ON THE DEVELOPMENT OF A MICROFLUIDIC INSTRUMENT FOR PRECLINICAL CELL ANALYSIS

Marie Catherine Keays B.Eng(Hons) Mech.Eng

A thesis submitted for the degree of Doctor of Philosophy at the Faculty of Science and Engineering, University of Limerick, Ireland

Supervisors

Dr. Tara Dalton

Stokes Laboratories, Department of Mechanical Aeronautical and Biomedical Engineering, University of Limerick

Dr. Patrick Kiely

Graduate Entry Medical School and Health Research Institute, University of Limerick

Submitted to the University of Limerick, June 2017

The substance of this thesis is the original work of the author and due reference and acknowledgment has been made, when necessary, to the work of others. No part of this has previously been accepted for any degree nor has it been submitted for any other award.

_____

Marie Keays Candidate

_____

Dr. Tara Dalton Principal Adviser

Abstract

The evolution of a new drug is initiated by testing new compounds in a preclinical laboratory setting. The methods for discovering new drugs involve scrutiny of a drug's efficacy on a cell model of a disease. Current methods of creating these cell culture models are highly dependent on consumables and manual time. New methods such as high-throughput screening of drugs has progressed the ability to test multiple drugs on cells. However, current high-throughput methods lack the ability to create live cultures that are biologically relevant and can be manipulated non-invasively. The research presented here furthers drug analysis technology by creating a microfluidic instrument for the generation of individual reactions under 1ul in volume. This is a continuous system that can create an unlimited amount of live cell cultures. Validation of this system is both the identification of antibiotic resistance and creating cancer models in microfluidic droplets and being able to monitor these cultures non-invasively in real time. For biologically relevant models a new method of forming 3D cell cultures within the microfluidic droplets is presented. These form structures that are equivalent to micro-tumours within a 600nl reaction. A novel aspect of this research is that breast cancer models are treated within the microfluidic droplets and show the same gene expression signature as found in literature from larger cultures. This demonstrates the integrity of the new method in reducing the amount of cells and reagents required to perform reactions. The treatment and monitoring of the cultures within a bench top instrument allows for thousands of reactions to be evaluated for preclinical analysis.

Acknowledgments

I would like to sincerely thank both my supervisors Dr.Tara Dalton and Dr.Patrick Kiely for your guidance and support throughout this research it was very much appreciated. Being able to discuss concepts and develop them to real experiments was made a lot easier with your support.

A big thank you to all the people in stokes who make everything run smoothly Fionnuala and Paddy. Eric and Maurice who helped with all the materials and electrical guidance.

To all the members of Pat's lab past and present, Maeve, Catriona, Miranda, Edel, Susan, Liam, Anu, Bea, Sheri, Sylvia, Michael and Mary. You have all made this time more enjoyable and your help teaching me biology and answering all my basic biology questions was greatly appreciated.

To the gang in Stokes thanks for all the support over the years it will miss the 10AM Stables tea and chats.

To the gang who joined stokes from Stokes Bio, Paudie, Seanie, Dave and Chris your help with microfluidics and general lab banter will not be forgotten. To my former housemates Conor and Brian, our years as the Annacotty family will always be remembered.

To Lisa who literally crossed the t's and dotted the i's for this whole thesis.

To Paddy Keays without your insight and understanding of engineering I certainly would not have this thesis today. I can't thank you enough for your time and advice over the last few years.

To my family Richard, Jack, Laura and Joe who have supported me though this whole endeavour. Richard and Eleanor I cannot express how much we all appreciate never ending support for our education and how we have all benefited from this. I definitely would not have been able to do any of this without you both, thank you.

In memory of Patrick Keays

Contents

List of Figures

List of Tables

Nomenclature

Symbol	Description	Unit
Ca	Capillary number	-
d	Diameter	m
h	Film thickness	m
I	Intensity	V
l	Length	m
ln	Natural Log	
OD	Optical Density	-
r	radius	m
Re	Reynolds Number	-
T	Transmittance	-
u	Velocity	m/s
We	Weber Number	-

Greek

ρ	Density	kg/m^3
μ	Viscosity	Pa.s
γ	Surface tension	N/m

Glossary of terms

Symbol	Description
Ct	Critical threshold
cDNA	Complementary DNA
DAQ	Data Acquisition card
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
ECM	Extracellular Matrix
EGFR	Epidermal growth factor receptor
FBS	Fetal Bovine Serum
LB	Luria-Bertani
LED	Light emitting diode
mRNA	Messenger RNA
NTC	No template control
PBS	phosphate buffer saline solution
PCR	Polymerase Chain Reaction
PBS	phosphate buffer saline solution
RNA	Ribonucleic acid
UV	Ultra-Violate

Chapter 1

Introduction

Drug discovery is initiated by creating a cell model of a disease. Cell culturing and drug testing techniques have been the same for decades. Collectively, an estimated $50 billion is spent each year by large pharmaceutical companies on drug discovery (Paul *et al*., 2010). This investment has only been rewarded with 29 new drugs being approved by the FDA in 2009, only 10 of which went to market (Paul *et al*., 2010). Drug discoveries are instigated by having a disease or condition that is without suitable medical products (Hughes *et al*., 2011). The strategy for new drug development consists of phases. Initially drugs will undergo preclinical trials, which is estimated to take 3-4 years (Lipsky and Sharp, 2001). If a drug shows sufficient evidence that it can treat a disease in a laboratory setting on cells or animal models, an application to the FDA is made to start clinical trials and these trials can take approximately 5 years (Lipsky and Sharp, 2001). If the clinical trials are successful in showing a positive change to a disease, the drug can begin to be commercialised. The amount of drugs that are successfully commercialised is low, for example, only 5% of drugs with anticancer properties at preclinical phase are licensed (Hutchinson and Kirk, 2011). To prevent 95% of drugs failing in the clinical trials, it is

imperative to have preclinical methods that are representative of in-vivo conditions. Considerations in the design of experiments need to include in-vivo factors such as the size of the cells, how they grow within the body (hard bone cells or soft tissue cells) and the signals the cells will get naturally from their environment. The focus of the research presented here is to develop a new preclinical method of testing drugs on biologically relevant models. The preclinical phase encompasses identifying a top drug candidate from experimental methods such as efficacy, pharmacology and toxicology studies (Steinmetz and Spack, 2009). These systems require a living cell model known as a cell culture. A cell culture model allows researchers to test at a molecular level how a prospective drug will react.

To create a new preclinical test method, it is necessary to have a better understanding of the cell culture process. Cells are the most fundamental part of the human body and every organ within it. From a single initial cell division, the cell will begin to

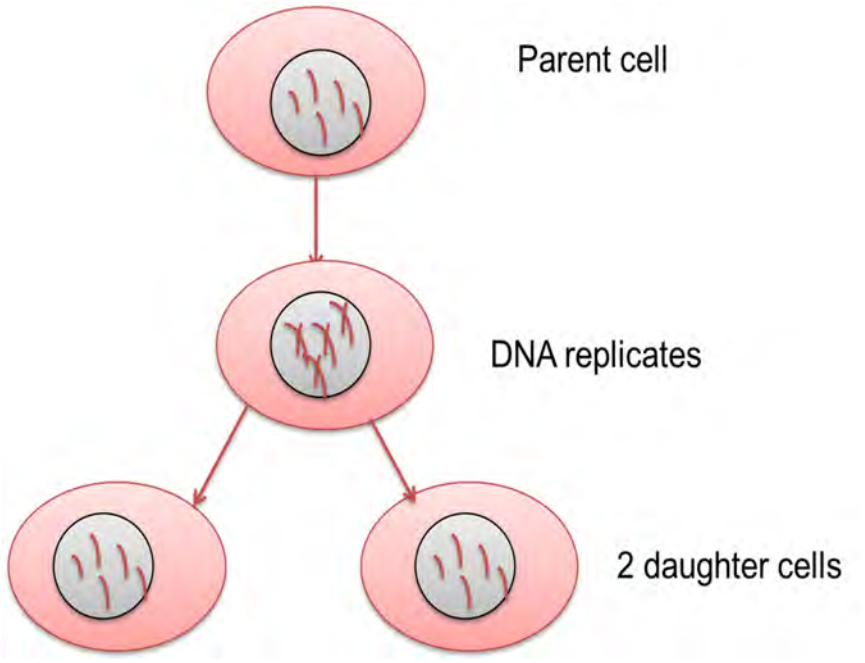


Figure 1.1 Schematic of cell division from parent cells, mitosis forms two new cells

grow and divide to form all the organs within the body. Each organ functioning correctly is dependent on all the cells working together. The fate of the cells and the organs they become is determined by their internal communications, cell to cell communications and the environment the cells grow in. The billions of cell divisions that take place in the body each day work together to form a fully functioning body. Cells will divide when they go through a process known as mitosis. Mitosis is cell division where one cell will divide into two daughter cells (Figure1.1) and involves the division of the cell's nucleus. Within a cell there are many organelles for example, the nucleus, mitochondria and chloroplast which are essentially the 'little organs' that determine the function of the cell. DNA is encapsulated inside the cell's nucleus. Once the cells have completed the mitosis cycle, the daughter cells will then go through the cycle again and this process will keep occurring. Cells will get signals internally to perform all of its processes including mitosis and eventually will get a signal called apoptosis which is the signal to die off. To understand at a molecular level what is occurring within the cell, gene expression is used.

Drug response is analysed on living cell cultures through gene expression (O'Neill *et al*., 2012). The rate of cell growth and the condition of a cell's morphology is dictated by its environment (Iloki Assanga, 2013), for example, a cell's metabolisms and growth rate are different in culture flasks, compared to in-vivo conditions (Shah *et al.,* 2011). For this reason, it is an essential requirement for a new preclinical method to mimic in-vivo conditions as closely as possible.

Mammalian cells models provide information about many diseases such as cancer (Neve *et al*., 2006), diabetes (Mathis *et al*., 2001) and lung disease (Eramo *et al*., 2007). Presently, the most common type of preclinical cell culture is two

dimensional (2D) (Ziółkowska *et al*., 2013). Preclinical cell culture is conducted on plastic culture dishes and well plates, where the cells adhere to a surface in two dimensions, whereas in the human body, the cell will grow in three dimensions (3D). 3D culture is more physiologically relevant than 2D culture (de Groot *et al*., 2016). Currently, the most common way to test mammalian cells is to place cells on a 2D cell culture flask and cover with growth media containing all the nutrients required (Heath and Kiss, 2007). The cells are incubated and monitored by taking the cells from the incubator and visually observing them under a microscope. This manual interaction continues as the cells are washed, nutrients replaced, when the cells have reached sufficient growth for testing, a drug is added. This is the process of setting

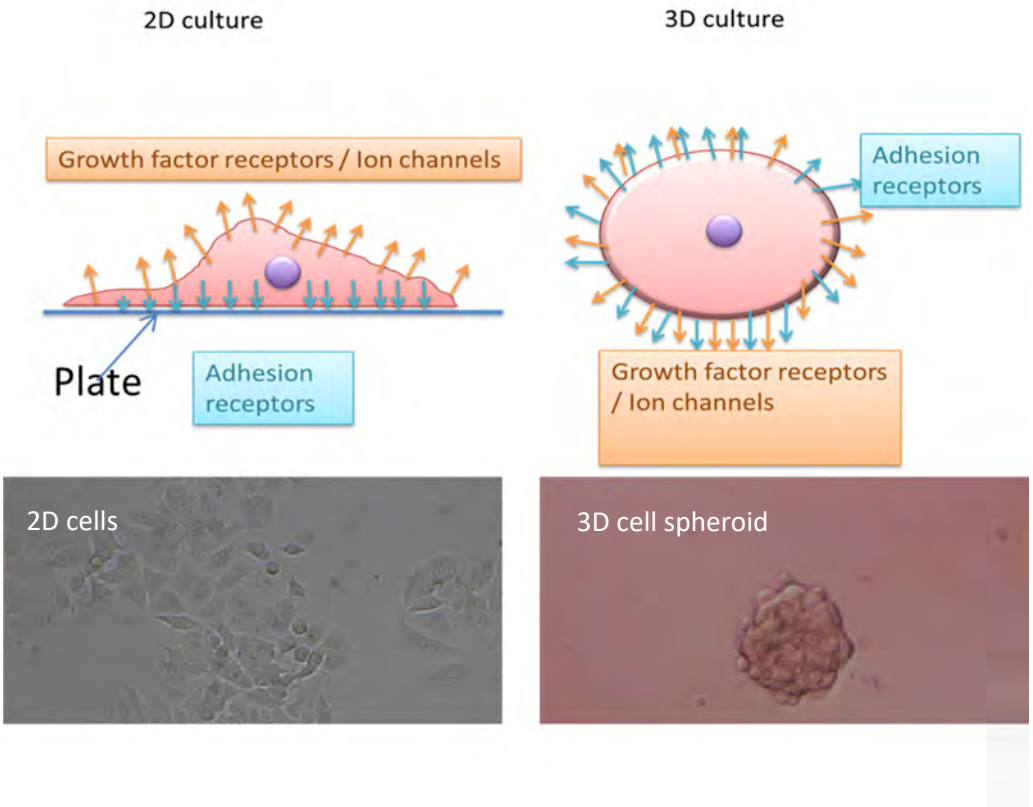


Figure 1.2. A schematic representation of growth factor receptors in 2D and 3D cell culture. Images show the physical difference of breast cancer cells in 2D flat cells and 3D spheroid culture

up a single 2D test and is repeated for each test required. Three dimensional culture has the potential to bridge the gap between laboratory testing and human clinical trials, which is currently being fulfilled by animal models. Recently, the emergence

of organs on chips is providing a method of placing multiple cells together to act like an organ on a microfluidic chip. This gives the ability to test what is happening to a whole organ model and not just one cell type (Bhatia and Ingber, 2014). With 3D culture being more biologically relevant characteristics such as viability, morphology, proliferation, differentiation, response to stimuli, migration and invasion of tumour cells into surrounding tissues and gene expression can be found without using animal models (Antoni *et al.*, 2015). The physical difference in 2D and 3D cultures is demonstrated in Figure 1.2, where an image of MCF-7 breast cancer cells that have adhered to a flask in 2D. Here it is clear, that the cells only have the ability to communicate in 2D, as they lay flat on the plate. The artificial polarity, which is the location of the receptors, is influenced by the 2D conditions, where all the adherent cells receptors have attached to the plate while the growth factor receptors are above the cell (Cukierman *et al*., 2001). The cells growth factor and adhesion receptors will only be able to communicate with other cells at the edges of the cells. Figure 1.2 demonstrates that the MCF-7 cells have formed a 3D structure, where the dispersion of the receptors throughout the cell is evident. These cells have communicated with each other to form a structure that is a representation of a tumour model known as a spheroid (Fennema *et al.*, 2013). Cell communication is imperative because it signals to the cells when to divide and the conditions of the environment around the cells. The receptors are important because they communicate to the cell nucleus the status of the conditions around the cell and how it should act. This informs the cell when the correct conditions to divide are present. Currently, there are many trends in forming 3D cultures from hanging droplet, scaffolds and extracellular matrix gels; and these can vary from batch to batch (Breslin and O'Driscoll, 2013). Differences in cell behaviour, such as gene

expression and proliferation occur between 2D and 3D culture (Fischbach *et al*., 2007). Proliferation rates are influenced by 3D culture (Pickl and Ries, 2008) for example, where SKBR3 cells are treated with a drug (transtuzumab) in 2D and 3D culture. The 2D culture demonstrates a slight reduction in proliferation, whereas a 48% reduction was recorded when cultured under 3D conditions. For this reason, drugs that have been successful in 2D preclinical phase have failed when in the clinical trial phase as the cells can react differently under different conditions. It has also been shown that cancer cells treated with anti-cancer drugs respond completely different when maintained in 2D or 3D culture (Tung *et al*., 2011). This highlights how the specific environment and cell-cell communication can influence the action of the cells. Sixty three percent of drug discovery cost is attributed to clinical trials (Du *et al.*, 2016) so it is imperative to have the correct drugs selected before this stage. A better system is required to get drugs to market and this can be enhanced by better scientifically relevant methods. Also, there are many limitations associated with these current methods, for example:

