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# On-Chip Living-Cell Microarrays for Network Biology

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## 1. Introduction

The recently developed field of systems biology creates a new framework for understanding the molecular basis of physiological or pathophysiological states of cells. Screening modalities that can be used on single cells are needed to study cellular systems biology. The recent development of cellular microarrays has provided a method for the complex molecular analysis of living, single cells (Chen & Davies, 2006). Unlike other high-throughput systems, such as gene expression profiling microarrays or protein microarrays, cellular microarrays use a printed pattern of geographically distinct spots to probe living cells, rather than cell lysates, or other non-viable samples. Among the most powerful tools to assay gene function on a genome-wide scale in the physiological context of intact living cells are fluorescence microscopy and related imaging techniques (Pepperkok & Ellenberg, 2006). To enable these techniques to be applied to functional genomics experiments, fluorescence microscopy is making the transition to a quantitative and high-throughput technology.

The combination of time-lapse microscopy, quantitative image analysis and fluorescent protein reporters has enabled observation of multiple cellular components over time in individual cells (Locke & Elowitz, 2009). In conjunction with mathematical modelling, these techniques are now providing powerful insights into genetic and proteomic behaviour in diverse microbial systems. Recently, a quantitative system-wide analysis of mRNA and protein expression in individual cells with single-molecule sensitivity using a yellow fluorescent protein fusion library for *E. coli* has been realised (Taniguchi *et al.*, 2010).

## 2. Microfluidic chips for cell microarrays

### 2.1 Cell assays and cell microarrays

A cell assay is defined here as a measurement and analysis of the cellular response, at a given level, to a chemical and/or physical stimulus (Barbulovic-Nad & Wheeler, 2008). Cellular responses are diverse, e.g. alterations of intracellular and extracellular biochemistry, cell morphology, motility and (de)adhesion, survival and apoptosis, and proliferation properties. These responses characterise single aspects of cell phenotype, and are typically monitored in a culture dish or a multiwell plate, while more recently microfluidic devices have been employed. While culture dishes require millilitre volumes of media and reagents, multiwell plates contain microliter volumes and enable simultaneous

analysis of multiple cell types or stimuli. Experiments with multiwell plates are typically integrated in a robotic analysis platform. Two major drawbacks of robotic platforms are the expense of the instrumentation, and the cost of experimental consumables.

The use of microarrays was first reported in 1989 (Ekins *et al.*, 1989). The variety and diversity of microarrays has become impressive. Three main types of microarrays have been developed: DNA microarrays, protein microarrays, and cell microarrays (Barbulovic-Nad *et al.*, 2006). Several different approaches to cell microarrays have been explored to investigate gene expression, cell-surface interactions, extracellular matrix composition, cell migration and proliferation, the effects of drugs on cellular activity and many other areas (Angres, 2005). There are two fundamental methods to produce cell microarrays: the indirect and the direct method. The indirect method – i.e. the “reverse transfection” method – was developed by Ziauddin and Sabbatini (2001). In the direct method, the cells are printed onto a substrate. In few cases contact-based microarrayers are used, but more often non-contact-based devices are used.

Miniaturisation of cellular assays via cell microarrays increases assay throughput while reducing reagent consumption and the number of cells required, making these systems attractive for a wide range of assays in drug discovery, toxicology, and stem cell research (Fernandes *et al.*, 2009). Cell microarrays have been developed for highly parallel, high-throughput analyses of cell phenotypes (Narayanaswamy *et al.*, 2006), assessing cell proliferation and morphology (Bochner *et al.*, 2001; Xu, 2002), protein expression levels (Schwenk *et al.*, 2002), and imaging of tissues (Kononen *et al.*, 1998; Radhakrishnan *et al.*, 2008) and single cells (Biran *et al.*, 2003). In these initially developed living-cell microarrays, microbial cells were printed on an agar growth medium and could grow as microcolonies, or cells were grown in multiwell plates and printed on a glass slide for imaging, or only short time analyses on living cells were performed. High-throughput experiments on a library of cells require on-chip cell culture. Microchip 2- or 3-D cell cultivation techniques can provide many advantages for cell culture systems because the scale of the cultivated environment inside the microchip is fitted to the size of the cells. Table 1 gives some examples of developed mammalian cell microarrays/wells.

## 2.2 Cell assays in microfluidics

Microfabrication technology originated from the electronics industry, where 3D micro-features for electronic devices were manufactured in the sub-centimeter to sub-micrometer range using lithography techniques (Franssila, 2010). Microfluidics emerged as an extension of MEMS (Micro Electro Mechanical Systems) technology at the beginning of the 1980s. Microfluidics is a technology that is characterised by devices containing networks of micrometer-dimension channels (Whitesides, 2006). It involves the manipulation of very small fluid volumes, enabling the creation and control of  $\mu\text{l}$  to  $\text{n}\text{l}$  volume reactors. Microsystems create new opportunities for the spatial and temporal control of cell proliferation and stimuli by combining surfaces that mimic complex biochemistries and geometries of extracellular matrix with microfluidic channels that regulate transport of fluids and soluble factors (West *et al.*, 2008). Further integration with bioanalytic microsystems results in multifunctional platforms for basic biological insights into cells and tissues, as well as for cell-based sensors with biochemical, biomedical and environmental functions. Highly integrated microdevices show great promise for basic biomedical and pharmaceutical research, and for drug discovery (Dittrich & Manz, 2006). Microfluidic “lab

