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# PROCESS OPTIMIZATION AND VALIDATION OF AN ASSAY FOR HIGH-THROUGHPUT SCREENING

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# ABSTRACT OF THESIS

# PROCESS OPTIMIZATION AND VALIDATION OF AN ASSAY FOR HIGH-THROUGHPUT SCREENING

A biological assay is designed to set up a rapid and robust drug-screening system on a small scale. An assay is considered as a single unit of a platform to screen various compounds for aiding in drug discovery. Each assay is carried out in a 96-well plate, each of whose wells consists of the biological component called the Spheroids. The value of each assay lies in it facilitating for versatile screening applications. The spheroid is considered as a micro-structural product. And the addition of various compounds for testing is performed in each well (consisting of the spheroids). The focus has been to put forth the production principles and validation strategies to run the biological assay and test its efficacy to be used for screening in high volumes. The assay development illustrates processing and validation techniques. The goal is to develop optimized standards to process the assay, addressing various quality control issues, from the raw material to the end-product stage. Such an approach also brings interesting analogies of biological process in a manufacturing scenario. The developed system incorporates a value stream approach, by pulling the product from the customer end.

The process involves simply encapsulating HUVECs (Human Umbelical Vein Endothelial cells) from the raw material stage, culturing to form the spheroid and transferring the component to assemblage in a 96-well format undergoing stages of heat treatments. The small scale screening system allows the use of small amounts of drug, which is especially essential for new drug synthesis or in rapid decision making to find out any unknown potent compounds. The design of optimal processes in product development of the spheroid assay is illustrated. Thus in light of the value of this assay, developing the production system has been pivotal so as to produce quality spheroids in the 96-well plate formats. The quantification of the stimulatory and inhibitory effects of the different agents is required to help understand the complex biological behavior involved. The goal is to validate the data using image analysis software. The image analysis helps determine the quantification to be accurate, objective, and consistent. The quality of the product is tested by the reproducibility and robustness of the assay.

Keywords: Optimization, Validation, Variability, FMEA, Angiogenesis, Assay, Spheroids, In vitro, High-throughput

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May, 2006

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# THESIS

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**The Graduate School**

**University of Kentucky**

**2006**

# PROCESS OPTIMIZATION AND VALIDATION OF AN ASSAY FOR HIGH-THROGHPUT SCREENING

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**THESIS**

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**A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Manufacturing Systems Engineering at the University of Kentucky**

**By**

**Padma Priya Ravindranath**

**Lexington, Kentucky**

**Director: Dr. Lawrence Holloway, Professor**
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**2006**

# DEDICATION

*To Thatha ,Pati, Amma, Appa and Anna*

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# CHAPTER I INTRODUCTION

## 1.1 Motivation

Drug screening business for identifying potential drugs has hit the market picture since a decade. There are many manufacturing models used for drug screening purposes which draw parallels in a manufacturing scenario. In a recent release this year, the Food and Drug Administration announced it would identify ways of modernizing the drug development process under its so-called "*critical path*" initiative to take a drug from laboratory to patient. With more than 90 % of drugs failing when tested in humans, scientifically engineered tools are considered to improve the way the drugs are tested [1]. Given the current scenario with the dramatic failure rate of drug screening in pharmaceutical companies, the government is taking efforts to develop assays to benefit the drug screening platform in the public sectors.

Drug screening comprises of generating an artificial scenario of a desired human functioning system. Thus developing this artificial scenario for a prototype model is the main manufacturing goal of this project. The basic processing of this system includes mimicking of the blood vessels formation using a generated biological component and then the drug treatment is added. The formation of the biological component to desired geometric shape is also attained in the process. Various models were experimented and analyzed for this purpose and an optimal model was implemented. The concepts of problem solving, production planning, inspection, statistical process control, variability assessment, data validation are used in the manufacturing process. This thesis brings into light a unique way of describing a biological process. Since the process involves assemblage of two major components it brings scope to depict it in a manufacturing scenario. There has been in depth analysis of statistical process control in this thesis, which in a manufacturing system works toward continuous improvement or in generating areas for continual improvement. The basic concept of studying variation and using statistical signals to improve performance can be applied to any area. Such areas can be

on the shop floor, a research laboratory or in the office. Some examples I would quote in support of the statement would be machines or equipment and product (performance characteristics), processes, bookkeeping (deviation and error rates), waste analysis (scrap rates), etc.

Historically, statistical process methods have been routinely applied to parts, rather than processes. Application of SPC to control output (such as the end product) of the system should be only the first step. The industrial revolution has taught us that until the processes that generate the output become the focus of our efforts, the full power of these methods to improve quality, increase productivity and to reduce cost may not be fully realized. To bring some traditional highlights into manufacturing, it often depended on production to make product and on quality control to inspect the final product and screen out items not meeting specifications. Work is often checked and rechecked to catch errors. Both cases involve a strategy of detection, which is wasteful, because it allows time and resources to be invested. It is much more effective to avoid waste by not producing the unusable output in the first place, which is a strategy of prevention. Thus the research laboratory adopts ways of identifying and preventing the error before occurrence through intense study, problem solving and balancing variability. The statistical process control and its validation are elucidated in chapter four and thus draw parallels of defining the manufacturing processes in biology.

Biologically speaking, the goal is to develop a toolbox to help better understand the inter-connected networks of molecules that comprise cells, their interactions, regulation, and the combination of molecular bonding that lead to disease. These events are studied herein specific to angiogenesis, which is the development of new blood vessels that branch out from existing vessel formations. This further relates back to the objective of producing an assay which incorporates the above mentioned events to the fulfillment of successfully screening and validating various drug compounds. And this process of systematically screening tens to thousands of compounds is known to be High Throughput screening (HTS) [2]. The complex high-content screening (HCS) information coupled with HTS has the incredible power of capturing every shape of biology to allow grounds for decision making in drug discovery based on prioritization. This dual HCS-

HTS system brings immense value to drug screening aiding in time saving, cost reduction and in capturing intellectual protection.

Considering such a need, the current thesis addresses one such path to develop a platform for high throughput screening of drugs. This involves high level design in consideration of the right testing model to give reproducible and validating results. There are various genetic, tumor and surgical models applicable to help in developing drug discovery. But the void being reported is the pace in drug discovery, a critical research within the laboratory has gone into defining and producing the right cell-based assay for the high throughput screening system. With the identification of the right assay for mimicking blood vessel formation, the development of the assay and image analysis using proprietary software for validating the assay results is illustrated herein. The image analysis gives scope to generating library of data containing various pattern behavior of spheroids under varied conditions (such as growth factors or drug compounds based on the hypothesis for the experiment) and extracting biologically relevant information by comparisons made with drug candidates of unknown mechanisms. Such an assay helps gain an understanding of the nature of chemical space and how it provides a structure for organizing and analyzing biological/pharmacological data. In addition, the scientists will gain a very basic understanding of how *multi-criterion decision making* can be applied to help prioritize compounds as potential drug candidates. This inter-disciplinary approach elucidates the roadmap for angiogenesis drug discovery platform. The tools developed in the research are important as they establish relation between biology and engineering disciplines.

Any product that is new and before it hits the market undergoes intense research and development. Be it a car or a biological product, a prototype model is developed, processed and validated for its performance. When such validation becomes high in numbers, technology comes into picture. Thus the high throughput systems make use of technology to address processing and validation of a product.

The high throughput systems are used to ensure the product's reproducibility and there lays the true value to keep up to the ever growing demand. Unlike the car manufacturing which falls in a macroscopic work structure, the product in this study has a microscopic work flow and is visible only under a microscope. This microscopic work flow is considered in drug screening because it saves time and money spent on testing animal models. Like the way all cars are checked for consistency in performance, the product in each of the wells during its validation stage needs to be assessed for its consistent performance.

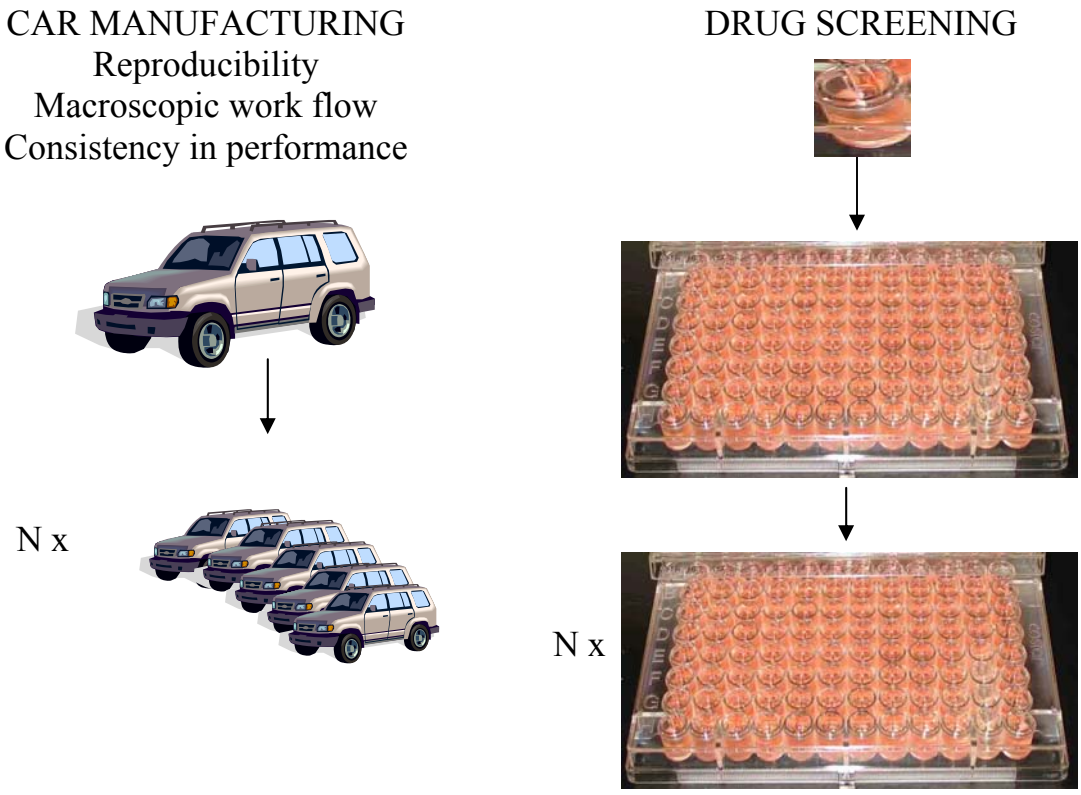



Figure 1: Illustration of high throughput systems

Each well of the 96-well plate is considered a product. This well will face the decision of the drug stating if it is potential or not. Thus the value lies in making it reproducible. The goal of the study was to develop the microscopic product, make it reproducible and validate its performance. We will see further where the product applies and its functions.

## 1.2 Angiogenesis and the Definition of Need

Angiogenesis is the formation of new blood vessels from pre-existing vessels. Angiogenesis is a normal physiological process that occurs in growth and development, as well as in wound healing. However, this is also a fundamental step in the transition of tumors from a dormant state to a malignant state and under excessive angiogenesis it is observed as abnormal rapid proliferation of blood vessels, which is implicated in many diseases, including arthritis, psoriasis and age-related macular degeneration [3].

When tissues need more oxygen, for example, they release molecules that encourage blood vessels to grow. The ability to inhibit angiogenesis and turn off the blood supply to tumors could potentially lead to a new generation of cancer therapies.

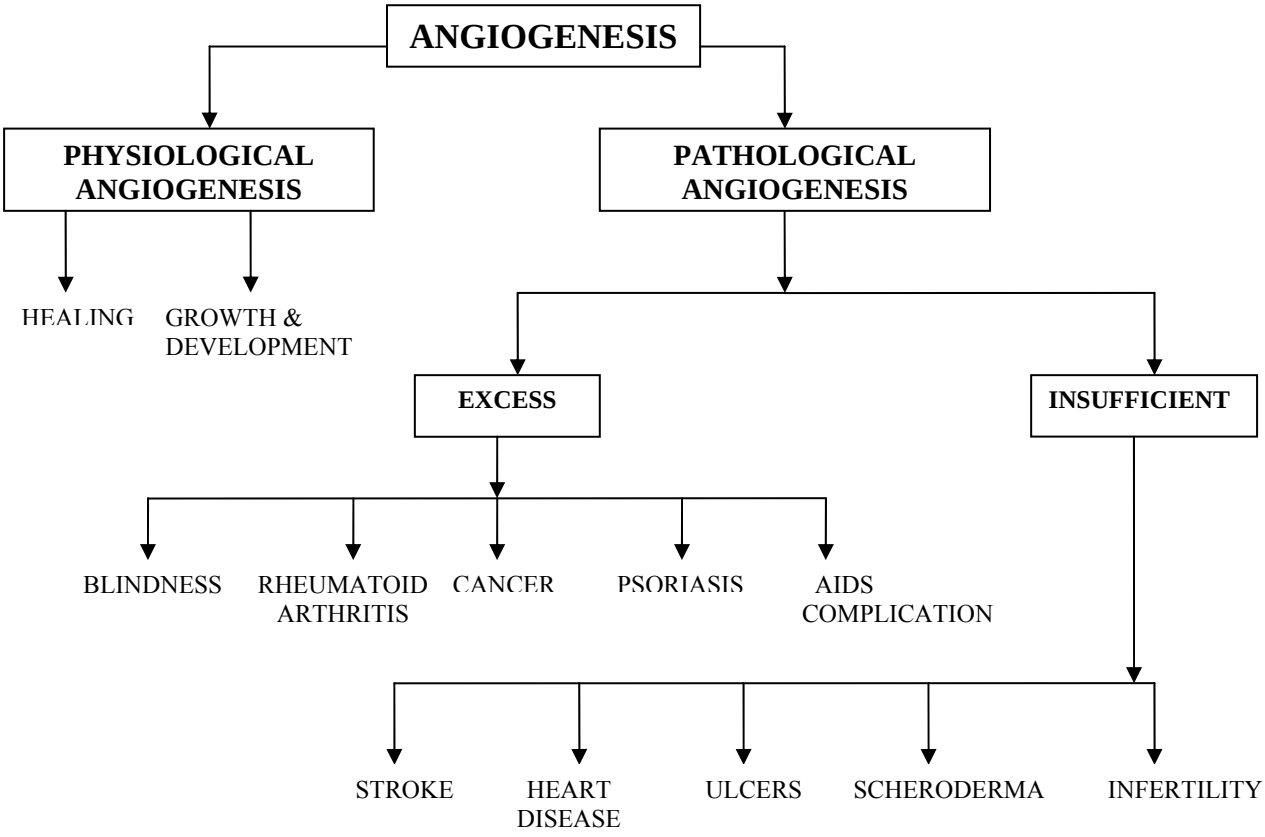



Figure 2: Angiogenesis schematic

*Sprouting Angiogenesis* Sprouting angiogenesis a cascade of morphological (structural organization) events as explained below is a common form of blood vessel growth. The Endothelial Cell (EC) is commonly known for inducing sprouts in-vitro*, indicating in vivo*-like tube formation. ECs line the entire circulatory system, from the heart to the smallest capillary [3]. In vivo, ECs form a monolayer of cells at the innermost surface of the blood vessels that attach onto an underlying basement membrane (a supporting structure that acts as vessel walls) forming an interface between circulating blood and the vessel wells.

* in-vitro: An experimental technique performed outside of a living organism or cell [3]

* in-vivo: A condition existing in a living organism or an experiment performed on a whole/living organism

The key events of angiogenesis, also called the angiogenic cascade, involve:

- The initial activation of the endothelial cells to escape from the vessel walls.
- Gradual migration from 2D monolayer to 3D macromolecular space into the surrounding matrix to form solid sprouts connecting neighboring vessels.
- With gradual cell migration and proliferation, these sprouts then become full-fledged vessel networks to slowly form new blood vessels and move in towards the angiogenesis stimulus (the tumor/damage site)[4, 5].

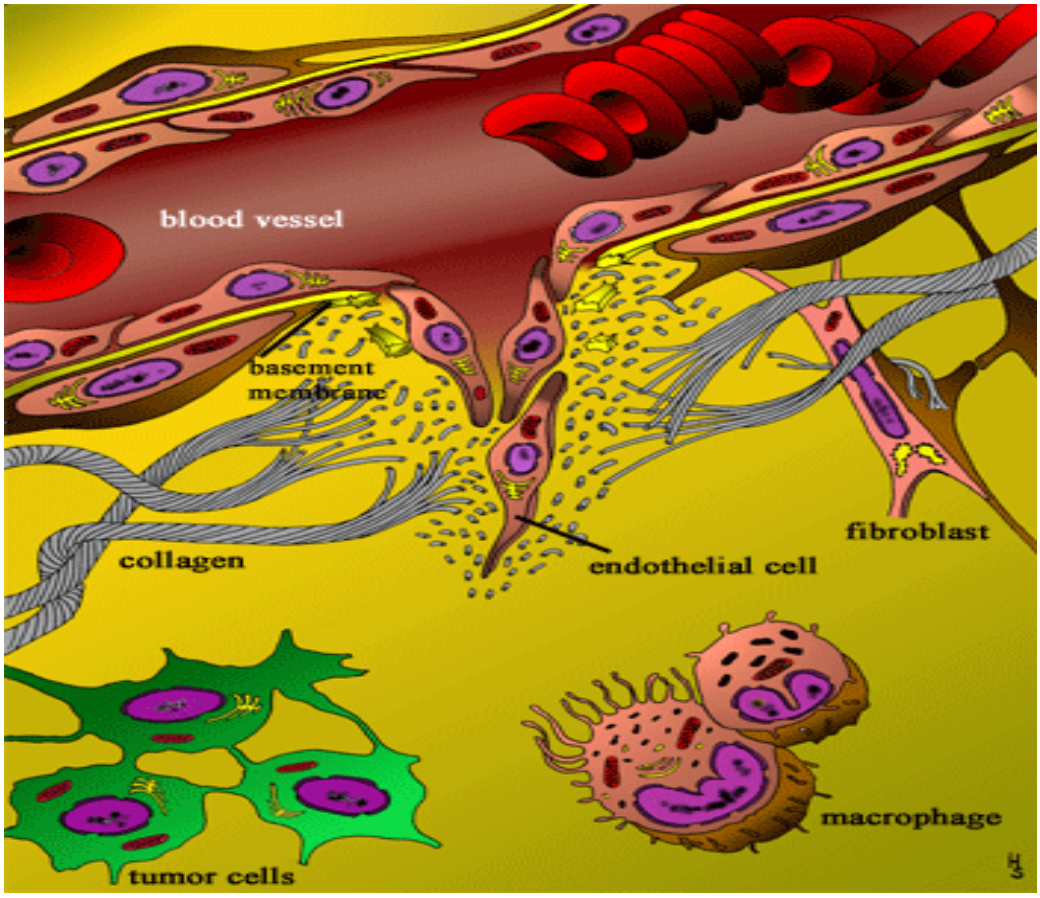



Figure 3: Angiogenesis pathway in-vivo (Reference: AMP lab GmbH http://www.amplab.de/angiogenesis.htm)

Given the potent therapeutic uses of angiogenic and anti-angiogenic factors in wound repair and cancer, there has been a need reported for reliable, quantitative angiogenic assays to screen for activity and to determine the biological mechanisms of action [6].

