked (PKH26; Sigma Aldrich) cells and channel 6 for green-marked (PKH67, Sigma Aldrich) cells. Channel 7 was set for a bulk dispense added with a port selection valve plus dispensing pump and acted as a medium donor. The deck reservoirs were kept separately to prevent contamination through this action. The cell suspension reservoir was changed after each family for the red- and green labeled cells. A family contented the cell cultivating of two AutoFlasks™ (greiner), first run with PKH26 fluorescent cells, the second with PKH67 fluorescent cells.

The needed solutions had to be located in modular reservoirs at the liquid handler deck (position P4: 15 ml PBS, 5 ml Trypsin; position 6: one empty reservoir for the cell solution, 40 ml isopropanol). The fluorescence marked monolayer was washed with 5 ml phosphate buffered saline (PBS; PAA) and harvested by using 2 ml trypsin-EDTA (Sigma Aldrich) solution. The enzymatic reaction was stopped using Dulbecco's modified Eagle medium (DMEM, Sigma Aldrich) after incubation of 4 minutes. The cell suspension was transferred into a modular reservoir. Fresh media (14 ml) and cell suspension (1 ml) were transferred into new AutoFlasks™ (greiner). Cultivation was done with a dilution of 1 to 2 in the Biomek® NX liquid handler. The cells were overnight incubated in the Cytomat (37 °C, 5 % CO_2; Thermo Fisher Scientific) [134].

Adherent Cell Cultivation and Seeding

The HeLa cells for manual handling were expanded in T75 cell culture flasks (greiner) for three weeks. The cells for automatic handling were seeded in an AutoFlask™ cell culture flask (greiner, Frickenhausen, Germany) and disseminated with the proprietary automatic cell culture system. The solutions had to be located in modular reservoirs at the liquid handler deck (position P4: 15 ml PBS, 5 ml Trypsin; position 6: one empty reservoir for the cell solution, 40 ml isopropanol). The port selection valve was connected with medium (200 ml). At a confluence of up to 90 % in the cell culture flask, cells were rinsed with 5 ml phosphate buffered saline (PBS, PAA) and were harvested using trypsin (2 ml, Sigma Aldrich). Medium (4 ml) was added to stop the enzymatic reaction after an incubation period of 3 min. The cell suspension was transferred to a modular reservoir and the cell count was evaluated using 500 µl cell solution detected by the Vi-CELL™ XR.

For bioscreening, cells were seeded in well plates (greiner) with different cell counts (1000, 2000, 4000, 6000 and 8000 cells/well) and incubated overnight (37 °C, 5 % CO_2). The manual steps were carried out with Eppendorf pipettes (Hauppauge, NY, USA). Automatic cell handling was enabled using the Biomek® NX liquid handler with steel cannulas. The automatic steps were programmed and

controlled with the SAMI® software (Beckman Coulter) combined with the liquid handler software [142].

Suspension Cell Seeding

The cell culture process contains two different automatic main processes (see figure 13-1). The solutions had to be located in modular reservoirs at the liquid handler deck (position 2: empty modular reservoir, 10 ml PBS, 40 ml Isopropanol; position 6: 4 empty modular reservoirs). First, the regular automatic cell cultivation was done. The Cytomat (Thermo Fisher Scientific) included the AutoFlasks™ (greiner) and the 96 well plates (greiner). The cell culture flask (greiner) with the suspension cells and the 96 well sheets (greiner) were transferred to the liquid handler deck by the lift and were positioned by the integrated gripper of the liquid handler. The 3D tilt rack in combination with the liquid handler allowed the transfer of 2 ml/well cell suspension into a deep well plate (DWP; greiner). The same volume (water) was transferred in a counter weight. The Vspin™ centrifuged the cell suspension (2,250 rpm, 5 min). The Biomek® NX liquid handler was associated with the port selection valve. The liquid handler removed the conditioned media (2 ml) and resuspended the cells in fresh media (0.5 ml). Finally, the cell suspension was pooled in a modular reservoir (Beckman Coulter).

Afterwards, the automatic dissemination of the suspension cells continued. The ViCell™ XR (Beckman Coulter) detected the viability and counted the cell number. After calculation of the exact cell number (50,000 cells/well), medium and subsequently the cell suspension were transferred into the 96 well plate with a volume of 150 µl. The well plates with cells was incubated by the Cytomat (37 °C, 5 % CO_2) [110].
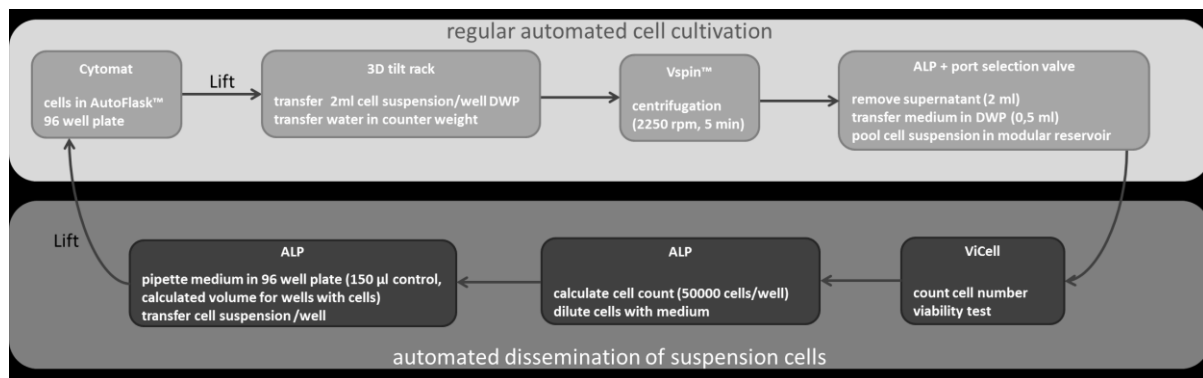
Figure 12-1 The automatic process for cell cultivation and subsequently dissemination of suspension cells [110]

The cell culture process for the investigation of suspension cells is separated in the regular automatic cell cultivation and the automatic dissemination of suspension cells.