Cell type	Batch/ microfluidics	Array size (wells, spots)	Characteristics	References
Carcinoma cells	Microfluidics	1	Cells immobilised with peptide gel	Kim <i>et al.</i> , 2007
Hepatocytes	Batch microarray	512	Dynamic monitoring of fluorescet probes in single cells	Roach <i>et al.</i> , 2009
Fibroblast	Microfluidics	4	Ligand labeling and cell binding analysis	Sui <i>et al.</i> , 2007
Fibroblast 3T3	Microfluidics	96	Response of single cells to different concentration of signalling molecule (TNF)- $\alpha$	Tay <i>et al.</i> , 2010
Fibroblast 3T3	Microfluidics	16	Perfusion culture for 3 days	Kim <i>et al.</i> , 2006
Fibroblast 3T3	Microfluidics	32	Quantitative interrogation of signalling networks	Cheong <i>et al.</i> , 2009
H 35 cells	Microfluidics	64/100	8x8 array with individually addressable rows/10x10 array	Hung <i>et al.</i> , 2005; Lee <i>et al.</i> , 2006
Hela-NF	Microfluidics	40	8x5 array: row with 5 wells is individually addressed; GFP-based gene expression	Thompson <i>et al.</i> , 2004
Hela-NF	Microfluidics	256	16x16 array; GFP-based gene expression	Wieder <i>et al.</i> , 2005
Human stem cells	Batch microarray	1700	Interaction of biomaterials with cells	Anderson <i>et al.</i> , 2004
Human stem cells	Microfluidics	96	Transient stimulation schedules on proliferation, differentiation and motility	Gómez-Sjöberg <i>et al.</i> , 2007
Human neural SC	Microfluidics	1	Growth and differentiation	Chung <i>et al.</i> , 2005
mESC	Microfluidics	16	Proliferation is flow rate dependent, 4 days culture	Chin <i>et al.</i> , 2004
mESC	Batch microarray	280	Cells immobilised in alginate gel spots	Fernandes <i>et al.</i> , 2010
Rat stem cells	Batch microarray	10000	Dimensions of the wells are tunable, diameter: 20 to >500 $\mu$ m, height: 10-500 $\mu$ m	Chin <i>et al.</i> , 2004

Table 1. Examples of mammalian and stem cell microarrays/wells.

on a chip" technologies have been used to track gene expression changes in individual cells, enabling large populations of cells to be monitored, and allowing precise control of the cell microenvironment (Breslauer *et al.*, 2006; Charvin *et al.*, 2009).

Conventional methods of fabricating microfluidic devices have centered on etching in glass and silicon (Pisani & Tadigadapa, 2010). Polymers have assumed the leading role as substrate materials for microfluidic devices in recent years (Becker & Gärtner, 2008). They offer a broad range of material parameters as well as material and surface chemical properties, which

enable microscopic design features that cannot be realised by any other class of materials. Today, the most preferred material for biocompatible microfluidic devices is poly(dimethylsiloxane) (PDMS) (Velve-Casquillas *et al.*, 2010), which was introduced as soft lithography by Whitesides (Anderson *et al.*, 2000). PDMS is soft, transparent, permeable to gases, for most, impermeable to liquids, biocompatible, nontoxic, and has a low electrical conductivity, making it a very suitable material for biological applications in microfluidic devices. Fabrication of microfluidic devices in PDMS by soft lithography provides faster, less expensive routes than these conventional methods to devices that handle aqueous solutions. Soft lithography refers to a collection of techniques for creating microstructures and nanostructures based on printing, moulding and embossing (Weibel *et al.*, 2007). It is based on rapid prototyping and replica molding. In rapid prototyping, a computer-aided design program is used to create a design for channels, which are printed at high resolution onto transparency film. The transparency film then serves as the photomask. The master molds are generated by using the photomask in contact lithography to produce a positive relief of photoresist. In replica molding, PDMS is poured over the master and heat cured to generate a negative replica of the master. The PDMS is then removed from the mold and sealed against a glass coverslip to form the device features and channels. Flows in microfluidic devices are mainly pressure-driven by using syringe pump, rotary pump, or electro-osmotic flow.

Microfluidic devices are advantageous for cell assays for various reasons. The most obvious one is the similarity in dimensions of cells and microchannels (10-100  $\mu\text{m}$  widths and depths). Another important advantage is flow: fluid flow in these small channels is laminar. Consequently, convection only exists in the direction of the applied flow, whereas in the direction perpendicular to the applied flow, diffusion contributes to mass transport. Although diffusion-based transport is slow across long distances, in microchannels diffusion enables rapid reagent delivery. In addition, the combination of laminar flow and diffusion makes the formation of highly resolved chemical gradients across small distances. This feature is particularly useful for cell assays as such gradients are common in living systems (but difficult to implement in macroscale setups). Another advantage is the increased surface-to-volume ratio, which facilitates favourable scaling of heat and mass transfer, as well as favourable scaling of electrical and magnetic fields that are used in electromagnetic cell analysis. Another consequence of the size regime lies in the concentration of analytes: as cells in microchannels are confined in sub-microliter volumes, relevant analytes do not become too dilute and can thus be more readily detected. A limitation of the high surface-to-volume ratio of microchannels is the adsorption of molecules onto channel walls that are generally hydrophobic. However, surfaces can be chemically treated to prevent adsorption of biomolecules (Velve-Casquillas *et al.*, 2010). Automated high-throughput experiments may be performed in a large number of repeating functional microstructures fabricated on a single chip. These microsystems can also monitor the time course of the release, which is difficult to measure by conventional batch cell culture methods. Microfluidic devices can be made transparent and the cells monitored in real time by imaging, using fluorescence markers to probe cell functions and cell fate.