The in-vitro assays represent the resting (2D monolayer) or sprouting (3D tube formation) phenotype reminiscent of their in-vivo counterparts that show both injury and repair features. Similar to the angiogenic cascade, the induction process of the in vitro sprouting assay incorporates:

(1) Preparation of aggregate of ECs (the spheroid) phenotype from 2D monolayer cobblestone structure,

(2) Preparation of a 3D matrix of collagen gel (from collagen I fibers to form the macromolecular space),

(3) Activating the resting aggregate of cells by the addition of a factor to be tested and

(4) Monitoring by quantification, the physical changes in resulting sprouting

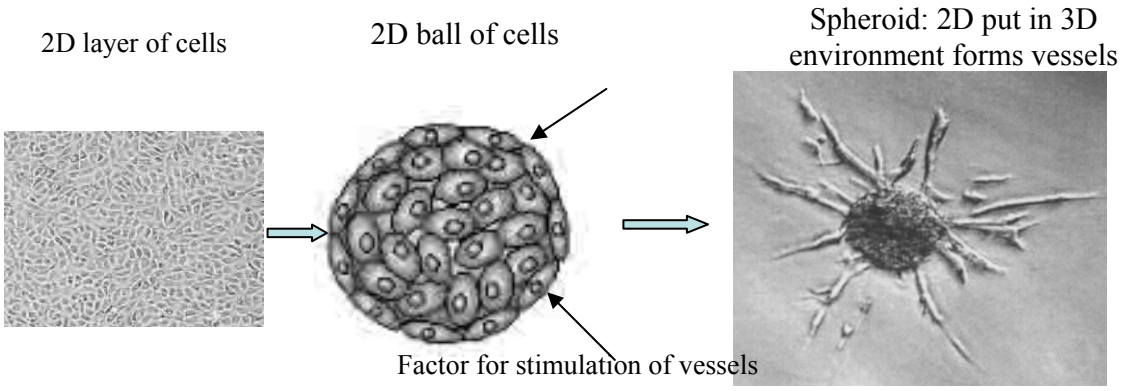



Figure 4: Simple sketch showing angiogenesis process from 2D monolayer to spheroid

The process involves getting the 2D layer of cells in a synthetic environment and processing them to form the aggregate of cells called the spheroids. This aggregate of cells is assembled in a 3D matrix and is then stimulated to form the vessel structures.

## 1.3. Research Objectives

In studying the various cases of defined angiogenic process and its modification, it was important to be able to develop a synthetic (in vitro) production model representative of angiogenesis. This is due to the reason that animal models are considered extremely complex and difficult to interpret for routine screening and also relatively expensive [7]. Addressing the current requirements, the in-vitro angiogenesis assay model was identified which was compatible with HTS. A production system is defined for this purpose. The spheroids which were the end product in this system were analyzed individually for detecting their performance parameters. This functions as a sensory assay to extract subtleties of high-content information using image processing software.

The purpose was to adapt production of spheroids to a high volume assay. This required optimal design of the processes to ensure quality control. This production system was aimed to address the processes, identify variability, make the product, and validate its efficacy. The quality of the spheroid was achieved with validating the processes at different stages incorporating various control techniques. Standardized procedures were documented to provide for reproducibility. The validation of the assay performance was determined by variability assessment from the data obtained using the image analysis software. The validation technique was used to evaluate the multi-functional uses of the product to prove its robustness for various applications.

Thus the critical part of the project being the production of the assay to repeatable numbers, also aims to illustrate the clear picture of software analysis for detecting the performance parameters of the product.

## 1.4. Thesis Layout

So far in the first chapter we stated the motivation for the current work and defined the need in the field of angiogenesis drug discovery. The thesis in the consecutive chapters defines the significance of the identified assay for the purpose of screening drugs in comparison to other assays. The detailed literature review on angiogenesis and the available assays for screening are elucidated. The uses of other assays and the versatility

of the spheroid assay in studying the behavior of spheroids to represent vasculature (blood vessel formation) are discussed. In detail description of the raw materials and their processing methods is illustrated. The material vendors have also been stated as a part of the assay protocol. Chapter three streamlines the protocols for the formulation of the assay from raw material to the screening stage. The chapter four continues to illustrate the value of the assay by showing the validation of the processes and also the powerful assay validation using the image analysis software. The chapter five discusses the results obtained from the analysis and their interpretation with respect to efficacy of the assay and its robustness. The assay application for screening a collection of plants is discussed in chapter six. It covers the validation of the assay when produced to screen a library of drug compounds for identifying any inhibitory activities.

# CHAPTER II THE BIOLOGICAL ASSAY TO MIMIC BLOOD VESSEL FORMATION

Bioassay or a biological assay is a type of in vitro experiment that is subjected to analysis. Bioassays are typically conducted to measure the effects of a substance on a living organism. Cell-based assays are one simplistic approach for such measurements. Bioassays may be qualitative or quantitative, the latter often involving an estimation of the concentration or potency of a substance by measurement of the biological response that it produces. Quantitative bioassays are typically analyzed using the methods of statistics [3].

## 2.1. Existing Assays

There is wide categorization of various in-vitro cell-based assays to assess response of EC to various agents and to study features mimicking in-vivo situation. A general classification of Cell-based assays for HTS [8]:

1. second messenger assays that monitor cellular signal transduction
2. reporter gene assays that monitor cellular responses at the transcriptional/ translational level
3. cell proliferation assays that detect induction or inhibition of cell growth.

Angiogenic cascade is a result of different steps. In this, the pre-existing vessels degrade their local basement membrane, migrate, proliferate, and subsequently align to form a network of new vessels which facilitates directional blood flow. These steps can be analyzed separately in different in-vitro assays [9].

In vitro angiogenesis assay system can be mainly categorized into migration, invasion and tube formation assays [10]. A brief description of the commonly used angiogenic assays is discussed further.

*Two Dimensional Assays*

Classical in vitro assays representative of cell migration and invasion are Boyden chambers and Transwell filter chamber systems. The assays consist of upper and lower chambers separated by a filter to separate the two compartments. The extensive use of these assays has provided with understanding of the composition and function of cell-cell and cell-matrix adhesions [11].

Migration Assay

*Migration (or chemotaxis) is the directional movement of cells in response to a concentration gradient of a soluble attractant*. In migration assays, test cells are seeded into the upper chamber, a chemoattractant is added to the lower chamber, and cells that have traversed through the filter are quantified after an appropriate time span [10, 11].

Migration is also detected by causing mechanical injury by scraping a confluent monolayer of cells. This mimics the lateral migration of EC from the wounded edge. This apparently is a classical non-directional chemokinetic experiment to determine the overall migration-regulating effect of a pharmaceutical test compound [9].

Invasion Assay

Invasion in addition to the migratory response requires for *cells to express significant quantities of matrix degrading enzymes that digest the matrix barrier*. This allows cell movement into and through the extracellular matrix. For observing invasion, the filter membrane is coated with extracellular compounds such as matrigel (which is a extracellular matrix from a tumor cell line [12]), so as to mimic the natural basement membrane (a surrounding environment in the matrix) [10, 11].

The established assay systems have some inherent limitations. Each is restricted due to the fact that they only study individual steps of the angiogenic cascade such as migration or proliferation, invasion, etc. It has been investigated that they are not quantifiable of the angiogenic cascade because that they are cellular specific such as in a 2D Matrigel alignment assay. The Matrigel 3D assay is one common form of endothelial tube formation [5]. The study of the events in angiogenic cascade in separation may lead to

loss of high content information capture. Furthermore in 2D assay, the EC monolayer even grown to super-confluence in density, have limited proliferation rates and do not become perfectly growth arrested in vitro in order facilitate formation of vessels [9].

These considerations trigger the need for developing novel in vitro angiogenesis assays that may functionally and molecularly be more representative for the angiogenic cascade than the previously established experimental systems. To address the same, the latest investigation is done on 3D assays [9].

*Three-dimensional (3D) assays*

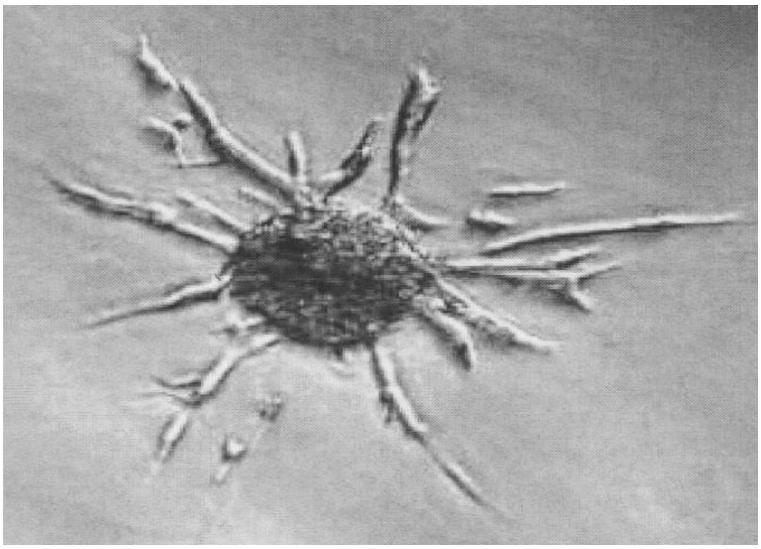


Figure 5: The biological product- spheroid sprouting stimulated with VEGF at 20ng/ml

The current thesis concentrates on 3D tube formation assays. As observed in the recent years, one major advantage of 3D cell cultures is their well-defined geometry, which allows for inter-relation of structure to function. And apparently, techniques incorporating spatial resolution interpret promising data which can be stored and retraced [7]. In order to better mimic the in vivo situation, 3D assays are preferred to 2D assays as, the molecular mechanisms of cell adhesion and its functional contribution to cell behavior differs dramatically in three-dimensional matrices [13]. In this type of new culture system, the cells can be observed in three-dimensional matrices composed of extracellular-matrix (ECM) macromolecules. The widely used reagents which facilitate

formation of the 3D extracellular matrix are collagen, fibrin gels, matrigel [14], etc. This assay allows for implementing such a system to cell growth as well as the tube formation. It has been known that for tube formation, cells are grown in spheroids either alone or in co-culture with other cell types [11].

It has been reported that the ECM provides the required scaffold for cells to organize and regulate their behavior [15]. Further investigation suggested that the physical state (such as structural and mechanical constraints) of the ECM and its molecular composition together, provides the relevant micro-environmental information regarding the cell-ECM interactions [16]. Recently simplified models of ECM using 3D matrices of type I collagen have been developed for understanding the ECM biomechanics [17]. In evidence, type I collagen polymerizes to form complex, 3D supra-molecular assemblies in vitro whose structure (in *axial periodicity*) is similar to those formed in vivo [18]. All these studies depict the relevance of in-vitro ECM to support the in-vivo like cellular phenotype.

## 2.2. Significance of 3D ECSA (Endothelial Cell Sprouting Assay)

In vitro primary EC usually cultured as two dimensional flat monolayers reflects most of endothelial in vivo phenotype properties. Yet in this phenomenon, the cells do not reach complete quiescence (stage representing cells resting phenotype, as mentioned in chapter one) which is needed for vessel formation. Studies have been performed to show that the multi-cellular aggregate spheroid model generates an inner area of quiescent cells. In order to keep the EC quiescent and to preserve their differentiation in vitro [3] the use of a novel method to culture EC as three dimensional spheroids has been adopted.

The three-dimensional (3D) endothelial cell sprouting assay (3D-ECSA) is a very promising in-vitro biological assay model to study endothelial cell morphogenesis in 3D extra-cellular matrices. As mentioned, there being many other assays researchers use for angiogenesis screening, the assay recapitulates the angiogenic cascade and features the differentiation of endothelial cells into sprouting structures within a 3D matrix of collagen I [20]. It has been known that the sprouting assay can bring high-content

information on different aspects of endothelial cell differentiation, migration, invasion, tube formation and branching.

The feasibility of this assay to be performed under well defined and controlled experimental conditions in order to analyse the EC functions makes it suitable for high-throughput screening purposes. The laboratory started with the use of the assay for one plant and has discovered the compound called Withaferin A from the herbal plant Withania Somnifera. The WA has shown to have potent anti-angiogenic activities [39]. Since then it has been challenging to optimize the assay for screening a library of extracts and the software analysis helps statistical validation of the screening data. Thus the chosen assay has great potential for drug screening and validation so as to fill the gap between animal models and monolayer culture. The 3D ECSA uses the cellular aggregates called spheroids to depict the angiogenic process in the presence of an ECM scaffold made using the collagen. Exogenous growth factors can be added to the 3D cell culture to stimulate the growth of vessel-like structures that grow out from the spheroid and the drugs that inhibit sprouting can be tested.

## 2.3. Materials

**Reagents, Material and Apparatus used**

| S.No. | Reagent Name | Concentration | Company | Catalogue # |
|---|---|---|---|---|
| 1 | Medium 199 | 1X | GIBCO | 12350-039 |
| 2 | Antibiotic/Antimycotic | 100X | GIBCO | 15240-062 |
| 3 | Trypsin-EDTA | 1X | GIBCO | 25300-054 |
| 4 | Low Serum Growth Supplement(LSGS) | 50X | Cascade Biologics | S-003-10 |
| 5 | Phosphate Buffered Saline | 7.4,1X | GIBCO | 10010-023 |
| 6 | Paraformaldehyde | 32% | Electron Microscopy Sciences | 15714-S |
| 7 | Fetal Bovine Serum | | GIBCO | 26140-079 |
| 8 | Methyl Cellulose/Methocel(MCS) | 4000 centipoise | FisherScientific | M352-500 |

Table 1: Reagents used

| Plastic/Glass Ware | | | |
|---|---|---|---|
| S.No | Name | Company | Catalogue # |
| 1 | 60mmx15mm Tissue Culture dish | Corning | 25010 |
| 2 | 100mmx15mm Petri dish | Falcon | 351029 |
| 3 | 15 ml Centrifuge tube | Fisherbrand | 05-539-1 |
| 4 | 50ml Centrifuge tube | Fisherbrand | 06-443-19 |
| 5 | 115 ml filter system | Corning | 430944 |
| 6 | 50ml Reagent Reservoir | Costar | 4870 |
| 7 | 2ml Freezer vials | VWR International | 16001-102 |
| 8 | Boxed pipette tips rack 200-1000 µl | Genemate | P-3249-2 |
| 9 | Boxed pipette tips rack 0-10 µl | Genemate | P-3245-3 |
| 10 | Boxed pippette tips rack 20-100 µl | Genemate | P-3259-200 |
| 11 | Pipettes 0-2 ml | Falcon | 35-7507 |
| 12 | Pipettes 0-5 ml | Falcon | 35-7543 |
| 13 | Pipettes 0-10 ml | Falcon | 35-7551 |
| 14 | Pipettes 0-25 ml | Falcon | 35-7525 |
| 15 | Flat Bottom 96-well cell culture cluster | Costar | 3595 |
| 16 | 2 ml vials | Fisherbrand | 05-408-140 |
| 17 | 500 µl vials | Fisherbrand | 05-408-120 |
| 18 | 9" Pasteur pipets | Fisherbrand | 13-678-20D |
| 19 | Glass funnel | | |

Table 2: Plastic/Glass ware used

| Apparatus Used | | Company |
|---|---|---|
| 1 | Pipettors(2 µl,200 µl, 1000 µl) | Genemate |
| 2 | Multi-Pipettors(2 µl,200 µl, 1000 µl) | Genemate |
| 3 | Pipet-Aid | Genemate |
| 4 | 6R Centrifuge | Beckman Coulter-Allegra |
| 5 | Mixer | Thermolyne |
| 6 | Waterbath-Isotemp 20 | Fisherscientific |
| 7 | Stirrer/Heater | Thermolyne |
| 8 | Push button counter | |
| 9 | Hemocytometer | |
| 10 | Microscope | Nikon |

| Storage Equipment | |
|---|---|
| Liquid Nitrogen tank | Thermoforma |
| Laminar Flow Hood | Thermoforma |
| Humidified Incubator at 5% CO2 37°C | Thermoforma |
| - 86°C ULT Freezer | Thermoforma |

| *Miscellaneous* |
|---|
| Parafilm M |
| Scissors |
| Kimwipes |
| Nitrile Gloves |

Table 3: Equipment and other miscellaneous used

### 2.3.1. Biological Supplies

There are three biological materials which are processed separately toward the assemblage of all. They are the HUVECs, Spheroids, Collagen. Each one's preparation is discussed in detail in the methods section.

*HUVECs*: A major advantage of cell culture is the ability to expand the number of cells available by subculture. Cells supplied in 1ml vials are seeded on plastic substrata (60 mm cell culture dish) at a relatively low density to attach on the surface. After they attach, they progress from sparse (low density) to subconfluent (medium density) to confluent stages (high density) of increase in cell density. Human Umbilical vein Endothelial cells (HUVECs) were used in the assay because they are primary cells of human origin bringing relevance to disease and are readily available in large supply.

*Collagen*: Collagen is an inert, rigid protein structure, inextensible in nature with good tensile strength. Type I collagen is the major structural component of extracellular matrices found in connective tissue and internal organs, but its presence has been studied to be most prevalent in the dermis, tendons, and bone. Its ability to strengthen blood vessels marks its use in the assay. It can be stored at its liquid state at $4^{o}$ C and can be processed with the assay to spontaneously form the triple helix scaffold at a neutral pH at $37^{o}$ C [22]. This provides the solidified extracellular matrix for the spheroids in vitro.
In order to get the quality end product, all the process stages should compliment one another and there lies the true value of the assay.

## 2.4. Methods

### 2.4.1. Cell culture - HUVECs

HUVECs (Cambrex Bioscience) were cultured in 1X M-199 medium (Gibco), 10% Fetal Bovine Serum (FBS) (Gibco), 0.1 mg/ml of Antibiotic/Antimycotic (Gibco) and 0.2 mg/ml of 50X Low Serum Growth Supplement (Cascade Biologics), at 37 °C in a humidified incubator with 5% CO2 and 95% air.

This culture mixture was called as the complete medium. Cells were used within the sixth passage. Medium was changed every 2-3 days and confluent dishes were split at 1:2 ratio using 0.05% trypsin-0.02% EDTA in HBSS (Gibco)

### 2.4.2. Collagen Preparation

Collagen type I was internally prepared in the laboratory. Rat tails from healthy young rats were used. In a 1 ml beaker, three tails were immersed in 70% ethanol solution for 15 minutes and washed and re-immersed for a period of 1 hr. After they have been sterilized, using two forceps, one in each hand, the tail is held without exerting pressure. The tail is broken apart and pulled to separate in two parts. Tendons are extracted and immersed in clean ethanol for 20 min [9]. Ethanol is decanted carefully and the tendons are submerged in 250 ml 10% acetic acid. The solution is stirred thoroughly for 48 hours at $4^{o}$ C. Later the solution is centrifuged at 2500 rpm at $4^{o}$ C for 1 hr. The supernatant (without the debris) is decanted carefully and stored at $4^{o}$ C in sterile bottle. Concentrated stock solution of collagen is thus obtained. The solution was checked for its pH to range from 3.9-4.3 and was diluted as necessary in sterile acetic acid solution [5]. The collagen stock should be left to mature for 4 weeks before its usage so as to keep the EC baseline sprouting low [9].