Automatic 3D Formation

Alginate Bead Manufacturing

The automatic cell culture process was realized with the Biomek® Cell Workstation (Center for Life Science Automation, Rostock, Germany; see figure 12-2). The solutions had to be located in modular

reservoirs at the liquid handler deck (postion 2: 24 ml $CaCl_2$, 6 ml alginate, 40 ml NaCl; position P4: 15 ml PBS, 5 ml Trypsin; position 6: one empty reservoir for the cell solution, 15 ml sterile water, 40 ml isopropanol). The cell culture media were provided by the port selection valve combined with a peristaltic pump. The Greiner CELLSTAR® AutoFlask™ (greiner, Frickenhausen, Germany) were located into the Cytomate (37 °C, 5 % CO_2; Hotel 3; Thermo Fisher Scientific) transferred with a lift from the Cytomat to the liquid handler deck. The gripper of the liquid handler robot transferred the AutoFlask™ (greiner) to different positions within the workstation. HeLa cells were washed with 5ml PBS (PAA). The 3D Tilting-ALP enables the homogenous distribution of PBS (PAA) by different tilting angles. Cells were detached by incubation with Trypsin/EDTA for 4 minutes using an on deck incubator. The 2 ml Trypsin/EDTA were neutralized with 6 ml DMEM and the cell suspension was collected into a modular reservoir.

For the production phase I, the cell count was measured with the Vi-CELL™ XR (Beckman Coulter). The production phase II started with the transfer of the cell suspension (1 x 10^6 cells/well) into a deep well (greiner). After centrifugation, the cells were resuspended in alginate (500 µl, Sigma Aldrich) and dropped into $CaCl_2$ (1 bead/well, Sigma Aldrich) in a 96 well plate (WP, greiner). Alginate beads were washed one time in 200 µl NaCl and two times in 130 µl NaCl. Then, 200 µl/well fresh media was added [73].
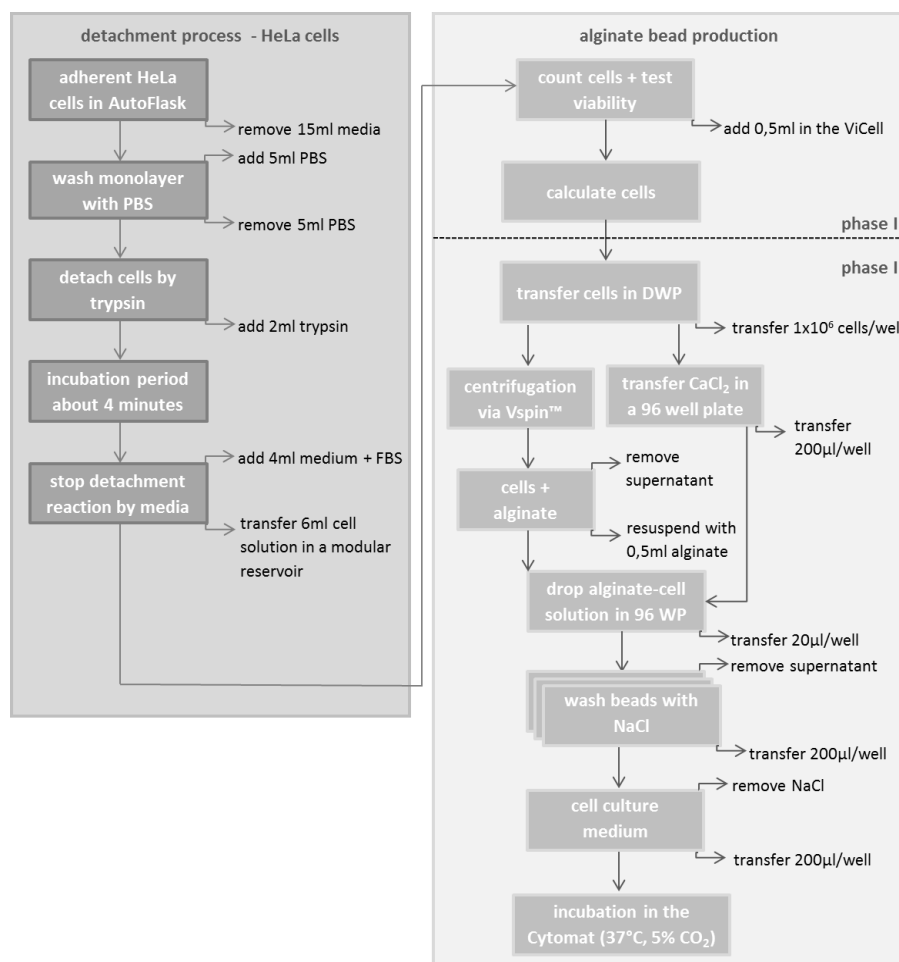

