DEVELOPMENT OF MICROFLUIDIC CELL GROWTH AND STRESS RESPONSE ANALYSIS PLATFORMS AT SINGLE CELL RESOLUTION

by
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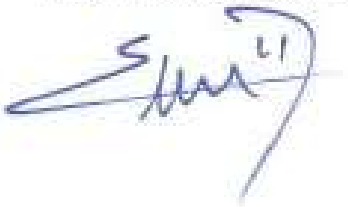

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ABSTRACT

DEVELOPMENT OF MICROFLUIDIC CELL GROWTH AND STRESS RESPONSE ANALYSIS PLATFORMS AT SINGLE CELL RESOLUTION

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Key Words: Cancer, Cell Culture, Microfluidics, Lab-on-a-chip, Gradient Generator, Simulation, and Single-cell Resolution.

Cancer, as one of the evolutionary diseases, is among the major causes of death all over the world. In order to obtain quantitative data about development of cancer and its underlying mechanisms, it is significantly important to understand interactions of cancer cells and their surrounding microenvironment at single-cell resolution. In this respect, microfluidic platforms are capable of mimicking tumor microenvironment, while representing a versatile tool for lab-on-a-chip (LOC) applications. In this thesis, new microfluidic tools were presented to investigate behavior of single cells and their response to different chemicals. The context of this thesis includes design, simulation, microfabrication and tests of these microfabricated devices using human cancer cell lines. One of these microfluidic platforms was a cell culture device to culture cells fed by medium flow, while allowing their live-cell imaging at single-cell resolution. The second one was a microfluidic gradient generator, which is known as Christmas-Tree chemical gradient generator, was used to create gradient of chemicals to be induced grown cell culture in the microfluidic cell culture device. These microfabricated tools were modular, therefore, they might be used as independent devices or they could be integrated. The designs of the microfluidic devices were simulated using COMSOL multi-physics program. Soft-lithography was performed for their fabrication. The DsRed fluorescent protein expressing breast cancer cell line (MCF7) was used to test the performance of the developed microfluidic devices. As a result of this study, a bubble-free single-cell loading protocol for LOC platforms was successfully developed. Furthermore, cells were cultured; their proliferation rate was obtained using image processing. Last but not least, the cells cultured in the microfluidic cell culture device were exposed to sodium dodecyl sulfate (SDS) using the microfluidic gradient generator. The dose-dependent, SDS-induced death was quantitatively investigated using the integrated microfluidic platform.

ÖZET

MİKROFLUİDİK HÜCRE BÜYÜME VE STRESLİ YANIT ANALİZ PLATFORMLARININ TEK HÜCRE ÇÖZÜMLEMESİNDE GELİŞİMİ

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Anahtar Kelimeler: Kanser, Hücre Kültürü, Mikroakışkanlar, Lab-on-a-chip, Gradient Jeneratör, Simülasyon ve Tek Hücreli Çözünürlük.

Kanser, evrimsel hastalıklardan biri olarak, dünyanın dört bir yanındaki başlıca ölüm nedenleri arasındadır. Kanser ve altta yatan mekanizmalarının gelişimi hakkında nicel veri elde etmek için, tek hücreli çözünürlükte kanser hücrelerinin ve çevreleyen mikro ortamın etkileşimlerini anlamada önemli önem taşımaktadır. Bu bağlamda, mikroakışkan platformlar, tüm-mikro ortamı taklit edebilmekle birlikte, laboratuvar-on-a-chip (LOC) uygulamaları için çok yönlü bir araçtır. Bu tezde, tekli hücrelerin davranışlarını ve farklı kimyasallara tepkilerini araştırmak için yeni mikroakışkan araçlar sunulmuştur. Bu tezin içeriği, insan kanseri hücre dizileri kullanılarak bu mikrofabrike cihazların tasarımı, simülasyonu, mikrofabrikasyonu ve testlerini içermektedir. Bu mikroakışkan platformlardan bir tanesi, tek hücreli çözünürlükte canlı hücre görüntülemelerine izin verirken, orta akışla beslenen kültür hücrelerine yönelik bir hücre kültürü cihazıydı. İkincisi, mikroakışkan hücre kültürü cihazında yetiştirilen hücre kültürü indüksiyonu için kimyasal gradyan oluşturmak için Noel Ağacı kimyasal gradyen üreteci olarak bilinen mikroakışkan bir degrade üreteci idi. Bu mikro-fabrikasyon araçları modülerdir, bu nedenle bağımsız cihazlar olarak kullanılabilirler veya entegre olabilirler. Mikroakışkan cihazların tasarımları COMSOL çoklu fizik programı kullanılarak simüle edildi. İmalatı için yumuşak litografi yapıldı. Göğüs kanseri hücre hattını (MCF7) ifade eden DsRed floresan proteini, geliştirilen mikroakışkan cihazların performansını test etmek için kullanıldı. Bu çalışma sonucunda, LOC platformları için kabarcıksız tek hücreli yükleme protokolü başarıyla geliştirildi. Ayrıca, hücreler kültürlenmiştir; Onların çoğalma hızı görüntü işleme kullanılarak elde edildi. Son olarak, mikroakışkan hücre kültürü cihazında kültürlenen hücreler, mikroakışkan gradyan üreteci kullanılarak sodyum dodesil sülfata (SDS) maruz bırakıldı. Doza bağımlı SDS ile indüklenen ölüm, entegre mikroakışkan platform kullanılarak nicel olarak araştırıldı.

"Our lives begin to end the day we become silent about things that matter."

- Martin Luther King Jr.

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ABBREVIATION AND SYMBOLS

μTAS : Micro-Total Analysis Systems

BE : Barrett's Esophagus

CAD :Computer Aided Design

CCLE : Cancer Cell Line Encyclopedia

CFD : Computational Fluid Dynamics

CTVT : Canine Transmissible Venereal Tumor

CVD : Chemical Vapor Deposition

DEP : Dielectrophoresis

DNA : Deoxyribonucleic Acid

EA : Esophageal Adenocarcinoma

EBL : Electron Beam Lithography

ECM : Extracellular Matrix

EGF : Epidermal Growth Factor

EGFR : Epidermal Growth Factor Receptor

eIFs : Eukaryotic Initiation Factors

FBS : Fetal Bovine Serum

FEM : Finite Element Method

FVM : Finite Volume Method

HPV : Human Papilloma Virus

LBL : Layer By Layer Deposition

LOC : Lab-on-a-chip

MNP : Magnetic Nanoparticles

MRI : Magnetic Resonance Imaging

PBS : Phosphate Buffered Saline

PC : Polycarbonate

PDMS : Polydimethylsiloxane

PEG : Polyethylene Glycol

PLA : Polycaprolactone

PLG : Polylactide-co-Glycolide

PMMA: Polymethylmethacrylate

PS : Polystyrene

PVA : Polyvinyl Alcohol

Rb : Retinoblastoma

RF : Release Factor

Re : Reynolds number

RFP : Red Fluorescent Protein

RNA : Ribonucleic Acid

SDS : Sodium Dodecyl Sulfate

Sh : Sherwood number

TGF : Transforming Growth Factor

UV : Ultraviolet

XRD : X-ray Diffraction

Chapter 1

LITERATURE REVIEW AND BACKGROUND

1.1 Introduction

The field of microfluidic devices for biological applications is positioned at the intersection of science and engineering. For this reason, its extensive interdisciplinary nature calls for a thorough research and understanding of several contextual fields. In this respect, this chapter is meant to provide a better understanding of biological systems and the origin of cancer. In addition, the principles of mammalian cell culture and the basics of laboratory environment will further be discussed. Finally, a brief introduction to the capabilities and potential of microfluidic devices in cancer biology will also be presented. In order to achieve the aforementioned objectives, the following is an attempt to briefly contextualize life at its very basic principles.

1.2 The evolution of life

The evolution of life can be interpreted as the accumulation of complexity. Although the generation of complexity is the first step towards life, the same forces that created these molecules can damage or distort them. Therefore, the sole generation of complexity is not sufficient for the emergence of life. In such an environment, a special feature would benefit a certain type of arrangement of molecules over the others through self-replication mechanisms. Mathematically, an "autocatalytic set" is referred to as an arrangement of values in such a way that this arrangement can be generated catalytically, and also lead to the production of a similar set. Chemical evolution can be modeled through autocatalytic sets in order to study the emergence of RNA life [1-3]. Today, the idea of emergence of a RNA life is generally agreed upon among evolutionary biologists, and more and more evidence supporting this hypothesis is accumulating [4-7].

After the establishment of reproducible molecular structures, the main governing force is "Natural Selection". This profound concept was first coined by the British scientist Charles Darwin, he indirectly challenged the perception of the universe in his era [8]. The natural selection is the simple idea that the species which best adapt to their environment are more

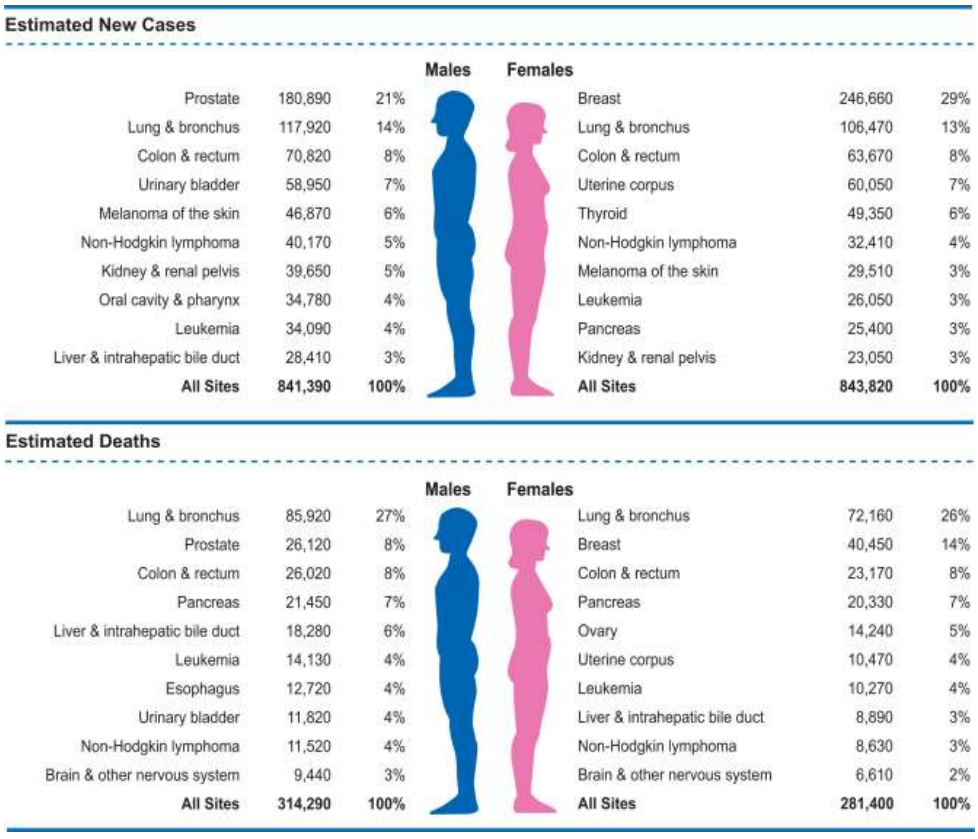
likely to survive and perpetuate their species. In other words, natural selection is the primary mechanism that identifies which organism is the most likely to survive among different species which find themselves in perpetual competition over material and energy resources. One other aspect of Darwin's findings is the "Evolutionary-tree". In this concept, the emergence of organisms occurs through mutations in heredity genetic information. Heredity is nothing but the the aforementioned autocatalytic set necessary for reproducible functionality. However, the general concept of heredity is not only limited to the genomic information, but one may also consider memory and learning as inheritable information which can contribute to the survival of one specie.

In the course of several billion years, life as we know it emerged on Earth. By tracing the tree of evolution, scientists speculate that all organisms have a last universal common ancestor (LUCA) which used RNA as its genome [9]. The evolution of photosynthesis changed the earth's biosphere from an anaerobic media, to an aerobic media by generating oxygen. As a result, during several thousand million years, this complexity increased, and many organisms came to existence and replaced the ones which became extinct [10]. This evolution which occurs over billions of years is referred to as "Macroevolution". In contrast, as complexity increased, the environment changed as well, and gave rise to new species. Therefore, it is also possible to face evolution within a population itself. For instance, the resistance of certain pests against a pesticide over a few generations, or resistance of bacteria despite antibiotics can be recognized as "Microevolution" due to their short evolutionary interval.

1.3 Cancer is a form of life

Cancer is a series of diseases which have an irregular growth pattern of cells in such a way that these cells are capable to spread and grow in the other parts of a eukaryote. In contrast to microbial diseases, cancer develops through mutations within a normal body tissue [11], similar to microevolution in any population. Although a cancerous cell type evolves in a relatively short time in the body, it must be treated as an invasive parasite. Canine transmissible venereal tumor (CTVT) is one extreme example of the parasitic nature of

cancer, since this cancer acts as a transmittable infectious disease. In addition, cancer is also defined as a genetic disease, and the reason for this is the accumulation of genomic mutations which leads to uncontrolled cell growth and metastasis. These mutations can occur due to environmental factors or hereditary mutations inherited from parents. However, unlike the processes pertaining to macroevolution, due to their microevolutionary nature, cancer cells do not have enough time to develop new biological pathways; instead, the vital mechanisms that control the cell growth and death become malfunctional.



Estimated New Cases

Males				Females		
Prostate	180,890	21%		Breast	246,660	29%
Lung & bronchus	117,920	14%		Lung & bronchus	106,470	13%
Colon & rectum	70,820	8%		Colon & rectum	63,670	8%
Urinary bladder	58,950	7%		Uterine corpus	60,050	7%
Melanoma of the skin	46,870	6%		Thyroid	49,350	6%
Non-Hodgkin lymphoma	40,170	5%		Non-Hodgkin lymphoma	32,410	4%
Kidney & renal pelvis	39,650	5%		Melanoma of the skin	29,510	3%
Oral cavity & pharynx	34,780	4%		Leukemia	26,050	3%
Leukemia	34,090	4%		Pancreas	25,400	3%
Liver & intrahepatic bile duct	28,410	3%		Kidney & renal pelvis	23,050	3%
All Sites	**841,390**	**100%**		**All Sites**	**843,820**	**100%**

Estimated Deaths

Males				Females		
Lung & bronchus	85,920	27%		Lung & bronchus	72,160	26%
Prostate	26,120	8%		Breast	40,450	14%
Colon & rectum	26,020	8%		Colon & rectum	23,170	8%
Pancreas	21,450	7%		Pancreas	20,330	7%
Liver & intrahepatic bile duct	18,280	6%		Ovary	14,240	5%
Leukemia	14,130	4%		Uterine corpus	10,470	4%
Esophagus	12,720	4%		Leukemia	10,270	4%
Urinary bladder	11,820	4%		Liver & intrahepatic bile duct	8,890	3%
Non-Hodgkin lymphoma	11,520	4%		Non-Hodgkin lymphoma	8,630	3%
Brain & other nervous system	9,440	3%		Brain & other nervous system	6,610	2%
All Sites	**314,290**	**100%**		**All Sites**	**281,400**	**100%**

Figure 1. 1 The major cancer deaths and the estimated cancer cases in 2016 [12].

In 2015, cancer was responsible for 8.8 million deaths globally, which means almost 1 out of every 6 deaths, and it is expected that during the next two decades the number of cancer cases will increase by 70%. In addition, the annual economic cost of cancer is estimated to

be over one trillion dollars worldwide. Dietary habits such as: consumption of tobacco and alcohol, excessive body weight, and lack of physical activity, as well as infectious viruses such as hepatitis and human papilloma virus (HPV), as well as environmental pollution can be described as the main causes of cancer [13]. Prostate cancer is the most common form of cancer in men, while breast cancer is the most prevalent type of cancer in women. However, other types of cancer such as lung and bronchus cancers were responsible for more than 25% of cancer deaths in the United States in 2016 (Fig 1.1) [12].

1.4 Cancer development

Cancer corresponds to any type of disease where tissue undergoes unusual proliferation with the potential of invading other tissues. However, cancer does not suddenly occur, it requires several steps prior to forming an invasive colony of cancerous cells. Neoplasm is referred to as the abnormal growth of tissue which is not necessary leading to cancer. It can be initiated through the transformation of cells in a certain tissue, which is also known as metaplasia. Metaplasia is a cellular response toward environmental stress to resist the superimposed stress. Nonetheless, the continuation of stress may result in dysplasia and eventually in malignancy in the form of neoplasm.

Neoplasm can be recognized as the earliest step towards cancer. It consists of different types, and it can also be referred to as tumor. One type of tumor is the benign neoplasm, such as skin moles which do not transform into cancer. In contrast to benign tumors, there are some neoplasms which are not malignant yet, however they are potentially capable of transforming into cancerous tumors such as colon polyps. The final category of neoplasms is cancer, in the form of an invasive type of tissue which lost its original functionalities. In addition, in case of metastasized cancer cells, the newly formed tumors are considered the secondary neoplasm. Moreover, based on the origin of the cell type that a cancer emerges from, cancers can be divided into carcinoma, leukemia, lymphoma, sarcoma, and germinoma.

1.5 Evolution in cancer

In order to transform a normal cell into a cancer cell its genetic structure must be mutated. The type of mutation which occurs in the differentiated cells is referred to as "Somatic Mutation". In the course of somatic mutation, the already existing functional genes go through mutations in such a way that they lose their original functionality. The most common type of mutation is the mutation of epidermal growth factor receptor (EGFR) gene. This gene produces a trans-membrane protein whose outer membrane site comprises a ligand site for epidermal growth factor (EGF), once it binds with EGF, it activates the protein-tyrosine kinase part of the EGFR inside the cytoplasm. Any mutation in the signaling pathway of EGFR, such as over expression in EGF, or EGFR, or any mutations that derive self-propelling signaling would lead to an abnormal growth of tissue and, ultimately, to cancer [14].

The rate of somatic mutation in healthy human fibroblast cells is around 2.8×10^{-7} per base-pair (*bp*) per generation which is almost a hundred times more than the mutation in germline [15, 16]. Considering the fact that human DNA consists of 3 billion bp, it can be concluded that each somatic cell is prone to almost 100 bp mutations during its life. However, only 1% of mutations of genes can cause cancer. Proto-oncogenes are the genes that through mutation can become oncogenes in such a way that they interfere with cell growth, differentiation and apoptosis [17]. Once a cell has gone through the genetic transformation which enables it to grow abnormally and form a neoplasm, it is still subjected to further mutations. In fact, the colonial evolution of cancer within a neoplasm is referred to as "Nowell's Hypothesis"[18].

Natural selection is the driving force in the evolutionary process within a neoplasm. There are three main parameters which define the rate of evolution in a colony; mutation rate, size of a colony, and domination of a selection. A higher rate of mutations and a larger original size of a neoplasm as well as high growth rates all contribute to a faster formation of a malignant tumor. In case of Barrett's esophagus (BE), which is a benign neoplasm, yet a pre-malignant form of esophageal adenocarcinoma (EA), it is possible to trace the genetic mutations in the colony. In the process of tumorogenesis in EA, the loss of several key genes can be traced back to the BE. As a result, it can be observed that, in BE, the loss

of p16 (CDKN2A/INK4A) is followed by the loss of p53 (TP53), both of which are among the most important genes in the cellular apoptosis (Fig 1.2) [19]. However, in this evolutionary process another phenomenon proceeds parallel to the natural selection in the neoplasm environment, which is due to the fact that the natural selection of a gene affects the frequency of the other neighboring genes. This phenomenon is referred to as “Genetic Hitchhiking” or “Genetic Draft”.

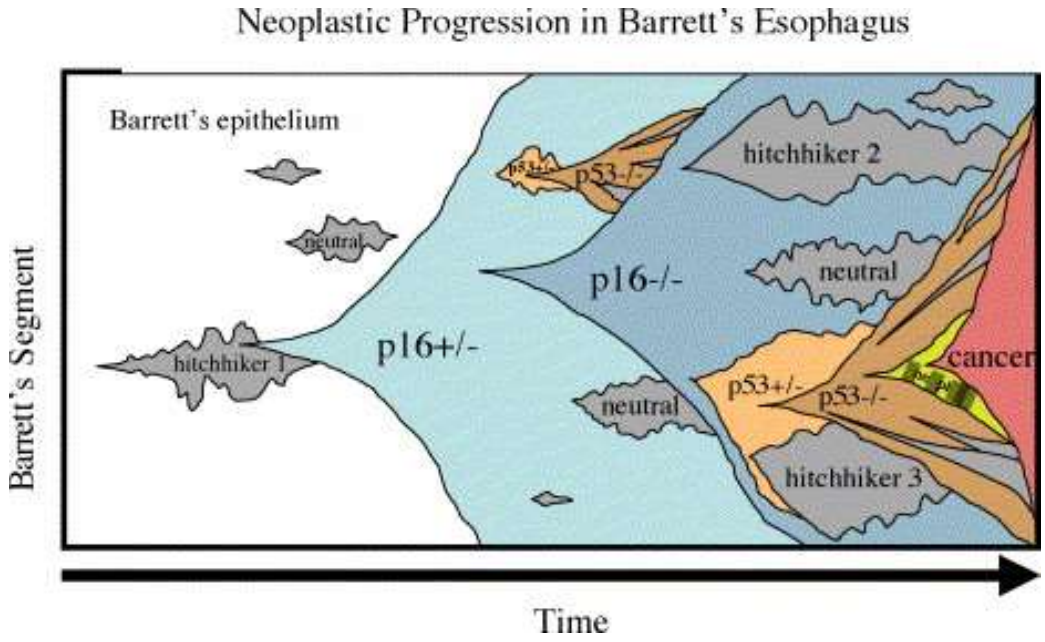


Figure 1. 2 Transformation of Barrett's esophageal (BE) neoplastic to esophageal adenocarcinoma (EA) during clonal evolution [19].

The structure of a tumor is heterogeneous as a result of multiple mutations in the neoplasm’s microevolution [20]. This intra-tumor heterogeneity, which can be revealed by means of a multi-region sequencing of the tumor, has been achieved through high throughput single-cell sequencing and mathematical algorithms. This insight into cancer evolution enlightens the strategies which must be considered in order to treat cancer from the phylogenetic point of view.