1. Multiple tests cannot be performed rapidly,
2. There are many human interactions with each culture increasing the possibility of human error,
3. Two dimensional culture is common practice,
4. 3D culture is expensive and relies heavily on the quality and batch specificity of extracellular matrices and components,
5. The volumes of the cultures is in millilitres and with the high cost of drugs this limits the amount of trials performed on cultures,

6. The current methods are not high-throughput as each experiment has to be on an individual dish, each dish has to be incubated and to perform thousands of tests could require many incubators.

In order to address the current preclinical shortcomings, high-throughput screening has been developed. High-throughput screening is the ability to conduct hundreds of experiments in parallel. This enables the researcher to do many experiments, analyse results and find out what result each experiment will have on a disease. High-throughput cell analysis has been used in investigations for diseases including diabetes (Amin *et al*., 2016) and cancer (Gupta *et al.*, 2009). This allows libraries of compounds to be tested on cells. However, most of these systems use well-plate technology and this limits the amount of experiments from 384 (Desbordes and Studer, 2013) to 1536 experiments per plate (Kang *et al*., 2008). Although these methods do increase the amount of tests possible they are finite, which limits the amount of data being generated. The cost is high to analyse libraries of drugs and reducing the amounts of reagents by using a smaller volumes causes the evaporation of reagents in the well plates (Du *et al*., 2016). Large compound libraries can contain up to one million compounds, thus more efficient methods are required to analyse these (Guo *et al*., 2012). Currently, the rise is microfluidics allows the reduction of volumes to the nano-litre range (Teh *et al*., 2008) and consequently this reduction allows the cost of each reaction to be decreased, therefore allowing for more reactions to be created, yielding more results and data.

Microfluidics has emerged as a technology that can accommodate the demand for increased throughput and reduced amounts of reagents. It utilises fluids in small volumes under the microliter range (Whitesides, 2006) and encompasses many disciplines, such as engineering, chemistry and as in this research is applied to a

biological application. Many biological processes have been generated using microfluidics including; biological bioreactors for PCR (Hayes and Dalton, 2015), protein crystallization (Squires and Quake, 2005) and bio-sensing (Teh *et al.*, 2008). The advantage of microfluidics is the significant reduction in the amount of reagents necessary to perform the assays (Ralf *et al*., 2012). Using microfluidic technologies allows the automation of the drug discovery process which not only reduces the user time, but also decreases the error between tests. A current state of the art commercial microfluidic culture instrument Callisto, produced by Fluidigm, consists of thirty two wells for mammalian cell culture on one plate, allowing multiple cultures in parallel. Steps can be pre-programmed by software, minimising the manual process time. However, the Callisto system is limited to thirty two reactions per plate. Another example of a microfluidic cell culture device is demonstrated in Figure 1.3, where the CellASIC plate (Millipore) has four wells that are used for long term culture. The microfluidics here allow the perfusion of nutrients to cultures for long term culture, however as can be seen is limited to four parallel cultures.

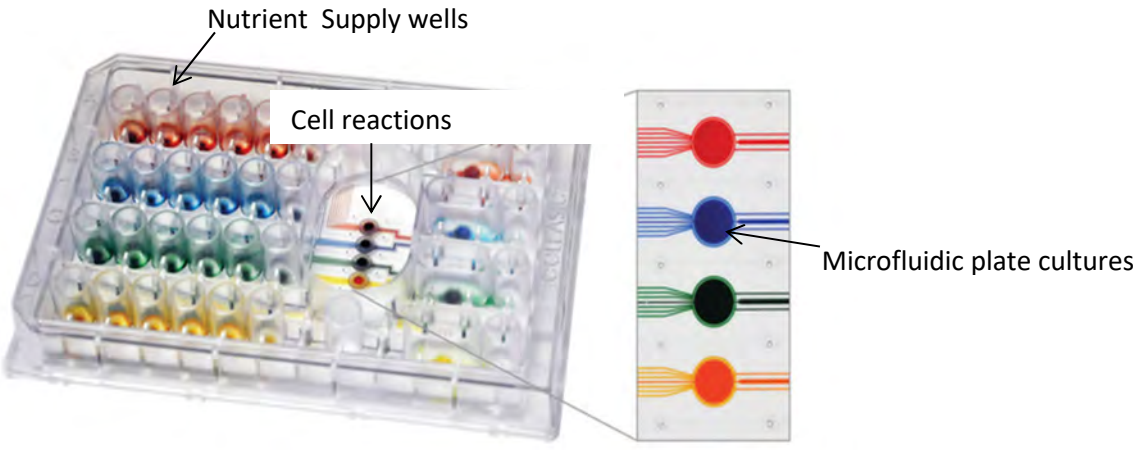


Figure 1.3 Image of a commercially available long term microfluidic cell culture system demonstrating the four plate culture region CellASIC ONIX (Millipore, 2016)

Microfluidics is also used for bacterial cell analysis as it provides a basis of miniaturising the current processing methods. An example of a miniaturised biological detection process is fluoresce detection. Fluorescence detection of bacteria was first developed for the detection of fluorescent proteins within a sixty nano-litre droplet (Martin *et al.*, 2003). This paved the way for many other microfluidic methods using fluorescence. Many of these methods still require additional equipment such as microscopes and lasers (Mohan *et al*., 2013). A system has been developed using a florescence assay to test the metabolic activity levels and antibiotic resistance using microfluidic droplets (Boedicker *et al*., 2008). More recently, Mohan *et al* (2013) used a bio-sensing method of antibiotic treatment of bacteria to successfully treat bacteria using green fluorescent protein. A multichip microfluidic method was developed by Churski *et al* 2012, where a microfluidic chip was used to generate droplets and mix them with antibiotics. Sequences of microfluidic droplets, containing various drug combinations, are incubated off the chip in a tube. The droplets of bacteria are then added to a detection chip, where fluorescent material is detected. Microfluidic techniques for mammalian cells have been developed for the diagnosis of cancer, where a cell's deformability was shown to determine the cell's health (Hou *et al*., 2009). Microfluidic systems have been used for 3D cancer models. A 3D cell culture microfluidic chip was developed by Yu *et al* (2010), where a droplet encapsulation system was used to create 3D spheroids on a hydrogel. The droplets are then moved through a microfluidic chip that has traps, this allow the alginate cultures to be maintained long term. Ziolkowska *et al* (2013) created a long term culture chip, where 3D cancer spheroids of HT-29 human colon carcinoma cancer cell were formed. Using the hydrophobic properties of PDMS chips, the cells attach to each other in each well within the chip,

forming a tumour spheroid structure. Cells are evaluated on the chip using a confocal microscope and fluorescent assay. Drugs were applied to the cultures and the diameter of the cancer spheroid is measured after treatment. The result shows dead cells detaching from the spheroid and thus reducing the size of the spheroid. The limiting factor for this method is that the number of cancer spheroids on a chip is dependent on the number of wells within the chip. Microfluidic methods are also used for developing 3D cultures, which involves techniques such as cell immobilisation using alginate (Yoon *et al*., 2013, Sakai *et al*., 2011) (Alessandri *et al*., 2013) or extracellular matrix gels (Guo You *et al*., 2011). 3D spheroids are generated using a microfluidic chip by aggregating the cells in one area (Ong *et al*., 2008) and testing drugs on these cells. Although these methods have advanced drug discovery research, a method that does not require additional florescent detection or create 3D cultures without the addition of gels or immobilising agents, to the authors knowledge, is currently not available.

The research introduced in this thesis investigates a method of cancer model spheroid generation that is simplified to culture media and cells alone. This new method generates individual bioreactors that are capable of being mixed with any drug. The research here develops a novel method for 3D spheroid production. This new method has the potential to provide high-throughput generation of miniaturised 3D cultures within individual reactions for drug analysis. Consequently it could change how preclinical trials are performed by creating a system that is miniaturised, more efficient and more effective in identifying new drugs.

There are two cell types investigated in this research; bacterial cells and mammalian cells. Bacterial cells were chosen as the initial test on the instrument as they permit rapid growth compared to mammalian cells. This will determine if the microfluidic

instrument is capable of cell culture and is biocompatible. Providing that the bacterial cultures are successful within the instrument, mammalian cell testing will then be pursued. The mammalian cells being used are breast cancer cells, which grow less rapidly than bacteria cells; it takes 18 hours for mammalian cells (ATCC) to double, compared to 20 minutes for bacteria cells (Sezonov *et al*., 2007). Cancer cells were selected for the microfluidic system as it a major area of medical research that affects fourteen million people worldwide (Stewart, 2014). Diagnosis is performed by taking a tissue biopsy and performing analysis to determine if it is cancerous (Yu *et al*., 2014). It is necessary to be able to test cancer cells in-vitro in a high-throughput manner to access more data rapidly. A system that is capable of creating high-throughput cancer assays, while reducing the number of cells required, as well as the volume of drugs necessary, could immensely improve the amount of tests that can be performed, hence delivering more data about the disease. Specific conditions are necessary to enable growth of the cancer cells and this will truly be the determining factor to reveal if the microfluidic instrument is successful in creating stable breast cancer models.

The development of a new system for cell analysis raises a number of engineering challenges:

1. Miniaturising the cell treatment process into individual reactions. Each reaction is required to be manipulated and this requires mixing the cell with drugs or antibiotics.

2. The health of the biological samples must be maintained for the duration of testing and cannot be compromised.

3. The cells must have the correct atmospheric conditions, namely temperature and CO_2 levels, which requires any system to be gas permeable and isothermal.

4. The cell samples are required to be monitored in real time.

5. It must facilitate the reduction in the volume of the culture to create a high-throughput system.

To address these challenges, a new method of cell culture and analysis is established in this research. To create cell models, the desired situation is to mimic in-vivo cell morphology, in a laboratory setting. The scale of microfluidics aims to provide an environment closer to the cell's own length scale, with the average culture flask being in the scale of millimetres and the scale of the cells 10-100um in diameter (Whitesides, 2006). The novel method proposed in this thesis shows how high-throughput preclinical cancer models and bacteria cultures are formed using microfluidics droplets. The instrument developed in this research uses continuous microfluidic droplets as bioreactors. The microfluidic droplets are shown in Figure 1.4.

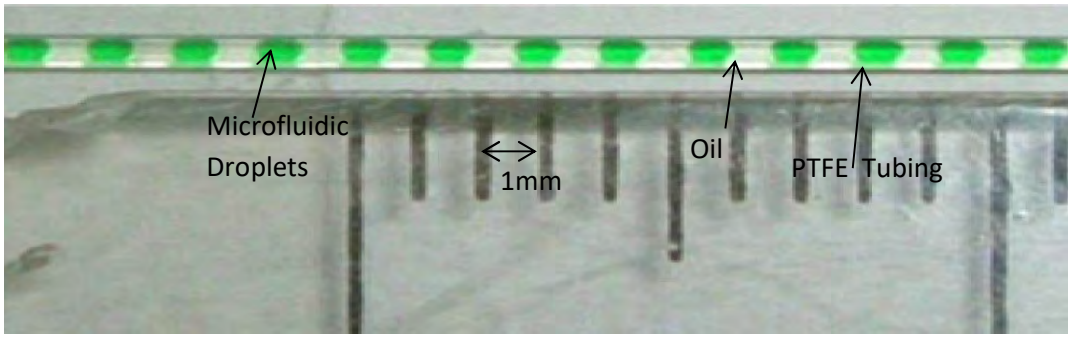


Figure 1.4 Image of a Droplet microfluidic system where aqueous green droplets are carried through the PTFE tubing with a transparent carrier oil. Droplets are approximately 1mm in length.

Each of these reactions can be varied in volume, size and content depending on the requirements of the test. This variability gives the advantage of setting up many experiments rapidly. This has the advantage that multiple droplets can be created and tested on the system at the same time. As the droplets are contained within a tube that is less than 1mm in diameter, the surface to volume ratio is increased, compared to current culture flask methods. This allows heat transfer and diffusion rates to be shorter thus reducing reaction time (Teh *et al*., 2008). This research demonstrates an innovative method where low volume samples are utilised to provide data for preclinical analysis. To challenge the system, it is necessary to form biologically relevant cell models and treat with drugs. The benefit of this is that a sample of the cells from the initial biopsy could be taken and tested against a wide range of drugs and combinations of drugs on a patient specific level. This could enable the clinician to have more detailed data on how a patient could potentially react to a treatment and also have the ability to test alternative treatments if a current treatment fails. This instrument creates a high-throughput way of creating cultures and analysing them all on a single platform. To the author's knowledge, this is the first time 3D cultures are created in gel free microfluidic droplets. A preclinical system that is continuously creating cultures provides endless possibilities on the amount of reactions that can be created and subsequently, the amount of data being generated.