Figure 12-2 Automatic alginate bead production, modified [73]

Spheroid Culture Manufacturing

The spheroid cultures were formed in Perfecta3D®Hanging Drop Plates (see figure 12-3). The solutions had to be stored in modular reservoirs at the liquid handler deck (position 2: 5ml medium, position P4: 15 ml PBS, 5 ml Trypsin, position 6: one empty reservoir for the cell solution, 40 ml isopropanol). The AutoFlasks® (greiner) with cells were transferred from the Cytomat (Thermo Fisher Scientific; Hotel 3) to the liquid handler deck by the lift. The integrated gripper of the Biomek® NX transferred the flask to the 3D tilt rack-1 (AIG, Rostock, Germany). The 3D tilt rack fixed and angled the flasks for the liquid transfer. The transfer of solutions was performed using steel cannulas, which penetrated through a septum. The consumed medium (15 ml) was removed and pipetted to the waste. The monolayer was rinsed with 5 ml PBS. The cells were detached with trypsin (2 ml) for a incubation period of 4 min in an on deck incubator (inheco). Subsequently, this process was stopped with 4 ml medium and the cell suspension was transferred into a modular reservoir (Beckman Coulter). The ViCell™ (Beckman Coulter) device enabled the counting and viability testing of the cells within 500 µl cell suspension (production phase I). The production phase II started with the dilution of the cells (40,000 cells/30 µl) with the specific medium (+P/S, -P/S). The cell solution was transferred into the Perfecta3D®Hanging Drop Plates (3D Biomatrix) by two channels of the liquid handler every second per well. The media was changed two times per week, whereby 15 µl medium were exchanged [52].
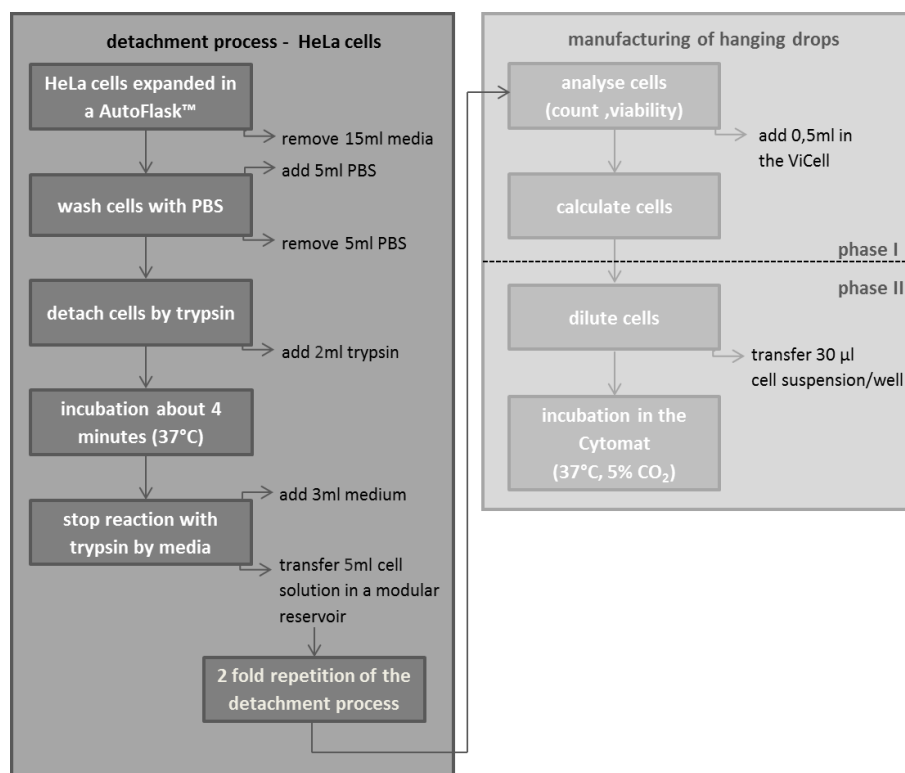

Figure 12-3 Automatic hanging drop manufacturing to form spheroid cultures, modified [52]

Pellet Culture Manufacturing

The Biomek® Cell Workstation was used to cultivate primary chondrocytes and afterwards produce pellet cultures automatically (see figure 12-4). The solutions had to be located in modular reservoirs