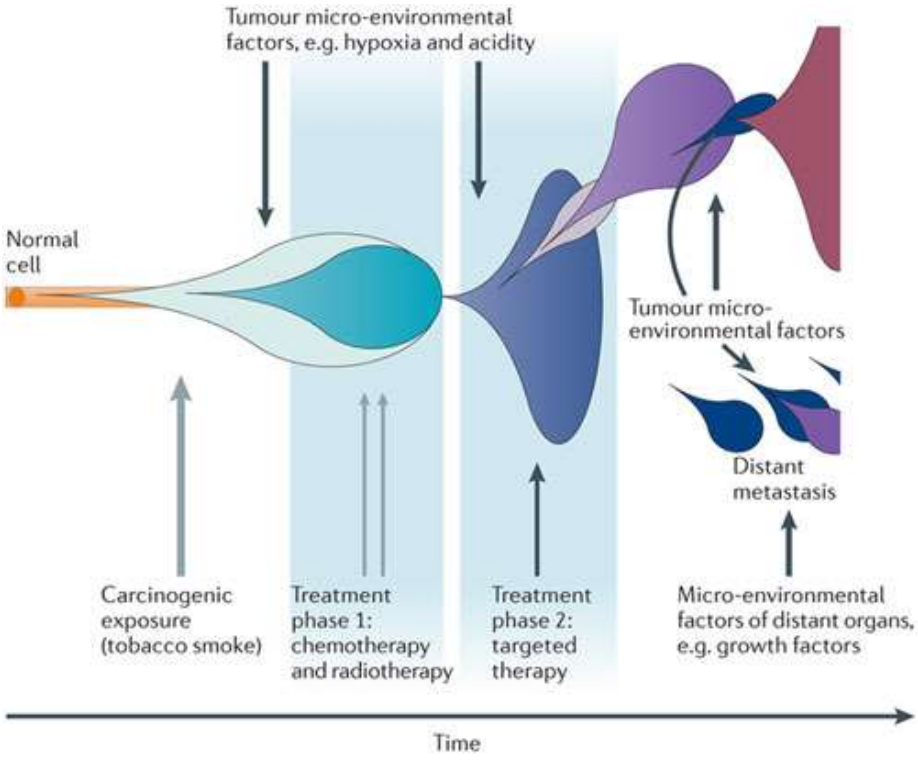


Figure 1. 3 Chemotherapy can play the same role as a microenvironmental factor in cancer development [21].

Surgery is a routine procedure to remove a tumor in cases of cancer other than hematological ones. In this way, the whole tumor and the area of organ which is affected by tumor cells, and sometimes the whole organ should be removed. The main reason for surgery is to remove the cells which can potentially disturb the rest of the tissue, or spread as metastasized cells. In addition, surgery is a means for pathologists to better estimate the tumor's advancement. Besides surgery, chemotherapy is usually applied in order to kill the metastasized tumor cells or undetectable secondary tumors. However, experiments have shown that cancer cells can also become resistant against single chemical agents (Fig 1.3) [21], similar to the antibiotic resistance in bacteria. Consequently, the genetic heterogeneity within a tumor increases the chance of tumor survival, and of its reoccurrence. Therefore, the combinational chemotherapy which is using different chemotherapy medicines with different mechanisms of action is more effective in cancer treatment than single agent chemicals.

1.6 The molecular biology of cell

Eukaryote cells are not only sophisticated machineries, but they are also capable of colonizing together to form macroscopic multicellular organizations. This microcosmos functions as a result of the harmony between different chemicals that form the components of the cell. Eukaryotic cells share many similar characteristics and components, which makes their study easier. These cells are believed to have originated from prokaryotes such as bacteria, and therefore they inherited many molecular mechanisms from unicellulars. A mammalian cell is the organization of several compartments with different functionalities which are all enclosed in a membrane (Fig 1.4).

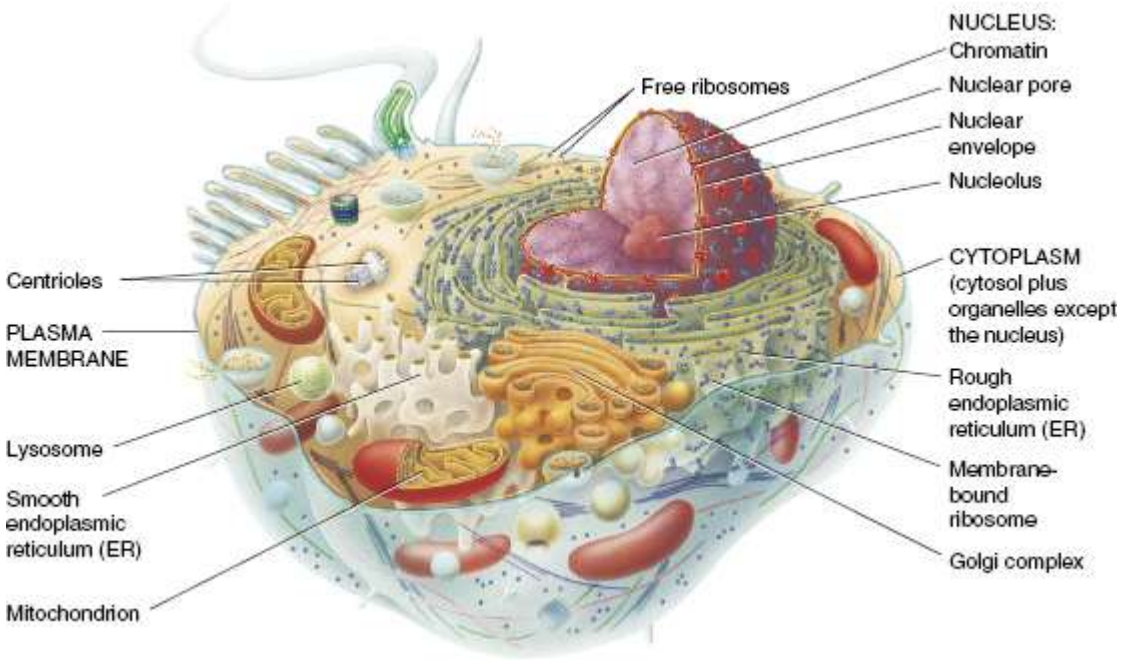


Figure 1. 4 The components of a mammalian cell [22].

The membrane or plasma membrane plays a key role in the separation of different chemicals, and in the formation of compartments or organelles. As a result of this barrier each part of a cell can be specialized in to perform different tasks. Furthermore, the structure of the membrane is organized in such a way that allows different organelles to merge together, or to form new organelles. The permeability of the plasma membrane is highly controlled due to its molecular structure. The membrane is impermeable to ions and

large molecules, and it only allows small molecules to pass through. This phenomenon occurs due to the structure of lipid bilayer membrane. The phospholipid has a super-hydrophilic head which is attached to a hydrophobic poly(hydrocarbon) tail. As a result, the hydrophobic parts of the phospholipid tend to self-assemble, while the hydrophilic part of it assimilates this process in an aqueous medium (Fig 1.5). Major phospholipids in a mammalian cell are phosphatidylcholine phosphatidylethanolamine, phosphatidylserine, and sphingomyelin. Additionally, glycolipids and cholesterol are the other components which are found in the membrane [23].

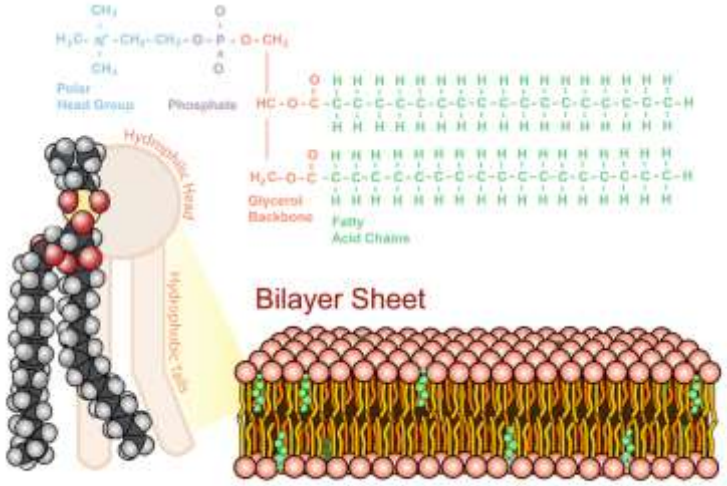


Figure 1. 5 The structure of plasma membrane [24].

However, almost half of the membrane weight is comprised of the proteins embedded in the membrane, which brings many more functionalities to the membrane. Membrane proteins are attached through ionic bonding (peripheral proteins) and hydrophobic interactions (integral proteins) with the bilayer membrane. Many of the integral proteins are trans-membrane and they are positioned to connect the inner and outer sides of the membrane [23].

Proteins are the main component of the cell machinery since they are involved in almost every process occurs in the cell. Proteins form enzymes for enzymatic reactions; they act as chemical switches, receptors, structural complexes, etc. Generally, proteins have 3D

structures at nano-scale, they form due to the folding of a linear polypeptide chain. This polymeric chain is comprised of covalent bonding of different monomers produced by the cell. These monomers are called proteinogenic amino acids and they consist of nearly 20 different types. The diversity of amino acids combined with their order brings the possibility of forming many different protein structures. The order in the protein sequence is referred to as the primary structure of proteins. Due to the Vandervells forces between different amino acids of a protein, some segments of a polypeptide fold together and form the secondary structures of a protein such as α-helix and β-sheet. The secondary structure of a protein forms rather spontaneously, and it is distinguishable through its repetitive sequence. Each polypeptide, besides its secondary folding, forms a structure through further molecular interactions, which is usually done with the help of a family of proteins referred to as "Chaperone proteins". The final folded structure of a polypeptide is its tertiary structure; however, as different polypeptides come together and form a protein assembly, this forms the quaternary structure of a protein (Fig 1.6).

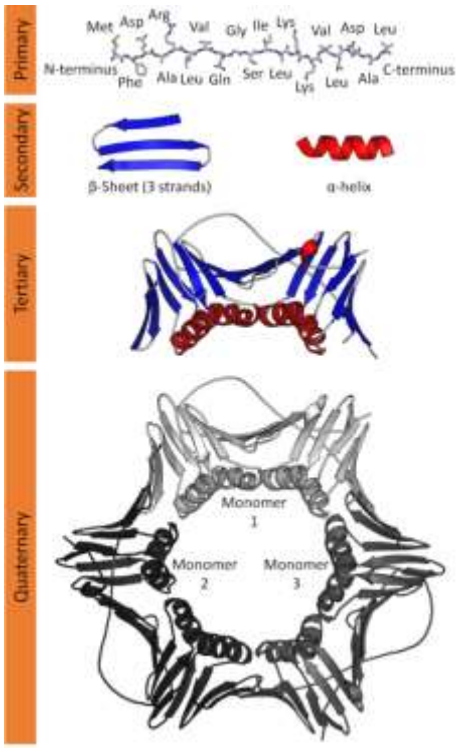


Figure 1. 6 The different substructures of a protein complex [25].

Similar to any other machine, the functional parts of cell machinery are exposed to damage and malfunction. Therefore, cells must reproduce and replicate proteins. The biosynthesis of proteins occurs through a process called translation (Fig 1.7). The polypeptide synthesis is a highly regulated mechanism, and even a minor error in this process will lead to a malfunction protein. Therefore, each sequence of a polypeptide is copied from a messenger RNA (mRNA) template. Each three-consequent-sequence on the mRNA is called a codon and corresponds to one of the 20 proteinogenic amino acids. These amino acids are attached to another RNA structure which contains an anticodon which exactly matches the corresponding codon on the mRNA. Therefore, this intermediating RNA is referred to as "transfer RNA" (tRNA). The appropriate amino acid is matched to the corresponding codon through two reactions mediated by aminoacyl AMP and aminoacyl tRNA synthetases to create the tRNA. There are 64 combinations for codons, 3 of which correspond to the stop-codons to halt the translation process.

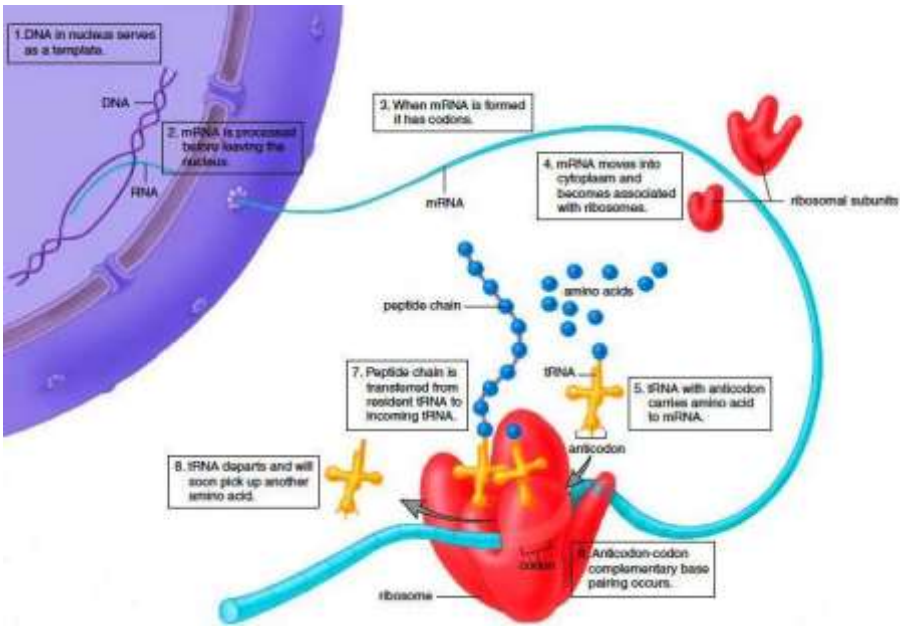


Figure 1. 7 Protein synthesis [26].

Ribosomes synthesize the protein and the number of ribosomes can reach 10 million in a growing mammalian cell, which indicates the importance of protein synthesis (Fig 1.4). Ribosomes can be free or located on the internal membranes, and their structure is

composed of tRNA and proteins. During the translation process, the mRNA reading is in the 5′ to 3′ direction, but the translation starts at a specific codon location. Most ribosomes can recognize the 7-methylguanosine cap at their 5′ terminus of the mRNA and then they scan the mRNA until they face an AUG codon to initiate the translation.

The translation process can be divided into initiation, elongation and termination. In eukaryotes at least ten proteins need to come together to initiate the translation process and this group of proteins is called "eukaryotic initiation factors" (eIFs). After the initiation, elongation continues by means of the polymerization of the polypeptide sequence through consumption of tRNAs. Eventually, the elongation process will be terminated once the ribosome complex encounters one of the terminating codons (UAA, UAG, or UGA), in which a release factor (RF-1 or RF-2) will release the polypeptide from the ribosome. After the translation process, mRNA tends to lose its protecting caps at the 5′ and 3′ ends. As a result, the mRNA will be exposed to different degradation mechanisms inside the cytoplasm.

Messenger RNA, as it is inferred from its title, is responsible for carrying a message. This message is coming from the cell nucleus where the vital genetic information is stored and protected from the rest of the cytoplasm in form of DNA. In fact, RNA originates from ribonucleic acid, while DNA originates from deoxyribonucleic acid, which indicates the presence of the ribose in the RNA structure. Moreover, the base uracil (U) only exists in RNA, while the base thymine (T) only exists in DNA, whereas the other bases, namely (adenine (A), guanine (G), and cytosine (C) are common in both. Although RNA and DNA have very similar structures, their structural conformations are different. In case of DNA, all of its bases are paired with a complementary DNA molecule in such a way that they form a double helix which increases its chemical stability. However, in the case of RNA, it does not form a double helix, and instead it can fold, similar to proteins [27].

Transcription is the process in which a part of DNA is transcribed into a RNA molecule by means of the enzyme RNA polymerase. Nucleoside triphosphates are consumed by the RNA polymerase to make a complementary base pair with the DNA sequences. In comparison to DNA, RNA molecules are usually much shorter and they depend on the length of the transcribed gene. One interesting difference between RNA and DNA

polymerization is the fact that RNA polymerization does not need a primer to start the polymerization. Similar to translation, transcription also consists of initiation, elongation and termination. RNA polymerase has a weak interaction with DNA and in order to start the transcription it requires a promoter. There are several promoters which are part of a family of proteins called “transcription factors”. They control the gene expression in the cell, besides coactivators, chromatin remodelers, histone acetyltranferases, histone deacetylases, kinases and methylases.

During the initiation phase, transcription factors bind to a specific promoter site on DNA and after RNA polymerase unwinds the double helix, they will be released and the RNA polymerase continues the transcription process. The RNA polymerization continues one base at a time and the energy for the process is supplied from the triphosphate bond of the nucleotides. Eventually, transcription will be aborted by means of transcribing a sequence of poly(adenine) nucleotides which detaches the RNA polymerase from DNA. The freshly synthesized RNA is called “precursor messenger RNA” (pre-mRNA) that will be later on tailored through RNA splicing. Splicing occurs inside the nucleus either during or after transcription by stitching some parts of RNA called exons and discarding the other parts called introns to form the final mRNA [28].

1.7 Cell cycle

The triumph of life is its self-replication, and it is achieved at cellular level. Cells go through a cell cycle, and during this process some cells are able to replicate, and some others sustain their living state (Fig 1.8). A cell cycle is comprised of five phases, namely referred to as: G_0, G_1, S (Synthesis), G_2, and M (Mitosis). G_0 is the state when the cell has just been divided and is able to undertake all the necessary functions. Most of the cells in the body are at this phase, and they may remain in this state indefinitely. However, some cells, like epithelial cells, do not remain in this state and they need to continuously replicate. In the case of the cells that need to replicate, during an interphase, they must collect all the nutrition required for the cell division, which usually takes almost 90% of the duration of cellular division. The period required from mitosis until the DNA synthesis

is called G_1, and it is the first period of the interphase. During this period, the cellular activity increases and the cell is prepared for DNA synthesis. During the S period, each chromosome is replicated by means of DNA polymerase, and two copies of all chromosomes exist at the same time in a cell. G_2 is the period when the cell grows sufficiently in order to provide enough supply of proteins for two cells. Eventually, at the M phase, the cell will divide into two daughter cells through five different steps [29].

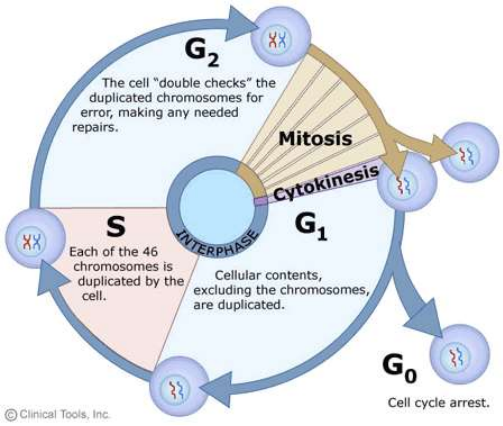


Figure 1. 8 Cell cycles during the replication process [30].

1.8 Hallmarks of cancer

In most cancers, the DNA damage caused by radiation, chemicals or other environmental factors must be prohibited from transferring to the daughter cells. The cell regulatory circuitry is a complex adaptive system with many interconnections. Thus, similar to any other dynamic system, many of the regulatory systems are based on negative feedback loops which suppress the cell proliferation mechanisms. Damages to these circuitries loops affect the cell cycle control and proliferation, which eventually can lead to a cancer (Fig 1.9). One of these pathways is the Ras pathway that is responsible for 30% of cancers. Mutation in this gene negatively affects the Ras GTPase activity. Lack of Ras GTPase prolongs the activated form of Ras protein, and consequently its downstream signaling, which results in cell proliferation [31]. Similarly, PTEN and mTOR are other proteins which inhibit the activity of PI3K, and deletion or damage to their genome results in hyperactivity of PI3K [32]. P13K is a family of intercellular enzymes which are involved

in cell growth, differentiation, and mortality. Although disruption of negative feedbacks and over-expression in tumorigenic pathway increase the cell proliferation, some studies have shown contrary results. The over-expression in Ras pathway can also terminate the cell proliferation. This disruption in cell cycle usually leads to a bigger cell size without the capability of division referred to cell senescence [33].

The retinoblastoma (Rb) is a gene responsible for the suppression of a tumor. The pRb is a check point at G_1 which activates the cell senescence program irreversibly with the help of $p16^{INK4a}$ in humans [34]. The other important tumor suppressor is p53 and its corresponding gene, TP53. This pathway can not only lead to the activation of cellular senescence, but it is also in charge of cell apoptosis in response to several stress factors alongside p63 and p73 tumor suppressors [35]. In addition to the aforementioned tumor suppressors, which work internally, there are external tumor suppressors; Merlin is the product of the NF2 gene that exists in the cell to cell junctions as well as the cell to base-membrane junctions. Somatic mutations in this gene are observed in many types of cancer, especially in mesothelioma [36]. LKB1 is another gene which regulates the organization and polarization of epithelial cells, and disruptions in its pathway affect several other downstream tumorigenic pathways [37].