The aim of this research is to develop an instrument that can create a robust system for cell treatment. The uniqueness of this instrument is the miniaturisation of bacterial cell cultures and creation of miniaturised three dimensional mammalian cell cultures. The four main objectives of this research are:

1. To create an instrument that will generate cell models. This incorporates the design and building of the instrument that will generate microfluidic droplets.

2. To design a novel method of cell detection for bacterial cells that can accurately evaluate the rate of growth of the cells.

3. To validate this instrument using bacterial cells for the identification of antibiotic resistance.

4. The final objective is to validate the microfluidic droplets as bioreactors for mammalian cancer cell models.

This introduction has given an outline of the issues associated with current methods of developing high-throughput drug discoveries. Understanding cell reactions provides a method of drug screening for diseases. Microfluidics has provided a way of improving the process of cell preparation for analysis. The microfluidic droplet method provides a means of forming discreet reactions; however, they currently rely on florescence and laser equipment for the evaluation of the cells. Having reviewed the current methods and outlining the microfluidic methods, a great insight has been gained into the shortcomings of the current state of the art methods of cell analysis. This has shown a need for one complete high-throughput system that can create individual cultures that are biologically relevant, incubate the cultures and find a response to drugs; all on one system. Throughout this thesis, the development of a 'proof of concept instrument' will be discussed and how this instrument has the potential to establish cancer models, as well as a system that can rapidly detect antibiotic resistance in bacterial culture models, is demonstrated. Droplets are also generated to produce 3D breast cancer cell models. This high-throughput system has the potential to give more information about a disease, thus making this a new method of preclinical analysis.

The remainder of this thesis is divided into 6 sections:

Chapter 2: Biological Methods

In this chapter the biological processes required for general cell health are introduced. These include detailed descriptions of current cell culture methods for both bacterial and mammalian cellular analysis.

Chapter 3: Instrument Design

The design and development of the microfluidic instrument is described here. This includes the design of the fluidics and the temperature control of the cultures. The optical units integrated into the instrument for real time analysis of the cells are established.

Chapter 4 :**Instrument Optimisation and Pre-Testing**.

In this chapter the microfluidic instrument is optimised for bacterial and mammalian cell tests. Aspects such as temperature control, the number of cells within the droplets and where the cells will reside within the droplets are analysed here. The visual monitoring system of the cells is optimised and pre-testing of the cells within the droplets is conducted.

Chapter 5: **Bacterial Validation**.

Initial testing of droplets as bacterial bioreactors is introduced here. The method of microfluidic droplets as a viable alternative for bacterial culture is demonstrated. The microfluidic system is then optimised for the droplets in terms of droplet volume and velocity. The characterisation of bacterial growth, as well as the identification of antibiotic resistance is demonstrated in this chapter.

Chapter 6: Mammalian Validation.

This is split into four sections The first section verifies the droplet environment is capable of creating healthy cell bioreactors. The second section shows how long the cells will survive within the droplets. The third section shows the development of individual 3D cell cultures within the droplets and the final section demonstrates that with a miniaturised process cancer cells can be treated and a gene expression signature can be found using the microfluidic droplets.

Chapter 7*:* **Conclusions and Recommendations**.

This section reviews the whole thesis with recommendations made for future work.

Chapter 2

Biological methods

A main objective of this thesis is to validate the microfluidic instrument for cells and the identification of antibiotic resistance. Cell culture is necessary in biological applications as it provides an in-vitro model of cells and allows their examination under selected chemical and physical conditions. Cell models provide a system for understanding cells reactions to treatments such as antibiotics. From the limitations of current methods outlined in chapter 1, the focus of this research project is on the miniaturisation of the cell culture process into microfluidic droplets. The techniques to prepare the cells for testing on the new device are explained in this chapter.

Bacterial and mammalian cells follow the same trend in growth as in Figure 2.1. The cell cultures will begin to grow in lag phase. This is where the cells are getting accustomed to the environment. Once the cells have adhered to the flask or are settled in suspension they will begin to proliferate. Cell proliferation occurs at an exponential rate and this is known as the log phase. The log phase is where the cells

uptake most of the nutrients from the growth medium causing the cells to divide and multiply. The log phase provides the optimum conditions to examine the effects of how a drug will influence further growth or inhibition of the cells (Iloki Assanga, 2013). It is at this stage that testing or sub culturing should take place as the cells are at their most active. The time for each phase is defined by the cell type. Some bacterial cells will take hours to go through the growth cycle while mammalian cells can take days. Once the cells have reached capacity within the flask, they enter a plateau called the stationary phase. The plateau phase is where the cells remain healthy but do not divide anymore and equilibrium is achieved. After the cells have expedited all the nutrients at the stationary phase the cells begin to die and this is called the death phase.

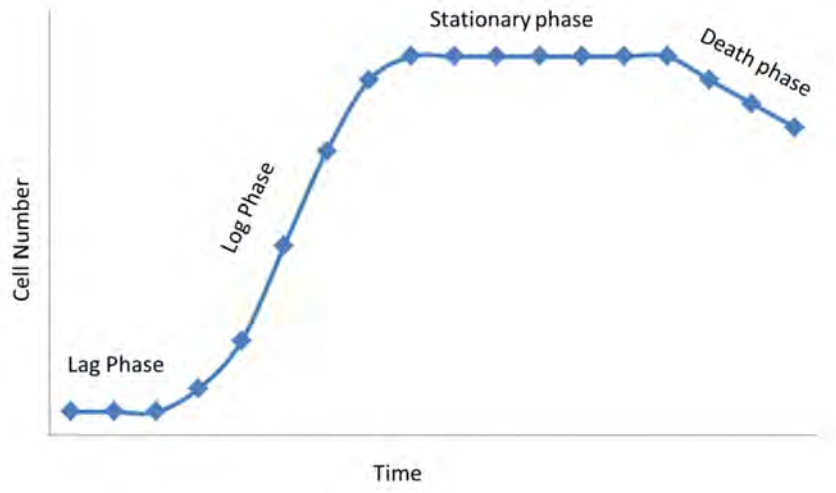


Figure 2.1 Representation of growth curve of mammalian and bacterial cells

There are two types of mammalian cell culture, adherent where the cells will need to attach to a surface or flask to proliferate an example of adherent cells are bone cells. The second cell type are known as suspension culture where cells do not require an adherence signal to proliferate in suspension such as blood cells. Sample preparation is different depending on the cell type, with a procedure to detach the cells from the

adherent surface necessary for cell maintenance. Maintenance of the cells consists of providing the correct nutrients for growth of the cell and removing waste from the cells when in culture. Throughout this chapter the fundamentals of cell culture will be explained and procedure for maintenance detailed.

Genes within the cell will dictate how it will react to a drug or an antibiotic. Gene expression is used to quantify if there is a change in the expression level of a particular gene between treated and untreated cells (O'Neill *et al*., 2012). Genes encode the proteins within a cell and this in turn will dictate the cells function. The gene expression process is demonstrated in Figure 2.2 where DNA consists of a double strand of nucleotides and this forms a double helix shape. The p

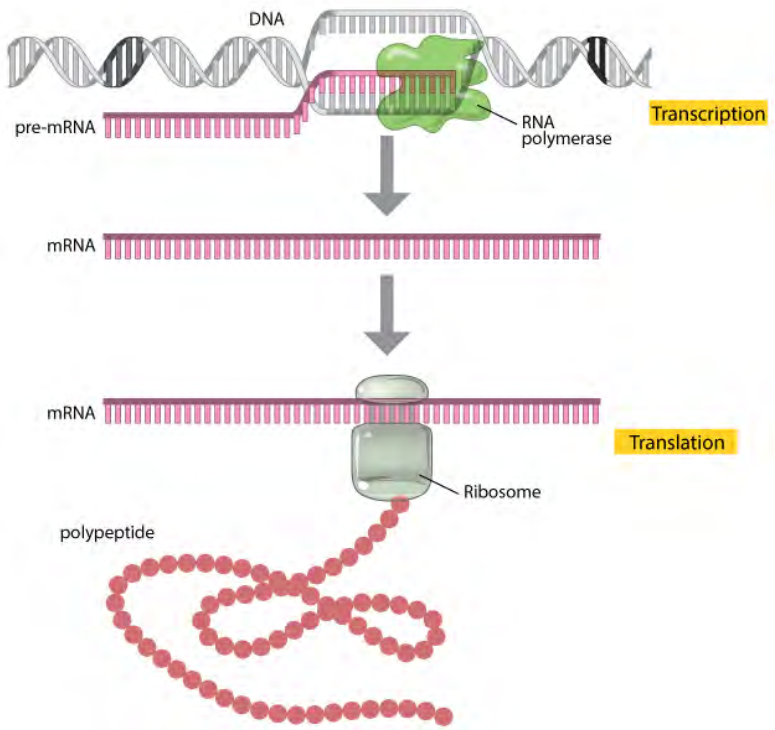


Figure 2.2 Clancy and Brown (2008) schematic of gene expression from transcription of the DNA to a single strand of mRNA translation of mRNA by the ribosome and hence the generation of polypeptides which form proteins.

rocess of gene expression consists of two steps transcription and translation. Transcription is the process of RNA synthesis. RNA is synthesised by unwinding the double helix to form a single strand. (Berg JM, 2002) This creates a template

known as messenger RNA (mRNA) that transcribes the information from the DNA. For the cell to perform a function the mRNA is required to be translated. The ribosomes function is to translate the mRNA information to polypeptides which form amino acids.(Clancy, 2008) The folding of the amino acids will dictate the function of the protein and hence the function of the gene. Analysing gene expression allows a snapshot in time of a particular cells state.

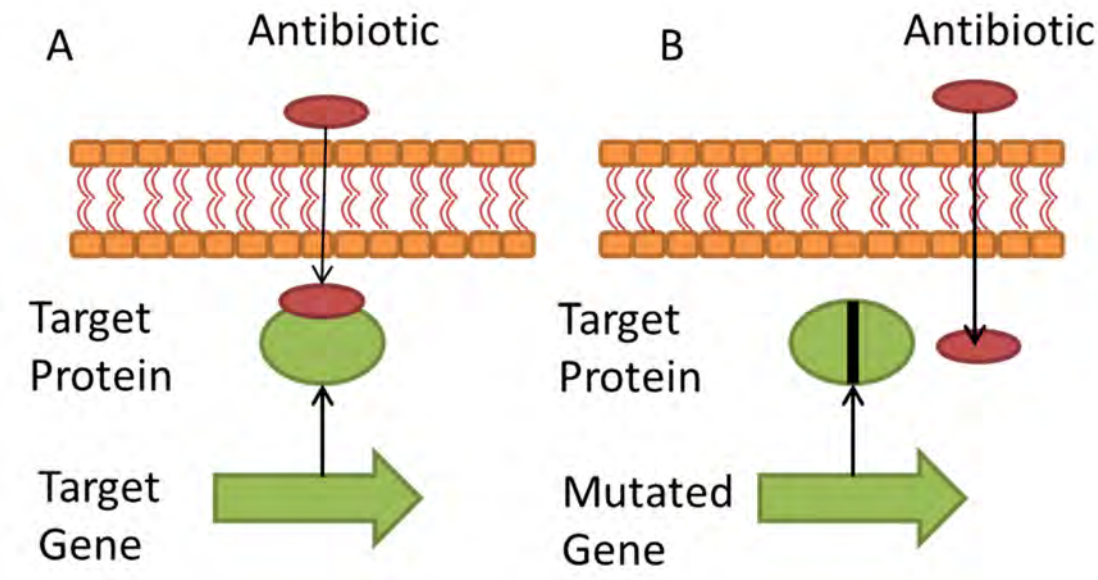


Figure 2.3 A. The antibiotic attached to a target protein to treat a disease. B. A target protein that has mutated which has prevented the antibiotic from reaching its target gene.

Cell resistance to drugs occurs at a molecular level where antibiotics bind to a specific protein. Figure 2.3 A depicts where an antibiotic will bind to a gene target. Once the antibiotic reaches the target this will signal to the cell how to react. However, these genes can mutate and this will inhibit the antibiotic from being effective. In Figure 2.3 B it is demonstrated how a mutation to the target gene site will prevent the antibiotic from reaching the target site. During infection there are large population of pathogens and if a single point mutation occurs within a gene this can prevent the gene to carry out its normal function and can confer resistance (Blair *et al*., 2015). This is what causes antibiotic resistance. Understanding gene

expression can gives a better understanding of the disease. The instrument developed through this research creates a system to form cell culture in microfluidic droplets and also allows the identification of antibiotic resistance within the droplets. Cancer cell models are developed within the microfluidic droplets and gene expression is examined. This gives an insight into conditions within a cell's molecular and physical reactions. Gene expression is quantified using real time quantitate Polymerase Chain Reaction (RTq-PCR). RTq-PCR amplifies DNA to a detectable level. The procedure for RT-qPCR is demonstrated in this chapter.

The remainder of this chapter details the biological methods for the growth, maintenance, quantification and treatment and gene expression of the biological cell samples.

2.1 Cell selection

The initial validation of the instrument in terms of biocompatibility is undertaken using bacterial cultures. *Escherichia coli (E.coli)* (BL21 L1191, Promega) is selected as the bacteria as it is well characterised in microfluidics (Wang *et al*., 2010) and grows relatively quickly with a doubling rate of every 20 minutes (Sezonov et al., 2007) compared with 38 hours for MCF-7 cells (ATCC) mammalian breast cancer cells. With the aim of treating bacteria effectively and identifying antibiotic resistance a second bacterial strain was required. An *E.coli* strain that is resistant to ampicillin is used in the identification of antibiotic resistance. This is used to potentially determine if the droplets could identify a specific treatment plan on a patient specific level.

Selection of mammalian cell models was focused on breast cancer cells. Breast cancer models were chosen as they are widely studied and understood. Previous

studies have focused on the development of 3D models *(Kenny et al., 2007, Gong et al*., 2015) of breast cancer cell lines, MCF-7 cells were selected as the model to base this on. MCF-7 cells are human carcinoma cells and are characterised as adherent cells. They are different to normal breast cells as they express oestrogen and progesterone receptors. These cells have been used in many characterisation studies of cancer (Hou *et al*., 2009, Oleksowicz *et al.*, 1995). MCF-7 cells are typically 10μm in diameter and the scale of microfluidics (>1mm) will challenge the cells at the same scale as the cells.