at the liquid handler deck (position 2: medium with supplements, position 6: one empty reservoir for the cell solution, 10 ml trypsin, 40 ml PBS, 40 ml isopropanol). The port selection valve was connected with medium (300 ml). The CELLSTAR® AutoFlasks™ (greiner) with human chondrocytes were stored in the Cytomat (37 °C, 5 % CO_2; Hotel 3) and transferred by the lift to the liquid handler deck. Chondrocytes were washed in 5 ml PBS, which was homogenously distributed with a 3D tilt rack. The detachment of the cells in CELLSTAR® AutoFlask™ (greiner) was realized using 2 ml Trypsin/EDTA followed by an incubation of the cells for 10 minutes in an on deck incubator. Fresh DMEM media (3 ml; Life technologies) was added for neutralization and the cell suspension was collected into a modular reservoir (Beckman Coulter). Referring to the automatic cell culture of chondrocytes, cell count was measured by the Vi-CELL™ XR (Beckman Coulter) at production phase I. The production phase II started with the transfer of 0.5 x 10^6 cells/ml into a deep well plate (greiner), followed by a centrifugation of the deep well plate by the Vspin™ at 2,250 rpm for 5 minutes. After removing the media, the cell pellets were resuspended in 200 µl medium with growth factors/well and transferred into a 96 well plate (greiner) by the liquid handler (Beckman Coulter) where cells organized themselves to pellets. The used supplements were previously described above. The process was promoted by a shaking period of half an hour by a thermomixer (Eppendorf) at 37 °C and 5 % CO2. The cells were incubated in the Cytomat (37 °C, 5% CO_2; Thermo Fisher Scientific).

For comparison, we investigated a total of 14 independent donors for our analysis (4 male donors, age: 65.1 ± 12.4 years; 10 female donors, age: 70.4 ± 9.1 years) [54].
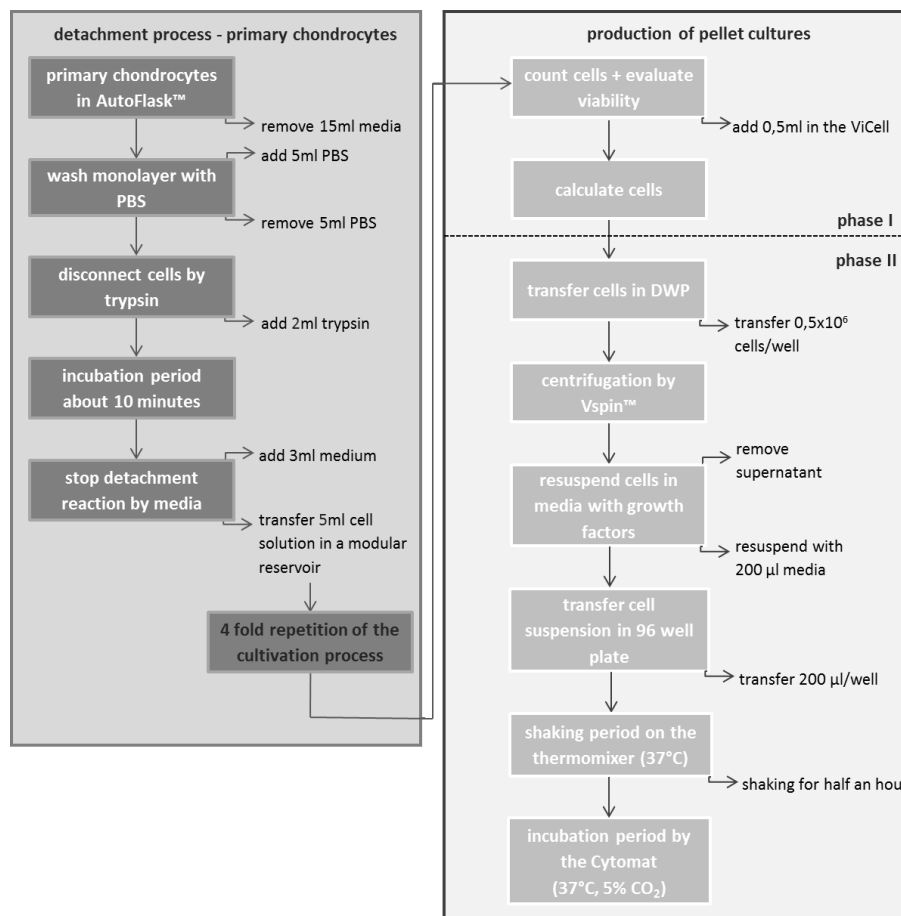

Figure 12-4 Automatic pellet culture manufacturing, modified [54]

12.1.2.2 Fluorescence Labeling (PKH 26, PKH 67)

The cells were adjusted for the appropriate cell amount for dyeing of 2 x 10^{-6} M of PKH (Sigma Aldrich) and 1 x 10^7 cells/ml serum-free medium. The cell suspension was washed with serum-free medium to remove FBS (Sigma Aldrich). After centrifugation the cell pellets were suspended in 1 ml Diluent C. Staining was carried out by resuspending the cell suspension 1:1 with PKH dye solution (Sigma Aldrich) under periodic mixing. After 5 minutes of incubation, the staining was stopped by adding FBS and incubating for 1 minute. The cells were centrifuged and washed three times with serum-containing medium. Every step of the procedure was performed at room temperature. The cells were transferred into AutoFlasks™ (greiner) with a result of three million cells dyed either with PKH26 or PKH67 per flask. The cells were rested for one day to ensure the adherence [134].

12.1.2.3 FACS Analysis

After automatic cultivation and 24 hours of incubation cell were harvested by 2 ml trypsin/EDTA-solution and washed two times with 5 ml PBS. Only the sample with PKH67 marked cells was analyzed using a FACSCalibur Cytometer (Becton and Dickinson, Heidelberg, Germany) since cross contamination events were expected. The data analysis was performed using the CellQuest software (Becton and Dickinson, Heidelberg, Germany) [134].