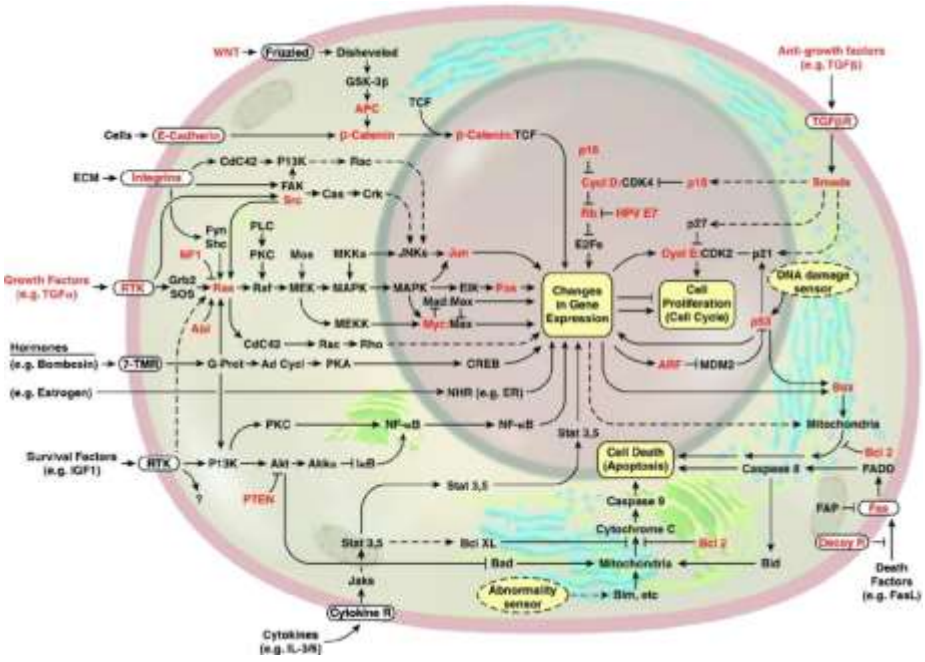


Figure 1. 9 Signal transduction in cancer [38].

Apoptosis is the most important mechanism to prevent the proliferation of malignant cells. An apoptosis pathway consists of pro-apoptotic and anti-apoptotic proteins, whose disruption is another barrier which cancer cells need to conquer before malignancy occurs. The over-expression of anti-apoptotic proteins such as Bcl-2 and Bcl-xL, or the under-expression of some pro-apoptotic proteins like Bax has shown a protective effect in some cancers [39]. Alternatively, autophagy through Beclin-1 protein is another mechanism by means of which cancer cells can be inhibited through the slowing down process of cell growth and self-digestion. However, it seems that autophagy has a paradoxical effect as well, and under some conditions it can promote tumors [40]. In contrast to apoptosis and autophagy, necrosis pathway for cell death is induced by the neighboring cells. Necrosis is regulated through RIP1 protein and it can be triggered through many stress factors such as TNF, TRAIL, and LPS, oxidative stress, or DNA damage. Necrosis is associated with the inflammation of cells and consequently activation of macrophages and dendritic cells. Paradoxically, necrosis is observed in the fast growing tumors, occurring as a result of the growth stimuli factors such as TNFα, IL-6, TGFβ [41].

As the neoplasms grow, they require a further supply of oxygen and nutrition. Interestingly, neoplasms are able to supply blood vessels by means of employing the angiogenesis mechanism, which is similar to a normal tissue. Angiogenesis is another barrier toward malignancy, and neoplasms which do not have this possibility stagnate. There are several proteins promoting or inhibiting the angiogenesis. Proteins such as VEGF and bFGF promote this process, while some others such as angiostatin, endostatin, interferon, platelet factor 4, thorombospondin, prolactin 16 kd fragment and metalloproteinase inhibit the angiogenesis[42].

All of the characteristics obtained by the cell toward hyper-proliferation simply cause tumors which can in many cases be removed through surgery. What makes a tumor lethal is the capability to form secondary tumors in the other tissues by means of metastasis. Cells are normally attached to each other by cell-to-cell junctions, as well as cells-to-extra-cellar-matrix (ECM), which are a group of proteins such as those stemming from the cadherin family. In carcinomas which originate from epithelial cells, as the cancer cells obtain more mobility and tissue adaptability so their characteristics shift toward adult stem

cells, or mesenchymal stem cells (MSC). Therefore, cancer initiating cells are referred to as “cancer stem cells” (CSC), and the process of transformation is named “epithelial to mesenchymal transition” (EMT). As the EMT proceeds, the expression of some cell surface markers such as CD44, CD24 and CD104 (ITGB4) changes, and through these markers, it is possible to identify in which state cells would exhibit CSC behavior (Fig 1.10) [43].

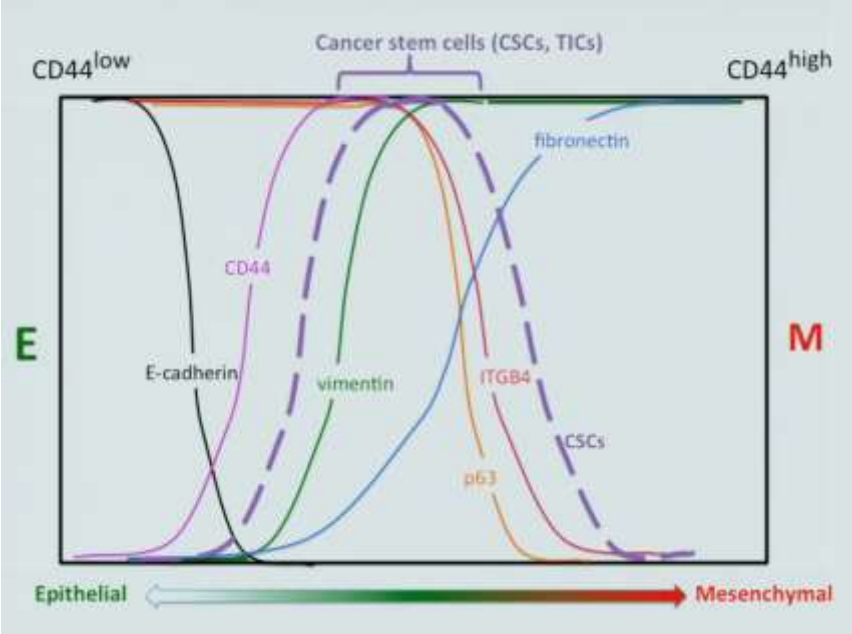


Figure 1. 10 The optimal condition for cancer stem cell behavior during epithelial to mesenchymal transition.

Complementary to the required characteristics for a cell to become malignant is the suppression or evasion of the immune system. The major mechanism of immune system suppression is believed to be mediated by $CD4^{+}$,$CD25^{+}$, and $FoxP3^{+}$ regulatory T cells (Tregs) [44]. Myeloid cells, modulated dendritic cells (DCs) and alternatively-activated M1 and M2 macrophages also promote the tumor development by means of inflammatory response [45]. Additionally, transforming growth factor (TGF)-β produced by the cancer cells facilitates the immune suppression as well as its contribution to the metastasis [46]. The factors which affect the immune response to cancer cells are depicted in Fig 1.11.

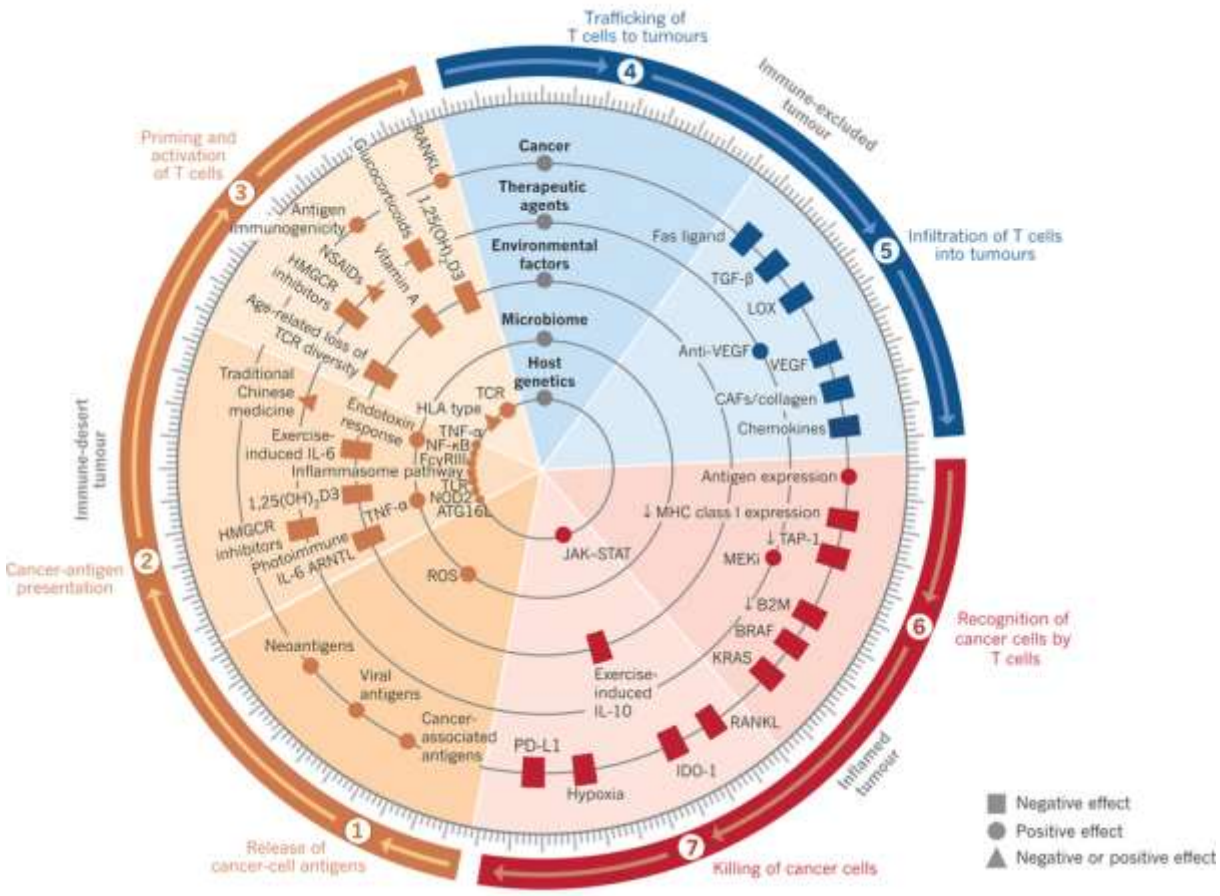


Figure 1. 11 Steps and parameters of killing cancer cells by immune system [46].

1.9 Application of cell culture for mammalian cell lines

In the previous section it was demonstrated that a biological system, such as a cell, functions as a result of many complex dynamic interactions. Contradictory to some scientific fields such as mathematics or engineering, biological sciences usually revolve around the discovery and respective understanding of natural physiochemical phenomena which are not comprehensively known. Therefore, the observation of a biological system is fundamental requirement in biology to discover the underlying mechanisms. In order to proceed with observation, it is necessary to establish a system in which an organism can be studied. Generally, a scientist starts from real tissue samples. Tissue can be both studied in a living body of humans or animals through some techniques such as physical appearance, X-ray radiography, Magnetic Resonance Imaging (MRI), endoscopy, etc.

However, some experiments and studies such as microscopy imaging require a sample tissue to be removed from the body. In this regard, some studies can be conducted that normally result in killing the cell. Nevertheless, sequencing techniques, XRD of proteins, fixed sample optical microscopy, electron micrograph imaging, and others, the data of only the present state of a cell is collectable. Alternatively, it is possible to keep the tissue alive out of the body through artificial adjustment of the living environment, which is referred to as "mammalian cell culture". The applications of mammalian cell cultures do not only have an extensive role in research, but they also have a significant role in biotechnology as well as in bioprocessing. The history of cell culture goes back to 1885, and since then the field of cell culture has been constantly evolving (Table 1.1).

Table 1. 1 History of the animal cell culture

Year	Significant work	Scientist
1885	Maintained embryonic chick cells in a saline culture	Roux
1897	Demonstrated the survival of cells isolated from blood and connective tissue in serum and plasma	Loeb
1907	Cultivated frog nerve cells in a lymph clot held by the "hanging drop" method and observed the growth of nerve fibers *in vitro* for several weeks	Harrisone
1911	First liquid media consisted of sea water, serum, embryo extract, salts and peptones	Lewis and Lewwis
1916	Proteolytic enzyme trypsin for the subculture of adherent cells	Rous and Jones
1923	T-flask as the first specifically designed cell culture vessel	Carrel and Baker
1948	Isolated mouse l fibroblasts which formed clones from single cells	Earle
1949	Polio virus could be grown on human embryonic cells in culture	Enders
1952	Continuous cell line from a human cervical carcinoma known as hela (helen lane) cells	Gey
1955	Nutrient requirements	Eagle
1964	Hat medium for cell selection	Littlefield
1975	First hybridoma capable of secreting a monoclonal antibody	Kohlar and Milstein

Red biotechnology is the terminology used for the application of using organisms for improving the medical processes. Thus, red biotechnology is the production of therapeutics and vaccines as well as tissue engineering and DNA manipulation, or other similar applications (Fig 1.12). Living cells which reside inside the body receive a constant supply of nutrition, oxygen, adjusted pH level, adjusted temperature, and are subjected to the removal of cell byproducts. Once cells are removed from the body, some problems related

to the cell survivability and reproduction arise. The biggest difficulty is the growth of primary cells from patient, and keeping the phenotypic similarities when these cells are transferred to an artificial culture environment. Slow growth rate, slow productivity, sensitivity toward shear stress, and complications in growth medium are among the challenges associated with cell culture. Since mammalian cells are derived from eukaryotes, they still uphold many of their programming in a multicellular environment. Some of these attributes include the apoptosis and telomere shortening of cultured cells. Genetic modification helped the field of cell culture overcome the aforementioned problems, and modern bioreactors are capable of producing bio-products on a large scale [47].

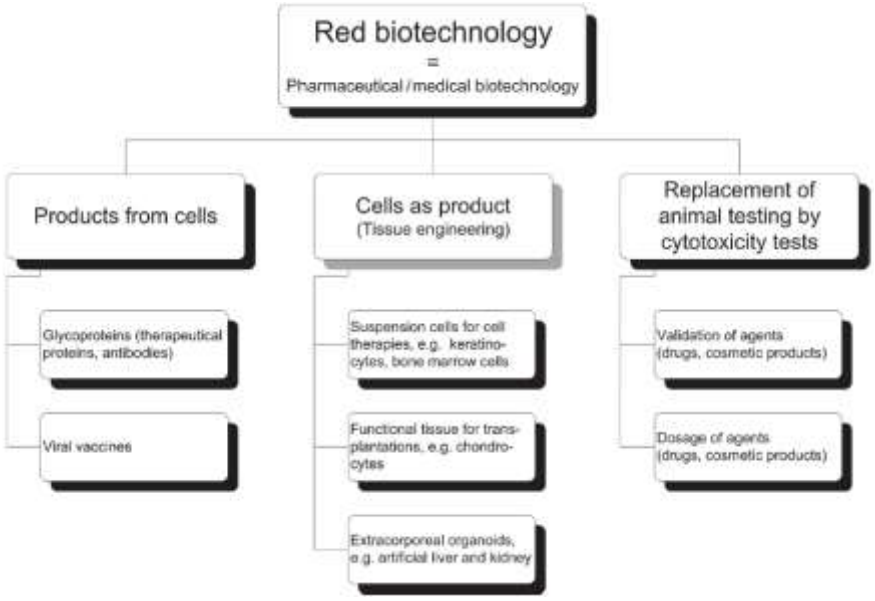


Figure 1. 12 Applications of cell culture in Red biotechnology [47].

1.10 Materials and protocols for cell-culture

In order to manage the handling of the living cells, a set of materials and equipment is needed. Laminar flow hoods are among the essentials while working with the cells. There are two types of laminar flow hoods, namely horizontal and vertical hoods. Both of these hoods, have a filtered air flow field to prevent the contamination of samples. The horizontal hoods are the best to work with cell cultures without the bio-hazardous concerns, since they blow the air toward the operator. However, vertical hoods must be

used when samples contain bacteria or viruses. These hoods have an internal air flow which isolates the internal and external working areas. All hoods are equipped with UV light to disinfect the working area before use, and safety regulations must be considered once the UV light is working. In addition, the air flow must be turned on 10-20 min before use, and the particle filter must be replaced after certain working hours, or according to the air flow sensor [48].

Cells need to be incubated in a suitable atmospheric environment, and this environment is provided by CO_2 incubators. In case of human cells, an incubator is able to maintain the temperature at 37°C all the time. Moreover, a water container is placed in an incubator in order to maintain the relative humidity as close as possible to 100%. The reason for this is the evaporation of the aqueous medium in which cells are kept alive, and consequent changes in chemical concentration which might be harmful for the cells. After all, the optimum pH level for the most cell lines is around 7.4, and therefore, the CO_2 is kept around 5% because of the bicarbonate/carbonic acid buffer in the aqueous medium [48].

In addition, cells need to be kept in an artificial cell growth aqueous medium to grow, generally referred to as medium. This medium must contain a buffering capacity, be isotonic, provide the source of energy and other nutrition, and it must also be sterile. Most mediums contain glucose, fructose, and amino acids as sources of energy. There are two general categories of cell culture mediums: natural and artificial mediums. Natural mediums contain naturally occurring biological ingredients like clots, biological fluids and tissue extracts. In contrast to natural mediums, the artificial mediums require a fine adjustment of chemicals and their concentrations in order to maintain the cell viability.

However, it is possible to optimize the material and expenses in the artificial medium according to the application. Basal media is an aqueous solution containing the minimum chemicals necessary for cell survival, such as: inorganic salts, vitamins, amino acids, anabolic precursors and energy sources as well as trace elements. In addition to this basal medium, supplements can be used to adjust the medium according to each cell type. Supplements may include fatal bovine serum (FBS), antibiotics, hormones, growth factors, transport proteins, and attachment factors (Fig 1.13) [49].

Table 1. 2 Cell dissociation methods and materials.

Procedure	Dissociation Agent	Applications
Shake-off	Gentle shaking or rocking of culture vessel, or vigorous pipetting.	Loosely adherent cells, mitotic cells
Scraping	Cell scraper	Cell lines sensitive to proteases; may damage some cells
Enzymatic dissociation	Trypsin	Strongly adherent cells
	Trypsin + collagenase	High density cultures, cultures that have formed multiple layers, especially fibroblasts
	Dispase	Detaching epidermal cells as confluent, intact sheets from the surface of culture dishes without dissociating the cells
	TrypLE™ dissociation enzyme	Strongly adherent cells; direct substitute for trypsin; applications that require animal origin-free reagents

The cells which are cultured immediately from the original tissue are called “primary cell culture”. These cells, especially in the case of epithelial and fibroblast cells are normally attached to an extra-cellular-matrix which needs to be dissociated. The cell dissociation procedures can be enzymatic or mechanical (Table 1.2). After dissociation, depending on the protocol, cells can be centrifuged in order to remove the enzyme or they can just be diluted in complete medium to be cultured. The subcultures of a tissue are referred to as “subculture” or “passaging”, and depending on the cell line, they will replicate indefinitely, or for a limited number of passages.

The vessels being used for the cell culture process can be made of any biologically inert materials such as glass or polymers. However, in case of polymers, due to the hydrophobicity of polymer materials, they normally need surface treatment. Oxygen plasma by means of microwaves is one of the most frequently used methods to overcome this problem. Contamination of bacteria, fungi, mycoplasma and chemicals must be avoided, so the cell culture environment must be checked for these contaminations routinely [48].

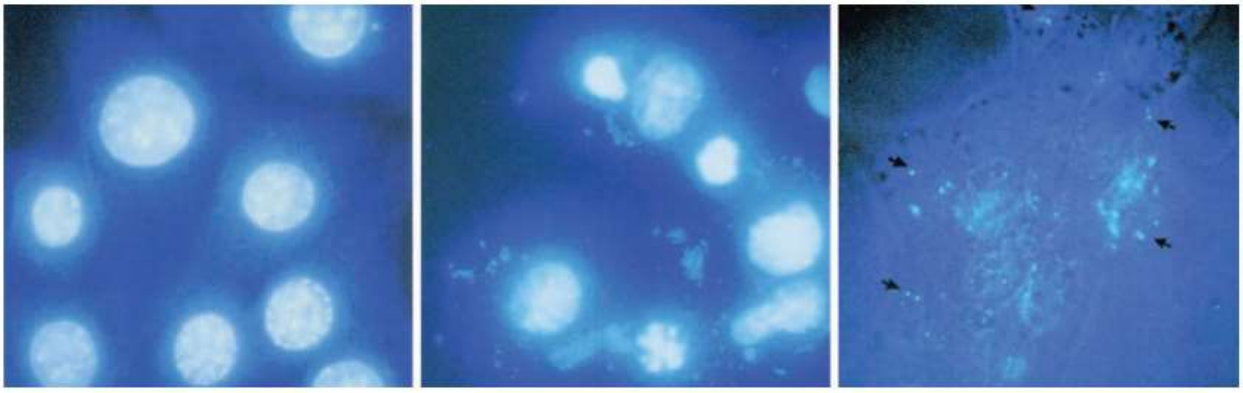


Figure 1. 13 The effect of mycoplasma on cell culture under UV microscopy of nuclei. (A) is the mycoplasma-free sample, (B) and (C) contaminated samples.

1.11 Cancer cell-lines in cancer biology

The developments in the field of mammalian cell culture during past few decades have paved the way for the cancer cell biology through cancer cell lines. HeLa cell line is the oldest human cell line which has been cultured since 1951. HeLa cells were collected from a cervical tumor belonging to Henrietta Lacks, an African-American lady, and taken without her consent. This cell line helped the development of polio vaccine and the discovery of telomerase enzyme, as well as almost 75,000 publications [50]. HeLa cells are still proliferating and they have been for more than 6 decades thanks to the telomere preservation. Nowadays, there are around 1000 cell lines belonging to many different cancers types, and their genomic analysis is available at the cancer cell line encyclopedia (CCLE). Although cell lines do not exactly mimic the characteristics of primary cancer cells, they are a versatile tool for pharmaceutical and cancer studies [51].

1.12 Application of microfluidic devices in cell biology

Understanding the inherent complexity in tumor and its interactions with the healthy tissue as well as with the immune system is the ultimate goal in cancer biology. Thanks to the emergence of micro/nano fabrication and the consequent development of microfluidic devices, the field of lab-on-a-chip (LOC) came to existence in order to provide a more

accurate insight into cancer biology. The original definition of LOCs referred to the downsizing of a laboratory into a chip-sized device. However, this definition can also be extended to micro-total analysis systems (μTAS) which are used for simulating the cancer microenvironment. Therefore, LOCs encompass a range of microfluidic devices which can be used for analytical as well as observatory applications.