A second breast cancer cell model was used for gene expression analysis. Treating the cells within the droplets and evaluating the gene expression allows the system to be verified as a viable alternative to current techniques. BT474 breast cancer cell line was selected as a breast cancer model to reproduce a study performed by O'Neill *et al* 2012. BT474 cells are human breast cancer cells that are epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor (Her2) positive. Once the cell selection was completed, the processes of maintenance and treatment were initiated using conventional methods discussed below.

2.2 Bacterial Culture

2.2.1 Luria-Bertani preparation

Luria-Bertani (LB) is the culturing media for bacteria and contains the nutrients for the healthy growth of *E.coli*. LB was prepared under sterile conditions to prevent contamination from other bacterial sources. LB is a solution that is prepared in deionised H_2O containing NaCl, Tryptone and Yeast extract (table 2.1). NaCl contains sodium ions for osmotic balance.

Table 2.1 LB preparation concentrations

H2O	**500ml**
NaCl	5g
Tryptone	5g
Yeast extract	2.5g

Tryptone contains the proteins and amino acids that aid in the cells growth and repair. Yeast contains the vitamins required by the bacteria to proliferate. The solution is mixed until all constituents are dissolved in the solution using a magnetic stirrer. This LB is then sterilised within an autoclave at 121°C for 20 minutes. After sterilisation the solution is ready to be used as growth media for the *E.coli.*

2.3 *E.coli* Culture Techniques

Traditional culture methods were used to form the seeding cultures for the droplets. *E.coli* proliferation follows the same trend as in Figure 2.1. To prepare the *E.coli* a

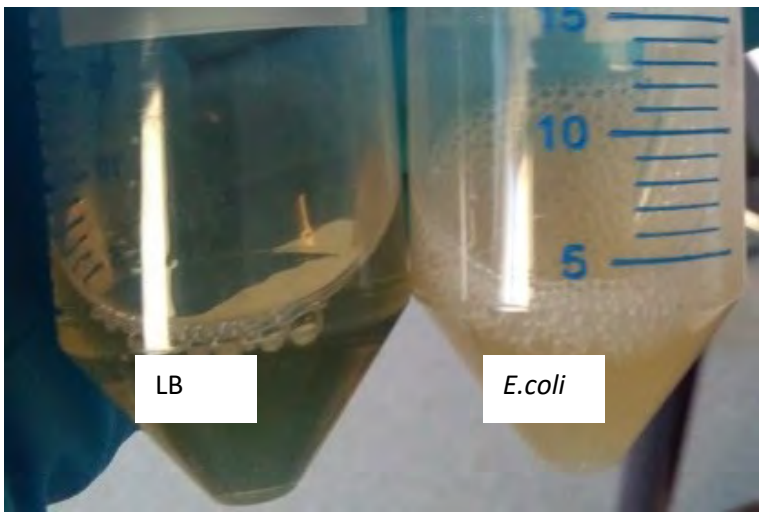


Figure 2.4 An image of translucent LB which contains no E.coli and cloudy LB which indicates the presence of E.coli

streaking from the frozen stock of BL21 cells is taken and placed within a centrifuge tube containing 5ml of LB. All steps are carried out beside an open flame to maintain sterility. The *E.coli* mixture is then placed within an orbital shaker incubator and incubated at 37^{o}C at 200rpm for 16 hours. Proliferation of the *E.coli* over the 16 hours is evident in Figure 2.4 where the LB on the left remains translucent and the addition of *E.coli* on the right has a cloudy appearance after culture. Due to the change in colour this indicates growth. Optical density (OD) readings are used to quantify growth where the change in absorbance of light through the sample is monitored. The theory behind the optical density measurements is discussed in chapter 3. Suspensions of *E.coli* to LB were made and seeding OD was obtained taking readings at the beginning of each test to monitor growth thereafter.

2.4 Mammalian Cells

2.4.1 Laminar flow hood requirements

Cell models are influenced by atmospheric conditions (Iloki Assanga, 2013) and it is necessary to keep all material around the cultures sterile as to not affect the conditions of culture and introduce contaminants. Cells were prepared under sterile conditions in a laminar flow hood (figure 2.5). This hood is initially sterilised with 70% ethanol applied to all surfaces and materials entering the laminar flow hood. To prepare the cells the hood should be switched on for 10 minutes prior to culture to ensure the pressure within the hood is slightly higher than the room pressure. This will make sure that all the flow of air through the HEPA filter will not be contaminated by anything within the cell culture laboratory. The equipment used to culture the cells is also required to be sterile. The laminar flow hood (figure2.5)

should contain individually wrapped serological pipettes, pipette controller cell and culture flasks. To dispose of cell culture waste Virkon (Sigma Aldrich) disinfectant

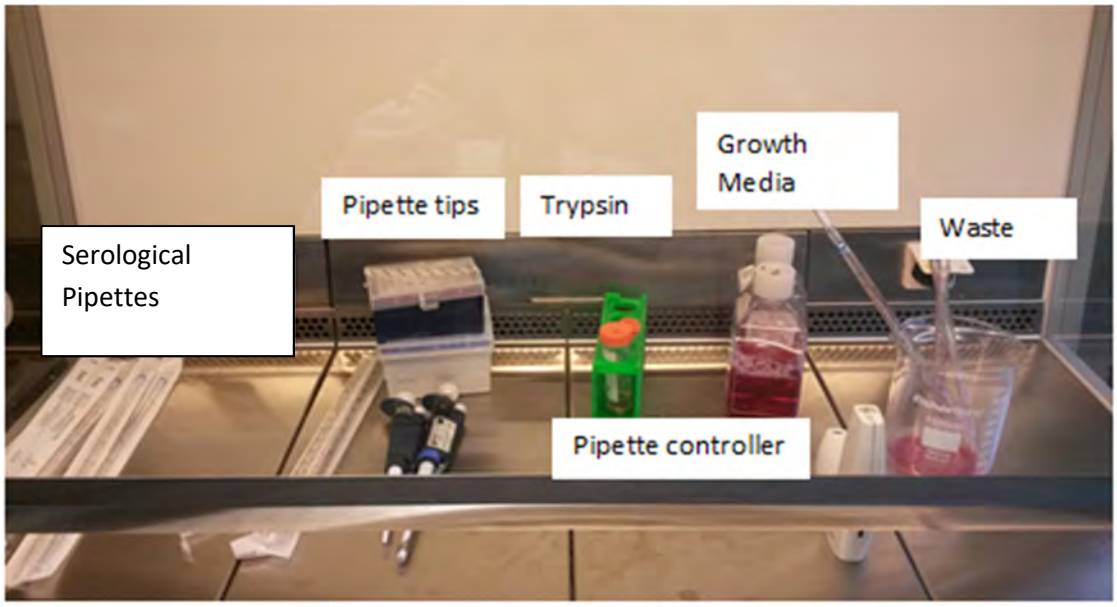


Figure 2.5 A working laminar flow hood with sterilised equipment for cell culture procedures

is diluted with water and all pipettes and waste are added to this after use. Once disinfected, all plastic waste and used pipettes are placed in an autoclave. The autoclave will sterilise the waste by heating to 120^{o}C to kill all contaminants within the plastic ware and this can then be disposed of. After culture is complete a UV light within the laminar flow hood is switched on and used to prevent contamination for the next use. Once every item is prepared the laminar flow hood is ready for use with cell cultures.

2.4.2 Mammalian cell culture

Cells will respond physiochemically (nutrients around the cell) and physiologically (physical environment the cell is contained in i.e. plastic dish or gel matrix) to their environmental conditions (Iloki Assanga, 2013). They are visually monitored daily using an optical microscope to track the growth and determine if the cells are confluent within the flask. Figure 2.6 A shows MCF-7 cells that are sparse within a

flask while in Figure 2.6B the cells that are confluent within the flask. Cell growth is dependent on cell to cell interactions and will grow at a slower rate if there is too much room for the cells (Nelson and Chen, 2002). The cells will also be prevented from growth if there is not sufficient room for growth, consequently, it is important to have the correct area for the cells to grow in the flasks. When cells are sub-cultured they are placed in a larger flask or split into two flaks. If cells are not sub-cultured they will be depleted of nutrients and begin to die.

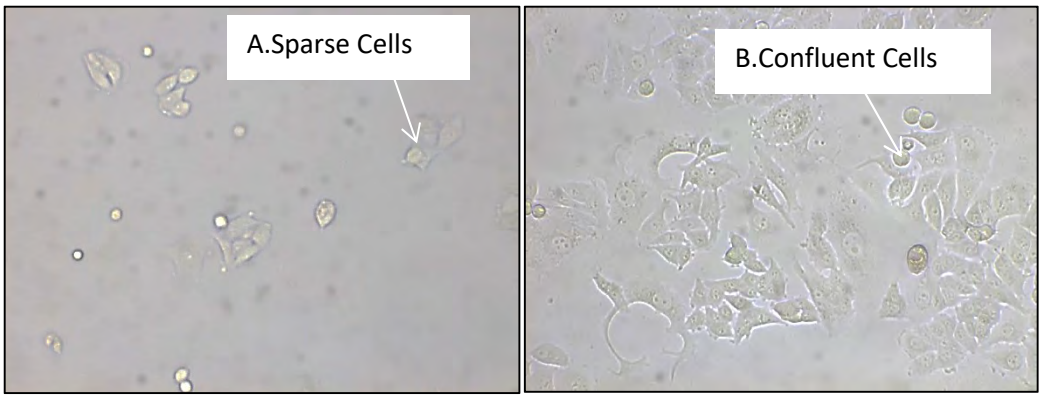


Figure 2.6 A. A 10x microscope image of MCF-7 cells sparse within a flask. B. A 10X microscope image of MCF-7 cells confluent within a flask

In general, cells are used in experimentation when the flask is 70% confluent and the cells are in their log phase. The rate at which the cells will reach all the phases is dependent on each individual cell line and this can be obtained from the cell line supplier. The growth rate for each cell line is quantified as the amount of time it takes for the cells numbers to double. The population doubling time for MCF-7 cells is 38 hours (ATCC) and these are required to be sub-cultured every 2-3 days. Each time the cells are sub-cultured and the passage number increases this allows the monitoring of the amount of times the cells have been manipulated. The steps for sub-culturing both cell models are similar with slight differences in the media preparation of the cells.

2.4.3 Media Preparation

The media that the cells are maintained in contains all the growth factors, proteins and nutrients for the cells to thrive and grow. Each cell type will have recommended media for the optimum growth of the cells and each media is supplemented accordingly. For MCF-7 and BT-474 cells Dulbecco's Modified Eagle's medium (DMEM, Sigma Aldrich) is used. DMEM is supplemented with 10% Fetal Bovine Serum (FBS) 1% Penicillin-Streptomycin and 1% L-gluatmine the BT474 cells were also supplemented with 1% Sodium Pyruveate. FBS provides proteins, amino acids and growth factors for cell growth. L-glutamine and sodium pyruvate provide energy to the cells while penicillin-streptomycin is an antibiotic to prevent the cells from becoming bacterially contaminated. If the cells become contaminated they are no longer suitable for use and new cells from stock need to be cultured. DMEM also contains phenol red as an indicator of pH, if the media becomes pink it is an indication of a basic solution while if it becomes yellow it is an indication of an acidic environment. If the pH changes it can affect and reduce the growth of the cells (Mackenzie *et al*., 1961). Once all the constituents are mixed this is used for all mammalian cell tests. Media is pre heated to 37^{o}C before application on the cells to avoid a sudden temperature change for the cells.

2.4.4 Cell Storage

Cells can be stored long term for future use by cryopreservation. To store the cells a freezing media is required. All cell samples used in this study originated from this process. This freezing media is made in a 10ml solution containing 5ml FBS, 4ml DMEM and 1ml Dimethyl sulfoxide (DMSO Sigma-Aldrich). DMSO is a cryo-protectant for the cells, it slows the freezing of the cells so that ice crystal formation

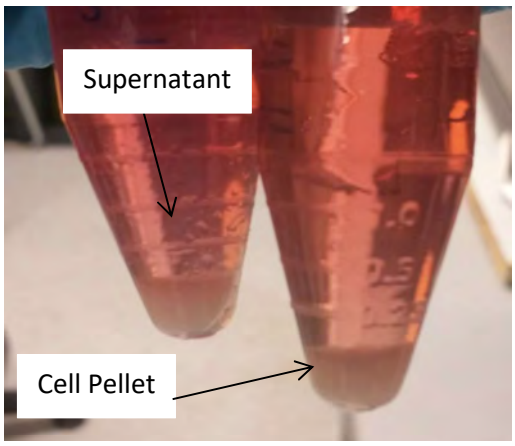


Figure 2.7 Two centrifuge tubes containing a pellet of cells at the bottom of the tube while a supernatant remains above the cells.

is slow within the suspension as this could damage or rupture the cells. To freeze the cells the following procedure was performed; Cells incubated in a flask are taken from the incubator. Cells health is monitored by microscope by visually seeing that the cells had adhered to flask (cell death is visually indicated by floating cells in the flask). The cells are washed with phosphate buffer saline (PBS) solution and a 3ml trypsin is used to detach the cells from the flask. The trypsin is neutralised by adding the equivalent amount of media to the mixture and then centrifuged at 1000 rpm for 5 minutes. A pellet is formed (figure 2.7) at the bottom of the centrifuge tube and the supernatant is removed from the cells. Freezing media is added to the cells and the mixture is aliquoted into 1ml tubes and immediately put on ice. The cells are then placed in a -80^{o}C freezer within a polystyrene container to make sure the freezing process is slow enough to not affect the cells. The cells are preserved in a freezer and thawed as required.