12.1.2.4 Compound Treatment + EC$_{50}$

The chemosensitivity of cervix carcinoma cells was investigated using the EZ4U proliferation test after cyclophosphamide treatment. To evaluate the growth rate, cells were seeded with a cell count of 10,000 cells per well and a ring of water to protect against evaporation. Then, cells were incubated at 37 °C and 5 % CO_2. The next day, cytostatic agent was diluted to a concentration of 35 mmol/l down to 0.27 mmol/l using the Biomek® NX liquid handler in a separate plate. Afterward, the Biomek® FX transferred the agent into the plate containing the cervix carcinoma cells. Dimethyl sulfoxide (DMSO) was used as negative control and cell culture medium as positive control. After two days of incubation, the proliferation rates were detected using the EZ4U proliferation assay [142].

12.1.2.5 Bioscreening

Cytotoxicity – AK Assay

The adenylate kinase cytotoxicity kit (PromoKine, Heidelberg, Germany) is based on the detection of ubiquitous adenylate kinase from cells with damaged membranes.

3D Cell Cultures (manual)

The 3D cell cultures were cultivated in 200 µl fresh medium/well over night. After preparing the reagent working solution, 100 µl of the supernatant of the alginate beads were transferred into a

new well. Then 100 µl of the reagent working solution were added to the supernatant, followed by an incubation of 5 minutes at 20 °C. The luminescence was measured with the PHERAstar microplate reader (BMG Labtech) [73].

Proliferation

EZ4U – Assay

The EZ4U (Biomedica Medizinprodukte GmbH & Co KG, Wien, Austria) is a non-radioactive proliferation and toxicity screening assay based on the conversion of tetrazolium salt to formazan, which results in a color change.

Adherent Cell Cultures (automatic)

For regular proliferation tests without agent, cells were seeded with a cell count of 1,000 up to 8,000 cells per well. The total volume of media and cell suspension was therefore 250 µl/well. After an overnight incubation at 37 °C and 5 % CO_2, the proliferation was detected using the EZ4U Kit (Biomedica Medizinprodukte) and the high-throughput screening system. The liquid handler added 25 µl of the reagent to each well. After 3 hours of incubation, absorbance (OD 450 nm) was detected by the PHERAstar reader [142].

3D cell cultures (manual)

The EZ4U Proliferation assay (Biomedica Medizinprodukte) based on the reduction of tetrazolium salt to colored formazan by vital cells. The proliferation was evaluated at two time points (14d, 35d). Alginate beads were transferred into a new 96 well plate (greiner) and 250 µl fresh media/well and 25 µl of the EZ4U reagent/well were added. After an incubation period of 3 hours at 37 °C, 200 µl/well of the supernatant was transferred into a new well and the absorbance was measured at 450 nm by the PHERAstar (BMG Labtech, Ortenburg, Germany) [73].

3D cell cultures - Automatic High Throughput Screening (EZ4U assay +AK-assay)

The automatic screening combined the proliferation and toxicity screenings in one method associated with the high throughput screening arrangement.

For the automatic bioscreening, the disposables were stored in the Cytomat-Hotel (Thermo Fisher Scientific). The well plates with the 3D cell cultures were incubated in the Cytomat (37 °C, 5 % CO_2; hotel 1, position 1: 3D cell cultures; hotel 2, position 1: AK assay plate; hotel 3, position 1: EZ4U screening plate). The solutions had to be manually located in modular reservoirs at the Biomek® FX liquid handler deck (position 1: 10 ml AK-reagent, position 2: low volume reservoir with 7.5 ml EZ4U reagent, position 14: empty reservoir for waste, position 15: reservoir with 12 ml medium). For automatic bioscreening, the Motoman (Yaskawa) transferred disposables and the well plates with 3D cultures to the shuttle of the Biomek® FX liquid handler (Beckman Coulter). The shuttle transferred the labware to the liquid handler deck and the gripper positioned the labware. The required reagents were provided in low volume reservoirs (greiner).

The automatic bioscreenings were performed in one method. The cytotoxicity assay used the supernatant and the proliferation assay the vital cells in fresh media. For the automatic toxicity screening 100 µl/well of bead supernatant and 100 µl/well reagents were transferred into a white well plate by the Biomek® FX (Beckman Coulter). After five minutes of incubation (20 °C), luminescence was detected using the PHERAstar microplate reader (BMG Labtech). Simultaneously, cell proliferation was evaluated by the transfer of 100 µl media into the waste and 250 µl fresh media/well to the 3D cell cultures plus addition of 25 µl reagent per well with the Biomek® FX (Beckman Coulter). After three hours of incubation (5 % CO_2, 37 °C), absorbance was detected with the PHERAstar microplate reader (BMG Labtech) at 450 nm [73].

WST – Assay

The WST reaction is a tetrazolium salt dependent reaction, whereby the colored formazan is the product.

Suspension cell cultures (automatic)

For the automatic proliferation screening by the WST assay (Roche) the disposables are stored in the Cytomat-hotel (Thermo Fisher Scientific; (hotel 8, position 1)) and the 96 well plates (greiner) with cells (150 µl) which are located in the Cytomat (37 °C, 5 % CO_2; Thermo Fisher Scientific; hotel 1, position 1). The Biomek® FX liquid handler (Beckman Coulter) deck was manually equipped with the reagent (2.5 ml) in a low volume reservoir (greiner) on position 2. The Motoman (Yaskawa) transferred the labware to the shuttle of the Biomek® FX (Beckman Coulter) where the integrated gripper positioned the disposables and plates. After loading the disposables, the Biomek® FX (Beckman Coulter) transferred 15 µl reagent per well. Then, an incubation period within the Cytomat (5 % CO_2, 37 °C) followed for 4 hours. The readout was realized using detection of the absorbance (450 nm, 630 nm) using the PHERAstar microplate reader (BMG Labtech).