In a microfluidic device for cell-based assays, adequate culture conditions must be maintained for the duration of the experiment, which can span several days. While being cultured, cells must be continuously perfused with nutrients and oxygen; in addition, constant temperature and pH must be maintained. In contrast to traditional batch cultures, miniaturised perfusion systems provide precise control of medium composition, long-term unattended cultures and tissue-like structuring of cultures (Heiskanen *et al.*, 2010). Adherent



cells must be detached from culture flasks and seeded or spotted into a microfluidic device while sufficient time has to be allowed to achieve proper cell attachment and reduction of stress induced by the transfer. Mobile cells in suspension are easier to handle and require less time to adapt to the new environment.

### 2.3 Single-cell analysis/monitoring in microfluidic devices

A fundamental goal of cell biology is identifying how cell behaviour arises from the dynamic collection of environmental stimuli to which the cell is exposed (Lee & Di Carlo, 2009). From a biosystems science and engineering perspective, there is great interest in how the cell behaves as a system that processes time-dependent input signals into output behaviour(s). Ideally, with knowledge of the history of the ensemble of environmental stimuli, one would be able to predict the precise behaviour that a particular cell would exhibit under a given stimulus. Unfortunately, cells under seemingly identical environmental conditions often display a distribution of heterogeneous behaviour(s) (Lidstrom & Meldrum, 2003). This appears to be partly due to probabilistic behaviour in the “decision” processes that connect input and output (Raser & O’Shea, 2005; Mettetal *et al.*, 2006). Underlying the links between inputs and outputs are systems of interconnected molecular interactions (signalling pathways). Signalling within one pathway as well as cross-signalling between pathways, localisation of reactions and the sometimes small molecule numbers involved in signalling contribute to stochastic behaviour in these systems (Raser & O’Shea, 2005; Kholodenko *et al.*, 2010), which in the case of stem cells may very well be an essential and necessary feature of their biology and enables them to transit from one state to another. Because of the meanwhile well-documented heterogeneity within such cell population, increased emphasis has been put on analysing a large number of single cells and determining distributions of responses (Cai *et al.*, 2006; Mettetal *et al.*, 2006; Yu *et al.*, 2006). New tools, based on microfabrication and microfluidic technologies, are now allowing improved dynamic control of environmental variables for high-throughput single-cell analysis. These experimental technologies combined with systems analysis of signalling pathways are expected to lead to an improved quantitative description of single-cell function (Lee & Di Carlo, 2009).

Several single-cell analysis techniques have been developed, which may be classified in terms of information content (number of elements capable of being studied simultaneously) and throughput (number of cells studied in a give time). The simplest and most widely used forms of single-cell analysis are fluorescence microscopy and flow cytometry. Automated microscopy techniques, often termed high-content screening (HCS) or “cellomics”, recently provided also quantitative insight into cellular behaviour and in most cases are applied to observe the response of the cells, e.g. to drug candidate molecules.

The utility of single-cell measurements with high temporal resolution has been demonstrated by bacterial studies, which used optical microscopy to observe *Escherichia coli* over long time periods and reveal interesting temporal fluctuations and cell-to-cell variability that would otherwise be masked by population-wide measurements (Pedraza & van Oudenaarden, 2005). A microfluidic microchemostat has been constructed and used to acquire single-cell fluorescence data from *Saccharomyces cerevisiae* over many cellular generations (Charvin *et al.*, 2009; Rowat *et al.*, 2009). One way in which cells can rapidly respond to environmental stimuli is to alter the localisation and abundance of proteins (Charvin *et al.*, 2009). In a microfluidic device, these aspects can be studied on the same cells under various growth conditions or in response to environmental insults.

## 2.4 Localisomics

Localisomics seeks to identify the subcellular location of all proteins in the cell, which can provide key insights into the cellular function of the individual proteins as well as their probable interacting partners (Joyce & Palsson, 2006). Protein localisation has to be described in intracellular compartments, e.g. the nucleus or cytoplasm, and also in organelles, as specialisation of cellular organelles defines the functional roles of proteins (Souchennytskyi, 2005). The most informative is data about protein localisation and its dynamics in a single, living cell.

Mostly fluorescence microscopy techniques have been used to monitor green fluorescent protein (GFP)-tagged- or yellow fluorescence protein (YFP)-tagged proteins in *E. coli* (Taniguchi *et al.*, 2010), *S. cerevisiae* (Huh *et al.*, 2003) and human cells (Shariff *et al.*, 2010). Visual interpretation of the fluorescent images, and more recently, automated image analysis, have been used to extract dynamic protein localisation data (Schubert *et al.*, 2006; Conrad *et al.*, 2011). Images from many studies are publicly available (Table 2).

Species (cell type)	Number of proteins	Tagging method	Website	Reference
Human (U-2 OS, A-431, U-251 MG)	> 6000	Immuno-fluorescence, immunochemistry	www.proteinatlas.org	Berglund <i>et al.</i> , 2008
Mouse (3T3)	>100	Internal GFP fusion	cdtag.bio.cmu.edu	Jarvik <i>et al.</i> , 2002
Human (HeLa) Monkey (Vero)	>1000	cDNA terminal GFP fusion	gfp-cdna.embl.de	Liebel <i>et al.</i> , 2004
Human (HeLa) Mouse (3T3)	>100	Immunofluorescence and genomic internal GFP fusion	murphylab.web.cmu.edu	Huang <i>et al.</i> , 2002
Human (H1299 carcinoma)	> 2000	YFP CD tagging	www.dynamicproteomics.net	Frenkel-Morgenstern <i>et al.</i> , 2010
Yeast	> 4000	cDNA C-terminal GFP fusion	yeastgfp.yeastgenome.org	Huh <i>et al.</i> , 2003
Human (brain) Various	Various	Various	ccdb.ucsd.edu	Martone <i>et al.</i> , 2008

Table 2. Publicly available microscopy images concerning protein localisation in cells (adapted from Newberg *et al.*, 2009).