### 2.4.3. Methocel Preparation

In a glass bottle, add 3 gm of Methyl Cellulose powder (4000 centipoise) to make up a total volume of 250 ml in Medium 199. Place a magnetic stirrer in the bottle, loosely close the lid and autoclave it under gravity mode in an autoclave. Later, add 125 ml of M199 to the bottle and let stir for 20 minutes. Then add 125 ml of M199 and put to stirring in the cold room (at $4^{o}$ C) for two hours. Store at $4^{o}$ C.

### 2.4.4. Cell count to generate spheroids

Once the HUVECs reach the sub-confluent to confluent stages of growth, they were considered ready to be utilized for spheroid generation. This monolayer of cells attached on the surface of the dish was dissociated from the substrate surface with addition of proteolytic enzymes contained in Trypsin-EDTA solution, which leaves the cells

themselves intact and afloat in the medium. The cells in the trypsin-medium were collected and counted for their total density on a Hemocytometer. HUVECs were re-suspended in the fresh complete medium containing of 10 % Methocel to generate the spheroids. Methocel acts as inert viscocity modulating substance by preventing cell-cell adhesion [23].

### 2.4.5. Spheroid Generation

The hanging drop method was used to generate a multicellular spheroid [24]. HUVECs suspension were diluted to obtain a concentration of $1x10^5$ cells/ml. HUVECs suspensions of 10 µl (containing 1000 cells) droplets were then spotted on a non-adhesive 100mm polystyrene Petri dish. Upon inversion of the dishes over the bottom plates containing 12 ml of sterile phosphate buffered saline (PBS) solution, the hanging drops were held in place by surface tension, and HUVECs were accumulated at the liquid air interface [25]. The spheroids were incubated for 24 hr at 37 $^oC$ in 5% $CO_2$.

### 2.4.6. Neutralized Collagen Mixture Preparation

The clear collagen stock solution was collected from different rat tails and these batches were stored at $4^o$ C. The collagen stock stored in a separate tube is degassed on the ice bath before every use to get rid of any dissolved oxygen content. To obtain a neutral pH collagen solution 10x M-199 medium (Gibco), 0.2 N NaOH, Collagen stock, 1:1000 diluted solution of sterile acetic acid and 50 mg/ml $NaHCO_3$ were gathered on an ice bath. Required numbers of 15 ml centrifuge tubes were pre-cooled for 2 min in the ice bath. The mixture of collagen is prepared by adding the reagents in the tube in the order mentioned above. While preparing the mix, reagents were thoroughly and gently mixed using the 1000 µl pipette (P1000) with the addition of every reagent, extreme care was taken not to generate any bubbles in the mix. The pipet-aid was set at low speed while making collagen mixture.

### 2.4.7. Three-Dimensional Endothelial Cell Sprouting Assay (3D-ECSA)

The endothelial spheroid assay [23] was adopted with some modifications to fit the experiment model. With the generation of spheroids and collagen mixture prepared, the assay was assembled with all components in the 96-well plate. A bed of 50 µl of

neutralized collagen I solution was formed in separate 96 well-tissue culture plates (Falcon) and polymerized by incubation for 30 min at 37$^{o}$ C. The spheroids were collected in PBS solution and centrifuged at 300 rpm for 3 min, the supernatant was removed and spheroids were carefully re-suspended in an ice-cold solution of neutralized collagen type I obtained from rat tails [17]. Next, 50 µl neutralized collagen-I containing equal number of HUVEC spheroids was then distributed over this solidified matrix bed and newly added collagen was polymerized at 37$^{o}$ C. An aliquot of 50 µl of culture medium containing 10% FBS was added to each well. To induce sprouting, 20 ng/ml of vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) (Sigma) was added to the medium. HUVEC-derived spheroids were incubated at 37$^{o}$ C in 5% $CO_2$ for 24 hr. Sprouting was visualized with an automated Nikon TE2000E2 inverted microscope, Photometrics digital camera and Metamorph software at 10X objective Digital images were imported to software for analysis of sprouting parameters.

### 2.4.8. Extracts from Plant leaves

Plant leaves from a wide range of species belonging to Kentucky native plants were collected from the Arboretum and stored at 4$^{o}$ C. Plant families belonging to Trees, Shrubs, Vines, Woods, Wildflowers and Prairie were collected and screened in the process.

The plant leaves were homogenized and dissolved in Ethyl Acetate to extract the supernatant. The ethyl acetate extract from all plants collected was dried using the Genevac Evaporator and further also vacuum dried. The dried extract was dissolved in DMSO and stored at -20$^{o}$ C.

*Supernatant: the clear fluid above a sediment or precipitate

# CHAPTER III ASSAY OPTIMIZATION

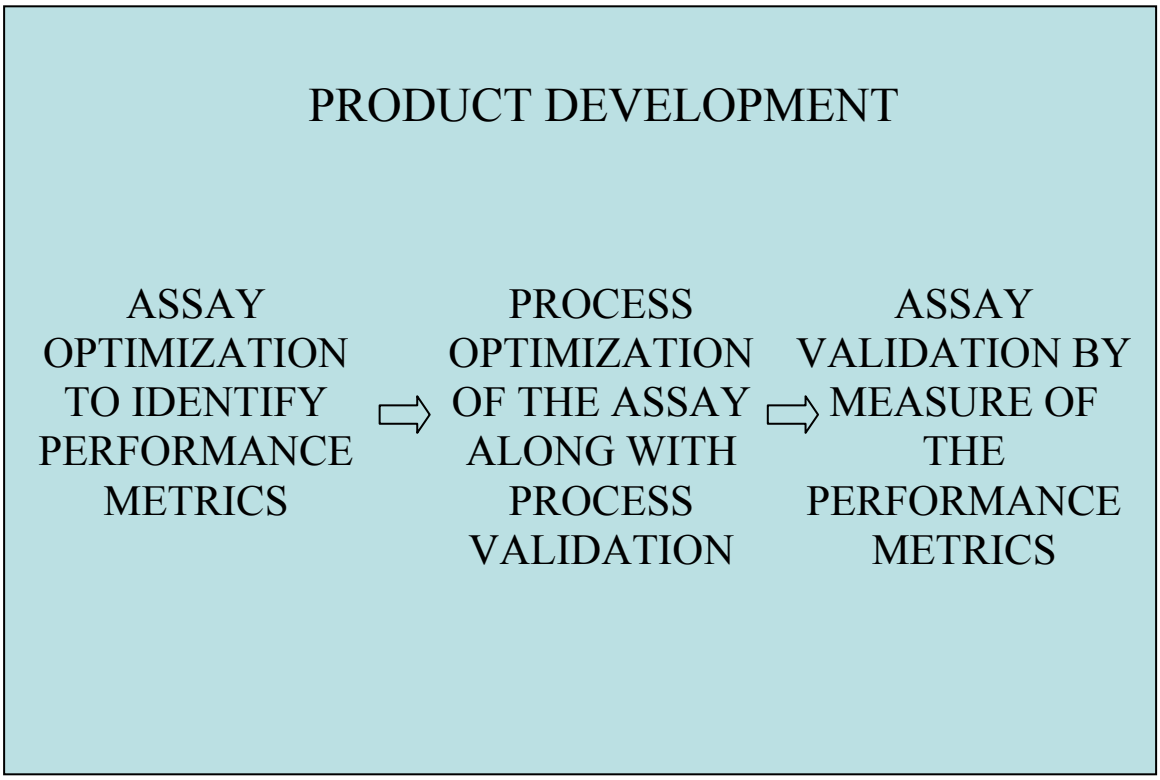



Figure 6: Product development schematic

## 3.1. Designing Process Development stages for Product Development

Attenuating concepts from general macroscopic view to developing microscopic work flow structure has been a critical part of the assay development. Assay developers quote that be it for basic research or clinical purposes, an assay's intended use becomes the anchor to which all optimization and validation activities are set. Optimizing an assay involves choosing its optimal format. Subsequently, the performance characteristics of an assay such as reliability, stability and repeatability need to be defined [26, 27]. With defining these parameters, the assay is checked for its optimization until the performance metrics are established and there is confidence in the results that are obtained from the assay. The results obtained proceeds to assay validation. A wide scenario sketching out the three main stages in product development is shown in the figure 6.

This strategy can further be categorized into four modules

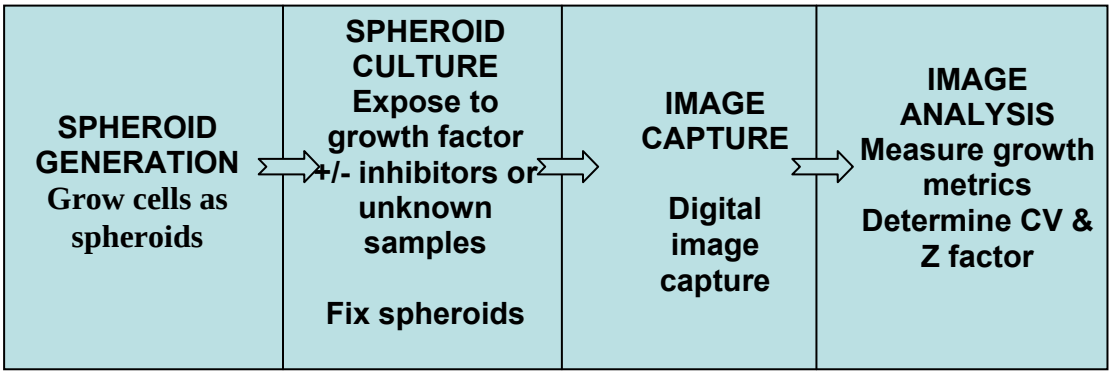



Figure 7: Schematic showing the overall research design and modules involved [28].

Product development consists of the certain phases. The first was to identify a model which fulfills the need. Addressing the first phase, the spheroid model using the HUVECs was adapted because of its compatibility to high-content screening and high-throughput screening. The stages of validating the reliability of the assay has been conducted which is discussed in the next chapter. The validation results certify that the assay is fit for use. Our purpose was to build a prototype of a powerful high content screening platform by interfacing high throughput screening capability with multi-parameter optical imaging of endothelial cell patterning in the 3D matrices. The multi-parameters established are discussed in the next chapter.

Product development can be associated to the process validation of a reagent or component. The process design helps in establishing user requirements throughout the development process. Product conformance to specifications is checked throughout all stages of development. Thus the process validation is made product specific [29]. **In our process, each well of the 96-well plate is considered a product.**

Once the process is designed, the process is validated statistically. Validation requires documented evidence that a process consistently conforms to the requirements. It is required to first obtain a process that can consistently conform to requirements and then run studies demonstrating that this is the case [30].

In the stage of developing the consistent process, experimentation and root cause analysis for problem solving is performed. A Failure Modes and Effects Analysis (FMEA) involve listing out the potential problems or failure modes and evaluating their risk in terms of their severity, likelihood of occurring and ease of detection. Where potential risks exist, the FMEA can be used to document which failure modes have been addressed and which still need to be addressed. As each failure mode is addressed, the controls established are documented [30]. The end result is a control plan in the form of Standard Operating Procedure (SOP). Addressing the individual failure modes will require the necessary theoretical and experimental analysis.

## 3.2. Standard Operating Procedures for Manual Processing

The major part of the thesis deals with processing the assay to optimize and adapt it to high throughput screening. The operating procedures for the product are discussed below:

- *Culture* HUVECs in 60 mm culture dish over a period of two days from the time of seeding them in the ratio 1:2. The cells reach approximately 70% confluence based on cell passage number and other intrinsic biological factors.

- *Trypsinize* HUVECs for 3 min in the incubator for the cells to dissociate from the dish surface. Later, perform 1:2 dilution of trypsin in complete medium to collect them in a centrifuge tube. Set the tube to centrifugation for 5 min at 750 rpm. Re-suspend in fresh complete medium and count for the concentration of cells. Then add Methocel (MCS) to make up the HUVEC medium.

  *Cell count*: After resuspending cells in complete medium, add a 20 µl droplet on one counting chamber of the Hemocytometer and center a cover slip between the mounting supports. Use the push button counter to count the number of cells on each of the 1mm x 1mm outer four squares. Take the average of the four readings and multiply the number to a concentration of $10^4$.

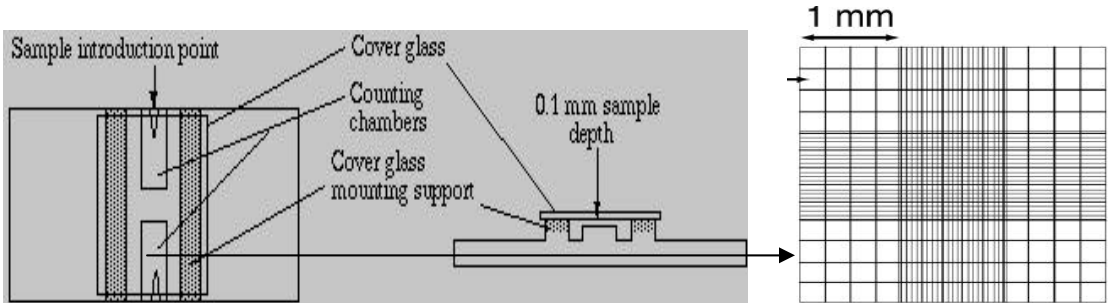



Figure 8: Hemocytometer showing the engraved cell counting surface on the chamber

- The usage of single plate cells per experiment is maximized to making optimal amount of HUVEC medium by diluting the cells in the medium to reach the concentration $1x10^5$cells/ml. This concentration gives the required 1000 cells per droplet. Make up the following volumes based on the confluent stage of the HUVECs and add 10 % Methocel. For a volume of N, it is necessary to use N-1 plates for generation of spheroids to avoid any deformed spheroids caused due to insufficient amount of cells in the droplet.

| 60 mm dish Confluence | Volume (N) of HUVEC medium for spheroids+ 10 % MCS to be added | Maximum number of spheroid plates (N-1) |
|---|---|---|
| 50 – 60 % | 6 ml | 5 |
| 70 – 80 % | 8 ml | 7 |
| 90 % | 10 ml | 9 |

Table 4: HUVEC medium chart for generating 10 µl spheroid droplets

- *Formulation of Cell Media* for Different Cell Concentrations/Optimizing to make up the Cell media to get 1000 cells per 10 µl droplet

  Required concentration of cells = 1000 cells/ 10 µl volume = $10^5$ cells/ml

  From every plate of cells being used for generating spheroids, calculate the dilution ratio from the cell density count performed using the Hemocytometer.

Dilution Ratio = $\frac{\text{Required concentration}}{\text{Cell density count}} = \frac{10^5}{X \times 10^5}$

Further, for 'Y' amount of medium used in resuspension, the volume of cells which gives the required $10^5$ cells/ml, calculate Z = $\frac{Y \times 1000}{X}$ ml of cells

Now, in total of Y ml, the spheroid medium constitutes of Z ml + 10 % Methocel

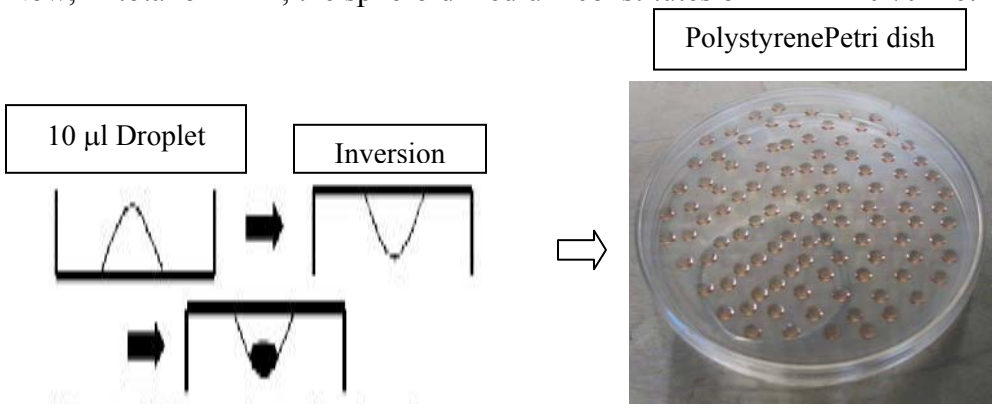



**Figure 9: Cells seeded in 10 µl droplets on Petri dish (6 nos. optimal) and inverted as hanging drop for generating spheroids to make up 1 96-well plate of spheroids.**

• Transfer HUVEC medium in a 50 ml reagent reservoir and using a multi-pipettor fill the surface of the lid of the Petri dish with 10 µl droplets as shown in figure 4.

• The bottom portion of the dish is filled with 12 ml sterile PBS medium to maintain a humid atmosphere during the 24 hr period of incubation.

• *Generation of Spheroids – Hanging drop model*

The cells are encapsulated in the form of a hanging droplet. A stable 3D multi-cell spheroid is formed within 24 hr of cultivation to the size of about 100-150 µm [22] in diameter. The spheroids organize over time (1d) to establish a core a quiescent unorganized cells and an outer layer of quiescent, non proliferating surface monolayer of endothelial cells [9].

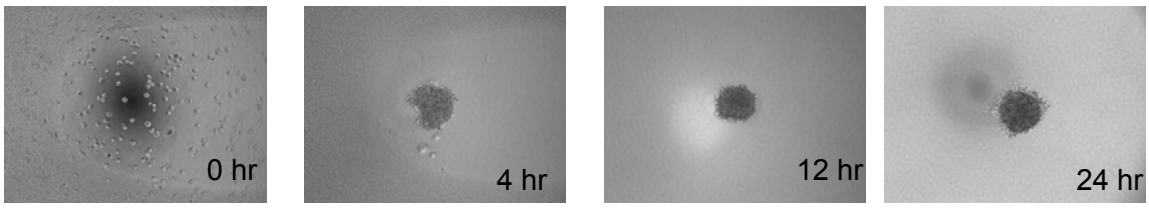



Figure 10: Spheroid shown with time course formation

• *Collagen mixture and plating primary layer of collagen on the 96-well plate*

It is preferable to set the pipet-aid at low speed while making the mix. Prepare Collagen mixture on ice as mentioned earlier, in appropriate ratios to make up the required volume for plating the 50 µl primary bed of collagen in the 96-well plate. Let plate to polymerize at 37$^{o}$ C for 30 min.

Collagen ratio chart is followed for making up different volumes (in µl)

| Ingredient / Ratio | A | B | C | D | E | F | G | H | I | J | K | L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 10X M199 | 100 | 200 | 250 | 300 | 350 | 400 | 450 | 500 | 600 | 700 | 800 | 900 |
| 0.2 N NaOH | 94 | 188 | 235 | 281 | 328 | 375 | 422 | 469 | 562 | 658 | 750 | 843 |
| Collagen Stock | 500 | 1000 | 1250 | 1500 | 1750 | 2000 | 2250 | 2500 | 3000 | 3500 | 4000 | 4500 |
| 1:1000 Acetic Acid | 500 | 1000 | 1250 | 1500 | 1750 | 2000 | 2250 | 2500 | 3000 | 3500 | 4000 | 4500 |
| Sodium Bicarbonate(1-10 µl) | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 5 | 5 |
| Collagen Mixture Sum | 1000 | 2000 | 3000 | 3500 | 4000 | 4500 | 5000 | 5500 | 7000 | 8000 | 9500 | 10500 |

Table 5: Collagen chart for making the collagen mixtures in required volumes

Based on the required final volume of the collagen for a single use, the respective ratio is selected (for one 96-well plate of 50 µl per well, ratio G is optimally used).