DNA – Isolation and Quantification (manual, automatic)

Alginate Beads, Spheroid Cultures and Pellet Cultures

For DNA isolation, five constructs of each 3D culture (alginate beads, spheroid cultures) were pooled into a 1.5 ml Eppendorf tube. Then, by the addition of lysis buffer, an incubation period of 30 min followed at 37 °C. After centrifugation for 10 min (900 rpm) the supernatant was removed using the Mini Spin centrifuge (Eppendorf). The 3D cultures were resuspended in PBS (200 µl) and stored at -20 °C. Two pellet cultures were pooled into a 1.5 ml homogenization tube containing steel beads and stored at -20 °C. The pellet cultures had to be homogenized (30 sec.; 3000 rpm) before isolation processes. The DNA was isolated by the peqGold Tissue DNA Mini Kit (PEQLAB Biotechnologie GmbH) according to the manufacturer's instructions. Subsequently, the DNA (manually and automatically processes) was quantified by the Quant-iT™PicoGreen®dsDNA kit (Life Technologies) according to the manufacturer's instructions. The high throughput screening system realized the automatic DNA-quantification. The needed PicoGreen® reagent (12 ml) was manually located at the Biomek® FX liquid handler deck (position 2). The tip box was stored at the Cytomat hotel (hotel 8, position 4). The

black well plates with the samples (100 µl) were located in the Cytomat (Thermo Fisher Scientific; hotel 1, position 1) and transported to the liquid handler deck of the Biomek®FX as well as the disposables, stored in the Cytomat-hotel (Kendro). The reagent (100 µl) was added, followed by an incubation period of 5 minutes (20 °C). Susequently, the fluorescence was detected using the Pherastar microplate reader (emission 520 nm, extinction 480 nm; BMG Labtech) [52].

12.1.2.6 Histological Working Steps

Safranin O Staining

Pellet cultures were fixed in formafix (Carl Roth). Alcohol was used for a dehydration of the samples, which were embedded in paraffin (Institute of Anatomy, University Rostock). After the histological sectioning by a rotary microtome (Leica), the 7 µm samples were transferred on glass slides (Thermo Fisher Scientific) and incubated on a heating plate (Medax Nagel GmbH). The sections were stored at 37 °C overnight. The dewax process was done by different alcohol concentrations. Afterwards, a Safranin light green-staining according to the manufacturer's instructions (Morphisto) was done. The stained sections were embedded in synthetic resin (Fluka), dried and microscopically documented (Leica) [54].

Alcian Blue Staining

The pellet cultures were fixed in formafix and embedded in paraffin (Institute of Anatomy, University Rostock) after dehydration with alcohol. The 3D cultures were sectioned in 7 µm slides using a rotary microtome (Leica) and dried by a heating plate (Medax Nagel GmbH) as well as incubated at 37 °C overnight. Before staining, the slides had to be dewaxed by solutions with different alcohol concentrations. Subsequently, the alcian blue staining followed according the manufacturer's instructions (Morphisto). Then, the sections were covered with synthetic resin (Fluka), dried and the documentation was done by light microscopy (Leica).

Papanicolaous Staining

Alginate beads were in 4 % PFA (Carl Roth, Karlsruhe, Germany), 0.1 M cacodyl buffer (pH 7.4) and 10 mM $CaCl_2$ at 20 °C for 4 hours. Ethylalcohol was used for the dehydration of the samples, which were embedded in paraffin (Institute of Anatomy, University Rostock). After the histological sectioning, the 7 µm samples were transferred on glass slides (Thermo Scientific) and incubated on a heating plate (Medax Nagel GmbH). The sections were stored at 37 °C overnight. The dewax process was followed by a Papanicolaous-staining after the manufacturer's instructions (Morphisto). The stained sections were embedded in synthetic resin (Fluka), dried and documented by light microscopy [73].

12.2 Expression of Thanks

My thank goes to Prof. Dr.-Ing. habil. Kerstin Thurow and Prof. Dr. Ing. Nobert Stoll. I would especially like to express my gratitude for Prof. Thurow and Prof. Stoll to give me the opportunity of this dissertation at the Center for Life Science automation (celisca) with the very interesting topic under the excellent working conditions as well as for the inspiring conversations and discussions.

Furthermore, I thank the whole team of celisca for the collegial cooperation and supports. My specials thank goes to Dr.-Ing. Thomas Roddelkopf, Dr.-Ing. Steffen Junginger, Dipl.-Ing. Lars Woinar Dipl. Ing. Hans Joachim Stiller and Heiko Engelhardt for their technical support and continuous inspiring exchanges. Especially, I would like to extend very, very warm thanks to the biological team Grit Koch, M.Sc. Carolin Gallert and Julia-Christin Severitt for any supports, conversations and the wonderful working atmosphere.

Furthermore, I thank Prof. Dr. med. habil. Dipl.-Ing. Rainer Bader and the Department of Orthopaedics, Biomechanics and Implant Technology Research Laboratory for providing human chondrocytes and the excellent expert advice.