2.4.5 Cell Culturing.

Initially, cells were thawed in a manner to ensure that the maximum amounts of cells are recovered after freezing. The frozen cells are placed in a water bath at 37^{o}C and remain there until they begin to thaw. Once the thawing process is in progress and

there is a small amount of ice left in the vial it is then moved to a laminar flow hood. The cells are transferred to the t-25 flask and 5ml of complete DMEM media is added. The flask is placed in an incubator at 37°C, 5% CO_2 and 95% humidity. After 24 hours of incubation and when the cells have attached to the flask the cells are washed with PBS and new media is added to the flask. The new media is added to remove the DMSO. DMSO can be toxic to cells and to minimise this effect the cells must be sub-cultured before use in testing. As the cell growth becomes confluent within the flask it is then sub-cultured into larger flasks t-75 and t-125 which have 75cm^2 and 125cm^2 growth area respectively. For optimum experimentation cells that are most active within the log phase of growth are required. Sub-culturing the cells at 70% confluency and replacing growth media ensures that the cells will remain in this phase. To sub-culture the cells MCF-7 and BT474 cells were prepared in a t-75 flask. Initially the culture media is taken from flask. After this the culture is washed with PBS, by applying 3ml of buffer to the cells covering all the cells and immediately removing the PBS. Cells are separated from the flask by using 3ml of trypsin EDTA (Sigma Aldrich) and incubating at 37°C, trypsin is an enzyme to separate the cells from the flask. The enzyme is neutralised by adding equal amounts of culture media to the cells. The suspension of cells and trypsin is then added to a centrifuge tube and placed in the centrifuge and rotated at 1000 rpm for 5 minutes. This will form a pellet of cells at the end of the centrifuge tube and a supernatant above the cells. The supernatant is then discarded as it contains the trypsin. The cells are then suspended in new culture media and quantified using trypan blue exclusion assay. The cells are then placed in a flask and incubated or frozen for the process to begin again as required.

2.5 Cell quantification

2.5.1 Trypan Blue

Correct quantification of cells is required to know how many cells are being sub-cultured and also for the addition of treatment to the cells. For this research it is necessary to know the amount of cells needed to form 3D cultures and for the synthesis of cDNA. To quantify the cells trypan blue (Sigma-Aldrich) is added in equal concentration to the cell suspension usually 50ul cells suspension to 50ul trypan blue. Trypan blue is an exclusion assay that excludes dead cells and is used to indicate cell viability. It works by a dye infiltrating the cells that are dead with a blue dye as they have damaged cell membranes. The live cells have intact cell membranes and the dye cannot enter these cells (Strober, 2001). These cells are visually monitored using an inverted microscope and can quantification is carried out using a haemocytometer. A haemocytometer Figure 2.8A is a grid that provides a way to determine a concentration of cells in 1ml from a 10µl sample and was used to determine all concentrations of cells for testing. Figure 2.8 B shows live cells are

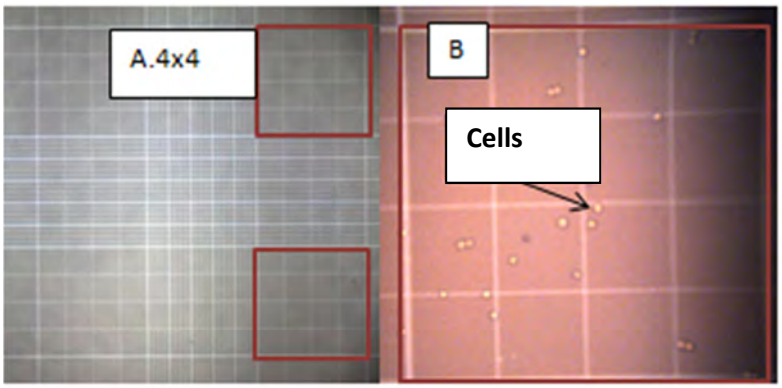


Figure 2.8 A 10x microscope image of a haemocytometer grid. B A 10x image of live cells on 4x4 haemocytometer grid after application of Trypan Blue exclusion assay.

bright and where dead cells present they would have turned blue. To count the cells, the number cells within two of the 4x4 grids are added together this value is then multiplied by 10,000 and this gives the concentration of cells per ml. Once the

amount of cells is verified, concentrations can be prepared for further testing. This type of exclusion will also give an indication of cell health as the amount of dead cells within a culture can be verified.

2.5.2 Cell Viability and Cytotoxicity

Cell health and viability is measured in terms of cytotoxicity. To continuously monitor the cells health alamar blue assay (Life Technologies) is used. As trypan blue is an end point assay, and is toxic to cells, it is not sufficient for ongoing monitoring of the cells continuously. A viability indicator is necessary in these cultures and alamar blue allows the measurement of the metabolic and proliferation

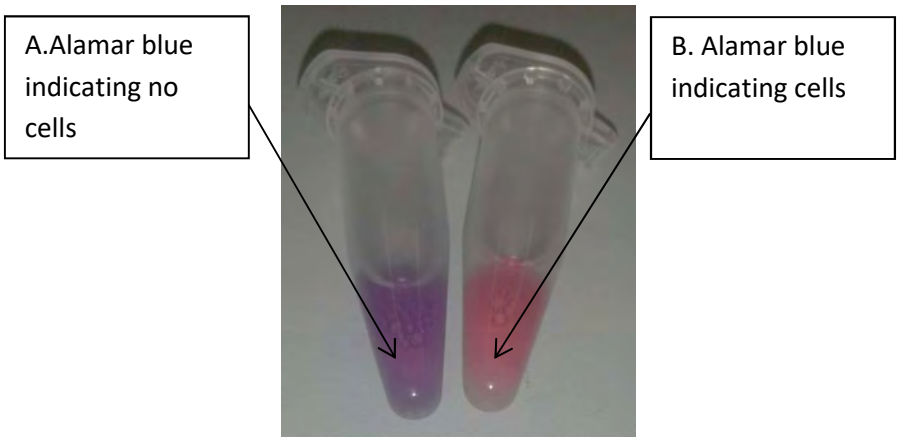


Figure 2.9 Two microcentrifuge tubes containing 10% Alamar blue, the left tube contains h no cells indicated by the blue/purple colour, live cells are indicated in the right tube as a pink colour is indicated

rate as it can be applied and monitored over a number of days without affecting the cells health. This method is used to measure the viability of cells within the droplets cultures and indicate if the cells are thriving within the droplets. Alamar blue indicator is measured by florescence on a microplate reader in a 96 well plate. Microfluidic droplets containing 10% Alamar blue and 90% cell culture mixture were incubated at 37°C and 5% CO_2. The cells were incubated over a number of days and when sampled, 5-10µl of the cell sample is mixed with 90-95µl cell culture media and then fluorescence at 540nm excitation and 590nm was measured. When

introduced to the cells the assay is blue as it contains resazurin which is a cell permeable compound. When cells are viable this compound will convert reazurin to resorufin or from blue to red. Resorufin is red fluorescent and this acts as an indicator of viable cells. Cells that are not viable will not convert to resorufin and remain blue. Figure 2.9 shows two samples incubated for 24 hours, on the 2.9A it is clear that the sample is blue and contains no cells while 2.9 B the suspension has been converted to red. This assay has allowed the cell health to be evaluated without influencing the cells growth patterns over time.

2.6 Cell Staining

Confocal microscopy gives a higher resolution and contrast that is not possible using a standard inverted microscope. This allows the imaging of three dimensional structures of the cultures and also determine the viability of the cells. Confocal microscopy provides a mechanism for the fluorescent stained cells to be imaged. MCF-7 cells are the cells investigated using this process as they can form three dimensional spheroids. Staining of the cytosol and cell nucleus is used to demonstrate the cells structure. Cells are incubated in droplets for 24 hours and then placed on a glass slide. The cells are left for 45 minutes to adhere to the glass slide. To fix the cells at a specific time point the cells are covered in Paraformaldehyde 4X concentration for 1 hour. The cells membrane is then made more permeable by adding 1ml PHEM buffer (0 mM Pipes, 25 mM Hepes, 5 mM EGTA, 1 mM MgCl, pH 6.9) and 1ul TritonX100 and left to cover the cells for 11.5 minutes. 500ul goat serum mixed with 9.5ml of PHEM is used to cover the culture to prevent nonspecific staining. The cells were placed in a humid environment that was dark as the stains were fluorescent. The antibody is then added, 1ml PHEM-goat serum mixture is mixed with 1ul Phalloidin and 3ul Hoechst and this is left for 45 minutes. Hoechest

stained the cytosol red and Phalloidin stained the nucleus of the cell blue. The cells are then washed for 20 minutes in PHEM to remove excess stain from outside the cells. Heated vinyl is used opposite each cells structure to mount them on the large glass slide. This is then covered and left at 4°C overnight and are then ready for imaging. Imaging was performed on Zeiss LSM 710 microscope. A laser to excite the staining causes fluoresces of the cells. Zen software 2008 LSM was used to process the images of the culture. Specific images of the cytosol and the nucleus could be achieved individually or in composite. This is used to give an indication if the cells were able to form a spheroid structure within the droplets.

2.7. Gene Expression Sample preparation

To perform gene expression a copy of the DNA is required. The DNA will give a reading of the genetic configuration of the cell. When treated with a drug the level of expression of some genes can change and this can be an indicator of the cells response to the drug. To copy the DNA, complementary DNA (cDNA) is used.

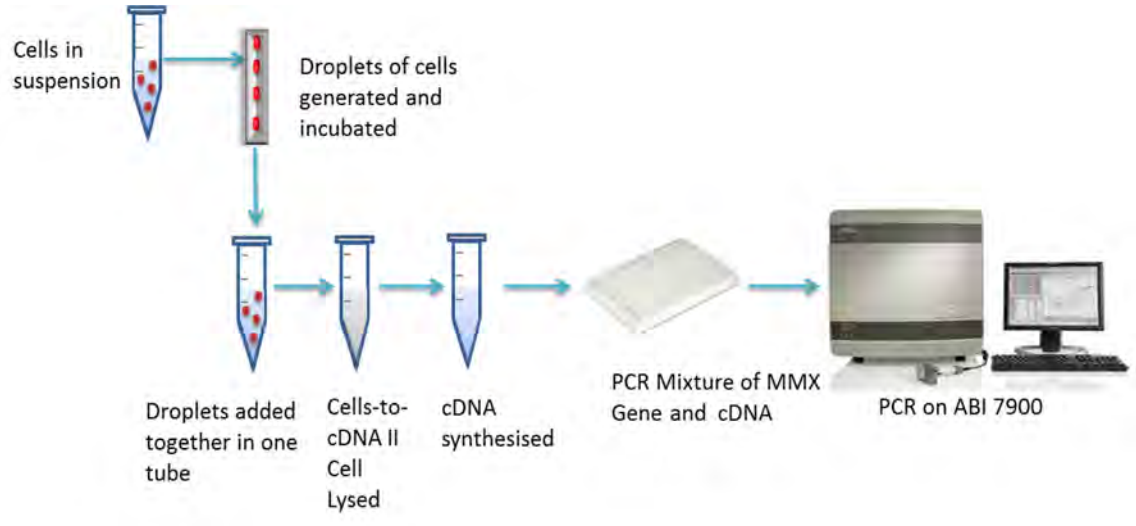


Figure 2.10 Schematic of process from droplet generation to gene expression

cDNA is a copy of the messenger RNA within a cell. To synthesise the cDNA Life Technologies kit Cells-to-cDNAii is used. Samples from both drug treated and untreated cells are used to make cDNA. After incubation the droplet cell samples are centrifuged at 1000rpm for 5 minutes. The supernatant is taken from the samples and PBS is used to wash the cells. The cells are then centrifuged again at 1000rpm for 5 minutes. The PBS is taken from the sample and 100ul of ice cold cell lysis buffer is added to the suspension and the sample was incubated on a thermocycler at 75^{o}C for 10 mins. Cell lysis buffer will open up and rupture the cells membrane to allow RNA to be released from the cell. After this the sample is placed on ice 2µl DNase and is then added to the 100ul sample and then the sample is incubated at 37^{o}C for 15 minutes. DNase will degrade the genomic DNA and this will ensure that the RT-qPCR step will amplify cDNA only to detectable levels as genomic DNA will be indistinguishable from those amplified from cDNA. This DNase is then inactivated by heating to 75^{o}C for 5 minutes. At this stage the cDNA will be released in the sample and the next step is to amplify this cell lysate to a detectable level using RT-qPCR.

Relative quantification by real time PCR (RT-qPCR) is the process of amplifying DNA to a detectable level. The method of reverse transcription is performed by adding 10µl of cell lysate, 4ul dNTP mix 2µl oligo(dT) primers and 16ul nuclease free water. This is incubated for 3 minutes at 70^{o}C and then the sample is placed on ice. Next, 2µl of reverse transcription buffer, 1µl M-MLV reverse transcriptase and 1ul RNase inhibitor are mixed together by centrifugation for 1 minute. RNase inhibitor is used to purify the samples. The sample is then incubated for 20 minutes at 42^{o}C and then at 95^{o}C for 10 minutes to inactivate the reverse transcriptase. The samples are then amplified to a detectable level. Amplification isundertaken using

TaqMan (Life Technologies) reagents via the Life Technologies PCR instrument AB7900HT. Taqman gene expression assays is set up in triplicate for statistical evaluation. Each sample contains 2.5µl TaqMan gene expression master mix 0.5µl cDNA from the prepared cell sample, 1.75ul RNase-free water and 0.25µl gene assay of interest. Gene assays were selected to replicate a previous study by O'Neill *et al* (2012). Each of the 5µl samples were pipetted into a 384 well plate. No template controls (NTC) which contain H_2O in place of the cells are also included in each experiment. The NTC ensures that there is no contamination from the preparation of the samples. The well plates are loaded into the AB7900HT. PCR is performed at 95°C for 5 minutes this is to denature the bond in the DNA strand. The next step is 95°C for 15 seconds and 60°C for 1 minute this makes a replicate of the cDNA and amplify each signal. This process is repeated for 40 cycles. The threshold was set at 0.2, where the expression above the threshold was evaluated.

$$R= \frac{E\ treated^{(CT\ gene\ of\ interest - C\quad housekeepe\quad gene)}}{E\ untreated^{(CT\ gene\ of\ interest - CT\ housekeepe\quad gene)}} \qquad (2.1)$$

The data was analysed using the ΔΔCt method (REST software) which represents the fold change difference between treated and untreated cells. The ΔΔCt method gives a relative change in gene expression of the treated and untreated cells in terms of genes of interest against a house keeper gene. REST software used equation 2.1 where a relative quantification ratio gives a fold change between samples (Pfaffl *et al*., 2002). This method was used for all gene expression analysis for this research.