Unlike the experiments carried on petri dishes, which can be categorized as 2D cell cultures, microfluidic cell cultures are also capable of performing 3D cell culture. A 3D cell culture is usually referred to as any cell culture that consists of more than one monolayer of grown cells. A 3D cell culture can be used to model a tumor environment, or for modeling the interaction of different tissues or cell types (Fig 1.14). Therefore, one of the most distinguishable features of LOCs is in the studies related to cell signaling. In addition, by modeling a tumor microenvironment, it is possible to have a better understanding of drug barriers, diffusion, and distribution within a tumor.

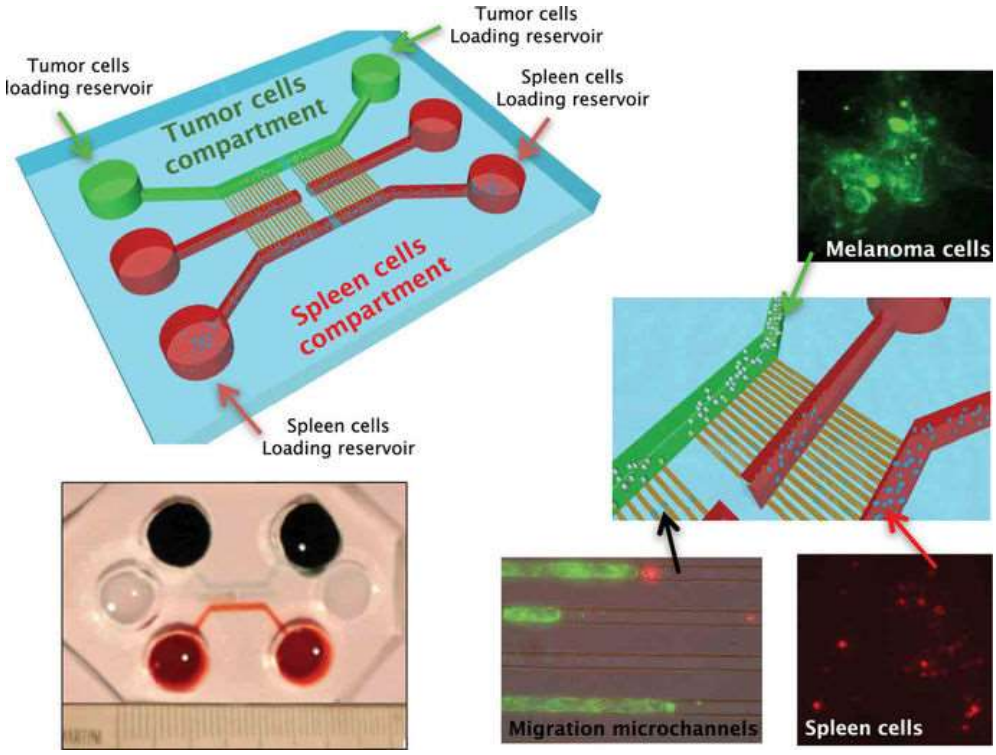


Figure 1. 14 The modeling and co-culture of cancer-immune cells [52].

As previously mentioned, one of the most critical stage in cancer is metastasis, and the formation of secondary tumors. Therefore, one of the most important applications of microfluidic devices in cancer biology is modeling the metastasis. In this respect, a microfluidic device can be equipped with specially designed surfaces and obstacles, special coatings, co-cultured and multi-layered cells, directional chemical gradient, polymer scaffolds, etc. As a result of these types of manipulation toolboxes at micro-scale, one can study the behavior of different cell lines such as: mobility, deformability, adherence, diffusion in tissue, as well as the effects of different hormones and the other parameters [53].

The other major application of microfluidic systems is for analytical applications. LOCs are often capable of single cell capture, manipulation and growth which can be combined with many analytical and diagnostic microdevices. Optical, magnetic, electrical, and mechanical manipulations can be applied to LOC to control a variety of processes. The application of optical mechanisms usually relies on the light absorption to detect chemicals, similar to the flow cytometery or Raman spectroscopy, as well as optical tweezers. The emergence of labeled magnetic nanoparticles (MNP)s is another mechanism which is usually used for cell sorting and separation. The electrical field is a label-free tool for cell manipulation which can be used for the movement of droplets through DC field, or for cell sorting through dielectrophoresis (DEP). Mechanical systems such as shear and inertial forces or even acoustic waves are yet another means of label-free cell sorting [54].

Chapter 2:

EXPERIMENTAL SECTION

2.1 Introduction to microfluidic cell culture

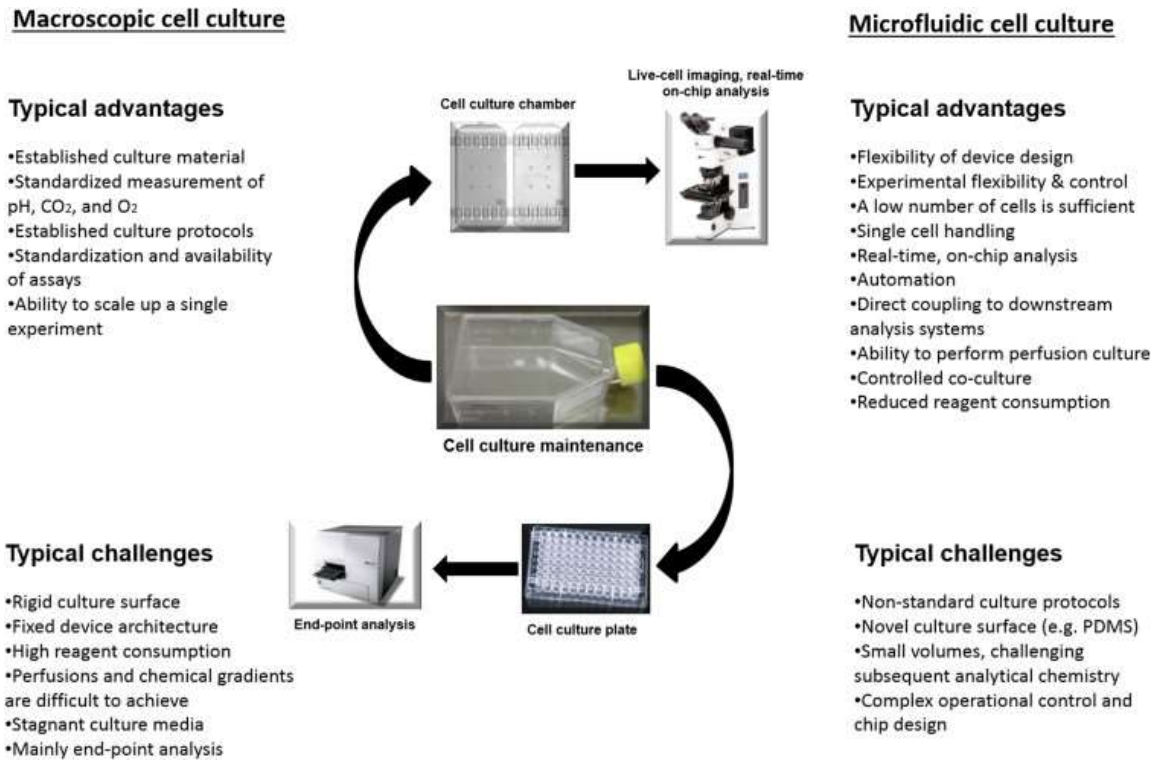


Figure 2. 1 Advantages of micro-scale cell culture versus macro-scale cell culture [55].

Microfluidic perfusion cell culture is a comprehensive way of studying cell biology; however, this technology is currently still under development. In most experiments related to mammalian cells, it is necessary to culture the cells in order to undertake any experiment. The main reason for this is that the adherent cells, including most of tumor cells, are normally static through cell-to-cell or cell-to-ECM junctions. Therefore, modeling of *in vivo* conditions usually requires the cells to form their junctions and means of cellular communication. In addition, once cells are being transformed into a new growth environment they are stressed mechanically or enzymatically. In a petri dish, cells start to attach to the surface after a few hours, and after a day, most of the cell can be used for the experiment. In contrast to petri dish cell culture, many challenges and complexities exist for the micro scale cell culture, which must be addressed accordingly (Fig 2.1).

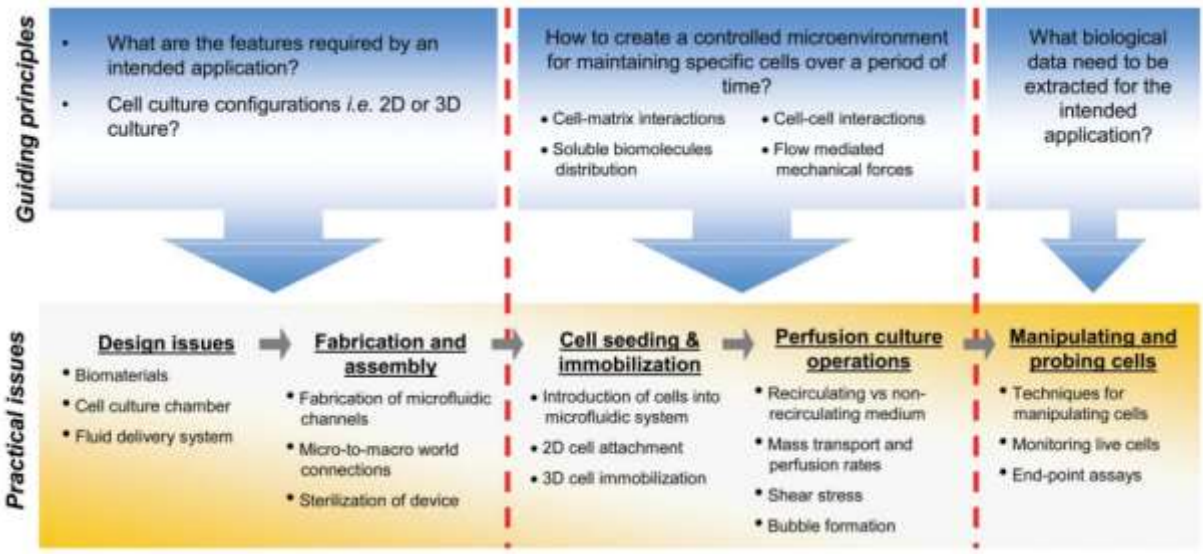


Figure 2. 2 Design and manufacturing considerations for microfluidic cell cultures [56].

2.1.1 *The design considerations*

Similar to the other fields of engineering, it is possible to segment the design and manufacturing process of microfluidic cell cultures. The first step towards the design of a microfluidic system is determined by the required features of the targeted experiment. Based on what type of experiment is going to be carried out, one should be able to find answers to some fundamental design questions (Fig 2.2), for instance, whether a 2D or 3D cell culture is needed; or what the geometry of the cell growth chamber is; or, what is the appropriate kind of material to be used for manufacturing? Also, what is the most effective and efficient way for manufacturing the device? Furthermore, one would also inquire how to proceed with sterilization, how to maintain the cells at the best living condition, and how to perform the experiment and monitor the variables.

During the process of drug design, the proposed medicine is first tried on a 2D monolayer cell culture, then on animal models, and finally in a clinical trial. However, only 10 % of drugs which are proven through 2D cell cultures and animal models show efficacy during clinical trial, which is a sign that *in vitro* experiments may often prove to be unrealistic. However, 3D cell culture in contrast to 2D cell culture has more resemblance to the *in vivo*

environment, since 2D tissue does not occur normally in a living body. 3D cell cultures can be established as an agglomeration of cells into a 3D form, or cells can be embedded into a matrix or scaffold. The scaffold-free 3D cell culture can be realized through forced floating, the hanging droplet method, and agitation (Fig 2.3). Biocompatible materials including naturally derived polymers, or synthetic polymers such as: polyethylene glycol (PEG), polyvinyl alcohol (PVA), polylactide-co-glycolide (PLG), and polycaprolactone (PLA) are usually used in case of cell culture equipped with a matrix or scaffold, which can be cast or bio-printed [57]. However, using 3D cell culture in microfluidic systems is an extra challenge owing to the inherent complexity in design and manufacturing. In addition, in cases of single cell studies, a 3D cell culture cannot be defined. Therefore, it is more desirable to avoid 3D cell culture in microfluidic design, unless it is an indisputable design requirement.

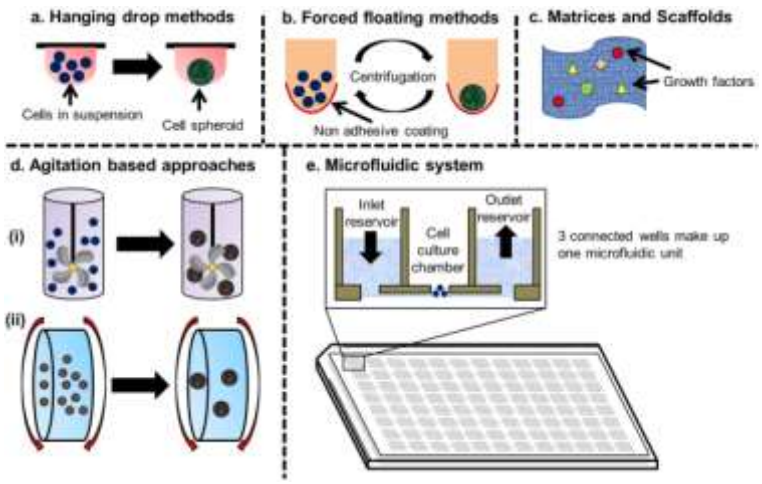


Figure 2. 3 3D cell culture techniques: a) hanging drop, b) forced floating, c) matrices and scaffolds, d) agitation, e) microfluidic system [58].

Bio-compatibility of materials being used for the cell culture is an out-of-question issue. The first generations of microfluidic devices were made of glass or quartz. These materials can be manufactured by means of standard lithography and thereafter wet etching. Although glass is the best material in terms of bio-compatibility, chemical resistance and

lack of deformation, the manufacturing process is usually expensive. Alternatively, polymers can also be used in manufacturing of microfluidic devices.

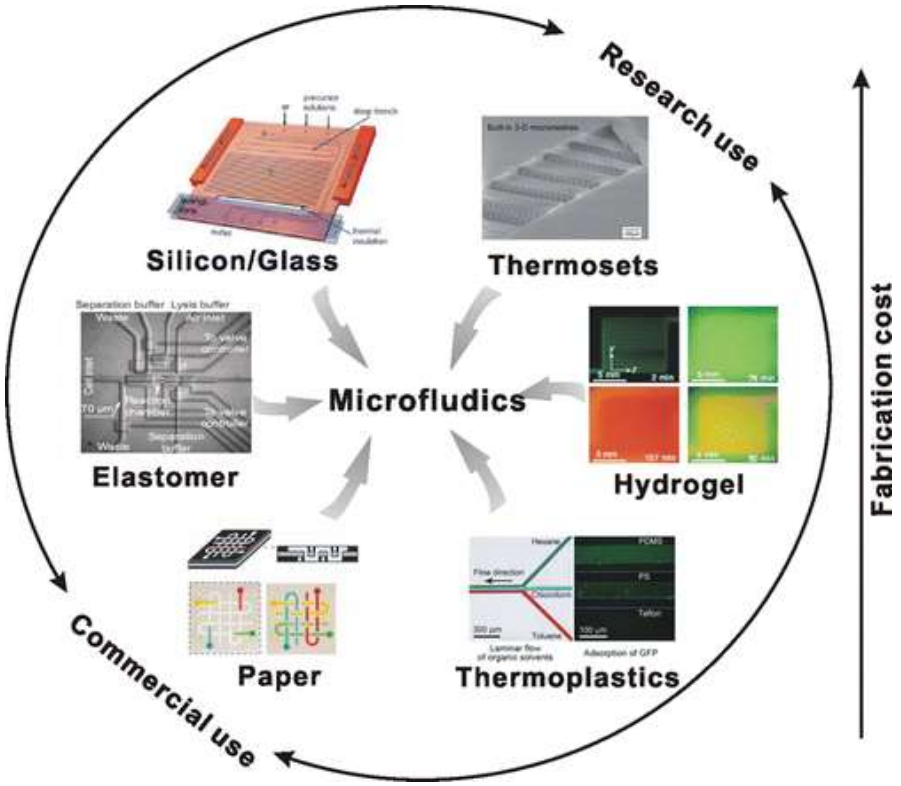


Figure 2. 4 Fabrication cost of bio-compatible materials for microfluidic systems [59].

2.1.2 *Materials for lab-on-a-chip applications*

Currently polydimethylsiloxane (PDMS) is the most prevalent elastomer in micro-fabrication due to its transparency, ease of use and low cost [55]. Moreover, PDMS is gas permeable which is necessary for a cell culture environment. The flexibility of this material is another advantage which allows use of flexible parts like valves and connectors. However, careful attention must be paid during PDMS preparation, since this elastomer is a fast cross-linking polymer, and if it does not cure correctly, the unreacted oligomers can interfere with the experiments. The manufacturing process of PDMS microfluidic devices

starts with the molding process and thermal curing. The obtained PDMS part can later on be attached to other parts by means of oxygen plasma or suitable adhesive.

In addition to PDMS, some other polymers such as polystyrene (PS), polycarbonate (PC) and polymethylmethacrylate (PMMA) are also being used in micro-fabrication, but like other thermoplastics they require a relatively high temperature, and thermally stable molds. Thermoset such as SU-8, and hydrogels for 3D cell culture are also categorised as polymer materials for microfluidic devices. Paper based materials can also be used in microfluidic devices; however, they are not very popular for cell culture applications (Fig 2.4) (Table 2.1) [59].

2.1.3 *Surface modification methods*

With the further developments in the field of micro/nano technology it is possible to add another level of manipulation to the microfluidic devices. Besides the main materials being used in LOCs, surface modification of materials is another appliance which brings an extra flexibility of design. Cells which reside *in vivo* condition are attached to an extra cellular matrix as well as to base membrane, which affects their general behavior. The embedding of nano-topography in cell cultures has shown that the surface adjustment at nano-scale could promote cell adhesion, elongation and migration. Random nano-topography can be generated through a chemical etching process, while self-assembly of co-block polymers would generate more organized structures; however, electron beam lithography (EBL) is usually used in case of extremely organized nano-structures [60].

The other series of versatile techniques which is very useful in changing the surface properties of microfluidic channels is chemical surface modifications. The main obstacle in using synthetic polymers is the surface hydrophobicity. Oxygen plasma is a general method of surface modification by means of which one of the oxygen radicals makes a covalent bond with the surface of the structural material while maintaining the other valence as a radical. Therefore, by using oxygen plasma not only it is possible to attach different surfaces together, but also it is possible to reduce the surface hydrophobicity. In addition, oxygen plasma can be used as a precursor for further surface functionalization.

Table 2. 1 properties of different materials for microfluidic devices [59].

property	silicon/glass[a]	elastomers	thermoset	thermoplastics	hydrogel	paper
Young's (tensile) modulus (GPa)	130–180/50–90	~0.0005	2.0–2.7	1.4–4.1	low	0.0003–0.0025
common technique for microfabrication[b]	photolithography	casting	casting, photopolymerization	thermomolding	casting, photopolymerization	photolithography, printing
smallest channel dimension	<100 nm	<1 μm	<100 nm	~100 nm	~10 μm	~200 μm
channel profile	limited 3D	3D	arbitrary 3D	3D	3D	2D
multilayer channels	hard	easy	easy	easy	medium	easy
thermostability	very high	medium	high	medium to high	low	medium
resistance to oxidizer	excellent	moderate	good	moderate to good[c]	low	low
solvent compatibility	very high	low	high	medium to high	low	medium
hydrophobicity	hydrophilic	hydrophobic	hydrophobic	hydrophobic	hydrophilic	amphiphilic
surface charge	very stable	not stable	stable	stable	N/A	N/A
permeability to oxygen (Barrer[d])	<0.01	~500	0.03–1	0.05–5	>1	>1
optical transparency	no/high	high	high	medium to high	low to medium	low

[a]Photosensitive glass can be considered as thermoset. [b]Most of the materials can be fabricated by laser ablation, but compared with those obtained with lithographic or molding methods the ablated features usually have a rougher surface and are often misshaped. [c]Excellent for Teflon. [d]1 Barrer = 10^{-10} [$cm^3 O_2$(STD)] cm $cm^{-2} s^{-1} cmHg^{-1}$.

Chemical vapor deposition (CVD) is another method in surface modification achieved through depositing a thin layer of polymer materials. In this method, usually a mixture of monomer, cross-linking agent and initiator is introduced under an inert atmosphere in order to form a thin layer polymer which is covalently attached to the surface. Some other techniques such as layer by layer deposition (LBL), use of surfactants, graft polymerization, and bulk addition of nano-materials are among the other surface modification methods. Additionally, if the aforementioned surface modification techniques are combined with patterning techniques such as UV lithography or PDMS stamp, it is possible to generate patterns with different physiochemical properties [61].

2.1.4 *Geometry design for microfluidic cell cultures*

Contrary to the limitations in material selection, microfluidic devices can benefit from a variety of geometrical design. Within the geometry design, mechanical, electrochemical and optical considerations can be applied to a microfluidic system. Therefore, the main design goals can be achieved through the correct selection of materials and chip design. As a result, today's microfluidic LOCs are capable of cell culture, treatment, selection, lysis, separation, and analysis (Fig 2.5). Each of these steps can be done separately, or they can be integrated into a single chip. Segmentation of an overall complex design into a set of simple designs is usually the best approach to design an integrated system. In this way, each segment of an LOC will be designed and experimentally optimized, and eventually each part will be integrated into a comprehensive chip depending on the design objectives. Although the physics and engineering behind different systems which can be integrated into a LOC is worth probing, the focus of this thesis is limited to the design of cell cultures.

A significant portion of studies focuses on single cell resolution in the field of mammalian cell culture on LOC. The single cell resolution LOC is usually distinguished through the capability of LOC to study each cell individually. Therefore, there is a variation of designs in microfluidic devices which can be recognized as a single cell. Nonetheless, all the single cell resolution LOCs have a common feature which is an enclosed micro-chamber to grow

the cells. The simplest chamber is a straight micro-channel, where cells attach to the bottom of the channel and grow. However, it is desirable in most of the designs to introduce some features to distribute, organize, and localize the cells (Fig 2.6).