• *Batch Harvesting*

After the 24 hour period formation of spheroids in the drops, collect spheroids from the dishes using 3 ml PBS per dish. Using a glass funnel, in batches of 3, transfer the spheroids with PBS into a 50 ml centrifuge tube for each set to make a 96-well plate. Centrifuge the tube(s) for 3 min at 300 rpm.

• *Seed Spheroids with Collagen*

Place the centrifuged spheroid tube(s) on ice. On ice, make the collagen mixture (avoid making any bubbles by setting pipette aid at low speed) in pre-cooled 15 ml tube for the required amounts to seed spheroids in a 96-well plate. Considering the spheroid delicacy and not to damage them, cut the 200 µl pipette tip to 5 mm distance. On the ice bath, collect the contents from the tube with a tip-cut 1000 µl pipette, using cold collagen and transfer onto a 50 ml reservoir. Using four 200 µl tip cut pipettes on a multi-pipettor, dispensing the collagen-spheroid medium from the well walls, seed the spheroid-collagen medium on the solidified primary bed of collagen in the 96-well plate. Extra care needs to be taken while working on ice to make sure collagen does not polymerize while in working conditions.

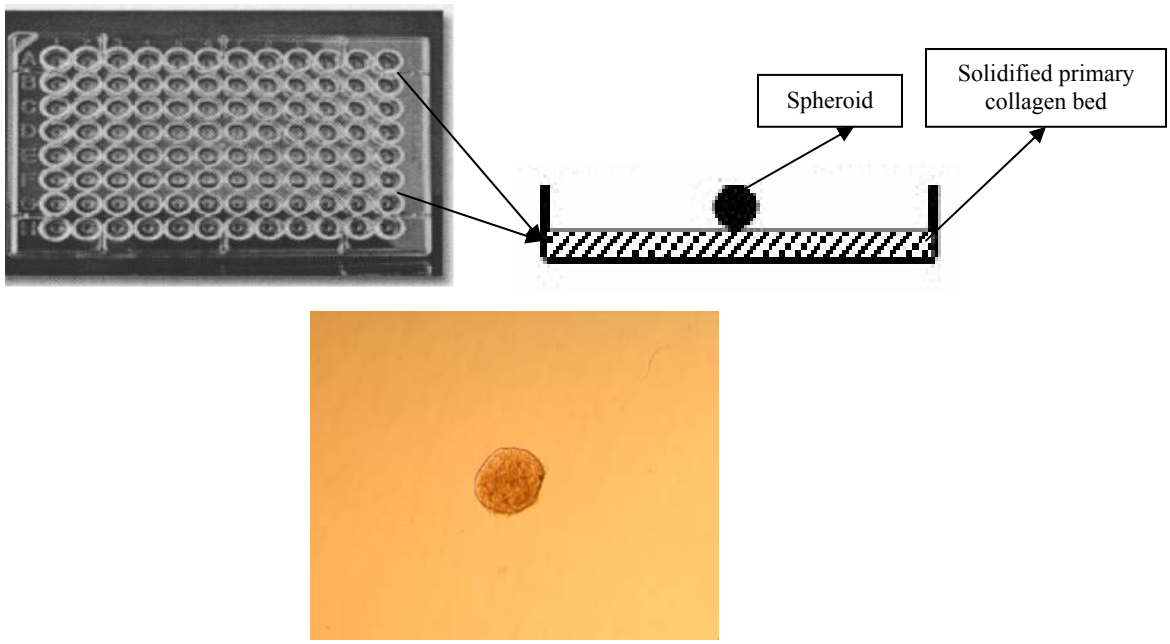



Figure 11: 96-well plate map, schematic and true color image of spheroid embedded in collagen

• Incubate the 96-well plate at 37$^{o}$ C in 5% $CO_2$ for 1 hr to ensure uniform collagen polymerization across all wells.

• Map the plate according to the treatments. Add respective treatments, in the wells to make up a total volume of 200 µl in each. Incubate for a period of 20-24 hr.

• Fix plate using 50 µl of 4 % paraformaldehyde and seal with strip of parafilm and store at $4^{o}$ C.

• Capture digital images corresponding to respective treatments in each well using the automated Nikon TE2000E2 inverted microscope, Photometrics digital camera and Metamorph software at 10X objective lens.

• Use image analysis software to process the images and collect data

It might of interest noticing the changes made with respect to the process that existed earlier. I have tabulated the previous to current processes as under

| **Previous** | **Current** |
|---|---|
| 4 plates of spheroids for 1 96-well plate | 6 plates of spheroids for 1 96-well plate |
| 40-50% loss in yield of spheroids | 50% loss in yield of spheroids |
| 20% loss of spheroids in the 96-well plate | less than 10% loss of spheroids in the 96-well plate |
| 30 µl of each collagen layer | 50 µl of each collagen layer |
| 20% of Methyl cellulose | 10% of Methyl cellulose |
| Transfer using tip-cut pipet | Transfer using funnel- Saves 50% time |
| Separate PBS used for washing | PBS from the bottom plate of petri dish used for washing |
| 96-well plate experiments | 96-well plate experiments and pilot studies on 384-well plate |
| | *384-well plate results:*<br>labor some manual image capture<br>volume of reagents reduced to 4 times<br>more bubble formation |
| | Pilot studies on: spheroid generation<br>U-bottom spheroids, V-bottom spheroids, Pipet hanging drop spheroids, Bead spheroids |

Table 6: Previous process and current and experimented processes

## 3.3. Safety

A scientist or a manufacturer should evaluate all factors that affect product quality when designing and undertaking a process validation study. Under this, the safety factors are considered such as air and water handling systems, environmental controls, equipment functions, bio-hazardous disposal and treatments along with other process control operations. A laboratory, similar to a manufacturing industry thus conducts vigilance on safety concerns for maintaining a contamination free environment in biological working conditions.

**Proper Hood Usage and Waste Handling to avoid contamination are listed as under**:

1. Make sure the blower is switched on while working and keep window up atleast 6 in.
2. Weekly empty the biohazardous material in aspirator flasks after treating with bleach.
3. Wipe hood area and glass shield with alcohol (70% ethanol) after every use while the blower is still running.
4. Spray alcohol on items that are placed in water bath before being used under the hood. To reduce corrosion and deposition of salts from water, occasionally change water in water bath.
5. Check on water in the humidifier container inside the incubator.
6. Empty all orange disposal bag containers and replace orange bags weekly.
7. Check on Nitrogen tank buffer so as to replace when the incubator runs low on supply level.

**Inventory Maintenance**

A minimum buffer of one was maintained for all reagents used by updating stock reorder requirement on a board accordingly

1. A minimum buffer of plastic supplies was maintained at 10
2. A minimum buffer of 3 vials was maintained for HUVECs

3. Liquid Nitrogen level was checked monthly and recorded on a chart every time it was filled.

# CHAPTER IV ASSAY VALIDATION

*Assay Validation*: Validation can begin only after the assay design is set and the test parameters have been established. In our assay validation, we have modules to separately validate the process and the product. Based on the data obtained during the development phase and with the help of sound judgment, a validation protocol is prepared. Various statistical practices in assay development reported that such a *protocol should include experiments that confirm those assay parameters deemed important during the design period and test whether the device meets the performance criteria for its intended use* [31].

## 4.1. Biological process validation

### 4.1.1. Variation in a manufacturing system

In every manufacturing operation there is variability. The variability becomes evident whenever a quality characteristic of the product is measured. Quality control systems focus on conformance of the product to its stated functionality and specifications. This is expressed in terms of statistical measures and the degree of variability is exhibited. The variability in measures of the physical characteristics of the product such as its dimensions, color, or structure, etc is viewed having two general causes: assignable and common. *Assignable cause variation is associated with one or more particular factors and the relationship is known in terms of both direction and magnitude for each of these factors. Common cause variation cannot be associated with particular factors instead is viewed as related to the collective impact of many factors. The relationship of any of these factors to a particular factor is not precisely known and cannot be directly controlled* [32].

It is important to learn how much of the product variability is actually inherent in the process. Many factors influence a process and each contributes to the inherent variation affecting the resulting product [33]. Common causes of variation are often considered to lie hidden within the system, and are sometimes assumed to be unavoidable. Yet it is very

possible, and often very rewarding, to improve processes and reduce common cause variation [34]. This is what has been aimed in my work. Typically, *this type of variation requires a long-term strategy to identify, understand and reduce it through on-going process management and improvement* (G.Tennent). With the required knowledge base amongst the people in and around the process, many ideas for improvements can be generated to make a significant impact. Two well-known ways to search for and remove common causes are experimentation and stratification [35] or a combination of both.

Under experimentation model, the PDCA (Plan-Do-Check-Act) cycle calls for creative thinking and analytic thinking, both essential to guide process improvement that brings the scattered pieces back together in a workable form. As it is usually used for process improvement, the search for common causes is just one of the many arenas in which the PDCA cycle can be used [35].

Stratification often needs to be done iteratively from a general level of information to the data at different levels, which brings visibility to the links between levels. This sorting of data into multiple levels of groups with shared characteristics, helps interpret the root cause of a problem. Even the cause and effect diagram could be used to build a tree of branching characteristics, each one being stratified further and further until root causes are reached [35]. We will see next, how incorporation of these methodologies can help identify process errors and validate them.

### 4.1.2. Process validation

The standardized process has been mentioned in the previous chapter. Here we discuss various parameters influencing each process and how it affects variability. Cause-and-effect diagrams permit assessment of factors important to measurement that may go beyond the process flow diagram. For each control point, various factors which can affect that control point with a resultant affect on the value of the product were identified using brainstorming sessions. Once all the elements of the diagram were identified, the ones which could be readily controlled were identified and results were measured [34].

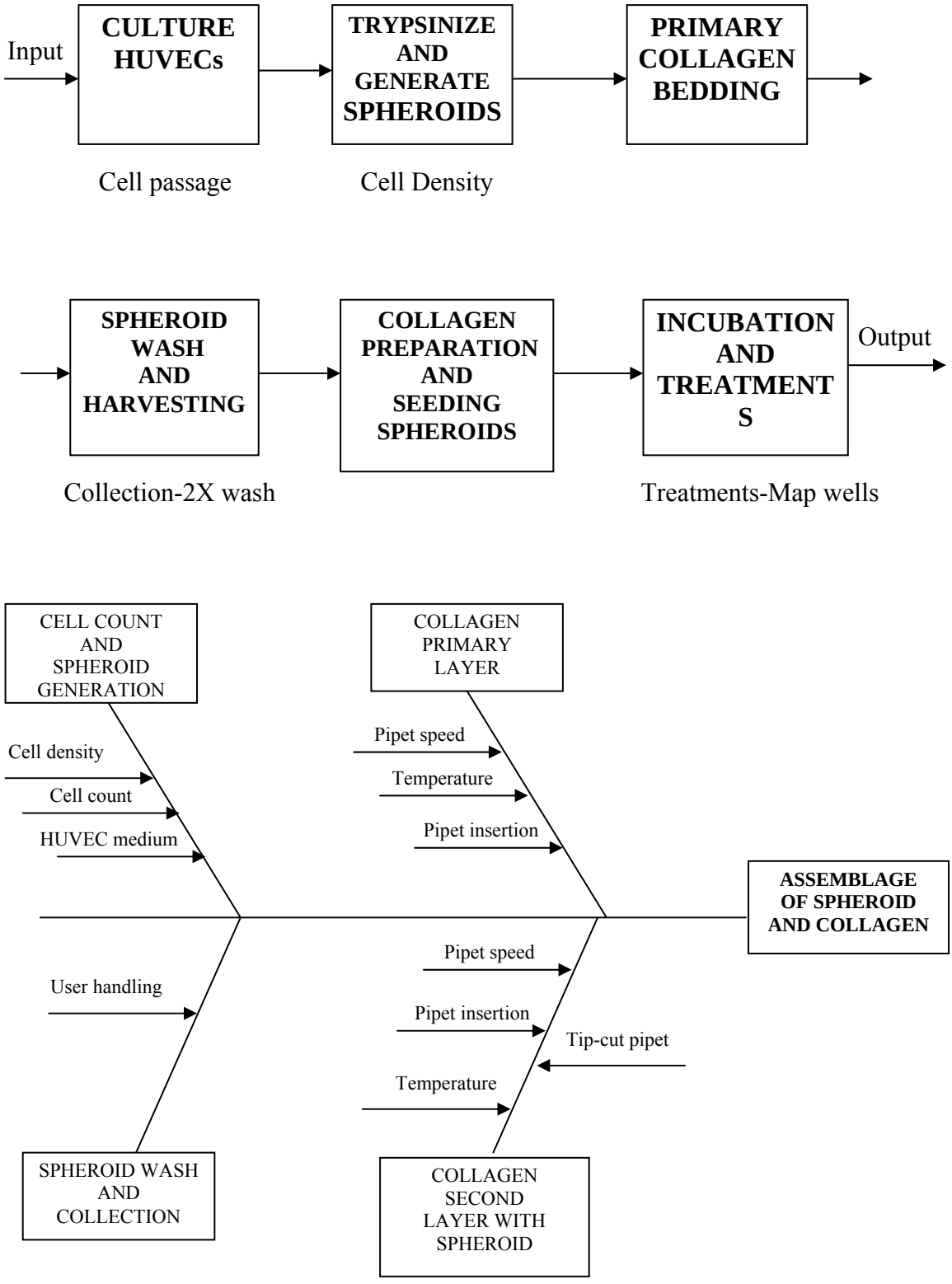



Figure 12: Process flowchart and cause-and-effect diagram labeled with the possible variable parameters at every process.

An FMEA is systematic analysis of the potential failure modes. It includes the identification of possible failure modes, determination of the potential causes and consequences and involves an analysis of the associated risk. It also includes a record of corrective actions or controls implemented resulting in a detailed control plan. FMEAs can be performed on both the product and the process. Typically an FMEA is performed at the component level, starting with potential failures and then tracing up to the consequences. This is suggested to thus be a bottom up approach [36].
The generation of spheroids has been optimized. Yet, the consideration for the yield of spheroid is not accounted in the sense that 40-50% of spheroids are lost during the wash and the transfer process to the 96-well recipient plate. This is an inherent overall loss, control of which has not been determined.

Though for validating the processes, pilot studies were conducted for the following processes:

**1. *Spheroid formation***

One of the main processes in the assay is to form the spheroid component. Hanging drop cell cultures were incubated. The gravitational force exerted on the cells in the hanging drop suspended cultures caused sedimentation to begin, and aggregates were formed within the first 24 hr. After sufficient sedimentation time, the resulting cellular aggregates were harvested. The two-dimensional aggregate prepared by hanging drop sedimentation assumes a three-dimensional spherical morphology after transfer to a collagen ECM [37].
Significance of wash process:

- Cleans spheroid from the extra cell debris
- Quality measure for spheroid formation

The quality of intact spheroid formation is tested in the process of washing and harvesting them. A comparison of 12 hour spheroid formation versus 24 hour spheroid formation was performed. It was observed that the 24 hour spheroid formation is more resistant to wash and a team member first observed there was a ring formation on the periphery of the spheroid surface. We consider this ring as a validating factor for the

spheroid formation. Few images of 24 hr spheroids under wash are shown below. With this process, only those healthy spheroids which survive the wash process are taken to the next stage thus ensuring a quality component flowing downstream.

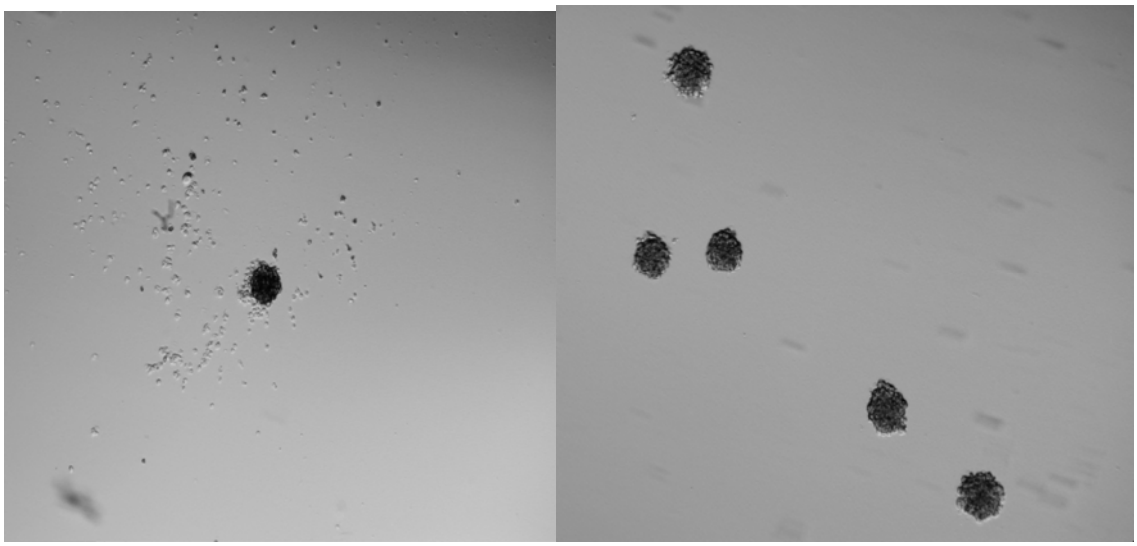


Figure 13: Spheroids shown in the wash and collection process

The spheroid(s) after the wash to get rid of the external debris of cells remain intact which can be transferred onto the 96-well receptacle plate

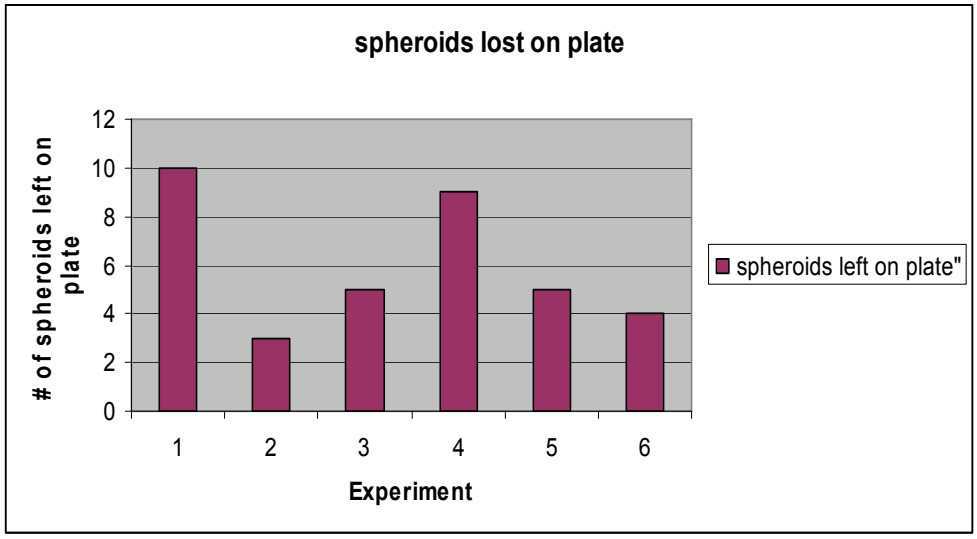



Figure 14: The number of spheroids lost on plate after single wash for different experiments is plotted here.