2.8 Chapter Closure

The focus of this chapter is to establish the methods that are used in the analysis of cells in microfluidic droplets. The cell growth cycle forms the basis of understanding when to sample the cells for testing. The selection of cells based on the application

within the microfluidic droplets were *E.coli* for bacterial culture and breast cancer models of MCF-7 and BT474 cells are selected. The nutrient media for both cultures were outlined and these will maintain the cells. To quantify the cells concentration trypan blue a live/dead assay is used. Viability assay alamar is used to examine the cells. Three dimensional biologically relevant cultures are developed within the droplets and to examine the structure of the cells, confocal microscopy and cell staining was required to visualise this. The ultimate test of this system is treatment of cells with anti-cancer drugs. To show these are effective, gene expression analysis was necessary. To perform gene expression cDNA was required and the method for synthesis is described here. The REST software analysis method is outlined for the quantification of fold change. These methods are necessary to prepare the cells and provide this droplet system with validation. The next chapter will discuss the design of the instrument to perform the biological analysis.

Chapter 3

Microfluidic device design

3.1 Introduction

This chapter describes the design of the microfluidic instrument used to perform all biological investigations. The conditions for cells to grow are outlined here and these are the most imperative factor in the whole device design. The cells will be processed in microfluidic droplets and the physical conditions that the cells will be exposed to are demonstrated. It is necessary to individually mix the droplets with drugs and the internal circulations within the microfluidic droplets are described in this chapter. Once the physical conditions are established the material components are justified to prevent adversely affecting the cells. The final part of this chapter details the whole design, from droplet generation to processing of the cells. This device is used for all testing for both mammalian and bacterial cells throughout the rest of this thesis.

3.2 Mammalian Cell Culture Environment

The critical consideration for cells is the environment they need to survive in. Cells respond to their physical and chemical environment(Iloki Assanga, 2013) and this is provided by the microfluidic droplets. It is imperative to mimic the cells in-vivo environment as closely as possible to maximise the growth of the cultures. The atmospheric conditions required for cell health are 5% carbon dioxide and 70% relative humidity to maintain a pH of 8.5. These conditions provide the most efficient cell growth, as 5% CO_2 allows the metabolism of nutrients which maintains the pH of the cells. Physical conditions such as the mechanical stimuli to the cells can determine how the cells will develop (Lim *et al.*, 2006). In-vivo conditions where the cells form structures within the body (organs) require both atmospheric and physical conditions working together in order to function correctly. The size of the mammalian cells used in this research is in the microscale ranging approximately 10-20um in diameter. Creating a platform that can mimic the cells own dimensions will allow cell division, cell to cell communication and consequently allowing lineage in an appropriate scale for preclinical analysis.

3.3 Microfluidic Droplets

Microfluidic droplets are used to create the growth environment for the cells. The most vital condition for the cells is that they remain healthy and avoid contamination.

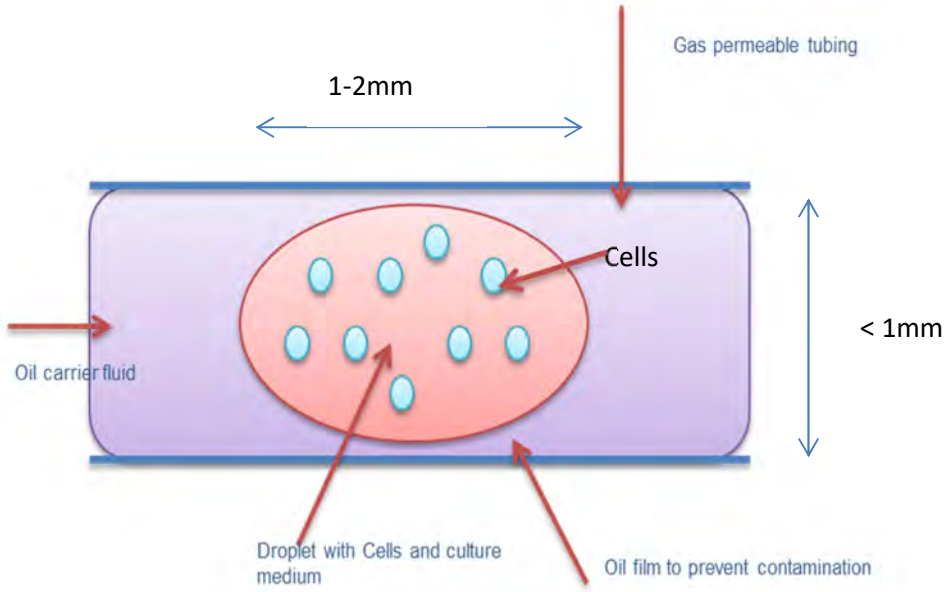


Figure 3.1 A schematic of a microfluidic droplets containing cells maintained within permeable tubing with oil carrier fluid.

The goal is to design microfluidic droplets as demonstrated in Figure 3.1. This shows the microfluidic droplet within a circular cross section tubing less than 1mm in diameter. Microfluidic droplets provide a robust environment for the cells conditions such as, viscosity, temperature, volume and atmosphere. Each droplet is a unique and discrete reaction that can be created continuously. This gives the advantage that a large number of reactions can be created without the addition of bigger equipment for example multiple incubators (Teh *et al*., 2008). The advantages of microfluidic droplets are that heat, mass transfer times and diffusion distances are shorter, creating shorter reaction times (Kaminski *et al*., 2016). Consequently, microfluidic droplets have been used for microbiology applications where yeast, algae and bacteria (Kaminski *et al*., 2016) have been investigated. Microfluidic droplets have also been used for the manipulation of mammalian cells from single cell analysis to complete cell culture (Barbulovic-Nad *et al*., 2010).

Microfluidic droplets are two phase liquid-liquid flow. The two phases are immiscible with an aqueous phase for the bio-reactor droplets and an oil phase

carrier fluid. Due to the two phase flow the interfacial tension between the aqueous phase and oil phase will affect the flow and determine if the droplets will stay as complete droplets or break up (Gu *et al*., 2011). The flow regime is determined by inertial forces divided by the viscous forces which are a dimensionless parameter known as the Reynolds number. The Reynolds number is calculated by equation 3.1 where the oil carrier phase density(ρ), u is the fluid velocity, d is the diameter of the tube and μ is the viscosity of the carrier fluid.

$$\text{Re}=\frac{\rho u d}{\mu} \tag{3.1}$$

Typically, due to the dimensions of microfluidics, the Reynolds numbers are so low the flow is laminar as the velocity is low. Viscosity will determine the type of carrier fluid used as it is desirable to have a low viscosity fluid to reduce viscous effects in the flow. As the viscosity of the fluid plays a significant role in the flow and the dimensions of the capillaries are so small the interfacial tension between the fluids needs to be considered using the Capillary number.

$$Ca = \frac{u\mu}{\gamma} \tag{3.2}$$

The capillary number gives a ratio of the interfacial forces to the viscous forces and is defined in equation3.2, where γ is the surface tension (N/m) between the carrier oil and the aqueous phase. A low capillary number indicates that the flow is dominated by capillary forces.

$$We=ReCa \tag{3.3}$$

The Weber number is used to evaluate if droplet breakup occurs within the microfluidic tube. The Weber number represents the ratio of disruptive hydrodynamic force to stabilizing surface tension force (Nomura *et al*., 2001). At

low Weber numbers the inertial affects will be negligible while above a critical Weber number (We=1.1) droplets will begin to distorted an break up (Nomura *et al*., 2001). This illustrates that it is important to know the physical conditions of the fluids before cells are exposed to this environment.

3.3.2 Film Thickness

An advantage of continuous droplets is that each droplet is fully wrapped in oil allowing each droplet to be an individual reaction. (Kashid *et al*., 2005). Fully wrapped droplets are not affected by the tubing or the carrier fluid. An oil layer is required to surround the microfluidic droplets and wraps them fully to form unique individual reactions. This oil layer is known as a film around the droplet. The capillary pressure to viscous drag forces determines the film thickness around the droplet (Baroud *et al*., 2010). The capillary number also influences the film thickness (h) and is defined by Bretheron law (Kashid *et al*., 2005) (equation3.4) where r is the radius of the tube.

$$h=1.34rCa^{2/3} \qquad (3.4)$$

For stable droplets the film surrounding the droplet needs to remain undisturbed. A length to diameter ratio to show the development of stable films around the droplets found that film thickness of L/d=1.89 forms an uniform film around the droplets (Mac Giolla Eain *et al*., 2013). If the film breaks down this would cause wetting of aqueous phase with the capillary and could be a cause of contamination of the droplets.

3.3.3 Internal Circulations and Mixing

Droplet movement through the system occurs throughout every process on this instrument. Internal circulations happen within the microfluidic droplets as they

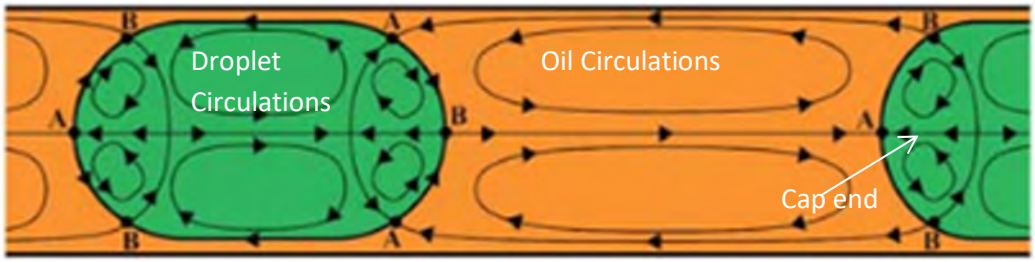


Figure 3.2 Baroud *et al* 2010 schematic of flow patterns in two phase flow within a microfluidic channel. Internal circulations indicated within the green droplets with separate circulations at the cap ends. Oil circulations are demonstrated in orange

move through the tubing. Circulations occur in the aqueous droplets as they will never move at the same velocity as the carrier fluid. This causes the droplets to move in a 'treadmill belt' fashion through the system with constant rotation. As the biological samples are cells, and as that are alive within the droplets it is necessary to understand the condition of cell within the droplets. Issues such as shear forces can negatively affect cells (Zhong *et al*., 1994) and it is essential to reduce this to limit damage to the cells. Shearing of cells occurs when two cells move past each other. The shear force applied to each of the cells on each other can cause the cells to rupture. Internal circulations within the flow will cause the cells to move within the droplets and it is vital that this does not negatively affect the cells in terms of increasing the amount of shear between the cells.

Figure 3.2 shows a representation of two phase flow, here Baroud et al (2010) represent the oil phase with orange and the aqueous droplet in green. Figure 3.2 also shows the regions of circulation within the droplet. Within the droplets there are two regions of circulation the middle region where the contents are constantly being

circulated and the leading and trailing cap end region, where two regions of circulation reside. An advantage of internal circulations is the mixing of fluids within a droplet. Tice *et al* (2003) show that the droplet length influences the circulation within the droplets and also shows that smaller droplets require a smaller travel length to achieve full mixing. One full circulation requires a droplet to move a length that is 4.5 times the diameter of the tube for perform complete mixing (Tice *et al*., 2003). Once full mixing is achieved the droplets will continue to recirculate. Longer droplets can take longer to achieve full circulation.

The physical attributes of the droplet such as size and velocity determine how mixing occurs within a droplet. Wang *et al* (2015) conducted CFD studies into the

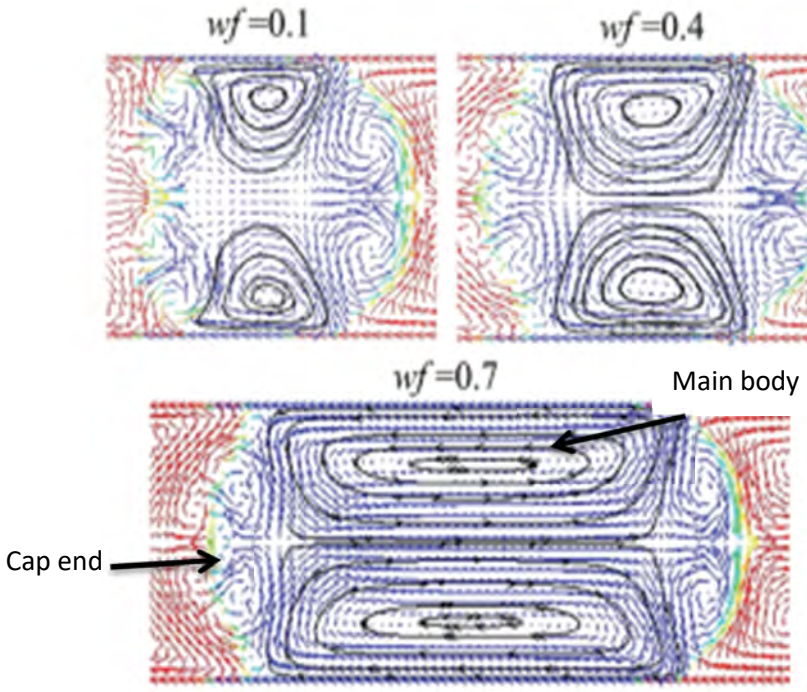


Figure 3.3 Wang *et al* (2015) CFD study of velocity profile of droplets of different lengths Where recirculation is demonstrated at the cap ends and within the main body of the droplet.

internal circulations within microfluidic droplets. Figure 3.3 shows the velocity profile of droplets of varying length in a straight cross-section. Two regions idetified within a droplet where the main body of the droplet and the leading and trailing cap ends of the droplets demonstrate recirculation (Wang *et al*., 2015, Kurup and Basu,

2010) Figure 3.3 shows that the cap ends region is decreased with increased length of droplet. The increased velocity shows a decrease in the length of the cap end region of circulation. The internal circulation may impact the location of the cells within a droplet. Knowing the circulation pattern within a droplet can aid in the design of experiments as it is essential to have sufficient movement within the droplet to provide circulation of nutrients to the cells. This will be achieved by having droplets that will move at least five times the diameter of the tube to ensure full internal mixing occurs. This will determine the size of the droplets reactions. Internal circulations within microfluidic droplets are verified experimentally by Kashid *et al* 2005. Particle image velocimetry (PIV) was performed on microfluidic droplets and compared to a CFD model of the same conditions. The PIV measurements are characterised and show internal circulations within the droplets at increasing velocity while at low velocity the particles remain stagnant within the flow. This shows that the system will have to have a velocity that will induce internal circulations and ensure that the cells do not remain stagnant within the droplet.