## 3. Computational methods for quantitative image analysis

Quantitative information from live cell microscopy can be obtained. To reach this goal, image analysis methods have to be used. These methods can provide quantified geometric, intensity, and motion properties, and these quantitative parameters can be used as input parameters for predictive systems biology models (Pepperkok & Ellenberg, 2006; Megason & Fraser, 2007; Bakal *et al.*, 2007; Verveer & Bastiaens, 2008).

Advances in imaging technology provide a huge amount of digital image data. A manual analysis is hardly possible. Additionally, 3D images over time are difficult to interpret manually and the result suffers from subjectivity. Therefore, computer-based image analysis is required to cope with the enormous amount of image data and to extract reproducible as

well as quantitative information (Peng, 2008; Zhou & Wong, 2008; Hamilton, 2009; Swedlow *et al.*, 2009; Rohr *et al.*, 2010).

Automatic analysis of multidimensional live cell microscopy images requires different computational methods. A general workflow for quantitative analysis of live cell microscopy images is composed of the following steps: (i) preprocessing, (ii) segmentation, (iii) registration, (iv) tracking and (v) classification (Rohr *et al.*, 2010).

### 3.1 Preprocessing

The goal of image preprocessing is to improve the quality of raw images prior to image segmentation and feature extraction. Applications include denoising for reducing the image noise, elimination of artifacts, intensity normalisation, contrast enhancement, and deconvolution for reducing the image blur introduced by the imaging process. Denoising methods use either linear or nonlinear filters to reduce noise in images and improve the signal-to noise ratio. For denoising images, a Gaussian filter is often applied (Rohr *et al.*, 2010). Filters that are not based on convolution are called nonlinear filters. A nonlinear filter that is often used to remove the pepper-noise generated by CCD detectors in optical fluorescent microscopy is the median filter (Zhou & Wong, 2008). This median filter can preserve high frequency information describing cell edges in high content microscopy images.

Deconvolution methods to reduce the image blur are relevant for both wide-field and confocal light microscopes (Cannell *et al.*, 2006). It is often assumed that the blurring of an image is caused by a linear process and thus can be presented by convolution with a point spread function (PSF). The aim of deconvolution is to reconstruct the original (true) image by reversing the effect of convolution and thus improving the resolution and contrast of the image (Rohr *et al.*, 2010). Examples of such approaches are the inverse filter, the Wiener filter, and the constrained least-squares filter.

### 3.2 Segmentation

Image segmentation is one of the most basic processing steps in many bioimage informatics applications. The goal is to segment out meaningful objects of interest in the respective image. In the case of microscopy images, one main task is to identify cells and to distinguish them from the background. Another task is to detect and localize particles in the image. Because particles are much smaller than cells and corresponded to spot-like image structures, different segmentation methods are required for cells and particles (Rohr *et al.*, 2010). Segmentation is a prerequisite for quantifying geometric properties of objects as well as for quantifying the corresponding signal intensities. Additionally, segmentation is often the basis for subsequent image analysis steps, i.e. for tracking.

#### 3.2.1 Cell segmentation

Cell segmentation can be categorised into two classes, i.e. nucleic segmentation and cytoplasm (or whole cell) segmentation. In recent years, there has been significant effort towards the development of automated methods for cell nuclei image and 3D cell segmentation have been developed (Ortiz de Solorzano *et al.*, 1999; Sarti *et al.*, 2000; De Solorzano *et al.*, 2001; Umesh Adiga & Chaudhuri, 2001; Malpica *et al.*, 1997; Belien *et al.*, 2002; Wählby *et al.*, 2004; Lin *et al.*, 2005; Lindblad *et al.*, 2004; Dufour *et al.*, 2005; Li *et al.*, 2007, 2008; Dorn *et al.*, 2008; Ko *et al.*, 2009). The main methods for cell segmentation can be



classified as: threshold-based segmentation, edge-based segmentation, region-based segmentation, and deformable models (reviewed in Rohr *et al.*, 2010).

### 3.2.2 Particle localisation

Often it is assumed that the intensities representing a fluorescently labelled particle resemble a 2D Gaussian function in which the peak intensity value of the particle differs significantly from that of the local background. A bottom-up or a top-down strategy is used to address the problem of particle localisation.

Bottom-up localisation schemes for fluorescent particles typically comprise three consecutive steps: image preprocessing, particle detection, and particle localisation (Rohr *et al.*, 2010). A common technique is to apply a threshold on the intensities of a (preprocessed) image to determine image regions that correspond to particles (Ponti *et al.*, 2003; Sbalzarini & Koumoutakos, 2005). Automatic schemes for determining an optimal threshold is required since manual determination is often impractical and can give inconsistent results.

Top-down approaches use model-driven strategies in which hypotheses regarding the possible configuration of the models are tested against the information found in the images. A 2D Gaussian function is typically used as a model for the shape and appearance of fluorescently labelled particles (Godinez *et al.*, 2007; Cortes & Amit, 2008).