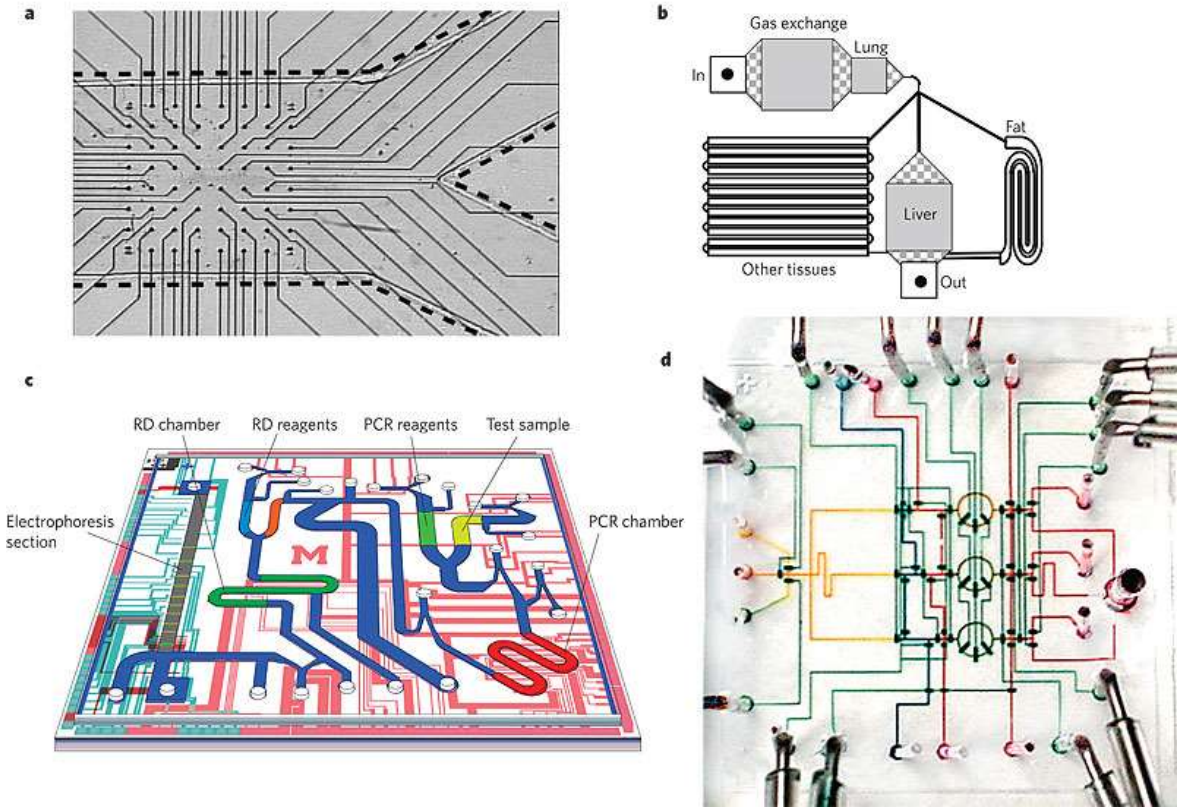


Figure 2. 5 Different complex lab-on-a-chip (LOC) designs. a) microelectrode array for neural activity measurement, b) an integrated organ on chip, c) DNA analysis LOC equipped with PCR and electrophoresis, d) DNA extraction and purification chip [62].

Although it is possible to sort the cells in microfluidic channels by means of optical, electrical, acoustic and chemical labeling, these methods are not considered as passive methods. In contrast, passive methods of cell sorting such as: size selection, gravitational/centrifugal forces, and hydrodynamic forces are less prone to introducing undesirable errors into the experiment. The size selection could be considered as the simplest method for cell trap in LOCs. In this method, cells are trapped by means of specially designed geometries which are capable of separating a cell or a few cells from the flow (Fig 2.7).

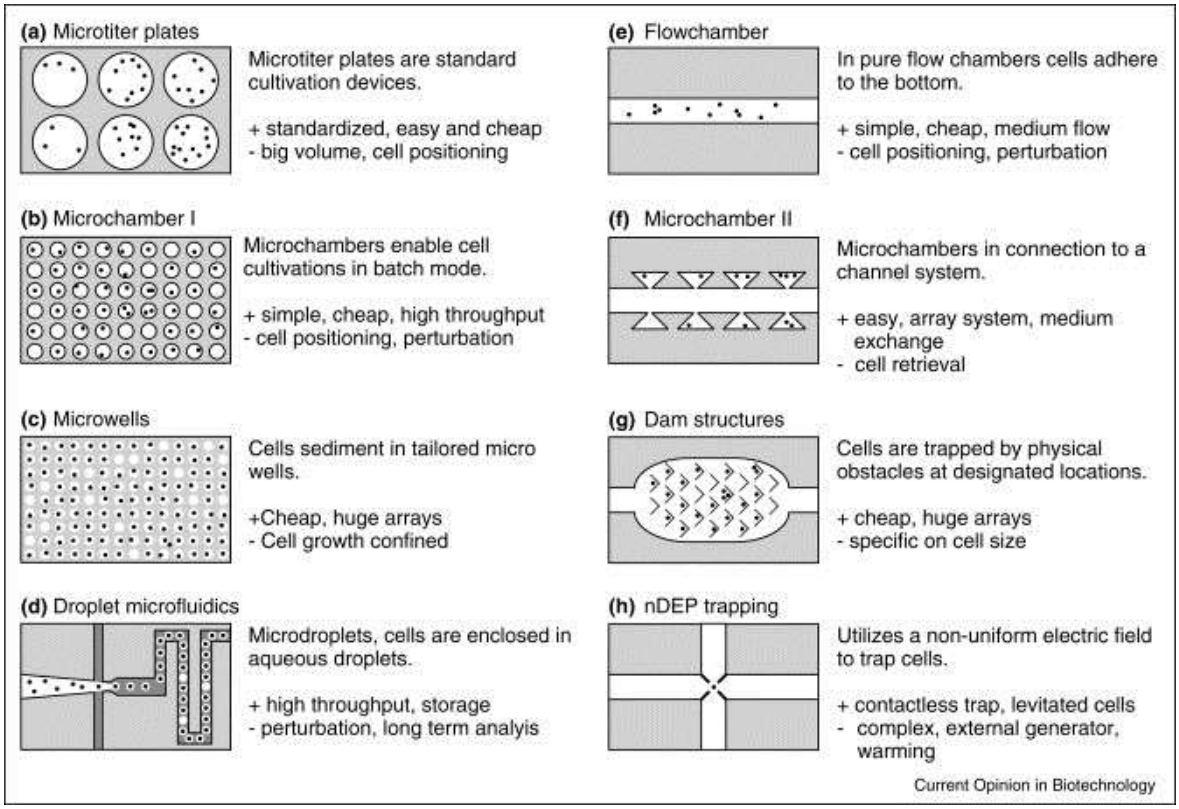


Figure 2. 6 The advantages of micro cell culture in micro-plates (a,b,c) versus microfluidic devices (d,e,f,g,h) [63].

However, one of the problems with this type of design is the efficiency in trapping. In addition, careful attention must be paid to the velocity field inside the chamber, since both the direction and magnitude of the flow affect the cell trapping. Furthermore, the shear stress, generated by the flow, must not exceed the limit for it leads to the distortion of the cell and its consequent damage. It is worth mentioning that the principle of design for gravitational/centrifugal cell trapping is also based on the device geometry, in which the flow can be generated by means of gravitational/centrifugal forces.

2.1.5 Hy*drodynamic forces in a microfluidic system*

The involvement of hydrodynamic forces in LOCs for cell culture applications is indisputable, since in all of these devices cells are suspended in a liquid medium. Therefore, understanding these forces is not only important to predict the forces being applied to cells, but it is also important to exploit these forces for cell trapping and other applications.

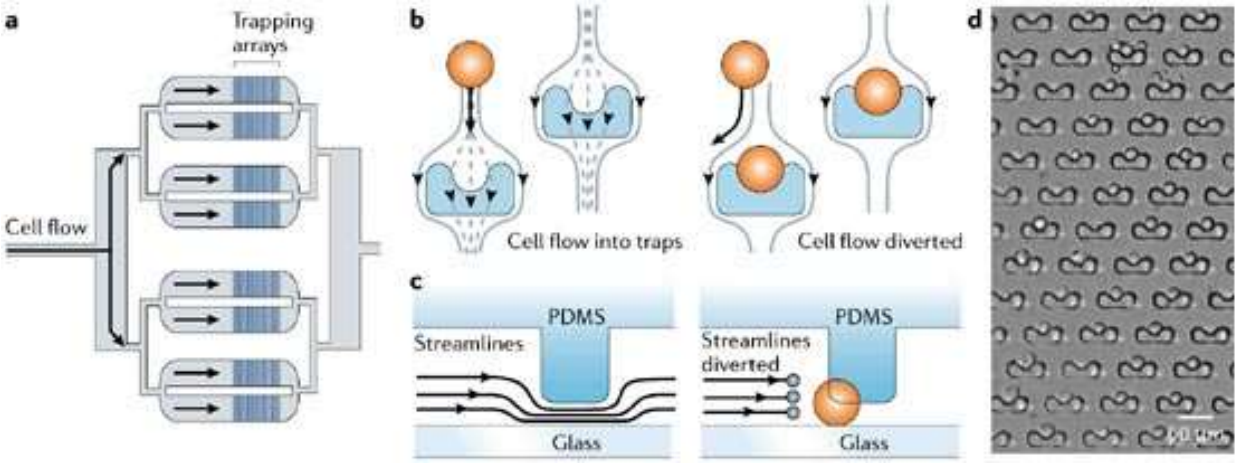


Figure 2. 7 Cell trapping by means of geometrical design. a) overall design, b,c) mechanism of trapping a single cell, d) micrograph of the device [64].

Inertial effects in a microfluidic system containing particles can be described by the Reynolds number ($Re=\rho UDh/\mu$), which is the ratio of inertia to viscosity. For a particle moving in a channel, two counteracting forces determine the position of the particle. One of these forces is the wall repulsion force which pushes the particle away from the surface, and the other is the shear-gradient force which tends to divert the particle away from the center line. Viscoelasticity is another phenomenon in non-Newtonian flows which, through its fluctuations, can allow for particle focusing in a micro-channel. In addition, cell deformability, which is defined by the ratio of cell internal viscosity to the viscosity of the medium ($\lambda=\mu_{in}/\mu_{out}$), is yet another means of cell manipulation. "Dean flow" is a pressure gradient generated in curved channels and through centrifugal force applying to a passing fluid inside the channel (Fig 2.8) [65].

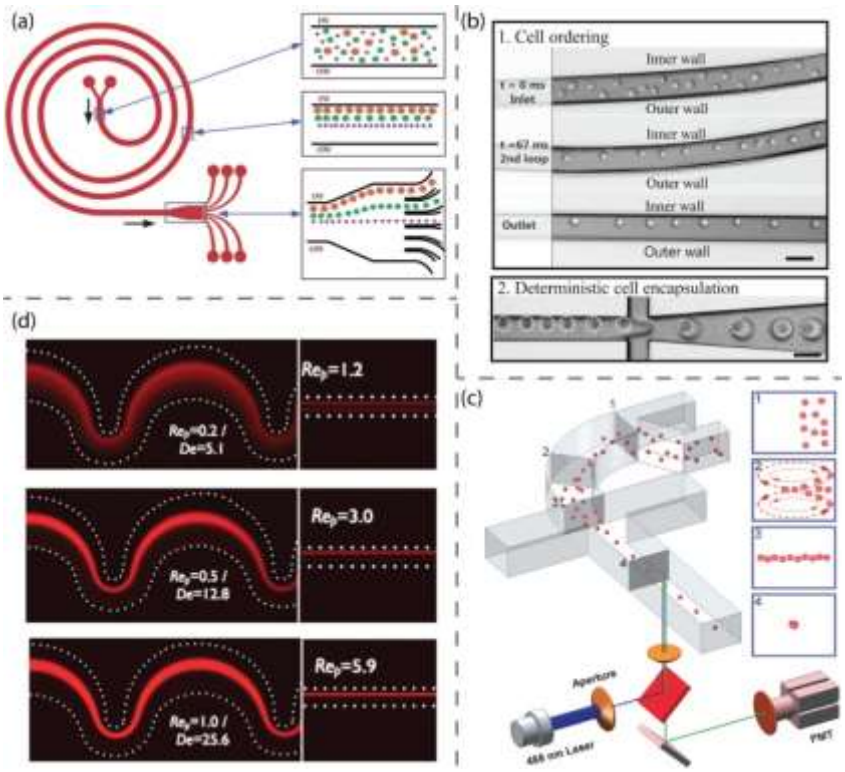


Figure 2. 8 Applications of Dean flow in microfluidic: a) particle separation, b) cell ordering for encapsulation, c) cell focusing combined with cell spacer for fluorescent microscopy d) cell focusing with respect to flow [65].

2.1.6 *Medium supply for microfluidic cell cultures*

The other criterion of design is the medium supply, used in order to provide a fresh medium and remove the toxic waste from the culturing area in order to mimic tumor microenvironment. Medium supply can be divided into cell seeding, cell culture and cell testing. The most critical step in any experiment is cell seeding, which refers to loading the suspended cells in medium into the growth chambers. The reason for this is in part the contamination concerns, which can be addressed by means of sterilization of LOC and tools, as well as tubing. The other reason is the challenges involved in the cell loading, occurring due to some factors such as undesirable particles, air bubbles, unconditioned cell environment out of the incubator, physical stress during loading, the efficiency of cell loading, etc. However, once cells are loaded, a continuous laminar flow medium can be supplied to the chip through diffusion, gravitational or injection pumping. During the first few hours, the flow can be as low as possible in order to allow cell to attach, and, later on,

it can be increased to the optimum flow rate. It is important to design the experiment and chambers in such a way as to ensure enough space for the cells to grow; otherwise, cells can block the medium flow or increase the shear stress. During the fluorescent imaging, a fluorescent die can be added to the medium. An important issue is that fluid connections need to be connected as gently as possible, since connections act as pumps, and cells can be damaged by exceeding shear flow [56].

2.2 Simulation and design

The main objective of this thesis is the development of a microfluidic cell culture array combined with a chemical gradient generator in order to study the effect of different drug concentrations on cancer cells at single-cell resolution. Therefore the design process is divided into two paths, the first one is design of cell culture array, and the second one is the gradient generator. The cell culture design mostly relies on the the laminar flow design, and on the mechanisms which enable cells to position and count, while the design of gradient generator is mostly concerned with chemical diffusion. Therefore, the following is a description of the employed design principles, and the simulation of proposed systems, before manufacturing and actual experiments.

In fluid dynamics the continuum approximation is the fundamental assumption that the properties of a fluid are the same in all directions. The continuum approximation remains valid for the calculations of microfluidic systems. In terms of the ratio of inertial forces to the viscous forces, the flow can be divided into laminar and turbulent regimes. This criterion is defined by means of the Reynolds (Re) number, which is a dimensionless number.

$$Re = \frac{\rho \times U \times D_h}{\mu} = \frac{U \times D_h}{\nu} \qquad \text{(Eq 2.1)}$$

In this equation, ρ *(kg/m^3)* is the density of fluid, *U (m/s)* is the velocity, D_h *(m)* is the hydraulic diameter, μ *(N.s/m^2)* is dynamic viscosity and ν *(m^2/s)* is the kinematic viscosity.

Therefore, a laminar flow satisfies the condition of Re < 2100. In fact, in most microfluidic systems, due to the small value of D_h, flow is considered as laminar flow and usually Re < 1.

Laminar flow is advantageous in terms of low drag forces and predictability of flow in most microfluidic applications. However, in the case of chemical reactions and mixture, laminar flow is not very useful and the mixing process usually relies on the diffusion transport process. Diffusive transport occurs due to molecular oscillation, and, as a result, a chemical gradient in the flow will be homogeneously mixed. Since the mixing phenomena in LOC devices is a combination of diffusion and the bulk convection of the fluid, it is possible to define the ratio of convective mass transfer to diffusive mass transfer. This dimensionless number is referred to as the "Sherwood number" (*Sh*).

$$Sh = \frac{k \times d}{D} \qquad \text{(Eq 2.2)}$$

Where k (m/s) is the convective mass transfer coefficient, d (m) is the channel diameter, and D (m^2/s) is the diffusion coefficient. For most of the microfluidic systems, the dimensions are very small, which results in the tendency of a mixing process mostly relying on diffusion rather than on convection. The diffusion in a liquid can be explained by Fick's law, which correlates molecular flux with chemical concentration, time, and location. In this formula, the flux of a chemical (J) is proportional with the chemical's concentration. In addition, the rate of change in concentration of chemicals in a specific location is proportional with the second derivative of concentration. In Fick's first and second laws (**Eq 2.3** and **Eq 2.4**), J ($mol/m^2.s$) is the diffusion flux, D (m^2/s) is the diffusion coefficient, C (mol/m^3) is the chemical concentration, x (m) is the position, and t (s) is time.

$$J = -D \frac{dC}{dx} \qquad \text{(Eq 2.3)}$$

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2} \qquad \text{(Eq 2.4)}$$

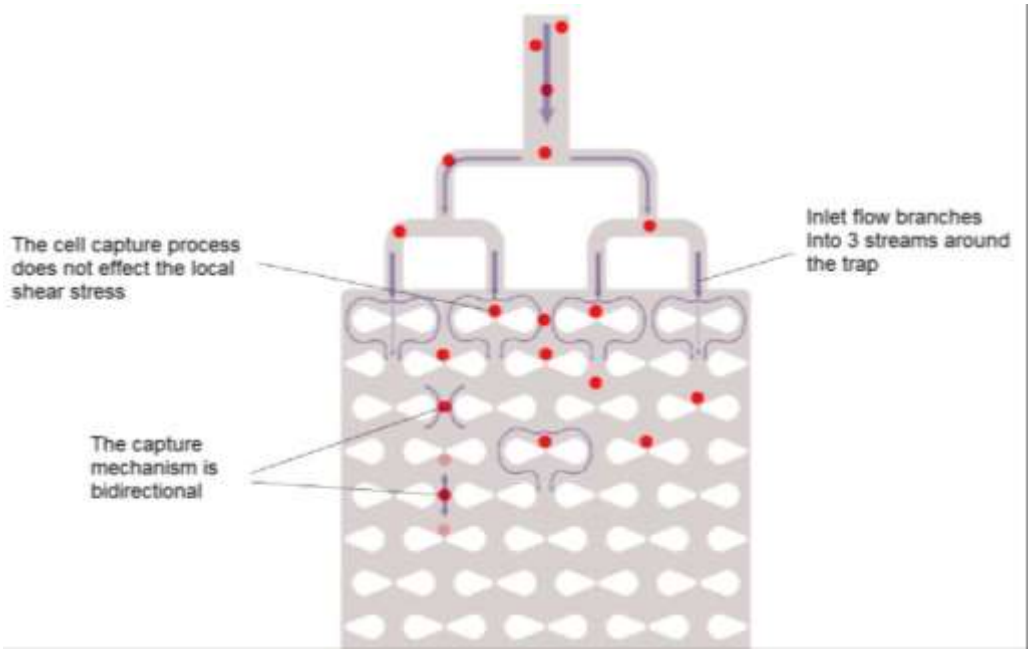


Figure 2. 9 The principle of operation of the first proposed micro-chamber cell culture design (red dots represent the cells).

2.2.1 *The first micro-chamber design*

In order to design the cell culture chamber at single cell resolution, two hypothetical designs were proposed. In the first design, a bidirectional single cell trapping mechanism was proposed in which each cell could be trapped by means of the direction of the medium (Fig 2.9). In this design, cells are branched into four micro-channels after passing through a 50 (μm) channel (Fig 2.11). The main inlet is meant to be relatively narrow in order to uniformly distribute the cells during branching. Each branch is 20 (μm) in diameter which is equal to the diameter of epithelial cells, and the reason for this is to focus the cell exactly at the trap position. Once a cell is positioned the trap is not theoretically capable of maintaining a second cell due to the asymmetrical flow distribution over the second cell. Therefore, the second cell inevitably circumvents an occupied trap, and while it is passing between two traps, it becomes focused again to occupy the next trap. This design is also meant to retain its cell trapping mechanism in case of an occasional reverse flow, which is very common during cell loading.

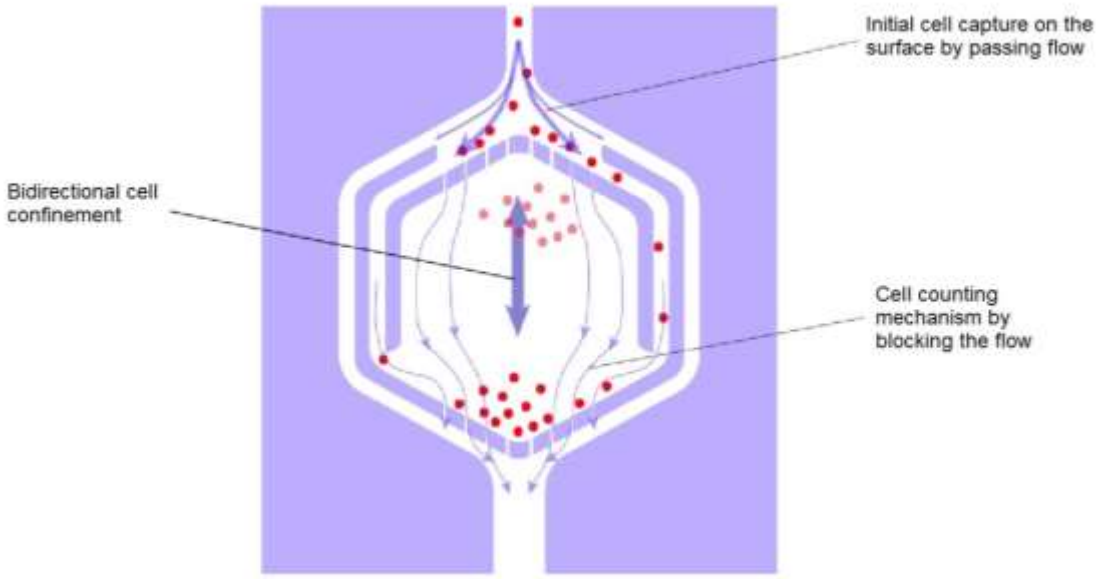


Figure 2. 10 The principle of operation of the second proposed micro-chamber cell culture design (red dots represent the cells).