Additionally, I thank Dr. Catrin Roolf from the Clinic for Hematology and Oncology of the University Hospital Rostock, Dr. Anika Jonitz-Heincke and Doris Hansmann from the Department of Orthopaedics, Biomechanics and Implant Technology Research Laboratory of the University Medicine Rostock for their excellent professional competence and support.

I thank Prof. Dr. Andreas Wree and Frauke Winzer of the Institute of Anatomy of the University Rostock to support the histological work.

Moreover, I thank Dr. Richard A. Wysk from the North Carolina State University for the support of the english dissertation.

Moreover, a great thank goes to my family. I thank my husband Stephen Lehmann very, very much for his unbroken motivation, support and love the last years of privation. I face the future with confidence and hope to be the best parents for our child. Additionally, a special thank goes to my parents for their relentless motivation and support to realize my carrier and wonderful life.

Last but not least, I thank my friends for the happy times and support over the long time of my education.

12.3 Declaration of Education Honesty

I declare that up to now I have processed no dissertation procedure and that I have not applied for a dissertation procedure before.
This dissertation thesis was never submitted to any other University or College.

Furthermore, I declare that this dissertation thesis "Evaluation of a flexible automatic system for 3D cell cultivation" was created single-handed by me. I have used no other sources and utilities than named in the thesis. Content, literally or with regard to, is marked as citations.
This dissertation was never used for another academic qualification.

Tessin, February 2015   Ricarda Lehmann

12.4 Curriculum Vitae

Personal details

Name	Ricarda Lehmann

Working experience

02/2012 - 03/2015	**doctoral studies** University of Rostock, celisca: Research Group "Life Science Automation - Processes" • Automatic Bioscreening, Automatic Cell Processing
10/2011 - 01/2012	**scientists** Medical University of Hannover, working group molecular perinatology • Cell culture of primary cells, migration- and proliferation tests
04/2011 - 09/2011	**student assistant** Leibniz university of Hannover, working group bioprocess technic • Cell culture of mesenchymal stem cells, Endnote
11/2007 - 03/2011	**student assistant** Department of orthopedics at the University Medicine Rostock, working group cartilage- and bone regeneration • Isolation and cultivation of primary osteoblasts, chondrocytes and 3D cultures
12/2006 - 10/2007	**student assistant** Bionas GmbH, lab for cell culture and sales • Cell culture, generation of leads, metabolic detection through Bionas® analyzing system
03/2004 - 09/2006	**laboratory assistant of biology** Bionas GmbH, lab for cell culture and sales • Organization of the lab, cell culture, supervision of students, generation of leads, GLP documentations
01/2003 - 02/2004	**laboratory assistant of biology** University of Rostock, center of pharmacology and toxicology • HPLC-Analysis, Therapeutic Drug Monitoring, agar diffusion methods
02/2002 - 12/2003	**laboratory assistant of biology**

Research institute for farm animals
- Molecular biology workings

Education

10/2009 - 09/2011	**Master of Science: medical biotechnology**

University of Rostock

Title of the master thesis:
"Characterization of subpopulations of mesenchymal stem cells of the cord under normoxic and hypoxic culture conditions"

10/2006 - 09/2009   **Bachelor of Science: medical biotechnology**
University of Rostock

Title of the master thesis:
"Examination of the vitality of cells from human cartilage or bone tissue in a 3D biocomposite matrix under using of the Bionas® cell chip technology"

08/2002 - 07/2005   **A level**
Night school of Rostock

10/1998 - 01/2002   **vocational education as laboratory assistant of biology**
Research institute for farm animals

Further Experiences

Languages   German – native language
English – Business fluent
French – Basic knowledge

Advanced training   certificate GCP

12.5 Theses

1. The increased aging population is associated with diseases (cancer, cartilage defect), whereby automation display the greatest potential to evaluate new therapeutic strategies.

2. Automation improves investigations in life science research and is associated with increased interests the last 10 years.

3. The combination of automatically cell processing and consequently bioscreening is new and could improve cell culture research.

4. The exclusion of contaminations of any kind (microorganism, cross-contamination) is especially important for automatic cell culture system and has to be investigated before automatically cell cultivation.

5. The development and integration of standardized decontamination and disinfection methods guaranteed the contamination-free cell cultivation by the Biomek® Cell Workstation.

6. The Biomek® Cell Workstation enables the processing of different cell lines (adherent cells, suspension cells) and the manufacturing of different 3D constructs (alginate beads, spheroid cultures, pellet cultures).

7. The Biomek® Cell Workstation is a flexible system for automatic cell cultivation and has the potential for further integrations of 3D cell cultures.

8. The 3D cell constructs should replace 2D cell cultures because the better simulation of *in vivo* conditions.

9. The automatically performed 3D constructs (alginate beads, spheroid cultures, pellet cultures) cover the most important cell systems for different cell lines.

10. The different 3D constructs are suitable for diverse cell lines (cancer cells, human primary chondrocytes) and applications, which can be bioscreening processes or tissue engineering for defect repair.

11. The consequently bioscreening depends on application and 3D cell construct.

12. The cell culture research should be performed with cells cultivated in media without antibiotic to prevent cross reactions with cells and compounds.