3.4 Material Choice

3.4.1 Selection of Tubing

As the droplets are formed from biological mixtures it is required to have materials that will not adversely affect the cells physically or chemically. PTFE tubing of circular cross section was selected as it is biocompatible (Chen *et al*., 2003), hydrophobic with a contact angle of 108^{o} (Zhang *et al*., 2004) and optically clear. PTFE tubing is transparent and allows optical access for continuous monitoring of cells throughout incubation and testing. Optical clarity is also required for optical density readings and microscope imaging of the droplet cultures. This transparency

also aids in verification of the droplet size as droplet length is measured through the tubing using a Vernier callipers. Another advantage of this tubing is that it is hydrophobic and this prevents the biological droplets from wetting the walls (Baroud *et al*., 2010) of the tubing and hence, stops the cells attaching to the walls and using this as an adherence signal for growth. A consequence of hydrophobicity is that thousands of droplets can be formed within the same tubing without adversely affecting each other and this allows the tubing to be used multiple times without contamination. This reduces the amount of consumables needed compared to other microfluidic culture methods where microfluidic chips have to be disposed of after use (Fiorini and Chiu, 2005).The tubing is gas permeable to CO_2 this gives the cultures a standard culture atmosphere (ZEUS). This tubing was used for all experiments using microfluidic droplets.

3.4.2 Selection of a Carrier Fluid

Oil is used as a carrier fluid and this forms a film around the microfluidic droplets.. To select appropriate carrier oil, characteristics like biocompatibility and fluid properties such as density and viscosity have to be considered. The selection of PD5 (Momentive USA) silicon oil was chosen due to its advantageous characteristics. Biocompatibility is required as the oil interfaces with the biological sample. PD5 oil is biocompatible and will not adversely affect the growth of the cells. Silicon oil has been shown to be biocompatible as it does not affect the adhesion or growth of the cells (Malchiodi-Albedi *et al*., 2002). This oil is sterilized by filtering through a 0.22um polyethersulfone filter (Millipore). Hydrophobicity of the oil aids in the encapsulation of the droplets and this creates a layer of oil around the droplets. The encapsulation of droplets within the oil allows each droplet to be an independent culture. PD5 silicon oil is also optically clear and this aids in the monitoring of the

cells. The viscosity of the oil is 4mPa.s this is close in viscosity to the biological samples which 1mPa.s. The low viscosity oil aids in the mixing of droplets when necessary as the oil around the droplets can breakdown when a mixing mechanism is introduced. The oil is permeable to CO_2 which allows the cells to maintain the required atmospheric conditions. Mammalian cells will not survive without a 5% CO_2 environment and if the silicon oil is not permeable this will show that the environment will not be conductive to cell growth. The density of the oil is also an advantage as it is necessary for the oil to be less dense than the biological sample. It is necessary to prevent the biological samples having contact with the atmosphere to reduce exposure to contaminants. The lower density oil provides a layer above the biological sample when creating the droplets and this prevents direct exposure to the atmosphere. As the oil is a commercial product the density given by the manufacturer is 920kg/m^3 and measured as 919 kg/m^3. The second phase fluid is aqueous based and predominantly cell culture media and the density is measured. The density is measured by weighing 10ml of media and is repeated five times. The density of 998 kg/m^3 was found making it denser than the oil, this is required to make sure the cells are always covered in oil.

3.5 Device Design

3.5.1 Fluidic Control.

It was necessary to have a system for smooth delivery of continuous individual bioreactors droplets within the carrier fluid. It is a requirement for the volume of each reaction to be precise. This is the only way to generate consistent droplets that are directly comparable to each other. To create the precise reactions a stable fluidic system is essential. There are many methods available to control the fluidics and these include a range of pumps from peristaltic pumps, syringe pumps to gear pumps. The pumping system selected was a Harvard apparatus PHD 2000 syringe pump. This pump has the capability to infuse and withdraw the fluid and this function is used to take the samples from the tubing after incubation. The syringe pump works under controlled pressure of the syringe and for this reason a glass syringe was selected. A Hamilton gas-tight syringe was used as the syringe will not deform as much as a plastic syringe under pressure. The precision in the pumping system occurs with the control over the plunger of the syringe where it can infuse or withdraw the fluid at a rate that is required. Tubing was connected to the syringe pump using lure lock (Upchurch scientific) fittings.

3.5.2 Generation of Droplets

The generation of droplets was performed using a dipping stage. The festo dipping stage was mounted on a bread board and the tubing was held in place by a metallic holder with a 1.3mm hole to allow the polyimide holding tube through. This holding tube is glued in place and the microfluidic tubing is fed through. The holding tube

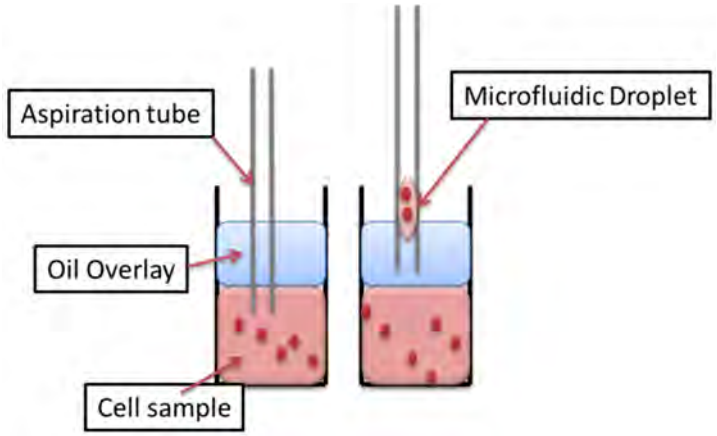


Figure 3.4 Schematic of droplet aspiration system where the aspiration tube is initially positioned in the cell sample then back into oi l creating the induvial droplets

was incorporated to keep the microfluidic tubing straight. This will ensure that the level of the tubing will always be controlled and that the depth of dipping will not need to be changed between samples. The biological sample was held in a 384 well plate. To maintain the dipping head in oil the 384 well plate was machined 5mm deep which allows oil to be placed above the biological samples at all times during the droplet generation process. The oil prevents air ingress to the system. In the interest of repeatability it is necessary to keep these plates at the same level for the duration of the test as the level of sample within a plate or tube is one of the factors that will determine the size of the droplet. Sample volume in the 384 well plate tube is typically 30µl in volume and is overlaid with 50µl oil. The droplets were aspirated into tubing of ID 584µm, 1050µm OD creating droplets of the same size repeatedly. Droplets are generated by moving the robotic stage holding the tubing into the cell

sample (figure 3.4). An example of a typical droplet generation consists of the tubing being immersed for the required time for example 2.25s at a flowrate of 15µl/min to generate a 600nl droplet. The tubing is held in place on the system and a festo robotic stage controller (figure 3.5). The stage is controlled by festo FCT software that interfaces the stage with a PC. The droplets move through the instrument demonstrated in figure 3.5and the droplets are then mixed with drugs or antibiotics.

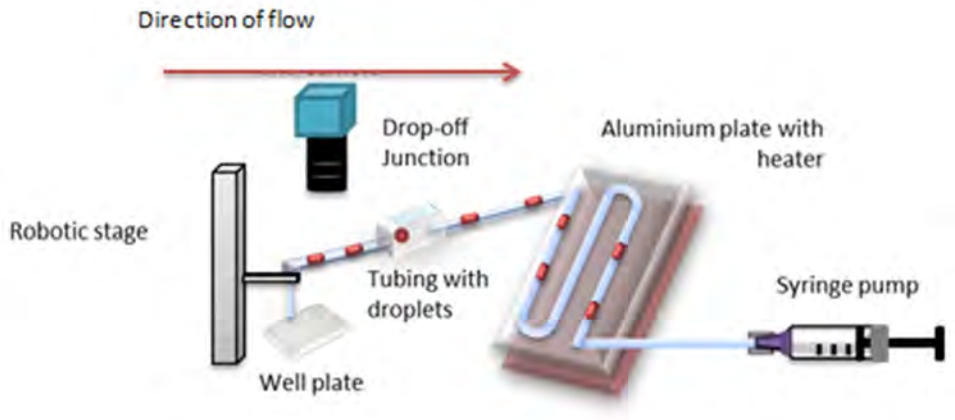


Figure 3.5 Schematic of microfluidic droplet system from droplet aspiration where the syringe pump is pulling a flow through the tubing the droplets are being generated by the movement of the robotic stage . The droplets are then imaged by the microscope camera and mixing with drugs occurs within the drop-off junction, when the droplets reach the aluminium heater plate they are maintained at 37°C

3.5.3 Droplet Mixing

Mixing comprises of combining two droplets together, one cell sample droplet and one droplet containing a drug, antibiotic, or compound of interest. Mixing of droplets is introduced so that the system can become multiplexed. It is important to have precise mixing as premature fusion of the droplets will result in unreliable data (Teh *et al*., 2008). Mixing for both bacterial and mammalian cells with drugs occurs in this research. This research aims to validate the effectiveness of mixing in droplets as an alternative to using individual flasks.

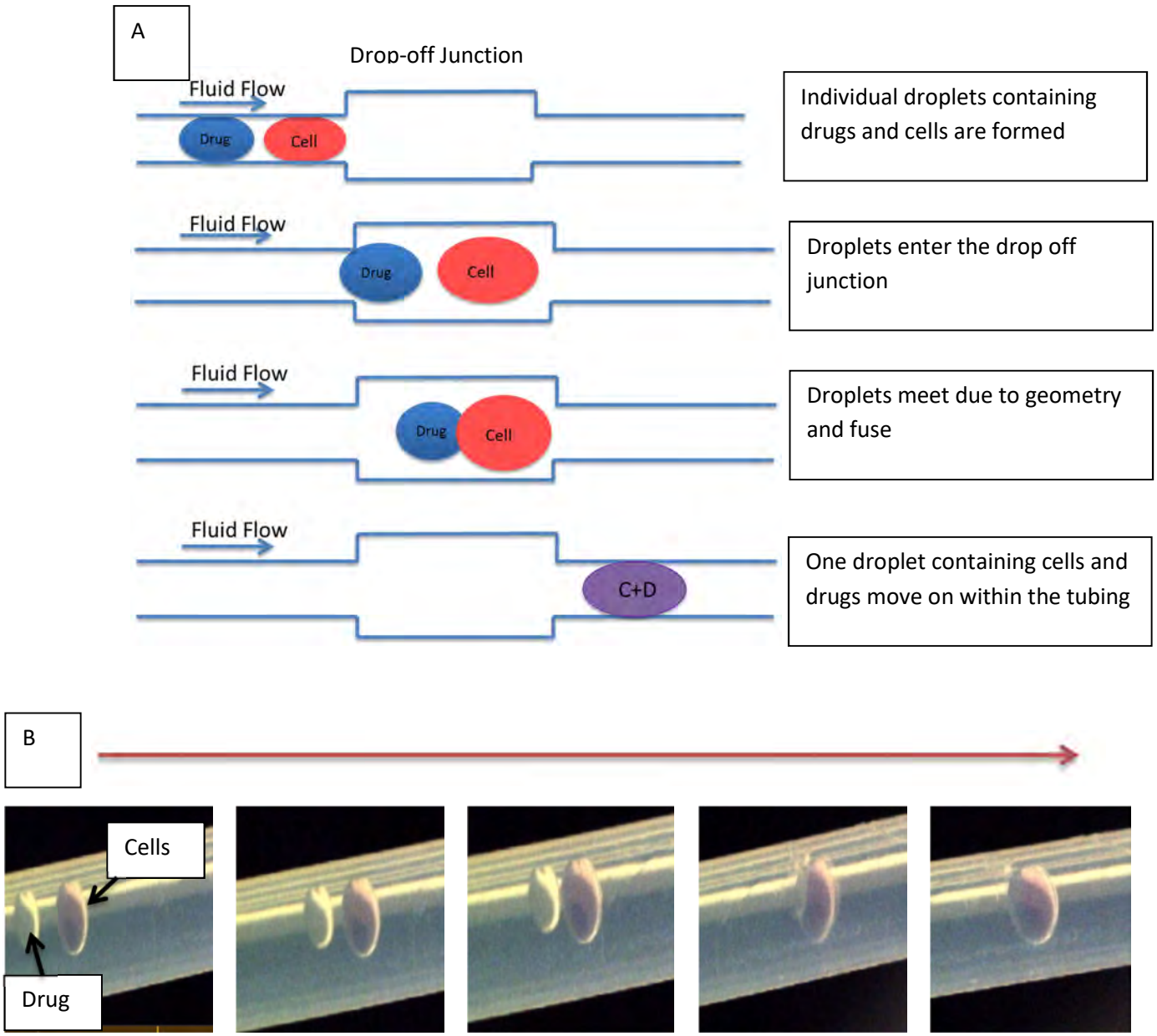


Figure3.6 A schematic of drop-off junction for mixing cells with drugs. Two droplets are generated one with cells and one with a drug. The droplets reach the drop-off junction and move with their own velocity as they do not reach the diameter of the tubing. Once the droplets reach each other instabilities in the film surrounding the droplets causes the droplets to merge. B. Images of drop-off junction mixing system where the small drug droplet and the large pink cell culture droplet are within a drop-off junction and merge once contact occurs.

The mechanism incorporated into this system for mixing was a drop-off junction (McCarthy, 2015). The method of mixing is droplet fusion, where droplets merge due to geometry of the tubing. Droplet fusion initiates when two or more droplets are brought close to each other until a thin film of fluid forms connecting the interfaces. This causes pressure to increase on the film and imbalance in the surface tension will cause the film to rupture and the droplets to coalesce (Teh *et al*., 2008). The drop-off

junction (figure 3.6A) takes advantage of the droplet geometry and physical movement through increasing the diameter of the tubing. To mix, two droplets are generated in tubing of 812µm ID, a large droplet containing cells and a smaller droplet containing a drug. The droplets move through the tubing as individual droplets until they reach the drop-off junction. From here the droplets transitions from tubing of diameter 812µm to 900µm for the mammalian cells and 812µm to 1200µm for the bacterial cultures. This transition is imaged in figure 3.6 where the mixing occurs by the geometry of the droplets changing when they enter the larger region. In this instance, both droplets are sub-spherical within the tubing due to surface tension forces dominating and do not reach the diameter of the walls. The flow rate of the droplets is no longer controlled fully by the set flow rate of the pump and the droplets move through the tubing by convection. This works to the advantage of mixing by allowing the larger cell droplet to move slowly through the drop-off junction. The smaller drug droplet moves at a faster speed due to its geometry than the larger cell droplet and this causes the droplets to meet and coalesce within the tubing. The droplets meet within the tubing as the first droplet that enters the tube will pause as the viscous drag forces are more than the force of the pump pulling the flow within the tubing. The second droplet then meets this droplet, and forms a larger volume making this new mixed droplet dominated by the flow of the pump and moves through the tubing again (McCarthy, 2015). Figure 3.6 B shows two droplets in a tube the large pink droplet is the cell culture media and followed the smaller drug droplet. The droplets then move onto incubation. This juction allows mixing of any two droplets containg any cells and any compound of interest. The drop-off junction can be incorporated at any point within the system allowing for mixing pre or post incubation.