### 3.3 Registration

The task of finding an optimal geometric transformation between corresponding image data is known as registration. Bioimage registration is essential in many applications that need to compare multiple image subjects of different conditions. Registration approaches can be classified based on the type of transformation model and image information used (Rohr *et al.*, 2010). The transformation model defines the degrees of freedom for geometrically matching two images, and a main distinction is made between rigid, affine, and nonrigid schemes.

Many of the 2D and 3D image registration methods proposed for medical image analysis, such as the mutual information registration (Viola & Wells, 1997), spline-based elastic registration (Rohr *et al.*, 2003), invariant moment feature-based registration (Shen & Davatzikos, 2002), congealing registration (Miller, 2006; Zollei *et al.*, 2005), etc., can be extended to align the molecular and cellular images (Peng, 2008). Nonrigid or elastic registration approaches are required to cope with the shape changes of live cells (Rohr *et al.*, 2010). An intensity-based nonrigid registration approach for cell microscopy images, which relies on an optic flow scheme and uses segmented images, has been developed recently (Yang *et al.*, 2008). An intensity-based approach has been used to register segmented 2D static images of different cell nuclei (Rohde *et al.*, 2008), and a biomechanical model has been used to register 3D segmented images of cell nuclei (Gladilin *et al.*, 2008). An intensity-based nonrigid registration approach that directly analyses the intensity information without requiring a segmentation step has been developed (Kim *et al.*, 2007). This approach relies on optic flow estimation and has been applied to register 2D and 3D time-lapse images of live cells for accurate analysis of protein particle movement.

### 3.4 Tracking and motion analysis

Dynamic cell population studies are becoming more and more important in understanding pathways and networks (Glory and Murphy, 2007). Live cell fluorescent video microscopy

offers a wealth of information on the dynamic organisation of proteins and subcellular structures that is unavailable in static 2D and 3D imaging. With the addition of time, organelle dynamics as proteins are recruited, transported and expelled can be viewed in detail and the passage through a cell of proteins and the structures that they interact with can be readily observed (Hamilton, 2009). Additionally, the addition of temporal parameters such as the change of size and size of nuclei and the duration between the different stages are important indicators of the cell division cycle (Zhou and Wong, 2006). There is also extensive work on analysing the behaviour of specific labelled proteins by tracking individual objects in time series images (Meijering *et al.*, 2006).

Tracking denotes the repeated localisation of objects in successive images. The aim is to establish temporal correspondences between objects to analyse object motion (Rohr *et al.*, 2010). Although finding correspondences is largely simplified when there is only one object in the images, this task is generally quite challenging when there are several or a large number of moving objects. Therefore, sophisticated multiple target tracking methods are required.

Object tracking from fluorescent video microscopy present many challenges (Hamilton, 2009). Objects viewed may join, split, disappear, change direction or substantially change their morphology, and there are technical challenges such as photobleaching and compromises between spatial and temporal resolution. Tracking algorithms developed in other research areas and adapted to fluorescent video microscopy tend to perform poorly and considerable research has gone into designing algorithms specific to fluorescent imaging (reviewed in Kalaidzidis, 2009).

### 3.5 Classification

A last step in image analysis is to distinguish objects into different classes. Automatic classification methods can be divided into supervised and unsupervised learning methods (Glory & Murphy, 2007). Supervised learning methods allow classification into predefined classes and require training of the classifier with a set of annotated examples. In unsupervised learning methods, the classes do not need to be known in advance. Supervised learning methods are used for cell microscopy since the classes are known in advance. Common used classifiers are artificial neural networks (Boland & Murphy, 2001), *k*-nearest-neighbour classifiers (Chen *et al.*, 2006), and support vector machines (Conrad *et al.*, 2004; Huang & Murphy, 2004; Harder *et al.*, 2008).

## 4. Biological network analysis

### 4.1 Network modelling

Network modelling is a key step for processing dynamic proteomics data, because a network model provides: (i) a means of understanding how detected proteins are associated with underlying network operations, and (ii) a platform into which other useful information, (such as protein abundances and localisation) can be integrated.

A cell is an enormous complex entity made up by myriad interacting molecular components that perform the biochemical reactions that maintain life. A cell can be described through the set of interconnections between its component molecules according to the network hypothesis (De Los Rios & Vendruscolo, 2010). The central dogma in molecular biology describes the way in which a cell processes the information required to produce the molecules necessary to maintain life and reproduce (Crick, 1970). In order to obtain a more complete description of the functioning of a cell, a deeper understanding of the manner in

which the sets of interconnections between these molecules are defining the identity of the cell itself is needed (De Los Rios & Vendruscolo, 2010). Therefore, it is important to investigate whether the genetic makeup of an organism does not only specify the rules for generating proteins, but also the way in which these proteins interact among themselves and with the other molecules in a cell. Networks provide a way to organise and regulate efficiently complex systems. In an effective network different parts are linked by reducing at a minimum the number of interconnections. A network is also a powerful method to represent the data in one object and to enable the quantitative assessment of the fragility or robustness of the system. The biological molecules in a cellular system are individual molecules, which affect each other by pairwise interactions (Chen *et al.*, 2009). A cascade of those pairwise interactions forms a local structure (i.e. a linear pathway or a subnetwork), which transforms local perturbations into a functional response. All linear pathways or subnetworks are assembled into a global biomolecular network, which eventually generates global behaviours and holds responsibility for complicated life in a living organism.