2.2.2 *The second micro-chamber design*

Alternatively, a second design was postulated in case of the failure of the first design (Fig 2.10). In this design, cells enter the chamber through a 50 (μm) (Fig 2.12); however, this design acts as a relatively microfluidic dam, and the chamber consists of an arrow structure, which is partially contained by a V-shaped structure. In this design, the inlet flow is divided into five streams, and as cells leave the inlet channel, the stream pushes them on an arrow structure which has flow passages smaller than the diameter of the cells. The reason for these passages is to keep the cells on the arrow structure on the one hand and on the other hand to confine the cell inside the chamber once they are trapped. Once cells adhere to the arrow structure, the flow will guide them into two symmetrical channels which lead the cells into the bottom of the V structure. The bottom of the V structure also has passages, through which the cells cannot pass. Therefore, once a certain number of cells occupy the bottom of the V structure, the flow in the central area of the channel will be blocked. Consequently, no new cells will be trapped, and the excess cells will bypass the trap toward the chamber outlet.

a)

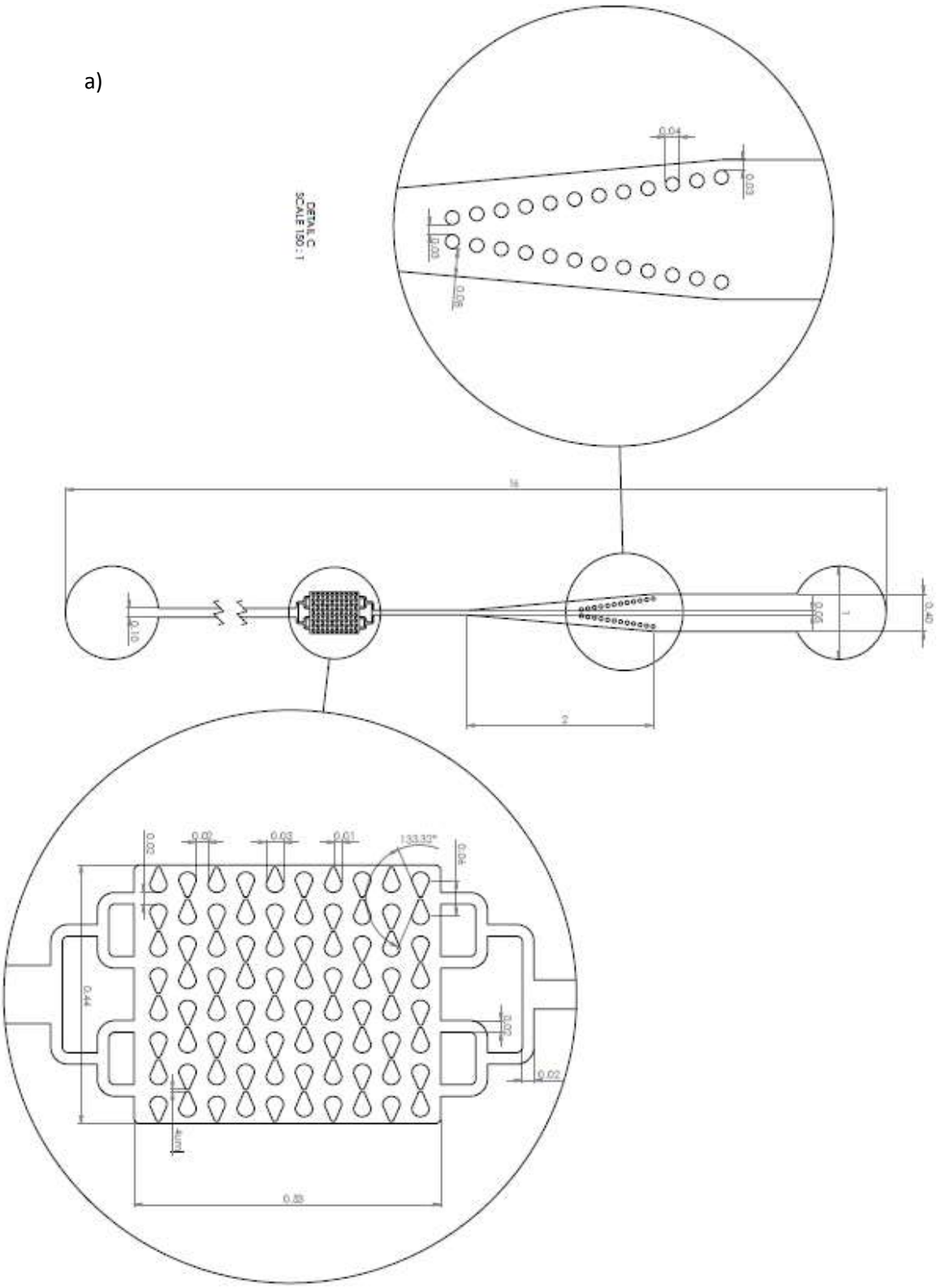


Figure 2. 11 Design specifications for the first micro-chamber design.

b)

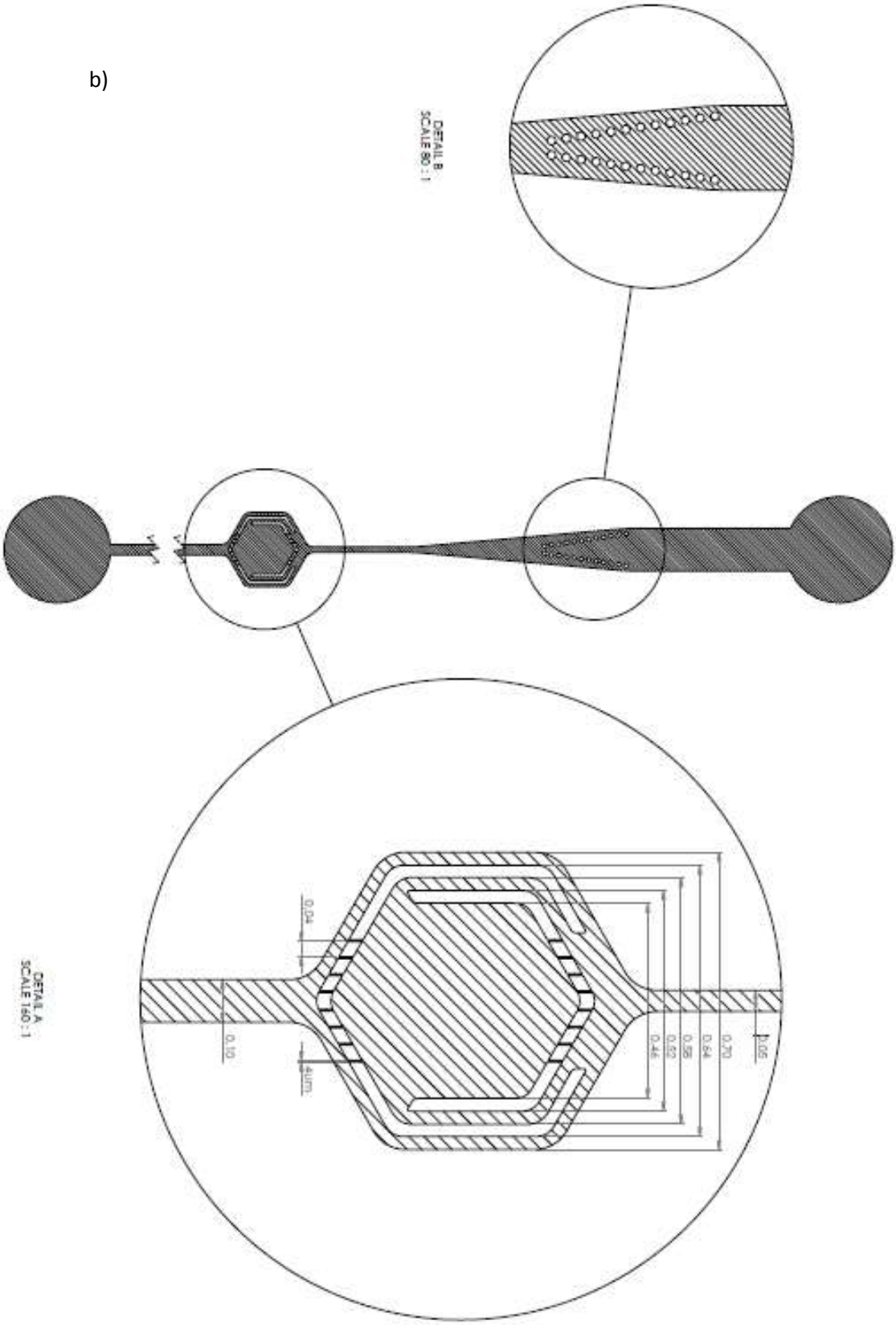


Figure 2. 12 Design specifications for the second micro-chamber design.

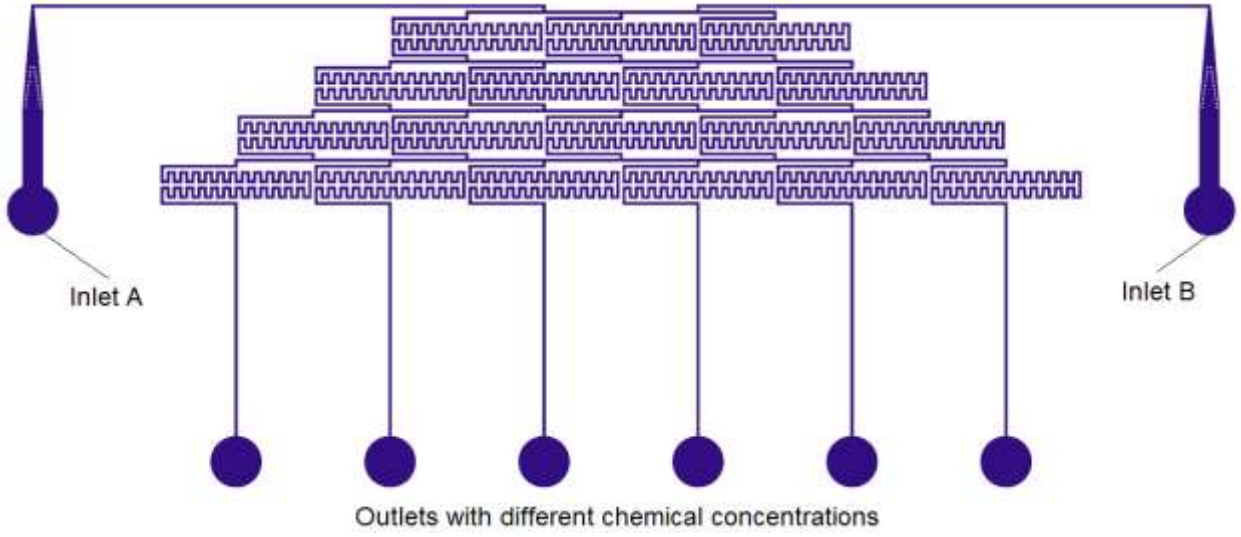


Figure 2. 13 The "Christmas tree" design for the cell microfluidic cell cultures.

2.2.3 *The design for the chemical gradient generator*

It is necessary to combine microfluidic cell cultures with a chemical gradient generator in order to undertake experiments which require applying a known chemical concentration to the cells. Therefore, the design for the chemical gradient generator was established based on the "Christmas tree" design, which is a well-known design due to its precise concentration dosing and gradient generation [66]. The designed platform is equipped with two inlets with different chemical concentrations, and six outlets with chemical concentration distribution of 0%, 20%, 40%, 60%, 80% and 100% in the outlet (Fig 2.13). The channel diameter in the whole chip was kept constant at 60 (μm) in order to prevent the variation of flow velocity across the chip. Similar to any other "Christmas tree" design, each inlet branches into two streams, and as two streams combine with each other, they generate a concentration which is the average of both streams. It is theoretically possible to generate an infinite number of concentrations by continuing the process of dividing and combining streams; however, the number of outlets will always be an odd number.

2.2.4 *The simulation of microfluidic systems*

The analytical method which was mentioned previously has a limited application in microfluidics due to the fact that in a microfluidic system the geometries are usually complex, and are coupled with the other parameters at the same time. In contrast, computational fluid dynamics (CFD) is able to solve complex fluid dynamics problems by using numerical analyses which are embedded in a computer program. A CFD software usually consists of a pre-processor, a solver, and a post-processor.

The pre-processor is the unit which connects the user to the solver. The simulation process starts with the physical portrayal of the original system in the software. The processing of a model depends on the computational power of the computer which is being used, as well as on the duration that computation takes place in. Therefore, it is always desirable to simplify the computer model. These simplifications can be achieved through segmentation of a model, use of 2D model over 3D, elimination by symmetry, etc.

Most of the computer-aided design (CAD) softwares today such as AutoCAD®, CATIA®, Autodesk®, SolidWorks® and others are capable of undertaking the 2D and 3D design and dimensioning. Once the geometry of a physical model is defined, this geometry needs to be divided into cells, mesh or grades in order to apply numerical methods. Meshing a model can be done automatically by the software, or it can be defined by the user. In order to completely define a physical model, the user must define the fluid properties, initial conditions, and the parameters of the solver.

The solver consists of algorithms which can solve the governing fluid dynamics equations in CFD. There are many numerical methods used for solving these equations, but the most prevalent methods are the finite volume method (FVM) and the finite element method (FEM). FVM is more suitable for solving CFD applications, while FEM is usually preferred for MEMS applications. Once the model has been solved for the given values, the obtained values such as velocity, pressure, concentration and others can be translated into visual graphs, curves and tables through post-processing [67].

COMSOL multi-physics is a simulation platform with CAD capabilities. This platform is capable of solving partial differential equations through the finite element method, and it is

also capable of solving fluid dynamics, chemical, electrical, optical, mechanical, acoustic, magnetic and other physical problems. This software is also able to connect to MATLAB and many CAD programs as well as custom-built physics interfaces. Therefore, due to the flexibility and ease of use, COMSOL multi-physics has been employed for the simulation of the proposed platforms in the present study.

2.2.5 *The simulation results in COMSOL*

The finite element modeling was performed using commercial COMSOL multi-physics software, version 5.1. The flow field and chemical concentration throughout the whole chip was calculated using the Navier-Stokes and Fick's law equations, predefined in COMSOL platform. Physical models in terms of laminar flow and chemical species were employed in the simulation through the stationary, single-phase, incompressible laminar flow, coupled with the transport of diluted chemical species physics.

The flow field and initial values for the solid walls were set in all directions, as 0 (*m/s*) and 0 (mol/m^3), with non-slip and no flux conditions in the case of the cell culture chamber. For the inlet boundary condition of the micro-chamber, the flow volumetric rate was considered 10 ($\mu l/hr$), and the outlet pressure was set to zero (*Pa*) for both designs. The simplified three-dimensional model was used to simulate the flow field and the velocity distribution (Fig 2.14) in the micro-chamber designs. The fluid was water, with a density of (ρ) of 10^3 (kg/m^3) and dynamic viscosity (μ) of 8.9×10^{-4} ($N.s/m^2$). The flow inlet was considered at the top, and the flow outlets ate the bottom of geometries.

In the case of the Gradient Generator, the inlet pressure was set to 10 (*kPa*), and the chemical concentration was 0 and 1 ($mole/m^3$) for the two water inlets of the gradient generator. The boundary condition of the outlet was set to be pressure-outlet with zero static pressure and outflow. By using convection and diffusion equations, the pressure and chemical concentration within the gradient generator were simulated. It is evident from the simulation that the uniform pressure distribution along the gradient generator leads to precise chemical concentrations of dilute species in the outlet ranging from 0, 0.2, 0.4, 0.6, 0.8 and 1 ($mole/m^3$) (Fig 2.15).

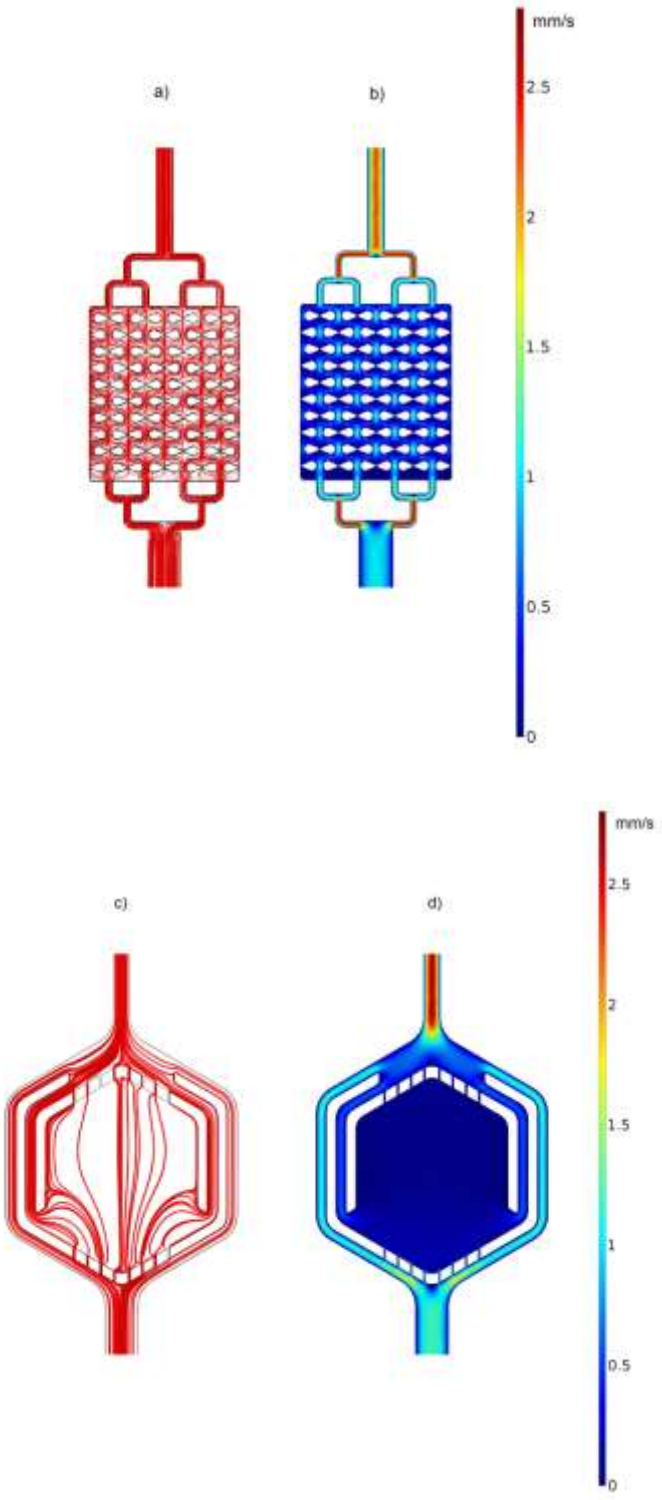


Figure 2. 14 a) and c) are flow fields in two designs to estimate the pathways that cells pass through micro-chamber, the red lines illustrate the streamlines of the flow, b) and d) are velocity distribution simulation for the microfluidic cell culture chamber to ensure where the cell reside shear stress is minimum, the velocity distribution is demonstrated with rainbow colors, red shows high velocity, and blue shows low velocity (mm/s).

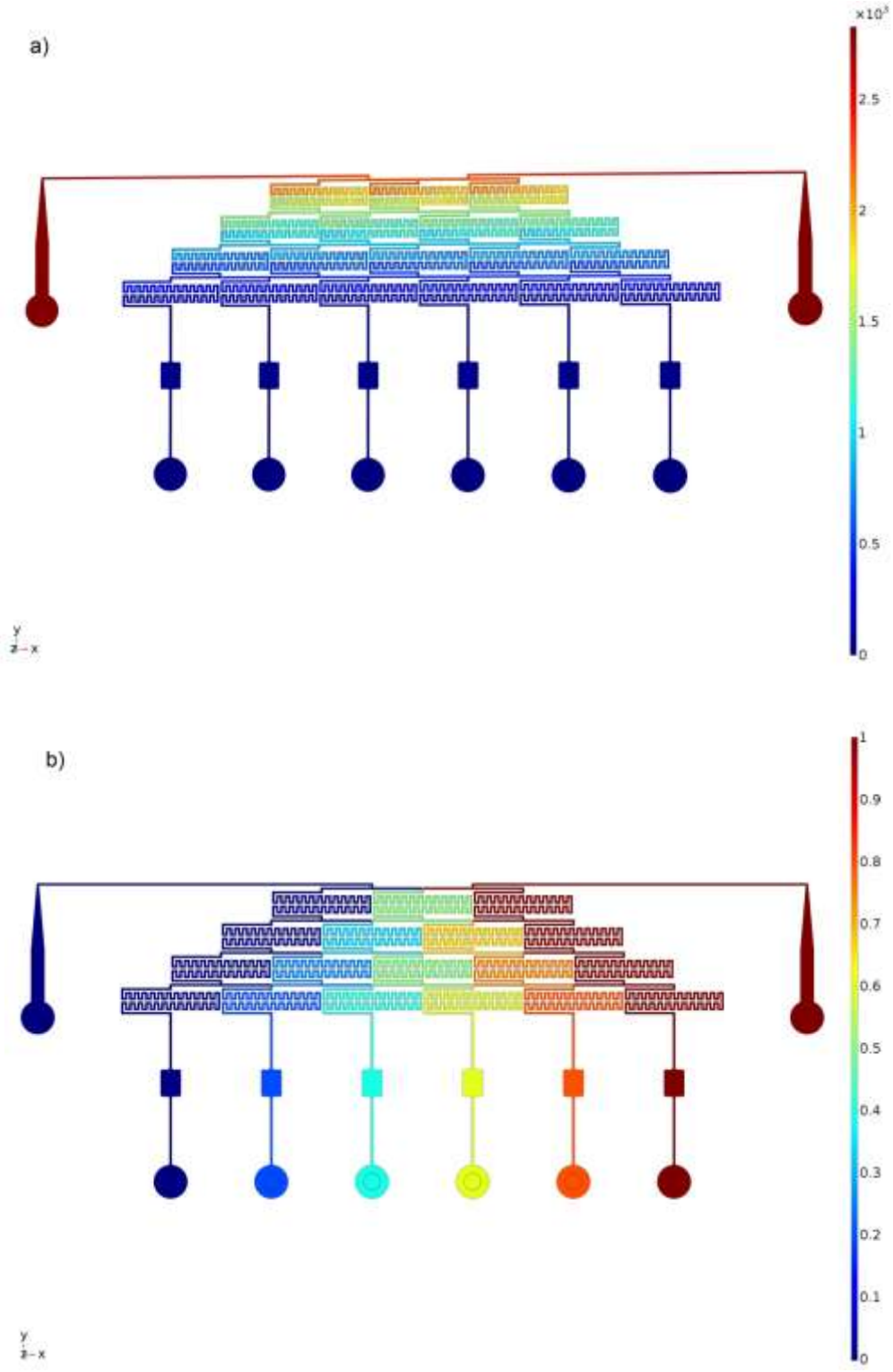


Figure 2. 15 a) Pressure (Pa) and b) chemical concentration (mol/m3) distribution simulations for the microfluidic gradient generator. The pressure and chemical concentration distribution is demonstrated with rainbow colors to ensure that the flow distribution is uniform in all channels, and device outlets can generate a uniform chemical distribution.