The number of spheroids lost on the plate varied depending on how well the spheroids were collected. Out of the 40-50% loss of spheroid yield, around 10% is loss in the harvesting process. Yet this loss does not influence the value of the end product. The return attained with the analysis of the end product was more than the investment in this process. The control was to rewash the plate to ensure maximum collection. Yet it was important to remember, the more aggressive the wash process, there was a probability to loose spheroids due to their getting damaged. The control of which was not determined yet but it doesn't affect the system overall and was less accounted as long as I (operator) was able to deliver quality spheroids onto the receptacle 96-well plate.

***2. Collagen polymerization***

In the subsequent stage, collagen mixture is prepared. Its polymerization is an irreversible process and thus the structure ceases to polymerize once disturbed. Temperature controls need to be maintained at $4^{o}$ C. Many controls have been incorporated as mentioned in the SOP in the previous chapter, to ensure uniform collagen polymerization. Collagen is a transparent layer in the 96-well plate which is gelled at $37^{o}$ C. Its polymerization level determines the quality imaging of the spheroids. A non-polymerized layer not only makes the spheroid view opaque under the microscope, but has also been observed to disrupt the sprout formation in some cases. Once the right working conditions are ensured, the use of high quality microscope at 10X magnification helps in good image capture of the spheroids.

Controls incorporated to ensure uniform polymerization are:

*the right insertion of pipettes in the multi-pipette holder

* maintain all material used for preparation of collagen bedding at or close to $4^{o}$ C

**3. *Bubble formation***

Forming bubbles in the collagen layer is another potential defect. Ensuring right working conditions (according to SOP) helps avoid generating bubbles. Though user variability has a lot to do with the process, one possible factor has been identified and validated. On

the same lines, statistically, it has been observed one out of 10 plates has bubbles in its wells and that one plate contains around 5-20% wells with bubble formation.

Controls:

- The collagen was degassed to remove any dissolved oxygen content.
- Dispense collagen at an approximate 45 degree inclination of the pipettor. For first layer make sure the collagen dispensed covers the entire surface of the wells. For the second layer, using tip-cut pipettes dispense collagen (consisting of spheroids) gently from the well walls and by trying not to touch the primary collagen bed with the tip. Usually I would recommend pipette introduction into the wells to distance not more than 15 mm.

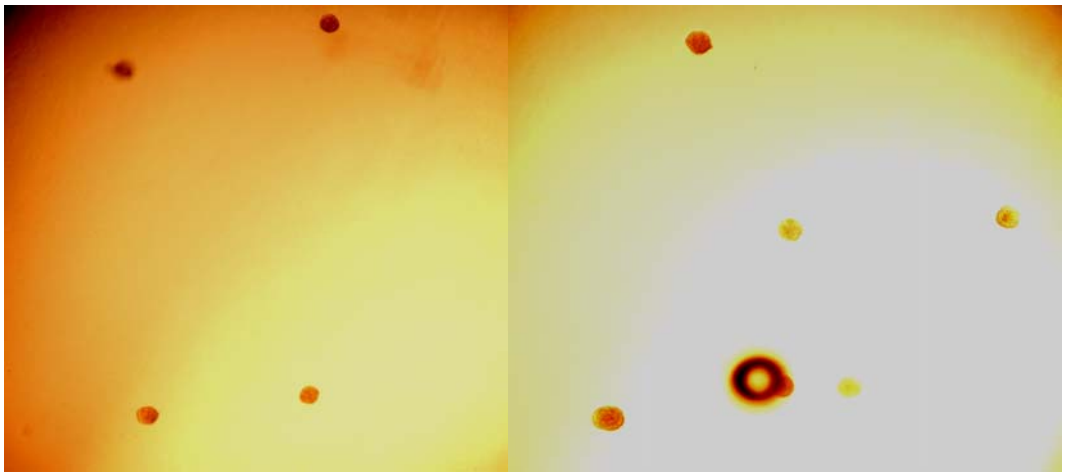


Figure 15: A well with no bubble and a well with bubble among the spheroids seeded in collagen

One way of validating the bubble formation was by the comparison of pipette tip-cut distances. There was a 40% decrease in the bubble formation when 5 mm distance tip cut pipettes was used in comparison to using a 10 mm distance tip-cut pipettes and with the

collagen dispensed from the well walls.

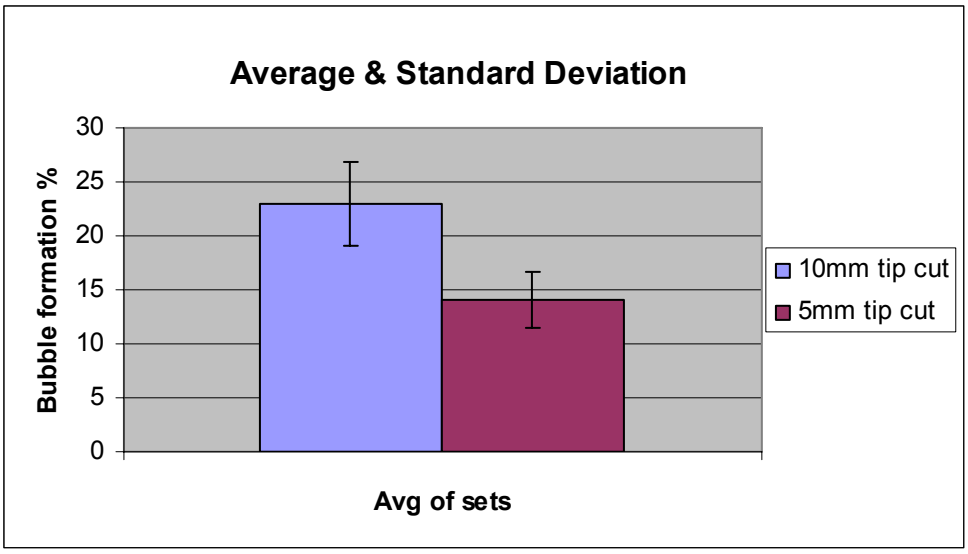



Figure 16: Bar graph showing 40% reduction in bubble formation when 5mm tip-cut pipette was used. Error bars show there is significant difference in values which is confirmed by Ttest with $p<0.05$

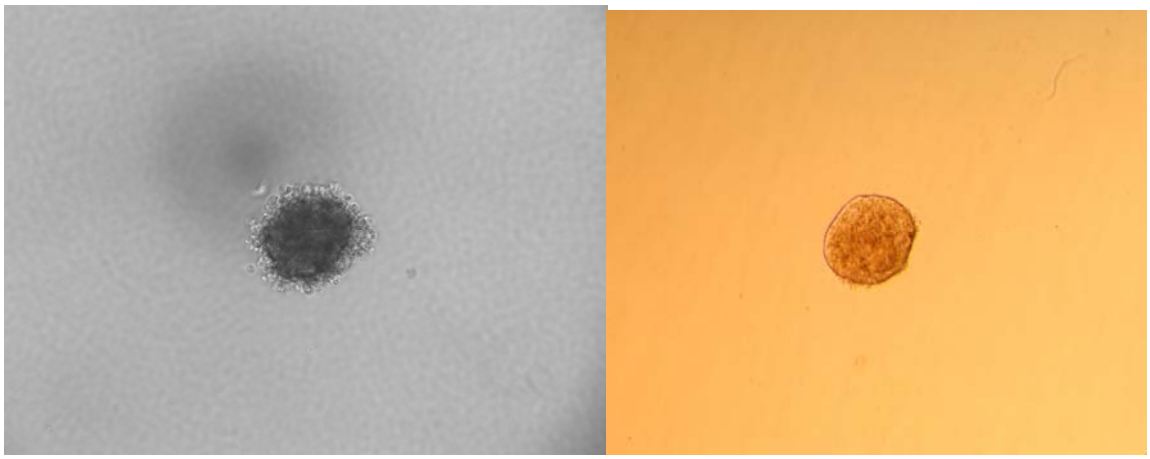
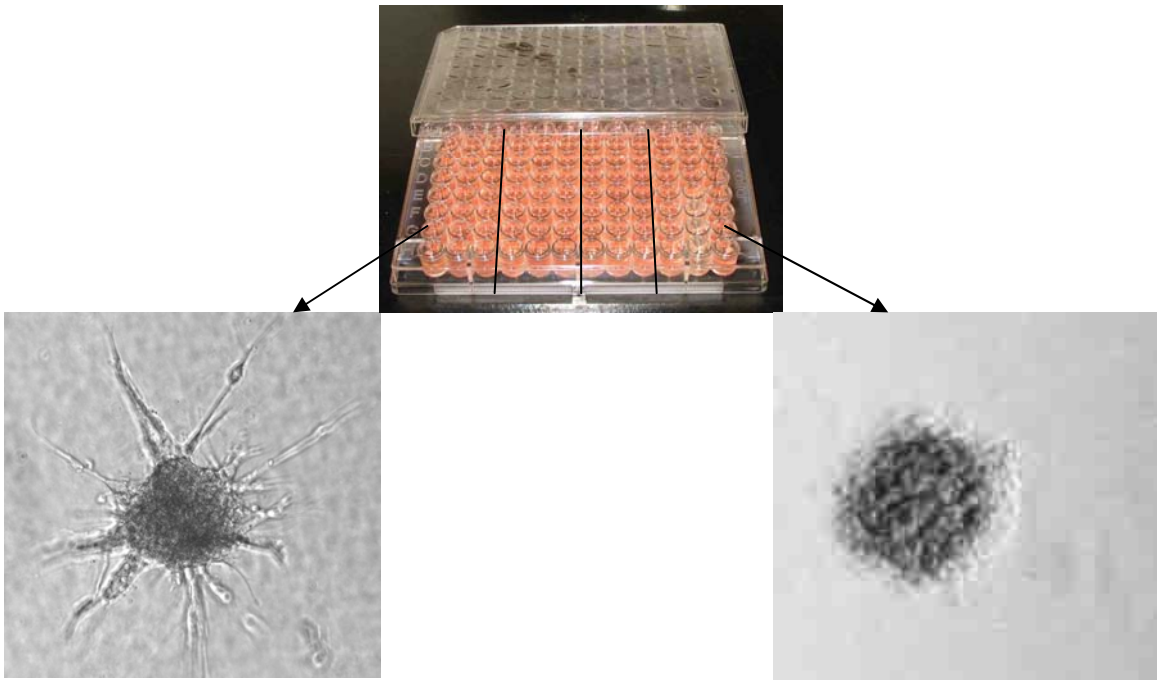


Figure 17: The figure above shows the spheroid from initial formation to the assembly stage in the 96-well plate.

Thus the problem solving helped optimizing the process at different stages and was checked for validation.

### *3. Screening stage*

In the final stage, the treatments were added per well with reagent mixture consisting of 1X M199, 5 % FBS and 0.025 mg/ml Antibiotic/Antimycotic. This is the stage that adds true value to the product with the addition of treatments based on a set hypothesis. This process needs to be quality controlled to ensure zero defects. Simple mistake proofing methods were adapted. The 96-well plate map was used as the visual control marking all the necessary treatments on the map and used as visual control during the addition of treatments. The Go, No-go gauges were the images of spheroids with sprouting and no sprouting. Typically for drug screening Go gauge is considered to be the spheroid with no sprouting. The images of the spheroids are shown below.



Sprouting No Sprouting

Figure 18: Treatments showing sprouting and no sprouting spheroid images

There is variation in a process even when all adjustable factors known to affect the process have been set and held constant during its operations [33]. The inherent variations result as the end product with the level of spheroid sprouting. The parameters

for the spheroid sprouting are measured using the image analysis software and the variation is determined using normal distribution graphs (shown in the Results section).

***Assay Lean features*:**

- Low lead time of four days
- The concentration of volume of reagents is optimized, as in a manufacturing process. Assay performance is also enhanced by the use of specially treated 96-well plate which has specific surface properties that assure even coating and minimize the meniscus formation [10].
- Replication allows a researcher to estimate experimental error. If the magnitude of experimental error is unknown (which happens if it cannot be estimated), then a researcher cannot determine whether any effects or interactions are statistically significant. This also allows a tolerance to the spheroids analyzed by eliminating the false positives *.
- The assay is not complete before the addition of necessary treatments in desired doses. This gives the assay a lot of variety to incorporate based on the experiment designed. The 50 µl of medium alone without any treatments can be added after the collagen second layer polymerization process. The addition of next 50 µl treatment variety is incorporated in the end. Thus, the assay is similar to having "postponement of variety approach".
- All the initial stages of the process are standardized and controlled and any inherent variability can be recognized by problem solving methods.
- The overall length of the assay was not be substantially shortened by this process, but the volume of samples that can be tested and analyzed using this strategy allows numerous conditions to be tested in a single run.

---

*False positive: The spheroid which turns positive for wrong reasons, such as due its health or interaction with the microenvironment (collagen matrix)

## 4.2. Software Validation

Simple measurement of the growth or shrinkage of spheroid sprouts was accomplished by microscopy and computer image analysis. A microscopy system was configured to reading the assay and taking the photographs (currently being worked in a manual mode). Our collaborator with his experience in pattern recognition, in the project has applied wavelet analysis and thresholding to get rid of noise components in the measurement of spheroid. The tubules of the spheroid were segmented from the background by searching the one-dimensional wavelet sub-bands looking for peaks in amplitude that exceed the noise level defined according to the largest radial rings. These signals out the noise were considered to result as tubule measurements.

With the individual tubule components identified, the collaborator and his team were able to monitor the growth metrics such as number of tubules, average and total length of tubules, average tubule width and the total area covered by the tubules. The software developed so far, does a good job in determining up growth parameters mentioned. Their analysis is further intended to trace for count on tubule branching, another growth metric which measures the spheroid behavior in the formation of vessels. The sprouts were imaged using an inverted microscope and digital camera to photograph spheroid in each well. The scripted software processed the images for number of tubules, length and width of sprouts and the total area [28].

The assay can be performed two-way. The one discussed was the regular method and involves rapid screening, by testing the effect of drug in the presence of any stimulating factors. If the drug compound overcomes the growth promoting action of angiogenesis stimulator, then inhibition of any possible tube formations was observed. The second one was significant for studying the effect of drug compounds on already existing vessel formations. In this the growth factors were incubated with spheroid for 24 hr to induce sprout formation and respective wells were photographed (without the fixation of sprouts), followed by the addition of drug compound for the next 24 hours. If the drug is a positive for inhibition then it is called a hit or active compound (where active means

some measurable activity below the control threshold of the assay) [38] and the pre-formed tubes are observed to collapse.

## 4.3. Variability assessment – inter plate, inter day, dose response assessment

A manufactured product needs to be assessed for the consistency in its repeatability. Usually in industries, products are checked to meet the specifications using the sampling approach. The variability assessment performed here also similarly samples out a bunch of spheroid data and gives a representative performance measure. With no automation involved in the product-development stage, the inter-plate, inter-day variability assessment consists of consecutive, independent experiments measuring assay responses collected for evaluating the coefficient of variation (CV). The most informative data will then come from experiments done on separate days with independently prepared reagents.

A typical layout for the 96-well plate is shown as under. Treatments were added in different wells as designed in singles or replicates, etc. and the plate is well mapped for identification of treatments and to label the respective images of spheroids for analysis based on well number as mapped.

| Well numbers by row, then column | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Column/ Row | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| B | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
| C | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 |
| D | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 |
| E | 49 | 50 | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 |
| F | 61 | 62 | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 | 71 | 72 |
| G | 73 | 74 | 75 | 76 | 77 | 78 | 79 | 80 | 81 | 82 | 83 | 84 |
| H | 85 | 86 | 87 | 88 | 89 | 90 | 91 | 92 | 93 | 94 | 95 | 96 |

Figure 19: A 96-well plate mapped with numbers for identification

For the assessments, we have sampled sets of spheroids to determine variation from well-to-well, plate-to-plate; two-day plate readouts and the metrics were calculated using the software analysis of the spheroid images.

As part of validating assay variability the assay reagents along the assay components need to be checked constantly. It is important to determine the stability of reagents under storage and assay conditions.
Reagent Stability and Storage Requirements

- For commercial products, use the manufacturer's specifications for the reagents.
- Identify conditions under which aliquots of the reagent can be stored without loss of activity. The fresh growth factors aliquoted in volumes of 10µl/vial were stored at -20$^{o}$ C. After the initial thaw they were stored at 4$^{o}$ C. After this, the growth factors cannot be used more than 3 thaw cycles.
- Test compounds/extracts were delivered at fixed concentrations in 100% DMSO (Dimethyl Sulphoxide), thus solvent-compatibility of assays in DMSO was determined. Typically the final concentration of DMSO in the assay was maintained below 2%.
- The drug compounds were stored at -20$^{o}$ C and thawed for stipulated time before every use.
- Be sure not to contaminate any reagent while thawing in the water bath.

## 4.4. Results on stimulatory responses

Variability assessment was performed for various assays on different days. The NIH manual for validating the assays was approached in our attempts. The following are the results from the assay plates. As a part of quality control measure for the spheroid culture module, I sampled sets of spheroids to determine variation from well-to-well, inter-plate and inter-day. Various growth metrics identified for analyzing the spheroid behavior were total number of tubules, total length of the tubules and total area of the tubules, average length, maximum length and average width of tubules. These measures of the parameters

show the inherent variability in the product performance as defined by the above mentioned parameters.