Gene products, such as mRNA and proteins, are produced through the transcription and translation processes. Gene, mRNA, and protein are known as biological molecules or basic components (Chen *et al.*, 2009). The complicated relations and interactions between these components are responsible for diverse cellular functions. Transcription factors (TFs) are DNA-binding proteins that can activate or inhibit the transcription of genes to synthesise mRNAs by regulating the activities of genes. Since these TFs themselves are products of genes, the final effect is that genes regulate each other's expression as part of a transcription (or translational) regulatory network (TRN) or gene regulatory network (GRN). At the proteome level, proteins participate in diverse posttranslational modifications of other proteins or form protein complexes and pathways together with other proteins. Such local associations between proteins molecules are called protein-protein interactions (PPIs), which form a protein interaction network. The biochemical reactions in cellular metabolism can likewise be integrated into a metabolic network whose fluxes are regulated by enzymes that catalyse the reactions. In many cases, these interactions at different levels are integrated into a signaling network.

Multiple proteins in a cell are in dynamic interaction with each other, and these interactions provide functioning and behaviour of living cells (Terentiev *et al.*, 2009). Reversible protein-protein interactions are among other dynamic processes that proceed in a cell and contribute to cell functioning. The whole set of protein-protein interactions of a given organism is referred to as the "interactome". Structural organisation of interactomes and the total number of interactions in them are important factors that determine complexity of biological systems. The number of copies of a certain protein per cell can vary from several tens to millions (Ghaemmaghami *et al.*, 2003). Interactomes even of simple organisms can be formed by a rather large number of interactions. The determination of physically interacting protein pairs makes it possible to design interactome maps as graphs consisting of nodes, in which a particular protein is located, and of links between them that indicate paired interactions. The interactome maps are considered as keys to obtain knowledge on protein functioning (Rual *et al.*, 2005).

## **4.2 Integration of biological networks**

### **4.2.1 Network visualisation and analysis**

Many tools exist for visually exploring networks and network analysis, including examples such as Cytoscape (Shannon *et al.*, 2003), VisANT (Hu *et al.*, 2009), Osprey (Breitkreutz *et al.*,

2003), CellDesigner (Kitano *et al.*, 2005), BioLayout (Goldovsky *et al.*, 2005), GenMAPP (Dahlquist *et al.*, 2002), PIANA (Aragues *et al.*, 2006), ProViz (Iragne *et al.*, 2005), and Patika (Demir *et al.*, 2002). These systems play a key role in the development of integrative biology, systems biology and integrative bioinformatics. The trend in the development of these tools is to go beyond static representations of cellular states, towards a more dynamic model of cellular processes through the incorporation of gene expression data, subcellular localisation information and time-dependent behaviour (Suderman & Hallett, 2007).

Cytoscape is an open source software project for integrating biomolecular interaction networks with high-throughput expression data and other molecular states into a unified conceptual framework (Shannon *et al.*, 2003). In Cytoscape, nodes representing biological entities, such as proteins or genes, are connected with edges representing pairwise interactions, such as experimentally determined protein-protein interactions. Nodes and edges can have associated data attributes describing properties of the protein or interaction. A key feature of Cytoscape is its ability to set visual aspects of nodes and edges, such as shape, color and size, based on attribute values. This data-to-visual attribute mapping allows biologists to synoptically view multiple types of data in a network context. Additionally, Cytoscape allows users to extend its functionality by creating or downloading additional software modules known as “plugins”.

VisANT is a web-based software framework for visualising and analysing many types of networks of biological interactions and associations (Hu *et al.*, 2005). Given user-defined sets of interactions or groupings between genes or proteins, VisANT provides: (i) a visual interface for combining and annotating network data, (ii) supporting function and annotation data for different genomes from the Gene Ontology and KEGG databases, and (iii) the statistical and analytical tools needed for extracting topological properties of the user-defined networks. The new VisANT (v3.5) functions can be classified into three categories (Hu *et al.*, 2009). (i) Visualisation: a new tree-based browser allows visualisation of GO hierarchies. GO terms can be easily dropped into the network to group genes annotated under the term, thereby integrating the hierarchical ontology with the network. This facilitates multi-scale visualisation and analysis. (ii) Flexible annotation schema: in addition to conventional methods for annotating network nodes with the most specific functional descriptions available; VisANT also provides functions to annotate genes at any customized level of abstraction. (iii) Finding over-represented GO terms and expression-enriched GO modules: two new algorithms have been implemented as VisANT plugins. One detects over-represented GO annotations in any given sub-network and the other finds the GO categories that are enriched in a specified phenotype or perturbed dataset. Both algorithms take account of network topology (i.e. correlations between genes based on various sources of evidence).

Osprey is a Java-based network visualisation and analysis tool for protein-protein and genetic interaction networks (Breitkreutz *et al.*, 2003). Osprey builds data-rich graphical representations that are color-coded for gene function and experimental interaction data. Mouse-over functions allow rapid elaboration and organisation of network diagrams in a spoke model format. User-defined large-scale datasets can be readily combined with Osprey for comparison of different methods.

GenMAPP is a free computer application designed to visualise gene expression and other genomic data on maps representing biological pathways and groupings of genes (Dahlquist *et al.*, 2002). Integrated with GenMAPP are programs to perform a global analysis of gene expression or genomic data in the context of hundreds of pathway MAPPs and thousands of



Gene Ontology Terms (MAPPFinder), import lists of genes/proteins to build new MAPPs (MAPPBuilder), and export archives of MAPPs and expression/genomic data to the web. The main features underlying GenMAPP are: (i) draw pathways with easy to use graphics tools, (ii) color genes on MAPP files based on user-imported genomic data, (iii) query data against MAPPs and the GeneOntology.