2.3 Experimental section

2.3.1 *Fluorescent MCF7 breast cancer cell line*

MCF7 breast cancer cells stably expressing RFP vector were used to demonstrate the utility of the microfluidic cell culture platform. In order to create RFP expressing MCF7 (MCF7-RFP) cells, lentiviruses were produced by transfection of pRSI9-U6-UbiC-TagRFP-2A-Puro plasmid (Addgene plasmid # 28289) together with its helper plasmids psPAX2 and pMD2.G (Addgene plasmid # 12260 and # 12259) into HEK293T cells. Cell supernatants were harvested at 48 hours and 72 hours after the transfection. The mixture of collected supernatant were stored at -80 °C and used to infect the cells. MCF7 cells were infected at a 60 % confluence for 24 hours with lentiviral supernatants diluted 1:1 with full DMEM (Sigma-Aldrich) culture medium supplemented with 5 µg/mL polybrene (Sigma-Aldrich, H9268). After 24 hours of infection, the medium was replaced with a fresh medium. MCF7 cells stably expressing RFP vector were selected with 1 µg/mL concentration of puromycin in DMEM supplemented with 10% FBS (Sigma Aldrich), 1% Pen/Strep (Penicillin-Streptomycin, Sigma-Aldrich), *102* 1% L-glutamine (Sigma-Aldrich) for a month.

MCF7-RFP cells were grown in DMEM supplemented with 10% FBS, 1% Pen/Strep, 1% v/v glutamine both in 75 cm^2-flasks (Corning® T-75 flasks) and in the microfluidic platforms inside the incubator (NUVE EC160), at a temperature of 37 °C, while 5% CO2 and saturated humidity were provided. Trypsin-EDTA (Sigma-Aldrich) solution was used for the detachment of the cells from the flask and to inoculate them into the microfluidic devices.

2.3.2 *Micro-manufacturing process*

The manufacturing process of LOCs began with patterning the design on a thin film chromium deposited-photo mask (Cr-blank) using Vistec/EBPG 5000 plusES Electron Beam Lithography. In order to proceed with the SU-8 mold manufacturing, SU-8 2025 (SU-8® 2025, MicroChem) was spin-coated on a 4" silicon wafer to obtain a thin resin

film with 40 (μm) thickness [68]. Afterwards, the photoresist-coated wafers were soft baked at 65 °C for 3 minutes and at 95 °C for 5 minutes. Later on, the resin was polymerized by UV light 160 (mJ/cm^2), and using the prepared Cr-blank mask written by means of Midas/MDA-60MS mask aligner. After two consecutive post-baking processes took place at 65 °C for 1 minute and at 95 °C for 5 minutes, wafers were developed for 5 minutes using MicroChem's SU-8 developer. Finally, the developed wafers were rinsed with isopropanol to remove any residuals/chemicals from the mold.

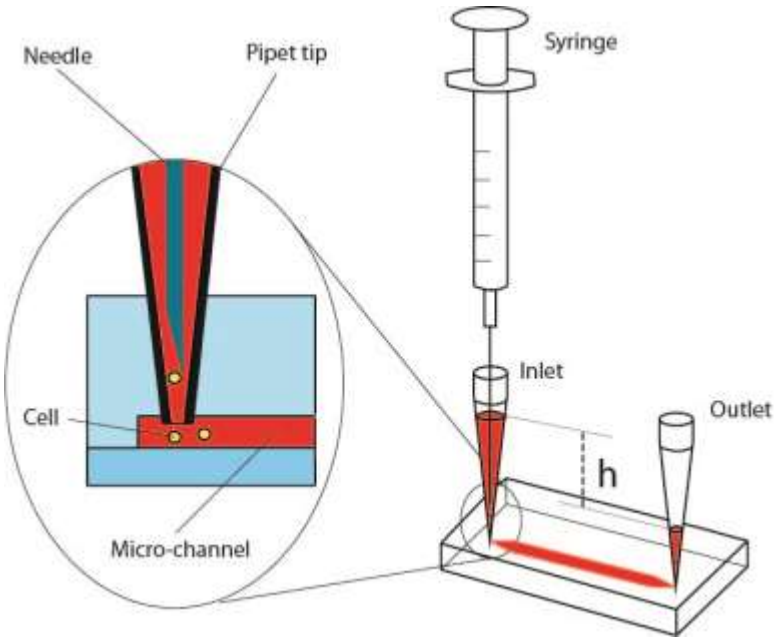


Figure 2. 16 Schematic of cell loading procedure in a microfluidic PDMS device (cells are in yellow).

The micro-manufactured SU-8 molds were used as a master mold for PDMS casting. PDMS resin and its curing agent (Sylgard® 184, Dow Corning, Midland, MI) were mixed carefully with w/w ratio of 10:1, and poured on top of a mold which was placed in petri dish. The bubbles generated during mixing were removed by applying vacuum on the whole setup. Next, the setup was placed in an oven at 75 °C for 50 minutes in order to cure PDMS. After separating PDMS from the mold, the generated patterns were punched, rinsed, scotch taped, and eventually bonded to a clean standard microscope glass slide by means of oxygen plasma (BD20-AC, Electro-Technic Products Inc.). The sterilized tubing (Tube Tygon s54HL .02X.06 500, Andwin Scientific) and metal couplers were autoclaved

*(*Hirmaya (HMC) HV-85L*)* prior to loading the microfluidic devices. Before usage, the medium and PBS were warmed in a 37 °C water bath. MCF7 cells were prepared in a complete medium. A new cell loading protocol developed due to the bubbling problem especially for the platforms dealing with a few cells.

2.3.3 *The cell loading protocol*

First the desired PDMS LOC was equipped in such a way that two pipet tips are simply located at the inlet and outlet of the LOC by pushing them directly, or with the help of an inter-connector. In order to establish the fluid inside the micro-channel and disinfect the inner surface of channels, similar to many other protocols for LOCs, a 70% aqueous ethanol was added to the inlet pipet tip. By applying pressure by means of a pipette, or any other device which can provide low to medium pressure, the ethanol solution is forced to go through the micro-channels and cavities of the device.

In our protocol, we avoided applying negative pressure at the outlet, which would have made the liquid flow through the chip, for two major reasons. The first reason is the fact that a negative pressure can lead to air leakage through connections, the interface of PDMS and glass, and the free volumes of PDMS. The second reason is based on Henry's law, which indicates that the amount of a gas dissolved in a liquid is related directly to the applied pressure.

After flushing the chip with ethanol solution, the chip was examined to ensure that there are no bubbles left. The excess ethanol from the inlet and outlet was sucked by means of the needle of a syringe and flushed with PBS. Later on, the inlet pipet was filled with fresh medium in such a way as to have a certain height (*h*) between the levels of the medium in the two pipet tips (Fig 2.16).

As a result, a very slow flow of the medium was established inside the micro-channels. Finally, the required amount of cells could be sucked into the needle of a Hamilton syringe (Hamilton, 100 µl SYR, #84884), which had already been degased by the same medium. Afterwards, a cell concentration of 1000-10000 (*cells/ml*) was directly loaded to the

bottom of the pipet tip at the inlet of the chip. The cells filled the chambers with single cell resolution through the designed cell trapping mechanism. Upon the microscope inspection (Carl Zeiss, Primovert Model Trinocular Inverted Microscope) of the cells in the microfluidic cell culture platform, we transferred the device into the incubator overnight to allow cells to adhere to the surface with an average of 4 (*μl/hr*) medium flow rate (Fig 2.17).

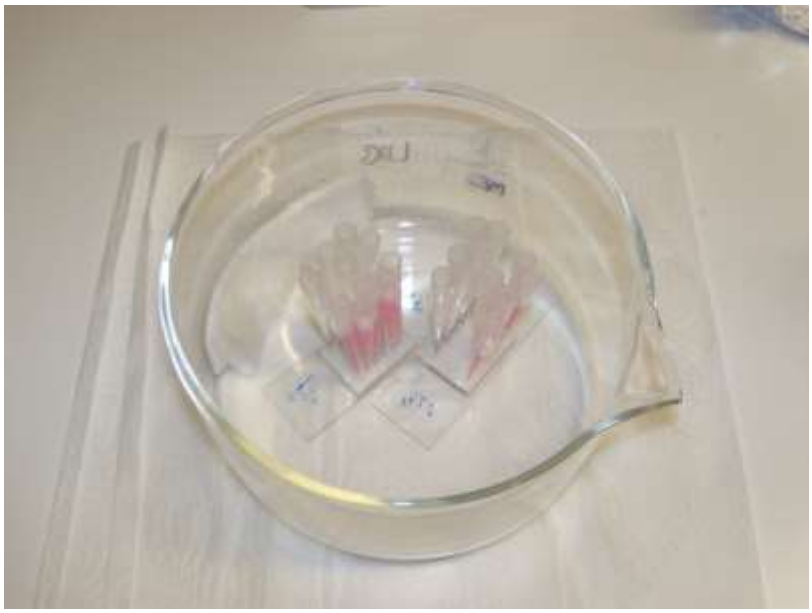


Figure 2. 17 The loaded micro-chambers with established flow and ready for incubation.

2.3.4 *Microscope imaging*

The images of the individual chambers within the devices were captured using 10x objective and combining phase and DsRed fluorescent channels using a Carl Zeiss, Axio Obserber Z1 motorized stage equipped with the AxioCam Mrc5 camera. We measured the total fluorescent intensity of the cells within the micro-chambers to quantify cellular growth using ImageJ software. To observe fluorescent intensity changes in the gradient generator, the imaging was performed with a green fluorescent filter using 400 ms exposure time. The composite image of the whole gradient generator was obtained via tiled images using Zen Pro microscopy software (Carl Zeiss). The fluorescent intensities were mapped to the 2.5D image (pseudo-3D image) using the same software.

2.4 Results and discussion

In this thesis, two microfluidic cell culture platforms with single cell resolution were designed, simulated and manufactured. Moreover, the performance of these platforms in terms of cell loading, cell culturing, and integration with a Christmas tree gradient generator were investigated. As an initial step, a very simple cell culture well was initially manufactured by means of punching a PDMS pillar and oxygen plasma bonding to a glass slide. The cells were loaded with the concentration of 200 cells per each well in a complete medium, and left inside the incubator for 24 hours. The aim of this experiment was to study whether cells attach themselves to the surface treated glass slide. After 24 hours, the cells showed elongation across the glass slide which is the sign of attachment and internal mechanical stress (Fig 2.18).

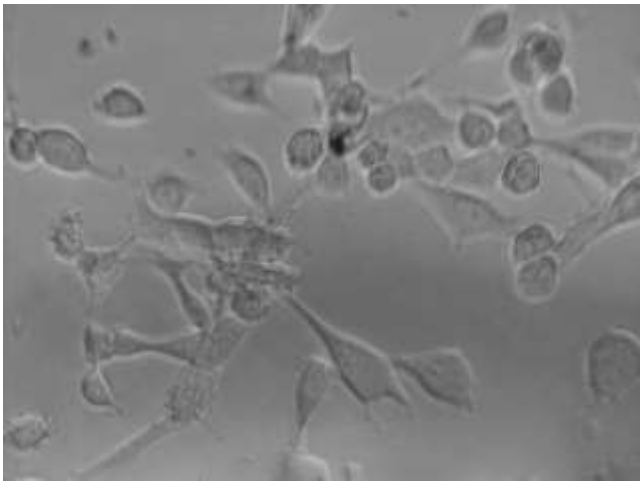


Figure 2. 18 Cell attachment on top of a glass slide after 24 hours of incubation.

The proposed chip designs were both successful and both performed as expected (Fig 2.19). Additionally, the developed protocol for bubble free cell loading proved to be a versatile method while being simple in nature. By observing the cell loading process, it was noticed that the first micro-chamber suffered from inefficiency in cell trapping. It can be postulated that the reason for this was the amplification of shear-gradient due to multiple expansion flows. Compared to the first design, the second design exhibited fewer

problems with the lack of efficiency in cell loading. However, it was observed that the cell counting mechanism highly depended on initial cell number, and higher cell concentrations would have resulted in more cells being trapped in the micro-chamber.

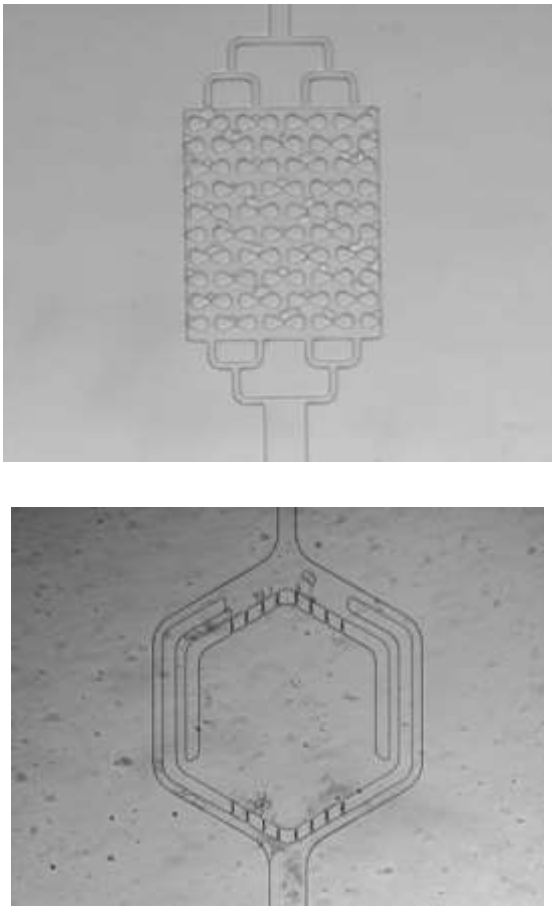


Figure 2. 19 The loaded first (top image) and second (bottom image) micro-champers.

It is not an exaggeration to rate the chip loading with the cells as the most exhausting part of the experiments. The reason for this is the fact that once everything is in micro-scale, miniscule issues can easily turn into big problems. Therefore, in order to overcome these issues a careful strategy and close attention are required. The process of loading cells starts with the preparation of a suspension of the cells in the medium. This process must be accurately done, otherwise cells can be damaged, not properly separated from each other, or they can create clusters. The main problem is the generation of PDMS debris due to connecting and other sources of contamination such as dust or dead cells. The presence of

micro-size debris is the main reason of microfluidic blockage and of the subsequent failure of the experiment.

Once chips were loaded they were immediately incubated for 2-4 hours in order to let the cell attach to the surface. Moreover, the consumed medium in each pipet tip was removed and fresh medium was added to the inlet pipet tip each 24 hours. Meanwhile, fluorescent micrograph images were taken every 12 hours in order to follow the cell growth pattern (Fig 2.20). The obtained images were transferred to ImageJ software to measure the overall light intensity which, in the case of MCF7 cells, it directly presents the number of cells and the fluorescent protein being expressed in them. The growth patterns for 36 hours indicated the incremental cell growth. The cell growth graphs belonging to the first and second growth chamber, and the petri dish bulk cell culture are depicted in Fig 2.21. It can be concluded that growth conditions for the cell inside a bulk cell culture are more favorable than a single cell resolution microfluidic cell culture due to the exponential growth rate in petri dish versus the linear rate in the micro-chamber. However, the low growth rate in microfluidic chambers should not be considered as a drawback, since micro-chambers in terms of constant supply of medium, diffusion of chemicals, and geometry have more resemblance to *in vivo* cell microenvironment.

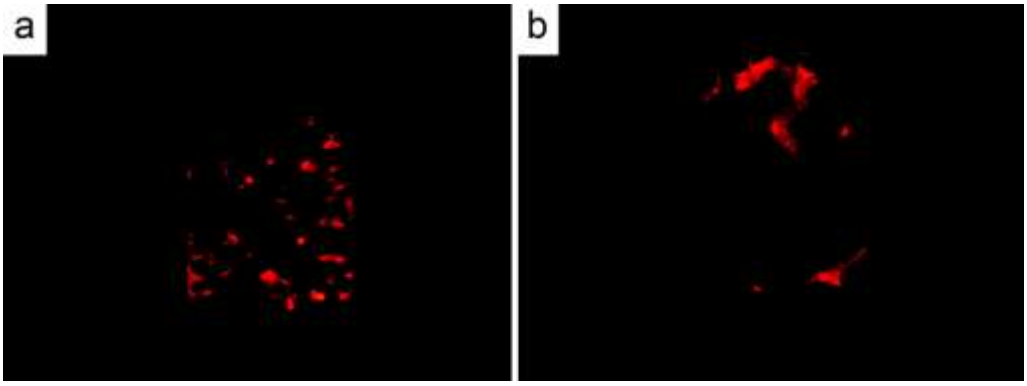
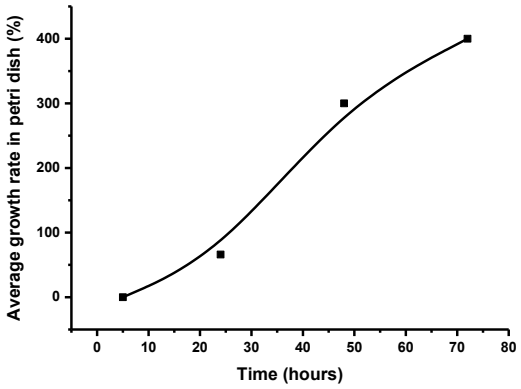


Figure 2. 20 The fluorescent microscopy of a) the first and b) the second micro-chamber cell cultures.

a)



b)

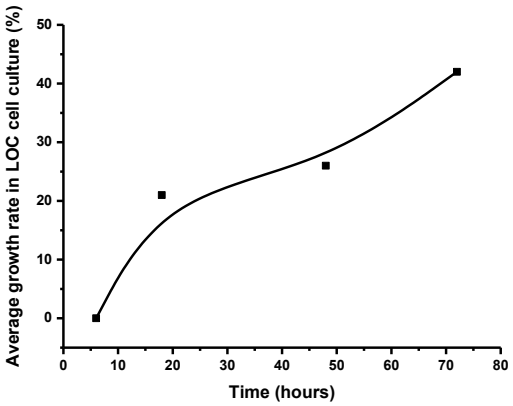


Figure 2. 21 The difference between growth rates a) in petri dish versus b) micro-chamber environment.

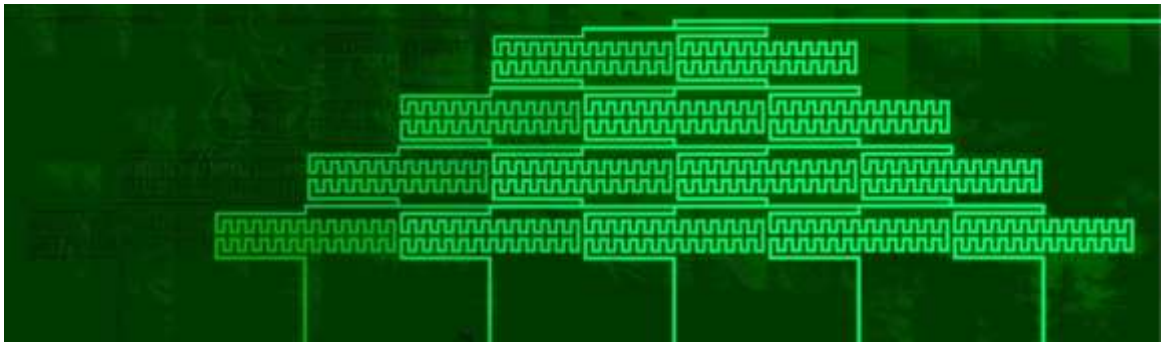


Figure 2. 22 The mechanism of action in the Christmas tree chemical gradient generator with two inlets and 6 outlets.

The examination of gradient generator was initiated by means of mixing two different fluid flows in each inlet and the use of fluorescent microscopy. In this experiment, 1 mg/ml of fluorescein isothiocyanate (FITC, Fluorescein 5(6)-isothiocyanate, Sigma-Aldrich) solution in carbonate-bicarbonate buffer (Thermo Scientific) pH 9 was prepared. Afterwards, the FITC solution was fed from one inlet, while introducing the carbonate bicarbonate buffer without FITC from the other inlet using two syringes mounted on a syringe pump (New Era Pump Systems, NE-1000). The flow rate initially was set at 300 (*μl/hr*), but upon establishing the steady chemical gradient it was reduced to 10 (*μl/hr*). The resulted images were tiled together and their fluorescent intensities were measured using the ZEN Pro 2 software (Zeiss) (Fig 2.22, 2.23). The measurements of the fluorescent intensity clearly show how the chemical concentration increases from 0% intensity to 100% chemical intensity as equal as the fluorescent inlet flow.