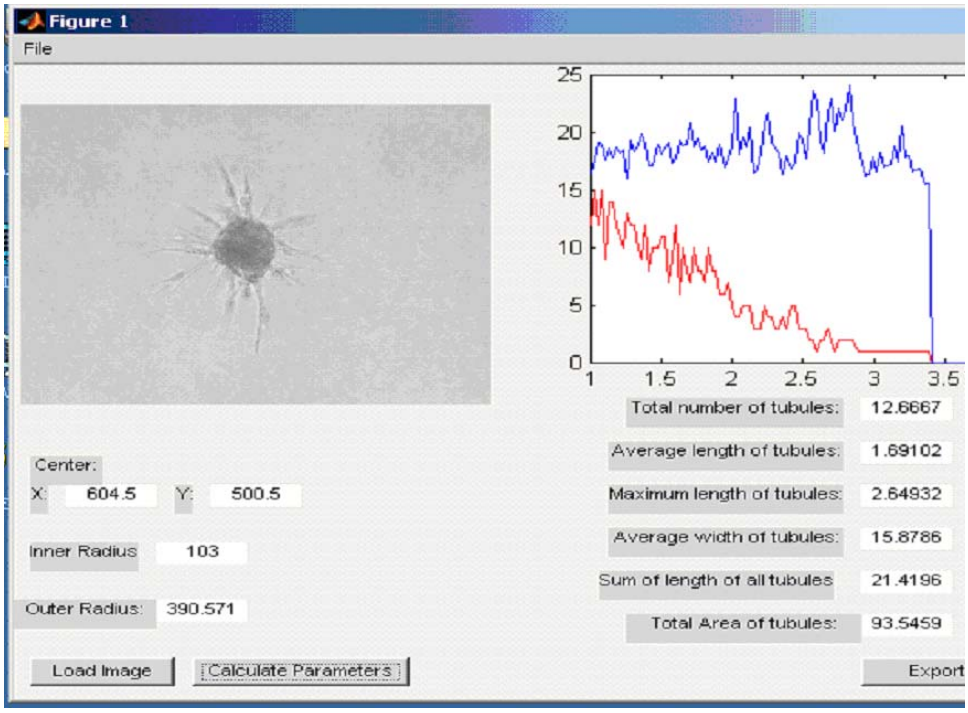
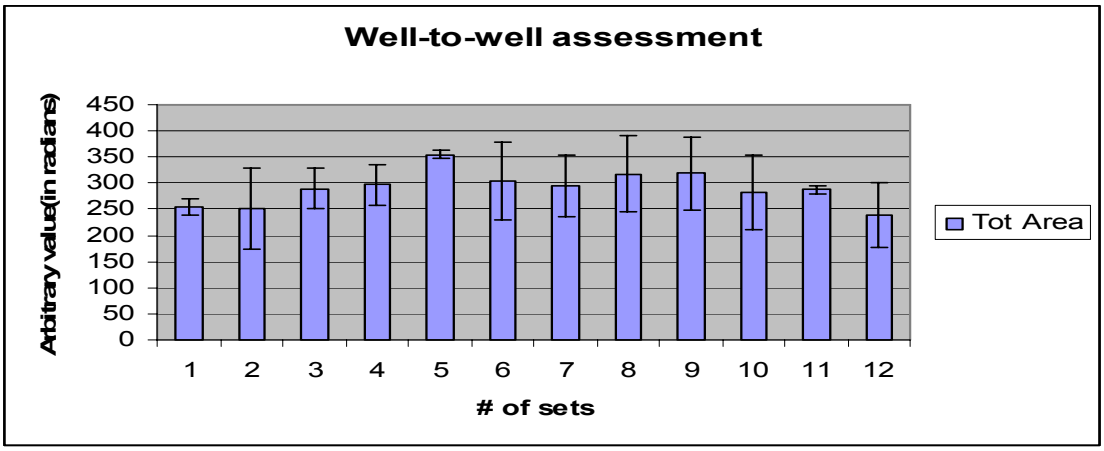
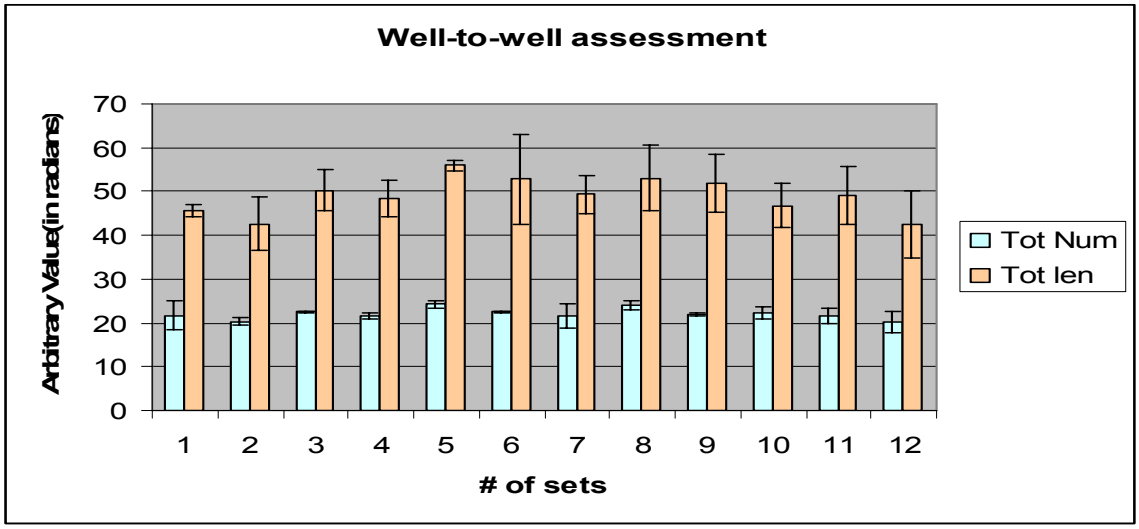



Figure 20: The graphical user interface of the software for calculating the performance parameters.

**1. Well-to-well (product to product) variability assessment**

Well to well variability assessment was performed to check for the consistency in the replicated spheroid samples in each well. The validation results show that there was consistency in values across all the growth metrics measured. The p-value for the parameters in the assay was calculated. In the data analysis values it was found that for average length, maximum length and average width of the tubules $p>0.05$ from the student's t-test which shows that there is no significant difference in those values of the parameters. It was preferred to consider the total number of tubules, total length of the tubules and total area of the tubule to be significant in terms of validation.



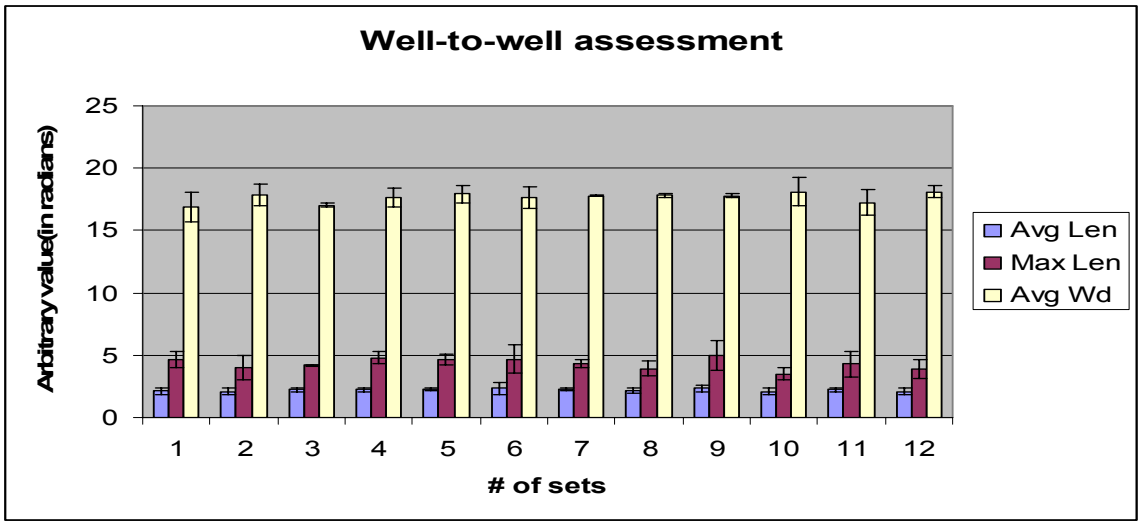




Figure 21: Graphs showing well-to-well variability assessments for all the defined growth parameters.

**2. Inter-plate variability assessment**

Initial plate to plate assessment was performed with inducing sprouts in spheroids to compare the two growth factors called the Vascular Endothelial growth factor (VEGF) and Fibroblast growth factor (bFGF). VEGF and bFGF are two proteins commonly known for sustaining tumor growth and are thus used as injury stimulating models in our in-vitro assay. This assay showed that statistically there was no difference in the performance parameters of the spheroids stimulated with either of the two growth factors. This was considered as the initial experiments of validating what was scientifically already known using the assay's performance parameters.

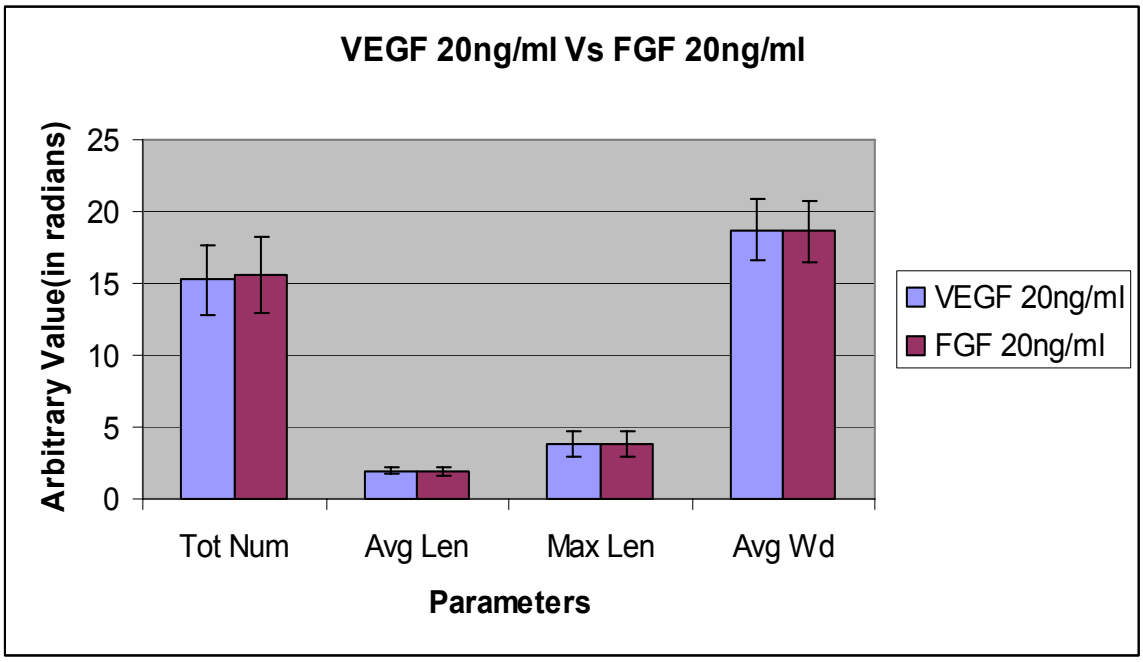



Figure 22: Statistical interpretation in comparison of two growth factors for stimulatory responses

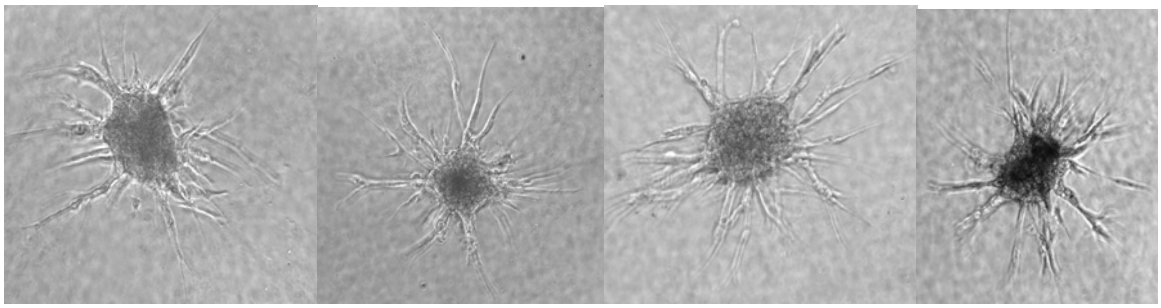
**3. Dose Response Assessment**

*Day 1: Inter-plate comparison – VEGF Dose response Vs bFGF Dose response*

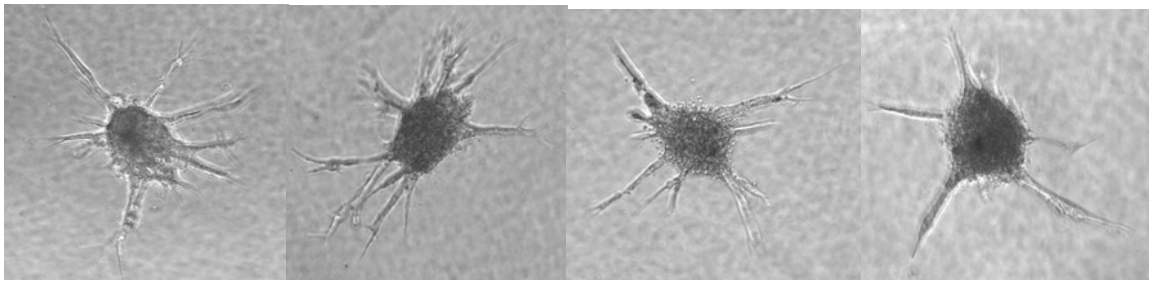
Dose variation experiments using VEGF and bFGF from doses 40ng/ml to 1.25ng/ml were compared to no growth factor (control) treated spheroids. There was significant increase in growth parameters when compared to the control and that is shown by *$p<0.05$. The different parameters are shown in respective separate graphical plots.

3.1. VEGF Dose Response Parameters

A set of doses representative images of spheroids treated with VEGF and the spheroid with no VEGF (control) are shown below.



VEGF 40ng/ml VEGF 30ng/ml VEGF 20ng/ml VEGF 10ng/ml



VEGF 5ng/ml VEGF 2.5ng/ml VEGF 1.25ng/ml Control

Figure 23: The dose response images of spheroids representative of VEGF doses from 40ng/ml to 1.25ng/ml and the control (no growth factor)

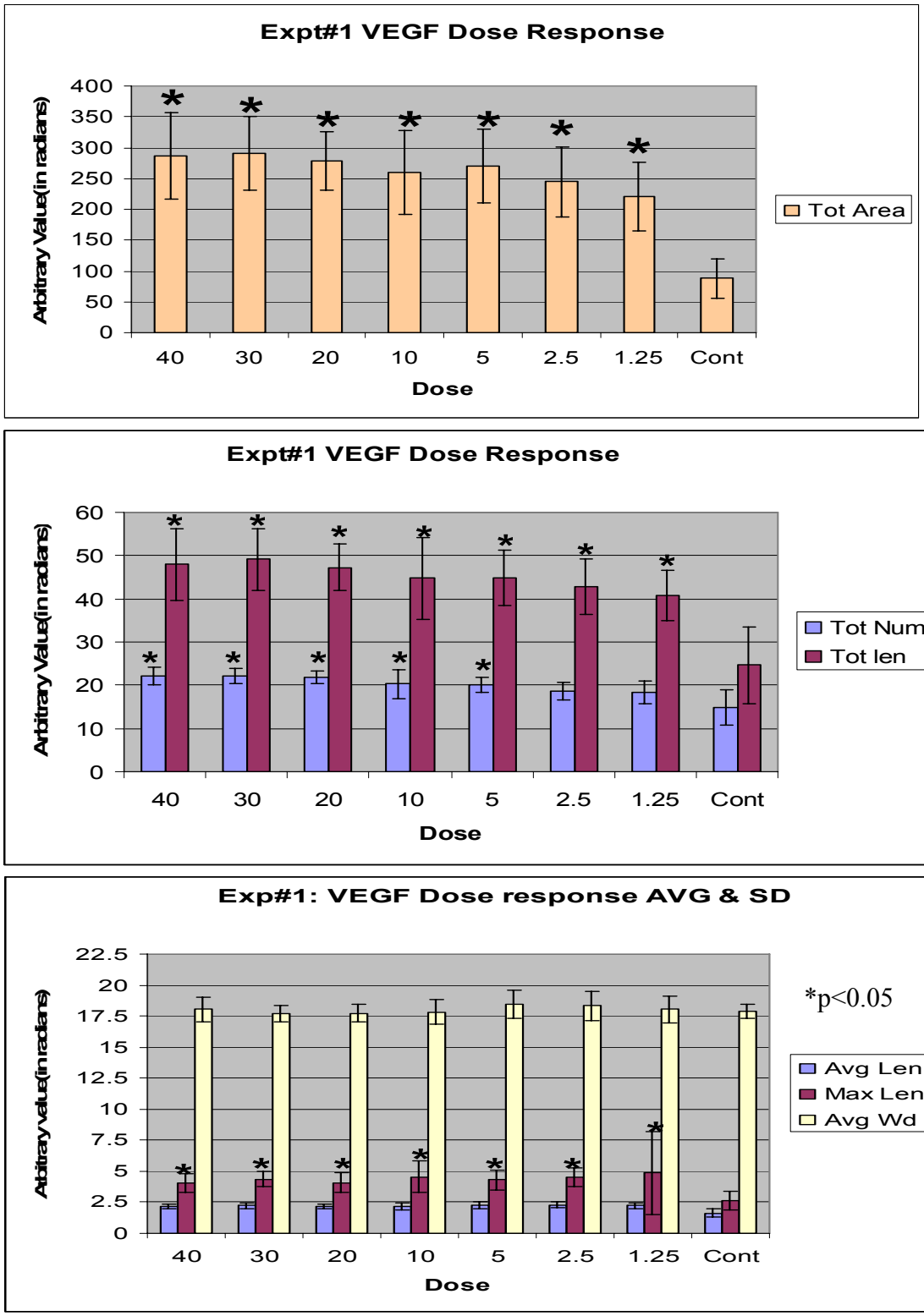



Figure 24: Graphs for VEGF dose response and comparison of growth parameters over the control.

The data from the above VEGF dose response graphs shows that for maximum length, total length and total area of tubules all the doses from 1.25ng/ml to 40ng/ml start to show significant differences over the control. This is not the case with average length and average width which show $p>0.05$ meaning there is statistically no difference in the dose response. For the total number of tubules the difference in values start showing up from the dose 5ng/ml over the control.