CellDesigner is a structured diagram editor for drawing gene-regulatory and biochemical networks (Kitano *et al.*, 2005). Networks are drawn based on the process diagram, with graphical notation system proposed by Kitano, and are stored using the Systems Biology Markup Language (SBML), a standard for representing models of biochemical and gene-regulatory networks. Networks are able to link with simulation and other analysis packages through Systems Biology Workbench (SBW). CellDesigner supports simulation and parameter scan by an integration with SBML ODE Solver and Copasi. By using CellDesigner, you can browse and modify existing SBML models with references to existing databases, simulate and view the dynamics through an intuitive graphical interface.

BioLayout uses a general approach for the representation and analysis of networks of variable type, size and complexity (Goldovsky *et al.*, 2005). The application is based on the original BioLayout program (C-language implementation of the Fruchterman-Rheingold layout algorithm), entirely re-written in Java to guarantee portability across platforms. BioLayout(Java) provides broader functionality, various analysis techniques, extensions for better visualisation and a new user interface.

PIANA (Protein Interactions And Network Analysis) facilitates working with protein interaction networks by (i) integrating data from multiple sources, (ii) providing a library that handles graph-related tasks and (iii) automating the analysis of protein-protein interaction networks (Aragues *et al.*, 2006). PIANA can also be used as a stand-alone application to create protein interaction networks and perform tasks such as predicting protein interactions and helping to identify spots in a 2D electrophoresis gel.

ProViz is a tool for the visualisation of protein-protein interaction networks, developed by the IntAct European project (Iragne *et al.*, 2005). It provides facilities for navigating in large graphs and exploring biologically relevant features, and adopts emerging standards such as GO and PSI-MI.

Patika (Pathway Analysis Tool for Integration and Knowledge Acquisition) is based on an ontology for a comprehensive representation of cellular events (Demir *et al.*, 2002). The ontology enables integration of fragmented or incomplete pathway information and supports manipulation and incorporation of the stored data, as well as multiple levels of abstraction. Patika is composed of a server-side, scalable, object-oriented database and client-side editors to provide an integrated, multi-user environment for visualising and manipulating network of cellular events. This tool features automated pathway layout, functional computation support, advanced querying and a user-friendly graphical interface.

#### 4.2.2 Subcellular localisation

Interesting tools that take into account the subcellular localisation are (Suderman & Hallett, 2007): the Cytoscape plugin Cerebral (Barsky *et al.*, 2007), Patika (Demir *et al.*, 2002; see 4.2.1), and Cell Illustrator (Nagasaki *et al.*, 2010). Cerebral (Cell Region-Based Rendering and Layout) is an open-source Java plugin for the Cytoscape biomolecular interaction viewer. Given an interaction network and subcellular annotation, Cerebral automatically generates a view of the network in the style of traditional pathway diagrams, providing an intuitive interface for the exploration of a biological pathways or system. The molecules are separated



into layers according to their subcellular localisation. Potential products or outcomes of the pathway can be shown at the bottom of the view, clustered according to any molecular attribute data-protein function, for example. Celebrial scales well to networks containing thousands of nodes.

Patika partitions the drawing space into regions corresponding to the subcellular localisations and then search for layouts where nodes are forcibly constrained to their respective locations (Demir *et al.*, 2002). It makes use of a modified force-directed algorithm to achieve this.

Cell Illustrator is a software platform for systems biology that uses the concept of Petri net for modeling and simulating biopathways (Nagasaki *et al.*, 2010). It is intended for biological scientists working at bench. The recent version of Cell Illustrator 4.0 uses Java Web Start technology and is enhanced with new capabilities, including: automatic graph grid layout algorithms using ontology information; tools using Cell System Markup Language (CSML) 3.0 and Cell System Ontology 3.0; parameter search module; high-performance simulation module; CSML database management system; conversion from CSML model to programming languages (FORTRAN, C, C++, Java, Python and Perl); import from SBML, CellML, and BioPAX; and, export to SVG and HTML. Cell Illustrator employs an extension of hybrid Petri net in an object-oriented style so that biopathway models can include objects such as DNA sequence, molecular density, 3D localisation information, transcription with frame-shift, translation with codon table, as well as biochemical reactions.

#### 4.2.3 Network integration for cellular microarray data using Cytoscape

Data from cellular microarray experiments include a list of differentially expressed proteins, i.e. changed fluorescence intensity (protein abundance), as a function of time and localisation in the cell. Integration of these data with other available biological network data for a specific organism can be performed using the above listed software platforms (see 4.2.1), e.g. by using Cytoscape supplemented with the available plugins.

The cellular microarray data can be mapped to the protein interactome. Network data related to these proteins can be imported into Cytoscape using three options: querying interaction databases using cPath (Cerami *et al.*, 2006), building an association network through text mining using Agilent Literature Search plugin (Vailaya *et al.*, 2005), and loading own network data from a text file. Additionally, pathways from repositories, such as KEGG (Wixon & Kell, 2000), Reactome (Joshi-Tope *et al.*, 2005) via the PSI-MI, BioPAX, or SBML data exchange formats (Strömbäck *et al.*, 2006), can be imported.