Interestingly, the chemical mixture could be observed at the presence of FITC solution, and at the point of mixing with the non-fluorescent flow (Fig 2.24). It is very clear that the 0% and 100% streams, as they face each other and integrate into the same channel, do not mix with each other through convention nor through turbulence. Instead, it takes both streams at least a quarter of length of channel to partially mix, and the whole length of channel to homogeneously mix together.

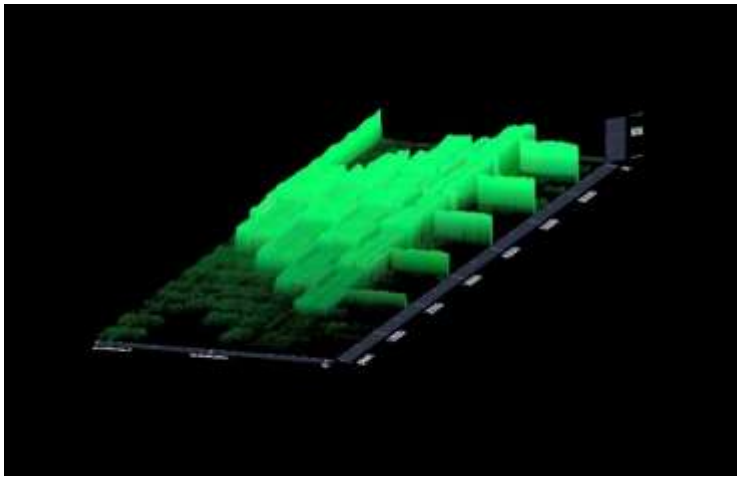


Figure 2. 23 The measurement of fluorescent light intensity and the incremental chemical concentration in the chemical gradient generator outlets.

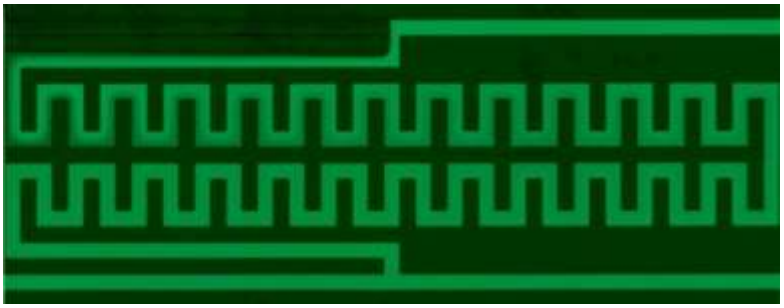


Figure 2. 24 The physical observation of mixing two streams with chemical gradient through almost pure diffusion.

Eventually, a series of six micro-chambers on the same galss slide were loaded with cells and left to grow for 72 hours. Meanwhile, a grdient generator chip was equipped with 6 plastic tubing and strelized internally and externally by means of 70% aquous ethanol, and consequent PBS washing. A mixture containing 0.005% wt/wt sodium dodecyl sulfate (SDS) in complete medium was charged into a 2ml syringe, and another 2ml syringe was charged with a complete medium without SDS. Both syringes were connected to the gradient generator and their flow was adjusted to 10 ($\mu l/hr$) before connecting it to the micro-chambers for at least 30 minutes. Just before the main experiment was conducted, images of micro-chambers were taken in order to compare the SDS effect on the cell viability. Afterwards, the inlet pipet tips from the cell culture chip were removed and the gradient generator tubings were connected instead (Fig 2.25).

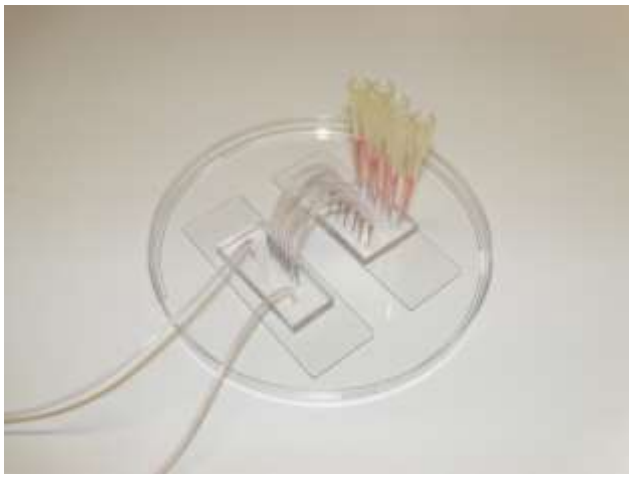


Figure 2. 25 Combination of gradient generator, micro-chamber cell cultures, and the medium supply.

After leaving the whole platform in the incubator and continuing the experiment for 30 minutes, the flow was halted and the tubes were replaced with the pipet tips containing fresh medium. In a separate experiment, a set of wells in a 96 well-plate was loaded with the same cell type and 200 μl medium and was cultured for 72 hours. The images of the wells belonging to the 96 well-plate were taken just before adding the SDS, and later on the medium was replaced with a medium which had similar SDS concentrations as the gradient generator. The experiment continued for 30 minutes in the incubator, and afterwards the cells were washed with PBS to remove the dead cells and deposits, and fresh medium was added to each well again. Subsequently, the images of both experiments were taken once again, and by measuring the light intensity of the cell cultures before and after applying SDS, it was possible to measure the cell viability with respect to chemical concentration (Fig 2.26). Later on, the images were taken to ImageJ software, and the light intensity of each chamber was measured and compared in order to determine the rate of cell death. In this method the dark background of each image was first subtracted, and the light intensity of the remaining area for each image was set to maximum. Therefore, the number of bright pixels in the image represented the area covered by the cells in each micro-chamber.

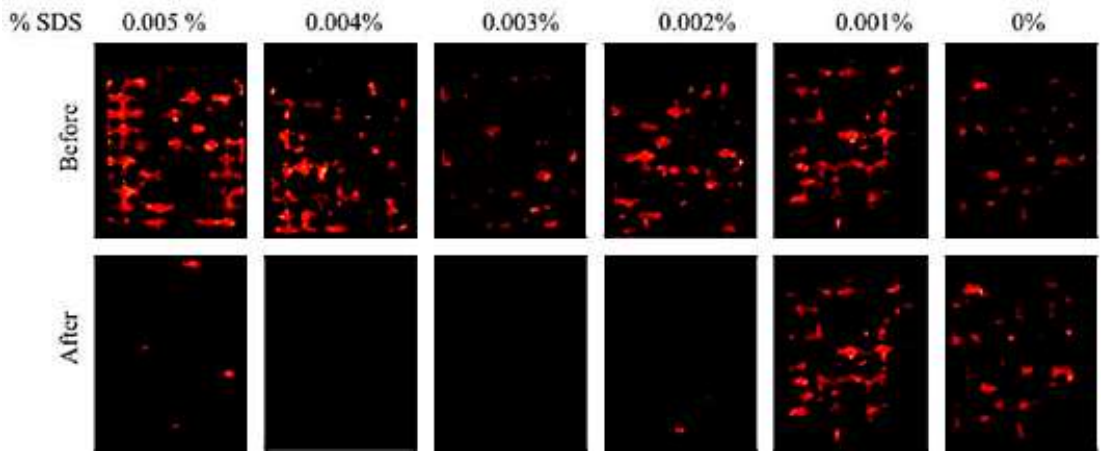


Figure 2. 26 Cell images taken before and after applying SDS at different concentrations.

By demonstrating the implications of chemical manipulation of cells at single-cell resolution, this experiment could lead to more promising results as opposed to the petri dish ones. In the cell viability experiment, a chemical threshold between 0.002 to 0.003 percent of SDS was observed in which the cell survivability suddenly dropped. When applying the same concentrations of SDS to cells cultured in a petri dish, due to its static condition and because of the reaction between the SDS and the cells, this threshold was detectable at 0.005% SDS concentration (Fig 2.27).

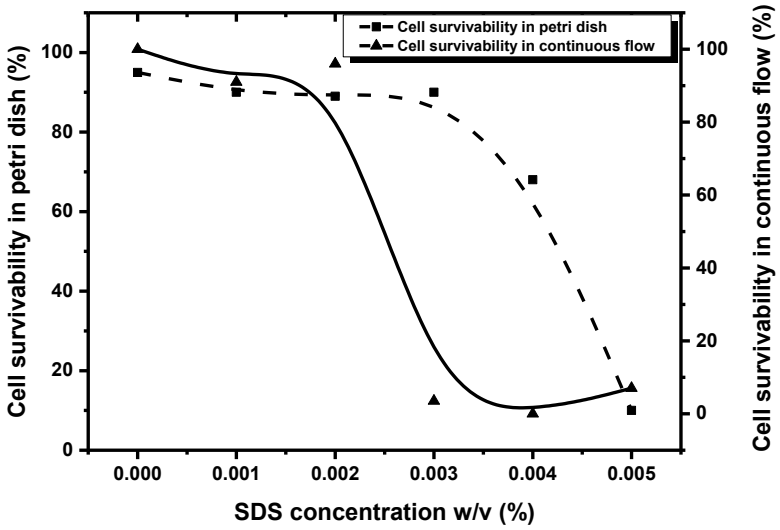


Figure 2. 27 The effect of SDS on cell viability at different chemical concentrations.

2.5 Conclusion

In this thesis, two hypothetical microfluidic cell culture designs were proposed in order to have a better insight into cancer cell biology at single-cell resolution. These designs were initially examined through CFD studies in COMSOL multi-physics to obtain a general idea about the shear stress inside the micro-chambers and cell trapping mechanisms. In addition, a Christmas tree type of chemical gradient generator was designed, and its capabilities were simulated in COMSOL multi-physics. The micro-fabrication by means of e-beam and UV litography techniques was undertaken to successfully make a SU-8 mold for PDMS with an aspect ratio of 1:10 and minimum resolution of 4 (μm).

A set of PDMS LOCs were manufactured and bonded to standard glass slides, and were used during several experiments. The methods of cell loading into microfluidic chips were examined and a facile cell loading technique was developed. In addition, the behavior of fluorescent MCF7 cancer cell line was studied both in the bulk cell culture and in micro-chambers. Moreover, the chemical gradient generator was successfully tested by means of fluorescent microfluidic microscopy. Eventually, the proof of concept of the platforms was tested by using a SDS solution.

Moreover, it was observed that the cell growth rates in microfluidic cell cultures are several orders of magnitude less than the petri dish cell culture. This phenomenon has tremendous potential for further exploring this kind of microfluidic cell cultures, or for studies which deal with the effects of physical parameters such as shear flow and geometry on cell growth, signaling, and other vital cellular interactions. Additionally, during testing the effect of SDS by means of gradient generator on the cell viability, the necessity of using continuous flow on the cell cultures was further proven. Lastly, one essential aspect that should not be overlooked in future studies is resolving the error of measurements associated both with microfluidic experiments and traditional cell cultures.

2.6 Future directions

In this thesis an overall knowledge of design, simulation, manufacturing and testing of microfluidic cell cultures was obtained which can be transferred to many other experiments and applications which involve singe-cell resolution cell culture. One of the most suitable applications for the current system is the growth of CTC or tumor biopsy samples and analysis given that their chemotherapy response would be obtained in a matter of days without changing the sample phenotype in comparison to the current techniques which require a few weeks. In addition, it is also possible to use this system for toxicity measurements of healthy tissue cells. Furthermore, some other applications such as studying the interactions between cancer cells and the immune system, as well as applications used for drug design can be further optimized by employing similar systems.

References

1. Hordijk, W., *Evolution of Autocatalytic Sets in Computational Models of Chemical Reaction Networks.* Origins of Life and Evolution of Biospheres, 2016. **46**(2-3): p. 233-245.
2. Stadler, P.F., *Evolution of RNA-Based Networks.* Quasispecies: From Theory to Experimental Systems, 2016. **392**: p. 43-59.
3. Adami, C., *Information-Theoretic Considerations Concerning the Origin of Life.* Origins of Life and Evolution of Biospheres, 2015. **45**(3): p. 309-317.
4. Patel, B.H., et al., *Common origins of RNA, protein and lipid precursors in a cyanosulfidic protometabolism.* Nature Chemistry, 2015. **7**(4): p. 301-307.
5. Root-Bernstein, M. and R. Root-Bernstein, *The ribosome as a missing link in the evolution of life.* Journal of Theoretical Biology, 2015. **367**: p. 130-158.
6. Root-Bernstein, R. and M. Root-Bernstein, *The ribosome as a missing link in prebiotic evolution II: Ribosomes encode ribosomal proteins that bind to common regions of their own mRNAs and rRNAs.* Journal of Theoretical Biology, 2016. **397**: p. 115-127.
7. Trifonov, E.N., *Tracing Life back to elements.* Physics of Life Reviews, 2008. **5**(2): p. 121-132.
8. Darwin, C., *On the origin of species by means of natural selection, or The preservation of favoured races in the struggle for life*1860, New York,: D. Appleton and company. 432 p.
9. Theobald, D.L., *A formal test of the theory of universal common ancestry.* Nature, 2010. **465**(7295): p. 219-U104.
10. Dobretsov, N.L., N.A. Kolchanov, and V.V. Suslov, *On the early stages of the evolution of the geosphere and biosphere.* Paleontological Journal, 2006. **40**(4): p. S407-S424.
11. Cairns, J., *Mutation selection and the natural history of cancer.* Nature, 1975. **255**(5505): p. 197-200.
12. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2016.* CA: A Cancer Journal for Clinicians, 2016. **66**(1): p. 7-30.
13. (WHO), W.H.O. *Cancer fact sheet*. 2017; Available from: http://www.who.int/mediacentre/factsheets/fs297/en/.
14. Normanno, N., et al., *Epidermal growth factor receptor (EGFR) signaling in cancer.* Gene, 2006. **366**(1): p. 2-16.
15. Milholland, B., et al., *Differences between germline and somatic mutation rates in humans and mice.* Nature Communications, 2017. **8**: p. 15183.
16. Scally, A., *The mutation rate in human evolution and demographic inference.* Current Opinion in Genetics & Development, 2016. **41**: p. 36-43.
17. Adamson, E.D., *Oncogenes in Development.* Development, 1987. **99**(4): p. 449-471.
18. Nowell, P.C., *Clonal Evolution of Tumor-Cell Populations.* Science, 1976. **194**(4260): p. 23-28.
19. Maley, C.C. and B.J. Reid, *Natural selection in neoplastic progression of Barrett's esophagus.* Seminars in Cancer Biology, 2005. **15**(6): p. 474-483.
20. Gerlinger, M., et al., *Intratumor Heterogeneity and Branched Evolution Revealed by Multiregion Sequencing.* New England Journal of Medicine, 2012. **366**(10): p. 883-892.
21. Yates, L.R. and P.J. Campbell, *Evolution of the cancer genome.* Nature Reviews Genetics, 2012. **13**(11): p. 795-806.
22. Tortora, G.J. and M.T. Nielsen, *Principles of human anatomy*. 11th ed2009, Hoboken, NJ: J. Wiley.

23. Cooper, G.M., *The cell : a molecular approach*. 2nd ed2000, Washington, D.C. Sunderland, Mass.: ASM Press ; Sinauer Associates. xxiv, 689 p.

24. *The Plasma Membrane*. Available from: https://www.ck12.org/biology/Phospholipid-Bilayers/lesson/Phospholipid-Bilayers-BIO/.
25. Shafee, T.; Available from: https://en.wikipedia.org/wiki/File:Protein_structure_(full).png.
26. Synthesis, W.I.P.; Available from: http://www.proteinsynthesis.org/what-is-protein-synthesis/.
27. Alberts, B., *Molecular biology of the cell*. 4th ed2002, New York: Garland Science. xxxiv, 1548 p.
28. Centre, V.G.E.; Available from: http://www2.le.ac.uk/projects/vgec/schoolscolleges/topics/geneexpression-regulation.
29. Smith, J.A. and L. Martin, *Do Cells Cycle.* Proceedings of the National Academy of Sciences of the United States of America, 1973. **70**(4): p. 1263-1267.
30. ; Available from: http://www2.le.ac.uk/projects/vgec/schoolscolleges/topics/cellcycle-mitosis-meiosis.
31. Fernández-Medarde, A. and E. Santos, *Ras in Cancer and Developmental Diseases.* Genes & Cancer, 2011. **2**(3): p. 344-358.
32. Carracedo, A. and P.P. Pandolfi, *The PTEN-PI3K pathway: of feedbacks and cross-talks.* Oncogene, 2008. **27**(41): p. 5527-5541.
33. Campisi, J., *Aging, Cellular Senescence, and Cancer.* Annual Review of Physiology, Vol 75, 2013. **75**: p. 685-705.
34. Takahashi, A., N. Ohtani, and E. Hara, *Irreversibility of cellular senescence: dual roles of p16(INK4a)/Rb-pathway in cell cycle control.* Cell Division, 2007. **2**.
35. Qian, Y.J. and X.B. Chen, *Tumor suppression by p53: making cells senescent.* Histology and Histopathology, 2010. **25**(4): p. 515-526.
36. Schroeder, R.D., L.S. Angelo, and R. Kurzrock, *NF2/Merlin in hereditary neurofibromatosis 2 versus cancer: biologic mechanisms and clinical associations.* Oncotarget, 2014. **5**(1): p. 67-77.
37. Hezel, A.F. and N. Bardeesy, *LKB1; linking cell structure and tumor suppression.* Oncogene, 2008. **27**(55): p. 6908-6919.
38. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
39. Wong, R.S.Y., *Apoptosis in cancer: from pathogenesis to treatment.* Journal of Experimental & Clinical Cancer Research, 2011. **30**.
40. Yang, Z.N.J., et al., *The Role of Autophagy in Cancer: Therapeutic Implications.* Molecular Cancer Therapeutics, 2011. **10**(9): p. 1533-1541.
41. Proskuryakov, S.Y. and V.L. Gabai, *Mechanisms of Tumor Cell Necrosis.* Current Pharmaceutical Design, 2010. **16**(1): p. 56-68.
42. Nishida, N., et al., *Angiogenesis in Cancer.* Vascular Health and Risk Management, 2006. **2**(3): p. 213-219.
43. Bierie, B., et al., *Integrin-beta 4 identifies cancer stem cell-enriched populations of partially mesenchymal carcinoma cells.* Proceedings of the National Academy of Sciences of the United States of America, 2017. **114**(12): p. E2337-E2346.
44. Cuturi, M.C. and I. Anegon, *Suppression and regulation of immune responses : methods and protocols*. Springer protocols2011, New York: Humana Press. xviii, 473 p.
45. Wang, N., H.W. Liang, and K. Zen, *Molecular mechanisms that influence the macrophage M1-M2 polarization balance.* Frontiers in Immunology, 2014. **5**.

46. Chen, D.S. and I. Mellman, *Elements of cancer immunity and the cancer-immune set point.* Nature, 2017. **541**(7637): p. 321-330.
47. Eibl, R., *Cell and tissue reaction engineering*. Principles and practice series,2009, Berlin: Springer. xi, 363 p.
48. Nema, R.a.K., S., *An animal cell culture: Advance technology for modern research.* Advances in Bioscience and Biotechnology, 2012. **3**: p. 219-226.
49. Bonifacino, J.S., *Current protocols in cell biology*, 1998, John Wiley: New York. p. v. (loose-leaf).
50. Callaway, E., *Deal done over HeLa cell line.* Nature, 2013. **500**(7461): p. 132-133.
51. Barretina, J., et al., *The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity.* Nature, 2012. **483**(7391): p. 603-607.
52. Agliari, E., et al., *Cancer-driven dynamics of immune cells in a microfluidic environment.* 2014. **4**: p. 6639.
53. Chaw, K.C., et al., *Multi-step microfluidic device for studying cancer metastasis.* Lab on a Chip, 2007. **7**(8): p. 1041-1047.
54. Hoyoung, Y., K. Kisoo, and L. Won Gu, *Cell manipulation in microfluidics.* Biofabrication, 2013. **5**(2): p. 022001.
55. Halldorsson, S., et al., *Advantages and challenges of microfluidic cell culture in polydimethylsiloxane devices.* Biosensors & Bioelectronics, 2015. **63**: p. 218-231.
56. Kim, L., et al., *A practical guide to microfluidic perfusion culture of adherent mammalian cells.* Lab on a Chip, 2007. **7**(6): p. 681-694.
57. Edmondson, R., et al., *Three-Dimensional Cell Culture Systems and Their Applications in Drug Discovery and Cell-Based Biosensors.* Assay and Drug Development Technologies, 2014. **12**(4): p. 207-218.
58. Gupta, N., et al., *Microfluidics-based 3D cell culture models: Utility in novel drug discovery and delivery research.* Bioengineering & Translational Medicine, 2016. **1**(1): p. 63-81.
59. Ren, K., J. Zhou, and H. Wu, *Materials for Microfluidic Chip Fabrication.* Accounts of Chemical Research, 2013. **46**(11): p. 2396-2406.
60. Yang, Y., et al., *Engineering of a microfluidic cell culture platform embedded with nanoscale features.* Lab on a Chip, 2011. **11**(9): p. 1638-1646.
61. Zhou, J.W., et al., *Surface modification for PDMS-based microfluidic devices.* Electrophoresis, 2012. **33**(1): p. 89-104.
62. El-Ali, J., P.K. Sorger, and K.F. Jensen, *Cells on chips.* Nature, 2006. **442**(7101): p. 403-411.
63. Schmid, A., et al., *Chemical and biological single cell analysis.* Current Opinion in Biotechnology, 2010. **21**(1): p. 12-20.
64. Dittrich, P.S. and A. Manz, *Lab-on-a-chip: microfluidics in drug discovery.* Nature Reviews Drug Discovery, 2006. **5**(3): p. 210-218.
65. Karimi, A., S. Yazdi, and A.M. Ardekani, *Hydrodynamic mechanisms of cell and particle trapping in microfluidics.* Biomicrofluidics, 2013. **7**(2).
66. Toh, A.G.G., et al., *Engineering microfluidic concentration gradient generators for biological applications.* Microfluidics and Nanofluidics, 2014. **16**(1-2): p. 1-18.
67. Geschke, O., H. Klank, and P. Tellemann, *Microsystem engineering of lab-on-a-chip devices*2004, Weinheim: Wiley-VCH ;. xi, 258 p.
68. MicroChem®, *SU-8 2000 Data Sheet (2025-2075)*.