3.2. bFGF Dose Response Parameters

A set of doses representative images of spheroids treated with bFGF and the spheroid with no bFGF (control) are shown below

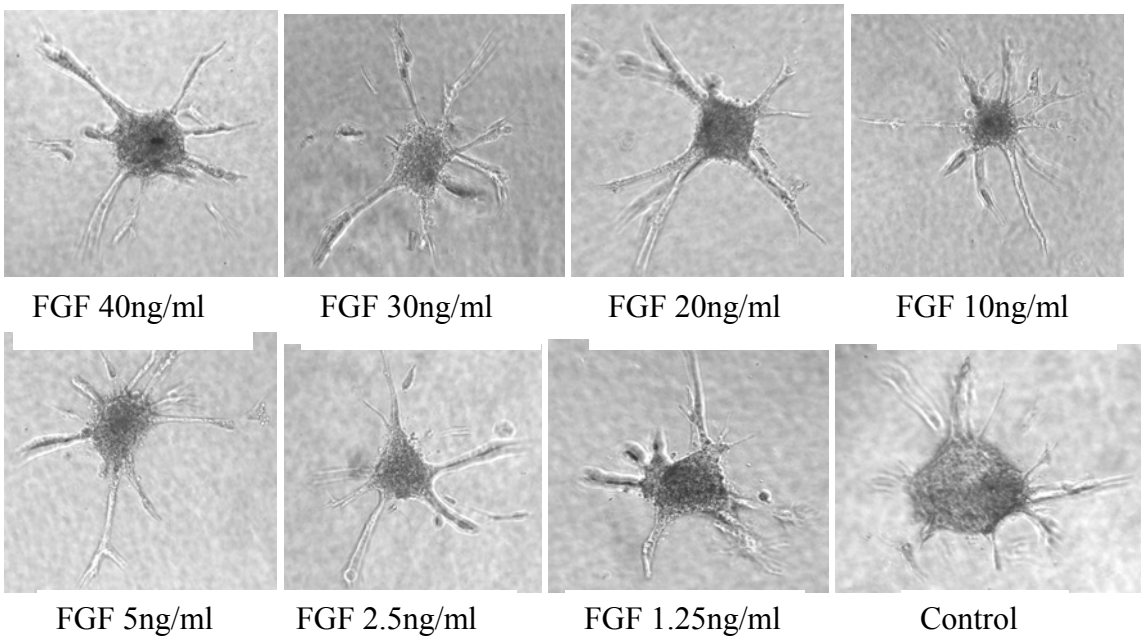



Figure 25: The dose response images of spheroids representative of bFGF doses from 40ng/ml to 1.25ng/ml and the control (no growth factor)

The statistical analysis of the data plotted for all the growth parameters is shown below. For total length and total area the difference in doses starts showing from 2.5ng/ml over the control. Average width and maximum length do not show statistical differences in the doses while total number of tubules show differences starting from 2.5ng/ml over the

control

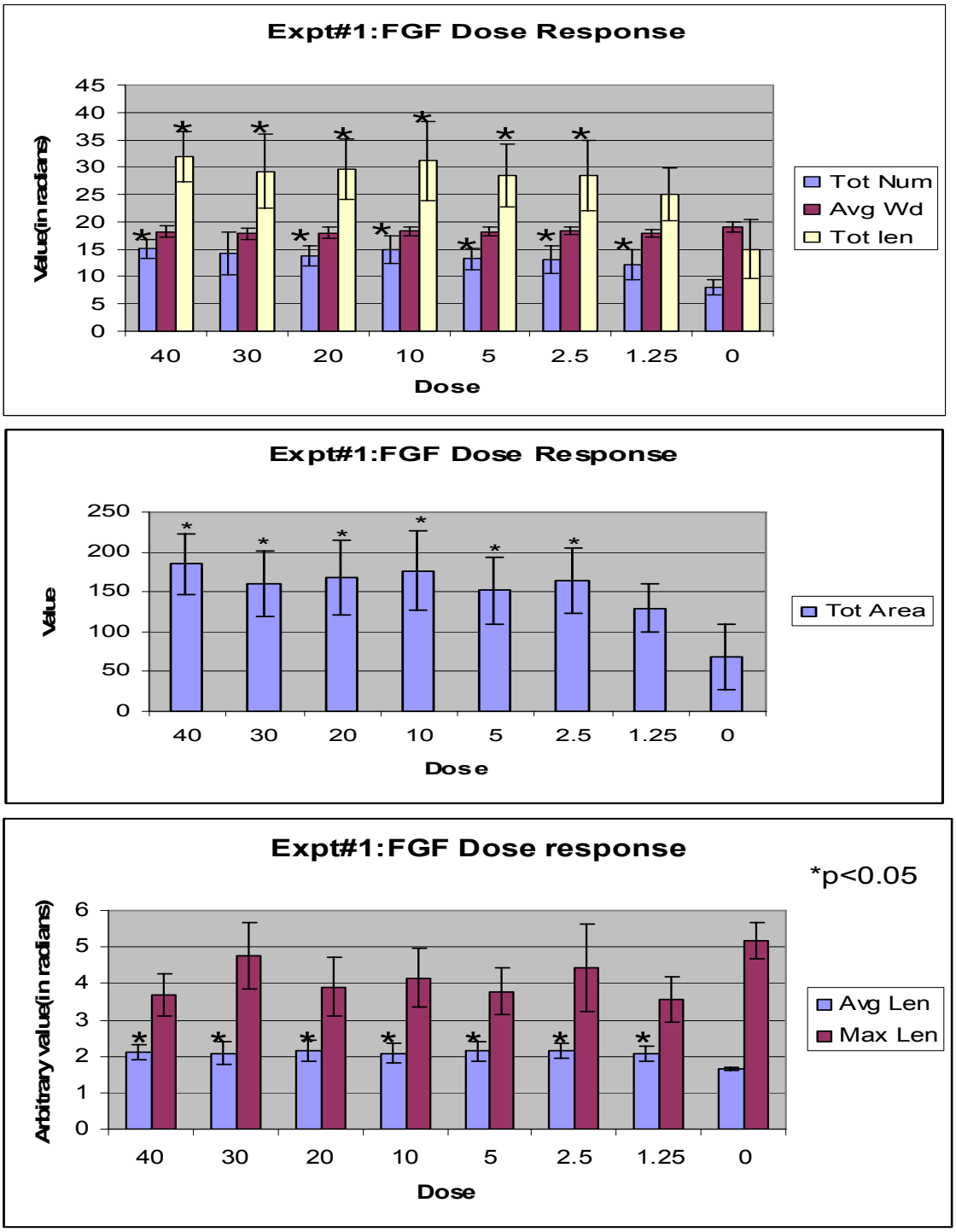
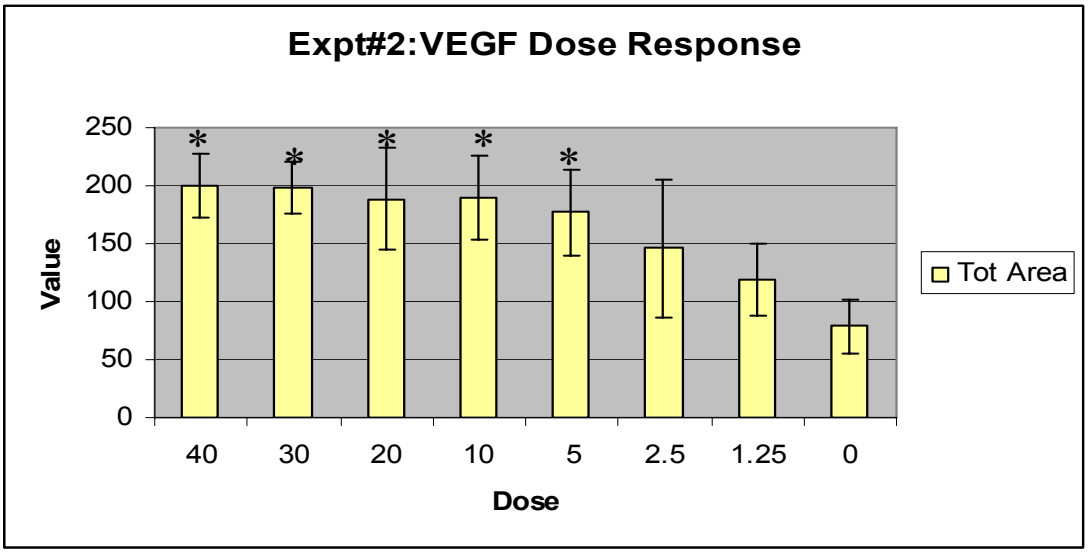


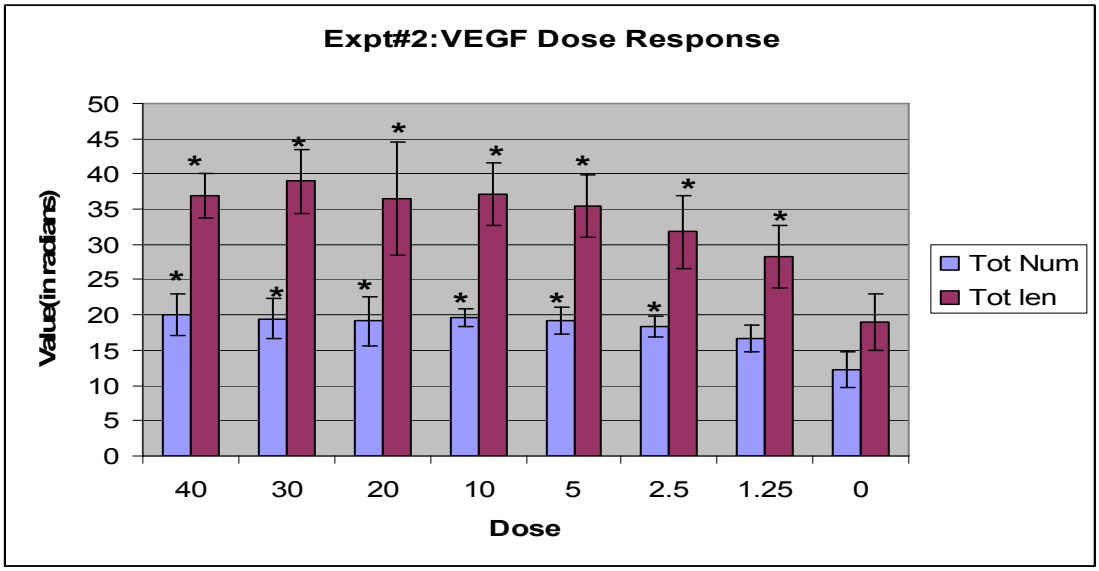

Figure 26: Graphs for bFGF dose response and comparison of growth parameters over the control.

*Day 2: Inter-plate comparison – VEGF Dose response Vs FGF Dose response*

A similar experiment was set up on day 2 to trace the plate-to-plate variability in the assay. The comparison of the assay performance parameters from day 1 and an assay from day 2 will also give us the inter-day variability.

3.3. VEGF Dose Response Parameters



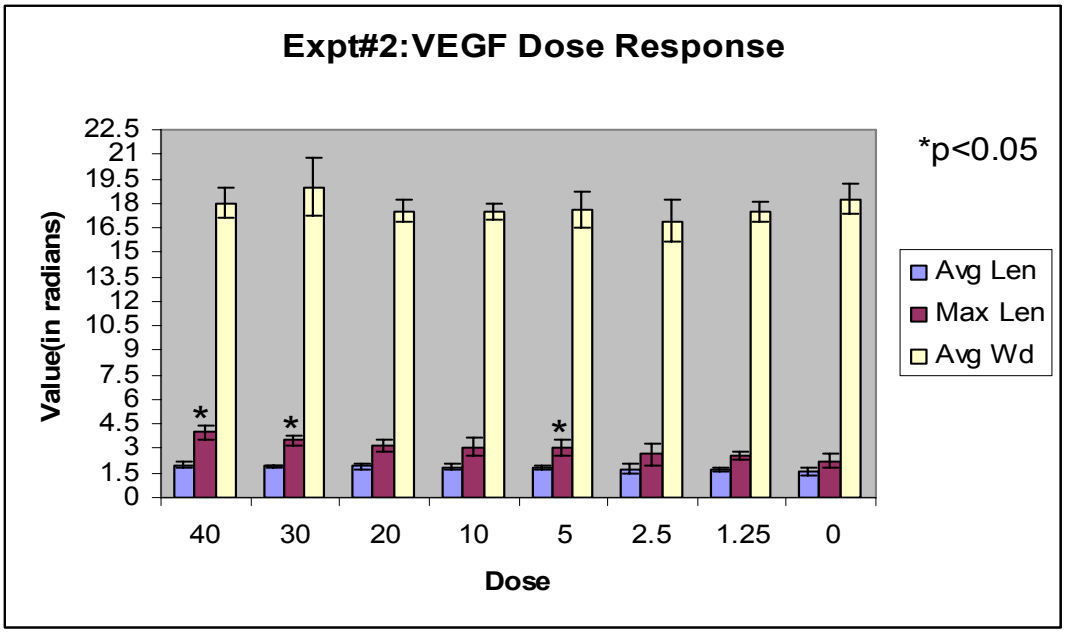
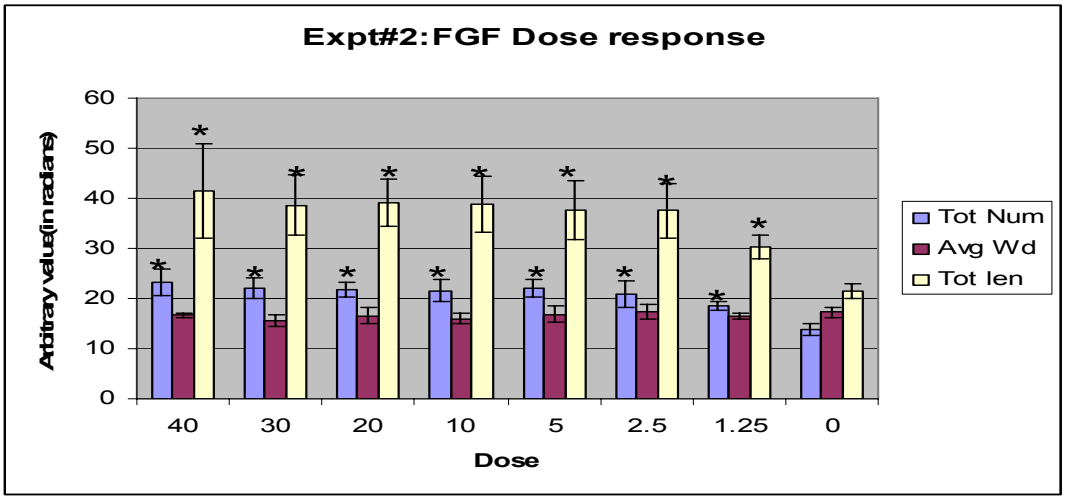


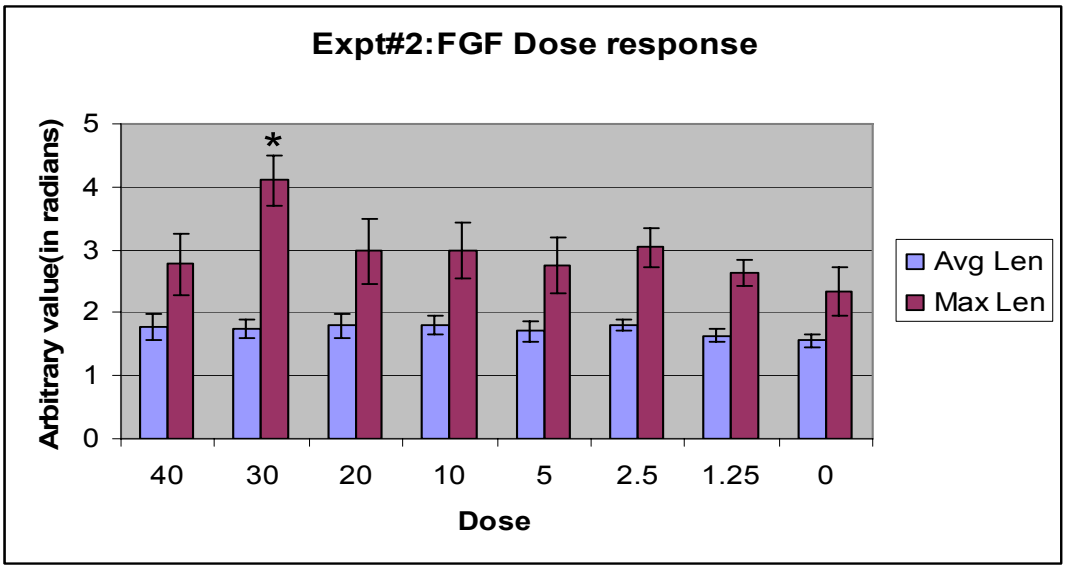

Figure 27: Graphs for Day 2-VEGF dose response and comparison of growth parameters over the control.

Doses 5ng/ml, 30ng/ml and 40ng/ml show statistical differences over the control when analyzed for the maximum length of the tubules. Average width and average length show no statistical difference. For the total area of the tubules, doses 5ng/ml to 40 ng/ml show difference over the control. For total number of tubules doses 2.5ng/ml to 40 ng/ml show difference over the control and for total length of tubules doses 1.25ng/ml to 40 ng/ml show difference over the control.

### 3.4. bFGF Dose Response Parameters

The parameters average width and average length show no statistical difference in the values. Total number, total length and total area differ over the control from 1.25ng/ml and above. Maximum length differs over the control only at the dose 30ng/ml. The graphs showing the results are plotted below.



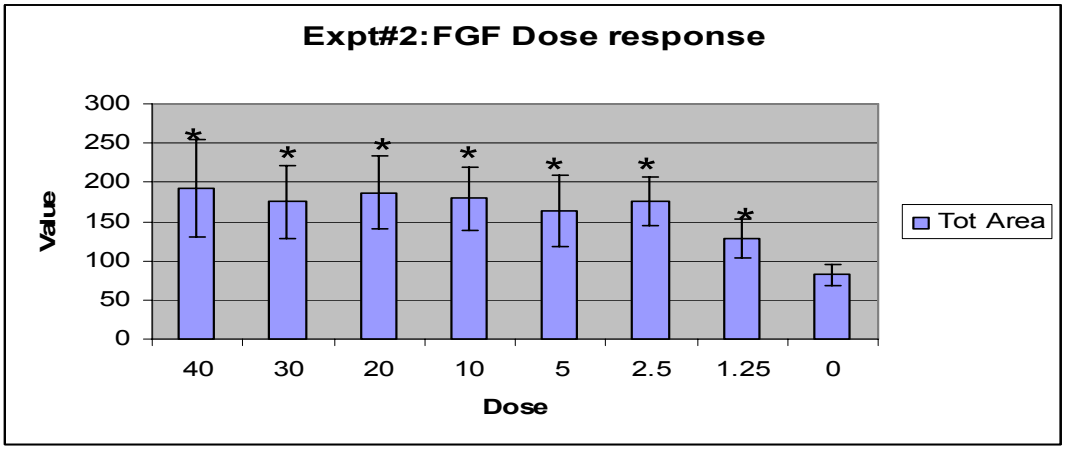
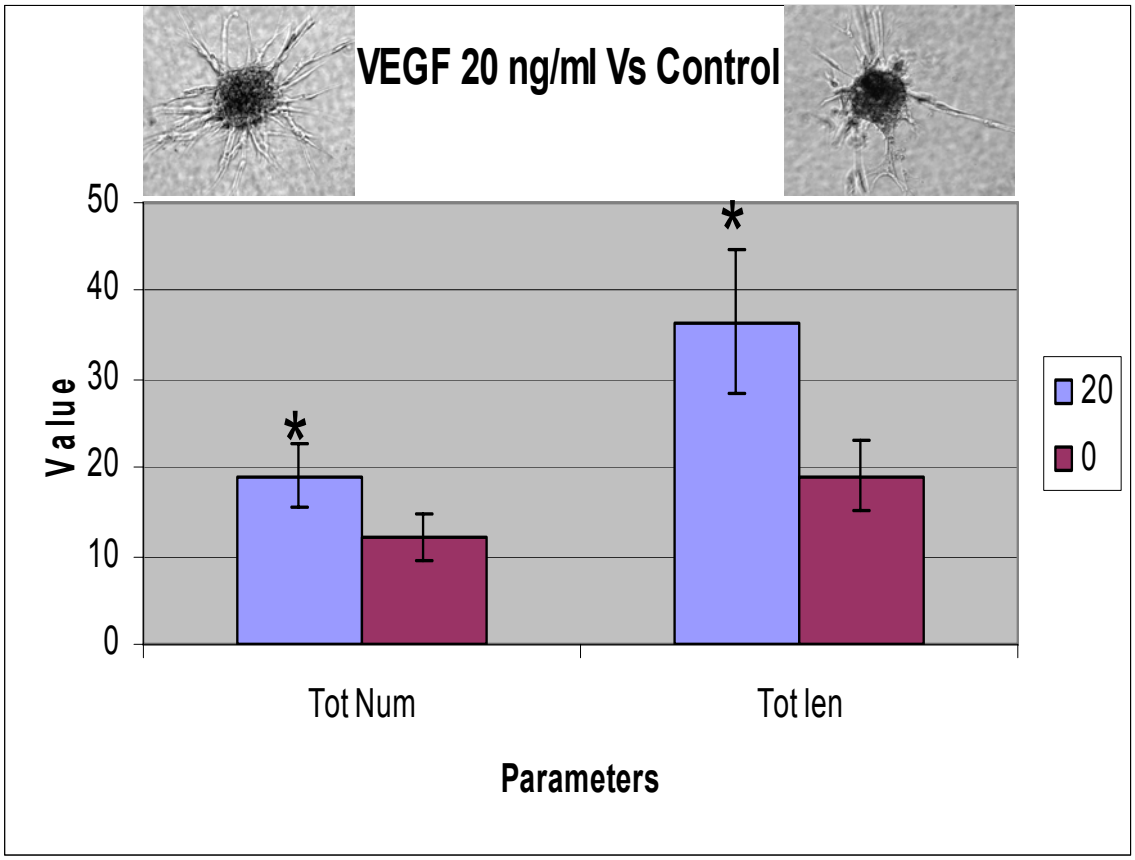




Figure 28: Graphs for Day 2-bFGF dose response and comparison of growth parameters over the control.