13. The Biomek® Cell Workstation enable the automatically cell cultivation in media without antibiotic realized using standardized decontamination and disinfection steps.

14. The automatically processed 3D-cell constructs enable the replacement of manually cell handling.

15. The manually bioscreening can be replaced by the automatically screening procedures.

12.6 List of Publications

12.6.1 Journals

1. R. Lehmann, T. Roddelkopf, S. Junginger, K. Thurow (2015) Comparison of the three dimensional cell cultures alginate beads and spheroid cultures containing of cervix carcinoma cell line; Journal Cytotechnology (in preparation)

2. R. Lehmann, J.C. Severitt, T. Roddelkopf, S. Junginger, K. Thurow (2015) Comparative Investigations of Manually and Automatically Manufactured and Screened Cervix Carcinoma Spheroid Cultures Formed in Perfecta3D® Hanging Drop Plates; Journal Cytotechnology (under review)

3. Lehmann R., Severitt J.-C., Roolf C., Thurow K. (2014) Role and Avoidance of Cross Contaminations in Automation Cell Culture Processes; Journal of the Association for Laboratory Automation (under review)

4. R. Lehmann, C. Gallert, T. Roddelkopf, S. Junginger, K. Thurow (2015) Biomek® Cell Workstation: a flexible system for automatic 3D cell cultivation; Journal of Engineering for Life Science pii: 2211068215594580

5. Lehmann R., Gallert C., Roddelkopf T., Junginger S., Jonitz-Heincke A., Wree A., Thurow K. (2015) Comparative Analysis of Manually and Automatically Produced Pellet Cultures of Human Primary Chondrocytes; Journal of Engineering in Life Sciences DOI: 10.1002/elsc.201500010

6. Lehmann R., Severitt J.-C., Roddelkopf T., Junginger S., Thurow K. (2015) Biomek® Cell Workstation: a variable system for automated cell cultivation; Journal of Laboratory Automation pii: 2211068215599786

7. Lehmann R., Gallert C., Roddelkopf T., Junginger S., Wree A., Thurow K. (2015) 3 Dimensional Cell Cultures: a comparison between manually and automatically produced alginate beads; Journal Cytotechnology DOI: 10.1007/s10616-015-9861-1

8. Lehmann R.; Vetter L.; Person L.; Thurow K. Evaluation of Antibiotic-Free Cell Cultures in Manual and Automatic Cell Handling. American Laboratory, (2013), online: http://www.american laboratory.com/914-Application-Notes/148434-Evaluation-of-Antibiotic-Free-Cell-Cultures-in-Manual-and-Automatic-Cell-Handling/

12.6.2 Proceedings

1. Lehmann, R., Thurow, K., Stoll, R., Weippert, M. (2014). Human vs. machine in life science automation: Comparing effectiveness of manual and automatic 3-D cell culturing processes. In F. Rebelo, Soares, M. (Ed.), Advances in Ergonomics in Design, Usability & Special Populations Part II. Proceedings of the 5th International Conference on Applied Human Factors and Ergonomics (pp. 45-52): AHFE International. ISBN: 978-1-4951-2107-4

2. Weippert, M., Rieger, A., Lehmann, R., Zhang, W., Thurow, K., Stoll, R. (2014). Physiological workload response of laboratory staff during simulated life science processes. In F. Rebelo, Soares, M. (Ed.), Advances in Ergonomics in Design, Usability & Special Populations Part II. Proceedings of the 5th International Conference on Applied Human Factors and Ergonomics (pp. 553-559): AHFE International. ISBN: 978-1-4951-2107-4

3. Gallert C., Lehmann R., Roddelkopf T., Junginger S., Thurow K. (2015). Biological High Throughput of 2D and 3D Cell Cultures for Future Industrial Upscaling, IEEE CASE conference, 24.-28.08. 2015, Gothenburg (S) (under review)
4. Gallert, C.; Lehmann, R.; Roddelkopf, T.; Junginger, S.; Thurow, K. (2015) High Throughput Screening System for Screening of 3D Cell Cultures. Proceedings IEEE I^{2}MTC, Pisa(I), accepted

12.6.3 Posters

1. Lehmann, R.; Junginger, S.; Roddelkopf, T.; Koch, G.; Thurow, K.: Automatic Hanging Drop Manufacturing. Poster, Status Seminar 2014, Junior Research Group, Life Science Automation – Systems and Process Technologies. Rostock (D), 02.07.2014.
2. Lehmann, R.; Gallert, C.; Junginger, S.; Roddelkopf, T.; Koch, G.; Thurow, K.: Automatic Produced 3D Cultures for Bioscreening. Poster, Forum Life Science Automation & Medical Automation Conference 2013, Rostock (D), 12.09.2013.
3. Lehmann, R.; Roddelkopf, T.; Junginger, S.; Koch, G.; Person, L.; Vetter L.; Thurow, K.: Do We Need Antibiotics in Cell Culturing? Comparison of Automatic and Manual Disseminated Cell Cultures. Poster, SLAS 2013, Orlando (USA), 15.01.2013, pp. 61.
4. Lehmann, R.; Roddelkopf, T.; Junginger, S.; Koch, G.; Thurow, K.: A new Method: Automatic Processing and Cultivation of 3D Cell Cultures – Alginate Beads. Poster SLAS 2013, Orlando (USA), 15.01.2013, pp. 50.