Networks can be analysed further using topologic information, and using combined information of various types, such as GO annotations and known pathways. Network modules enriched by GO terms and pathways (functional enrichment) can be identified. Therefore, the Cytoscape plugins BiNGO (Maere *et al.*, 2005) and DAVID (Dennis *et al.*, 2003; Huang da *et al.*, 2009) can be employed. GO Biologic Process (GOBP) trees with nodes corresponding to GOBP terms is generated using the BiNGO plugin. GOBP terms that differ in terms of their degrees of enrichment can be identified, as can sets of network nodal proteins belonging to such GOBP terms. Pathways enrichment analysis can be performed for network nodal proteins using DAVID.

Network structures and active subnetworks can be explored using the Cytoscape plugins MCODE (Bader *et al.*, 2003) and jActiveModules (Ideker *et al.*, 2002). The MCODE-plugin can be used to generate network clusters within which proteins are densely connected, whereas proteins across different network clusters loosely interact. Both the core network modules and their dynamic relationships can be identified by integrating time-dependent protein

abundance information. In addition, active networks can be identified among network modules using jActiveModules, which select networks with high collective abundances.

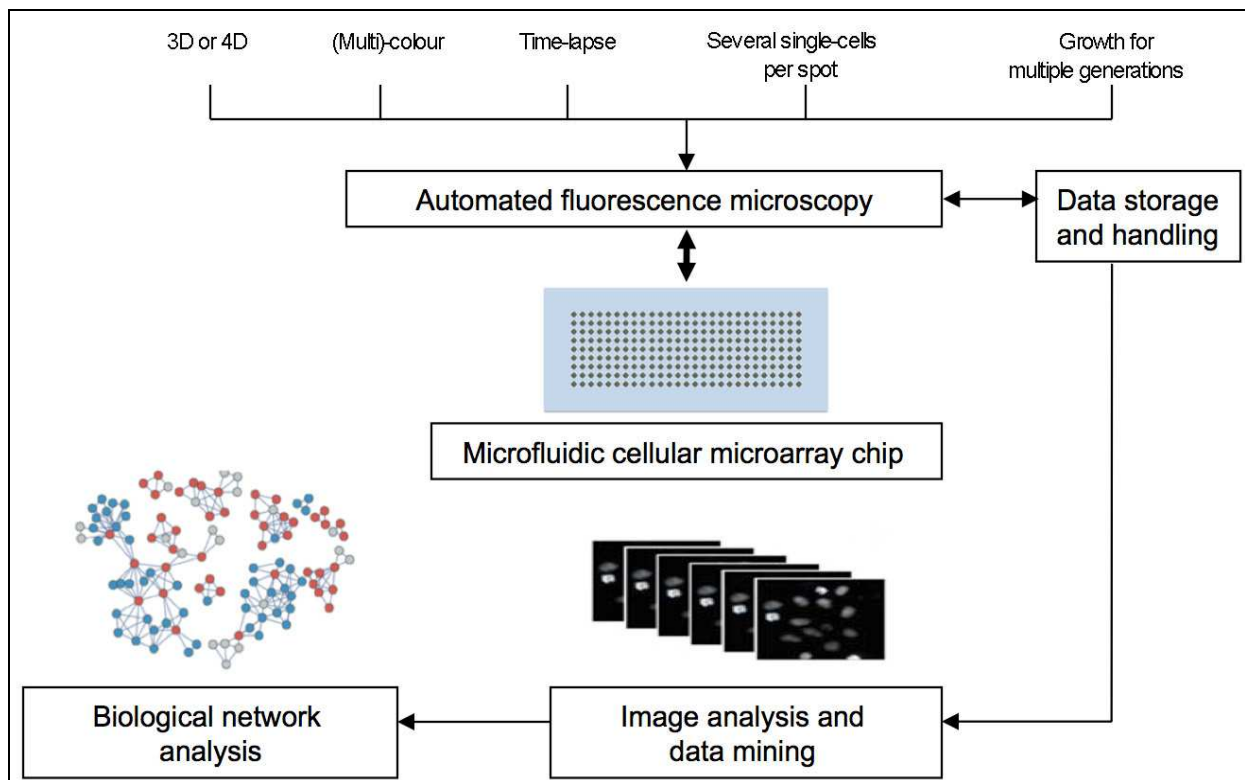


Fig. 1. Work scheme for on-chip cellular microarray screening and biological network analysis.

## 5. Summary

In this chapter, on-chip living-cell microarrays to study network biology is reviewed. A general work scheme is shown in Figure 1. Microfluidic technology holds great promise for the creation of advanced cell culture models. It can be used – in combination with time-lapse fluorescent microscopy, and image analysis and data mining – to observe multiple cellular components over time in individual cells, i.e. dynamics of a FP-tagged protein. Integration of dynamic localisomics data with other available biological network data allows performing a quantitative system-wide analysis for a particular cell.

Cell assays in microfluidic chips that have been used for cellular microarrays are discussed in detail in this chapter. Next, image analysis algorithms to extract dynamic proteomics data from cellular microarray experiments are reviewed. In the last part, the integration of the cellular microarray data into a network model, as well as network analysis options are discussed.

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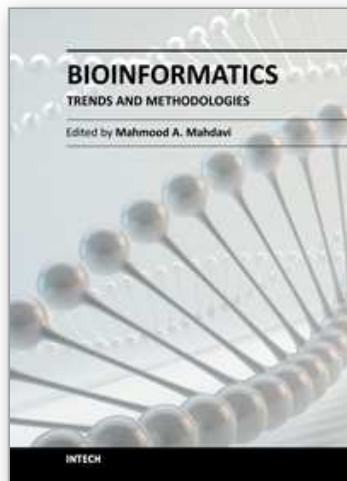
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