**4.** From the dose response curves obtained, an optimized dose of VEGF/bFGF was set to 20ng/ml. This dose was used for spheroids in treatment with drugs or plant extracts. The statistical measures are shown for a closer analysis on the total number and total length of tubules.



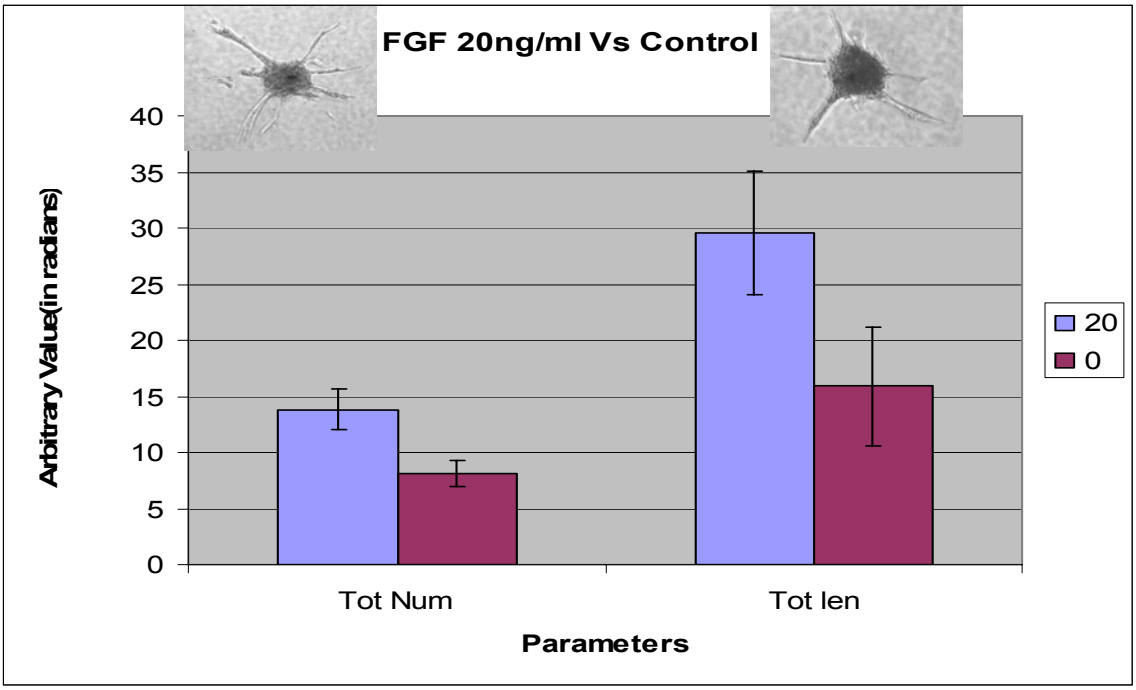



Fig. 29 The graphs above for VEGF and bFGF treated parameters, show the increase in total number of tubules and total length of tubules to being a two-fold and confirm the significant difference in 20ng/ml treated values over the control with p-value<0.05.

### 4.4.1. Summary

The assay was quality inspected using the software tool. Performance inspection was performed and variability assessment graphs have been plotted. Here they are summarized:

1. The assay was put to test by treating with two different growth factors at same concentrations and checked for the consistency in results.
2. The assay was further tested for well-well variability assessment which showed that the spheroids behaved consistently when considered in large numbers.
3. Next step in the validation was to perform, Plate-to-plate assessment of sprout formation when treated with VEGF and bFGF
4. A dose response assessment was performed and the data showed consistent measurement in growth metrics for doses range between 40ng/ml and 2.5ng/ml.

This showed that even with a least amount (1.25ng/ml) of VEGF, tube formations are stimulated. This concentration response curve can be used for setting up an optimal amount of the growth factor for inducing sprouts when introducing various drug compounds.

5. Growth assessment was performed with setting up a dose of 20ng/ml of the growth factor in comparison to no growth factor treated (here the control).

The above data shows *the stimulation responses* of the assay in presence and absence of a growth factor.

6. On a different angle, the assay can be used in interpreting *the inhibitory responses*. We can induce the spheroid assay with growth factor on the identified optimal dose (i.e 20ng/ml-call this the control here) and then compare the growth to a drug treated sample set of spheroids to see if there is any reduction in growth parameters.

For screening purposes in the assay there are minimum two spheroid sets, one the control with stimulators (or the growth factors) alone and the other with the drug/sample additions. Here the screening mode can be split into two-levels:

i) On a basic level or 24 hour treatment. One set with only the growth factor for stimulating sprouts, without the drug/sample treatment (the control for the experiment) and another set with growth factor and the drug/ sample together. The second set of spheroids was compared with the control to analyze the change in spheroid pattern.

ii) On the second level or the 36 hour treatment, the spheroids were treated with stimulators alone for the first 20-24 hr period. Once the vessels are grown, the drug/sample treatments were added along with stimulators. This was to validate if the drug/sample can be effective on already existing vessel formations (representative of in vivo situation)

Another way of testing the assay was to subject spheroid assay to a treatment which restricts its growth. The next chapter shows one assay to study the behavior of the spheroid when treated in high glucose which causes suppression of tube formation and

compared to the treatment with introducing a drug along with high glucose, which rescues the spheroid and causes tube formation.

As mentioned in the first chapter, different levels of angiogenesis in vivo leads to varied responses of the blood vessels. The tube formation is responsible in growth and development, in excess angiogenesis causing abnormal formation of vessels leading to tumors and also in insufficient angiogenesis. All the cases of angiogenesis are thus studied in this assay and prove its versatility thereby making it multi-functional.

# CHAPTER V ASSAY APPLICATION

## 5.1. Plants library

The production of this assay was used to screen plant extracts which helps identify plants with inhibitory, stimulatory and cytotoxic effects. Natural products having pro-angiogenic activity also demonstrate high degree of chemical diversity.

Use of natural products to study protein function has also witnessed an increasing interest in identifying novel chemical agents from plants [21]. This product can be extended for its use to screen natural products to identify new classes of relevant non-toxic extracts to discover angiogenic inhibitors/drugs and cell-permeable reagents.

Plant leaves belonging to Kentucky native plants were collected from the University of Kentucky-Arboretum along with a team member. An extract library of 221 plants was processed in the laboratory (stated in the methods section-chapter 2). The basic and second level screening approaches of the extracts were adapted for this application. In the primary screen, 44 test samples were identified to show complete and partial inhibition.

In the second level of screening on pre-existing vessels, the spheroid assay was treated with growth factor for stimulating sprouts and after a 24 hour period, they were treated with the sample concentration. The potent samples were considered to cause collapse of the pre-grown vessel sprouts.

The plants identified as positives (showing inhibition) from the two-level screening are considered for further analysis depending on what is already known of them and their relevance. From the secondary screening, 32 test samples were identified as specific inhibitors and 3 substances were identified as specific stimulators of VEGF induced sprouting and 3 were identified as being cytotoxic (causing death of the cells).This helps the scientists gain a very basic understanding of how multi-criterion decision making can be applied to help prioritize compounds as potential drug candidates.

Interestingly, in this blinded screening, a plant called Shrubby St. John's wort was identified. It has been reported for its anti-depressent behavior. This assay was validated because of its ability to identify potential samples. The purpose of optimizing the assay is thus fulfilled. And its reproducibility can target more such compounds to reach animal-model screens.

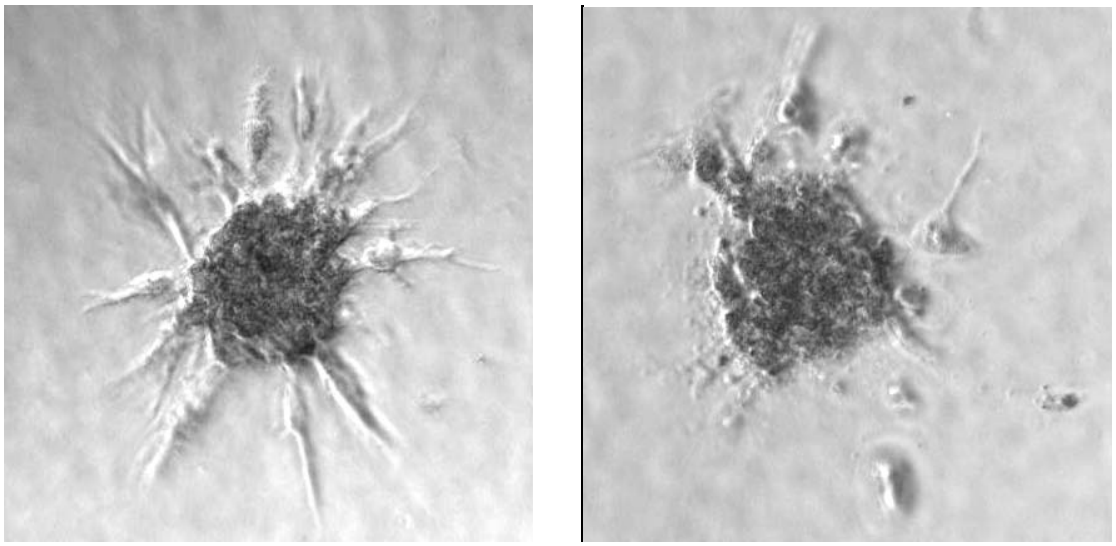


Figure 30: Growth factor treated spheroid for 24 hour, later with the shrubby St.John's wort for next 20 hours. The second image shows the inhibition of the already existed sprouts.

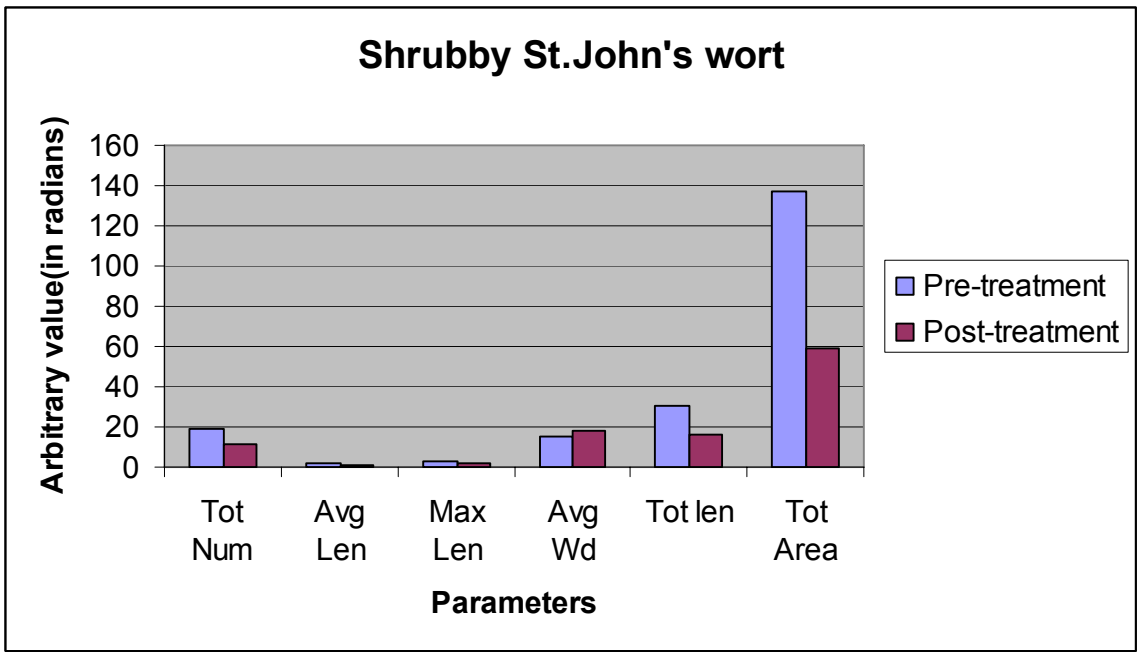



Figure 31: The graphical plot showing the difference in spheroid parameters after the treatment with plant extract over the pre-treated spheroid. There is almost a two-fold reduction in Total number of tubules, total length and total area of the tubules.

## 5.2. Drug Screening

One of the drug screening models is mentioned here and its assay validation is shown statistically. The spheroid assay was treated in high glucose of 25 mM concentration in the presence and absence of the drug called Benfotiamine, a potential new drug for diabetic treatment, at two doses of 50μM and 100μM. The researchers have reported that Benfotiamine halts much of the sugar accumulation [40]. The sugar accumulation in the experiment is shown at 25mM and there is no vessel formation (**). This model as mentioned earlier makes use of the drug to rescue the high glucose treated spheroids and foster them to new vessel formations.

Study Drug Stimulatory Responses

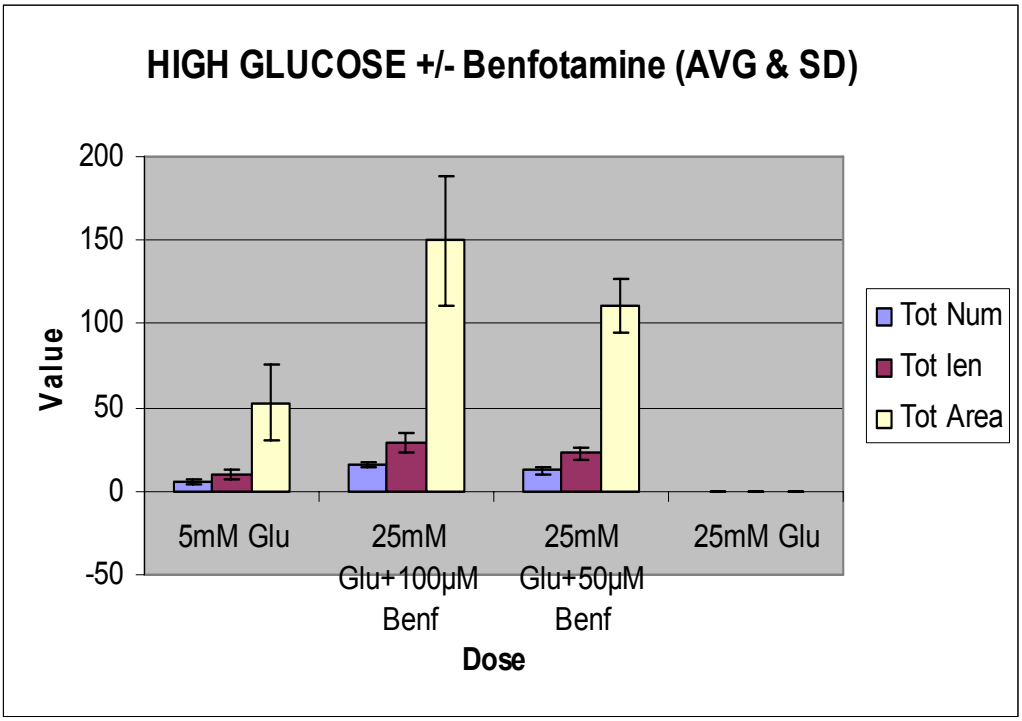



Figure 32: Stimulatory responses of Benfotiamine treated spheroids in the presence of 25mM glucose

This graph shows the 25 mM Glucose (control) treated spheroids with no growth (**) and the significant growth when treated with the drug at respective doses. At lower dose of high glucose i.e at 5mM, there is significant difference in performance parameters over the control. The validation shows that at 100µM of Benfotiamine there is increase in total number, total length and total area of the tubules in comparison to 50µM of Benfotiamine treated spheroids.

The above mentioned applications, validates the use of the assay for representing angiogenic (Benfotiamine) and anti-angiogenic (Shrubby St.John's wort) activities of the drug/ samples screened.

# CHAPTER VI CONCLUSION & FUTURE SCOPE

As very little is known of the cellular processes and molecular controls of sprout formation, much investigation is going into developing assays which can give predictable assessment about cell regulation under different drug and no drug conditions.
Optimized cell culture processing is one of the most important determinants of obtaining consistent data with cell-based assays, while at the same time the most labor intensive.
Traditional methods used bigger format (24 or 12 well) plates for screening at a random pace. The smaller wells in the 96-well plate format enable around five-fold less sample to be used, which in itself is a significant cost reduction to the laboratory.

The purpose of the thesis was to produce the assay, address its processing aspects, and the validation of various growth metrics which are the total number of tubules, average length of tubules and the average tubule width of the spheroid structure. In this thesis work, the assay was processed to an optimal stage. The product validation was performed and the coefficient of variation (CV) for the performance parameters was found to vary from 20-50 % for the spheroids under stimulation conditions using the growth factors. The assay was found to be robust for producing data for its analysis using the software. Thus the improved 3D spheroid assay, validated by the software for the first time can be quantified repeatedly.

Future work on processing the assay can be done when the system will be incorporated with instrumentation and controls for producing the assay with minimum user intervention and variation. Ideas and tools for automated image capture of the spheroids also need to be incorporated. Like any other new manufactured product that is prototyped by a supplier organization and approved by its customer before taking it to the production stage, this project after its prototype stage will address the advanced product quality planning (APQP) before taking a production phase.

On the biological value addition side, further study can be performed on screening various drugs with capturing any inhibitory effects on the spheroid assay to further elaborate its robustness on a high-throughput screening level. A system for such a

production can be streamlined and has a good scope to represent a pull system. Thus this can be extended to creating a library of data consisting of the behavior of spheroid under various drug effects.

On the software analysis side, in addition to direct extraction of growth parameters described in the chapter four, the sprouting tubules are analyzed using a parsing method. In this method, a tubule growing from the spheroid is completely characterized by a sequence of letters from A through F. This sorting can be used to search a database of previously analyzed assay images to identify specific growth patterns. The measurement of the growth metrics is translated to total number of sequences, average number of letters in each sequence, and the maximum number of letters forming a sequence. This side of research on the software development allows *building a database of assay sequences which allows applying statistical interference to identify specific relationships between assay tubule growth and the experimental conditions under which the assay was exposed* [28].

Further, to develop a robust HTS assay according to the modules mentioned earlier, we also need to consider the biological parameters which need to be optimized for the overall success. Ways to reduce the background sprouting to reduce noise, by adding reagents in the collagen mixture or by increasing the size of spheroid or any biologically significant ideas can be investigated for implementation.

Figure 33: Inventory of cells

Figure 34: Co-located material supply and equipment to the workstation

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