3.5.4 Droplet Incubation

To maintain cell health and for the cells to divide a stable temperature is required. It

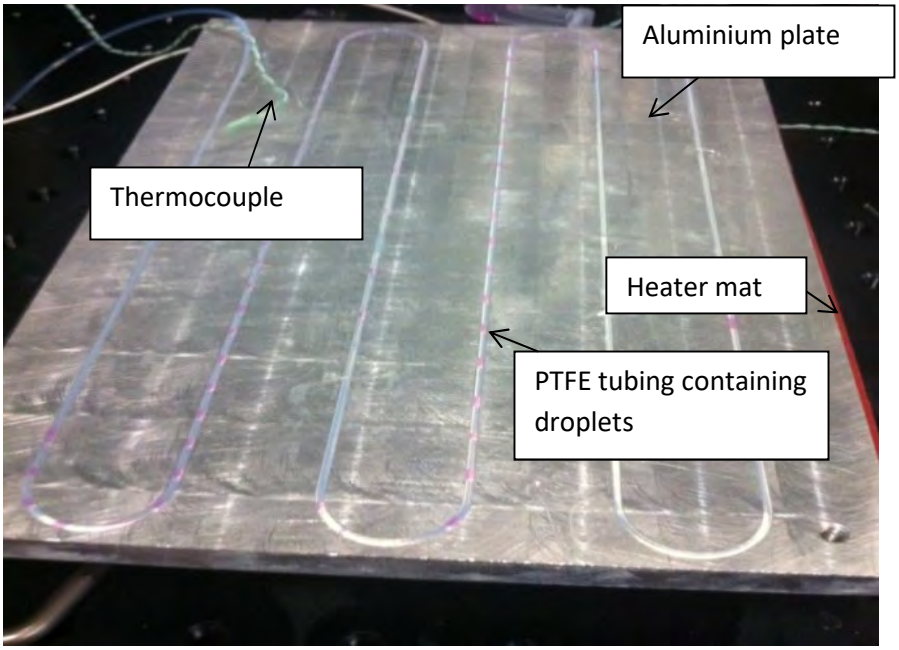


Figure 3.7 Aluminium heater pate with thermocouple attached to maintain the required temperature.

is necessary to keep both the bacterial and mammalian cultures at 37°C as it is body temperature. This temperature will give optimal growth of the cells and is representative of the temperatures the cells will experience in in-vivo conditions. To keep the cells at this temperature an aluminium heating block was designed figure 3.7. This is a 210x140mm aluminium block. Aluminium was selected due to its thermal properties as it is necessary to keep the cultures isothermal (Baroud *et al.*, 2010). Aluminium has a high thermal conductivity of 205W/mK and this will ensure the samples remain at the desired temperature for the duration of incubation. A serpentine channel of 1.3mm was machined into the plate this will allow the tubing to be embedded within and ensures maximum surface of the tubing was heated and also aids in mixing. A silicon mat heater (Radionics) was placed under the aluminium plate to heat the plate, it was selected as it gave the maximum coverage of the plate. The temperature was controlled by a PID (Eurotherm 2216E) controller.

A T-type thermo-couple was installed to feed back the temperature to the PID controller and this maintained a temperature within ±1ºC for the duration of each experiment.

3.6 Monitoring of Cells

Monitoring of the biological samples is necessary to confirm that the cells are held within the droplets and to track the cells health over the incubation of the cultures. Non-invasive visual access allows the same samples to be monitored meaning that continuous monitoring is possible instead of end point analysis. There are two types of monitoring system designed for this instrument, a visual monitoring unit using a microscope and an optical absorbance monitoring system using a photo diode.

Visual monitoring is performed non-invasively through the PTFE tubing. The optical unit design incorporates polycarbonate housing with glass slides 20 x 20mm top and

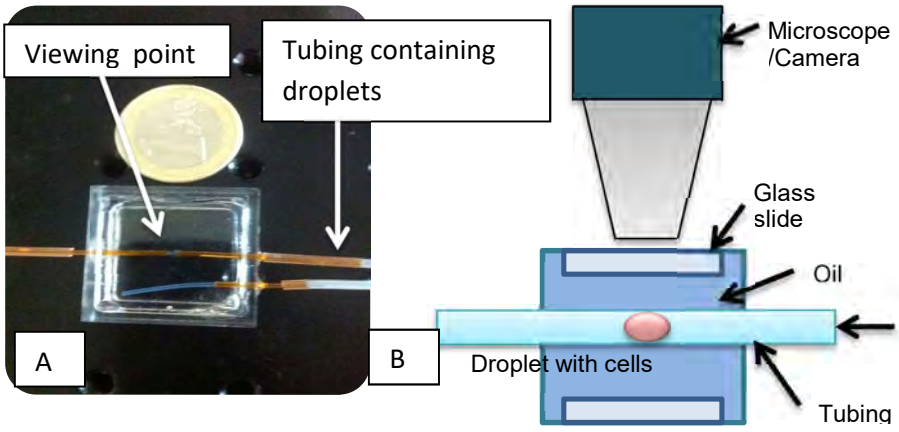


Figure 3.8 A optical monitoring unit for microscopic visualisation of the cells. where a polycarbonate housing has glass sides of visualisation of the cells . The microfluidic tubing passes through this unit allowing the cells to be visualised. B. A schematic side view of the microscopic visualisation unit this shows the droplet passing through the tubing past the microscope within the unit.

bottom of the housing Figure 3.8A. Polyimide tubing is used to keep the droplet tubing in place so that a small section is used for visualisation. Figure 3.8B shows a schematic of the optical monitoring unit. The glass allows clear visual access to the

samples. The tubing runs through this unit and has a diameter of 900µm ID and OD of 1100µm. This tubing was selected as it has a lower wall thickness allowing more visual access. The tubing is immersed in the PD5 oil, this reduces the curvature effect making imaging visually clear. The droplets move past the optical unit and can be imaged statically or while moving. The optical block is placed at the lens of an Olympus Inverted microscope CKx31 with a 10x lens is used to image the cultures. An Image source CCD camera DFK31AU03 is placed at the eye piece of the microscope and is connected to a PC and allows the cultures to be monitored on IC Capture at any point during the incubation without impacting on the cells.

As discussed in chapter two bacteria are approximately 1µm in size and double in population in 20 minutes this means that visual monitoring using a microscope is not sufficient as they are so small. Another system for non-invasive monitoring of bacteria is required. It is essential to have the ability to measure optical absorbance within the system in real time without impacting the cultures. An optical unit was designed for this system to continually monitor the cells. This has the advantage over

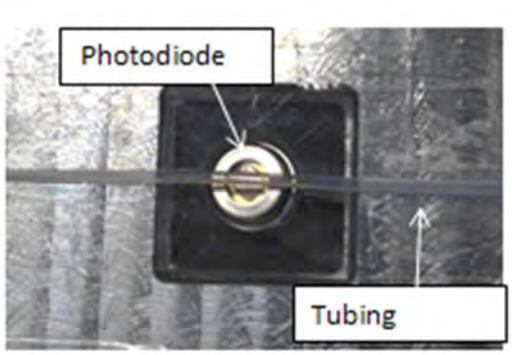


Figure 3.9 Image of Photodiode imbedded into the aluminium plate with the tubing over it. The photodiode allows the detection of optical density of the droplets.

current methods as the same sample can be measured each time and not just from a bulk sample. A second advantage is that these droplets can be maintained and used for further testing or post processing. The mechanism developed here is an optical density (absorbance) unit inserted into the aluminium heater plate. The absorbance

mechanism includes a 5mm cyan LED bulb of 505nm wavelength illuminating the tubing at a point directly above a photodiode. A 505nm was chosen as *E.coli* can be detected in the red light range from 400-700nm (Reller *et al*., 2009) Background light was eliminated in experimentation by a box covering the light source and photo diode at all times. The embedded photodiode underneath the tubing is demonstrated in figure 3.9. The photodiode (Thorlabs FGAP71) receives light within the range of 150-550nm. When a droplet passes the photodiode it absorbs some of the light. The photodiode changes the light signal to a voltage signal and this is amplified using an operational amplifier ±10v (Texas Insturments LM71) and the signal is monitored using with National Instruments PXI-6224 data acquisition (DAQ) card mounted on a PXI-1042 chassis. The National Instruments LabVIEW program monitors the signal in voltage and writes it to excel where results are interpreted. The acquisition of two readings per second allows for accurate monitoring of each droplet passing the photodiode. Optical density can be calculated using Beers-Lambert law. The transmittance of light is calculated by using equation 3.5

$$T=I/Io \tag{3.5}$$

Where I is the light intensity of the sample and Io is the reference blank in this system it was taken as the light intensity of the oil through the tubing. The optical density has a logarithmic relationship with transmittance shown in equation 3.6.

$$OD=-\ln T \tag{3.6}$$

The amount of bacteria within a droplet can then be evaluated using these equations. The optical density measurements are used to monitor all cultures on this system. Both methods provide non-invasive real time monitoring of cells.

3.7 Process Flow

Following the selection of material and assembly of parts the flow of the process is demonstrated in Figure 3.10. After sample preparation the droplets containing the

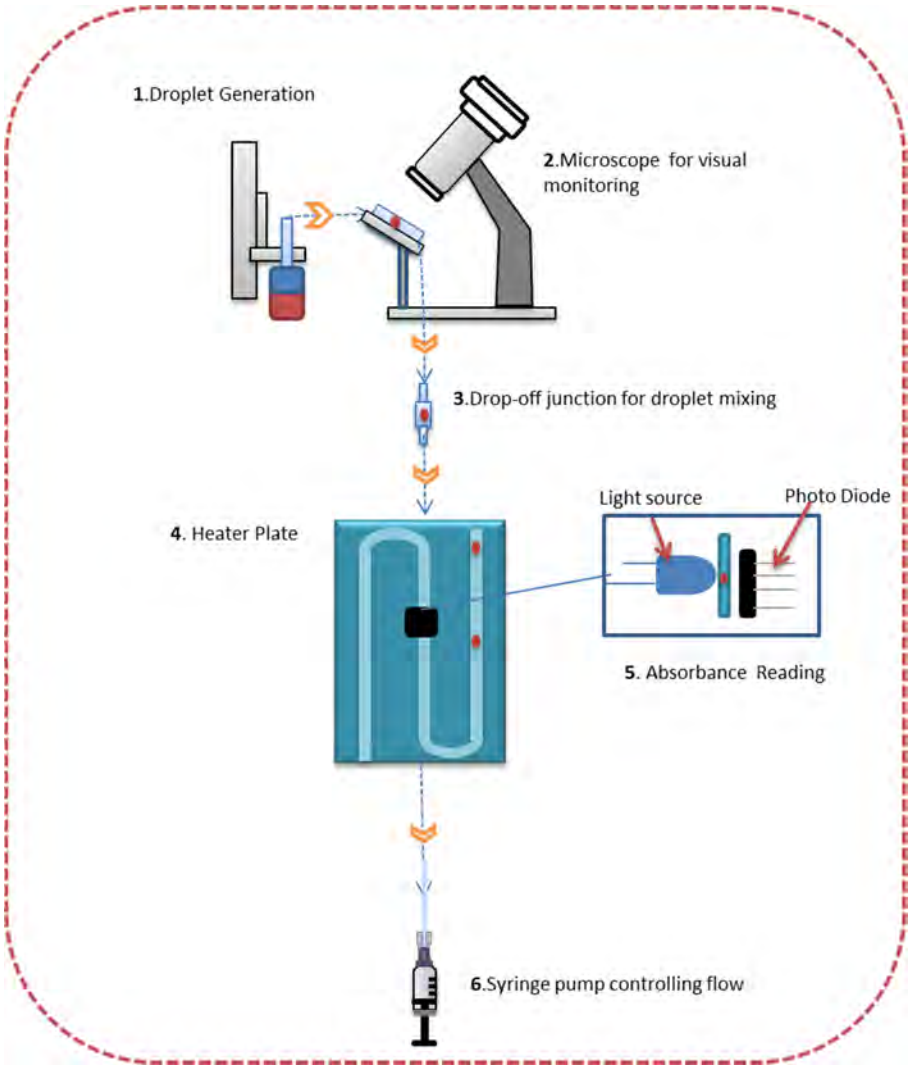


Figure 3.10 Process flow schematic of microfluidic instrument where the at 1. The droplets are generated ,the droplets then move onto point 2 where the cell droplets are imaged through a microscope. Droplet mixing occurs at point 3 using the drop-off junction. At 4 the droplets are incubated within this heater plate at 5 OD is measured and the pump controlling the flow is at 6.

cells are generated. These droplets then visually monitored using a microscope or the optical system at various stages through incubation. The droplets then move to incubation on the heater plate here the cells are monitored optically using absorbance. Finally the droplets are deposited or removed after incubation or testing.

3.8 Chapter Closure

This chapter outlined the design of the preclinical microfluidic cell analysis instrument. Each aspect of the design was identified and a solution was demonstrated. The selection of biocompatible materials such as the PD5 oil and PTFE tubing was justified here. The generation of the droplets is demonstrated where each droplet is an individual bioreactor. The establishment of correct thermal conditions for the incubation of both bacterial and mammalian cells is incorporated into the design. Other design elements such as the mixing system are demonstrated. The mixing system allows for multiplexed experiments to be prepared on this system. To monitor these reactions visual analysis of the cells is incorporated. The design of on-system optics is shown where a visual unit using a microscope is designed for mammalian cells. Bacterial analysis is monitored using optical density or absorbance measurements. The addition of the non-invasive absorbance readings gives this a novel aspect, as it can measure the same sample as many times as required and the sample can be used again for further analysis. This instrument forms the basis for all biological analysis and optimisation and validation of this system is discussed in the following chapters.

Chapter 4

Initial Testing and Instrument Optimisation

4.1 Introduction

This chapter focuses on preliminary tests that optimise the microfluidic instrument. Validation was performed to optimise the conditions for the biological samples. The initial test was to establish the correct temperature conditions for the cells on the instrument. Once this was verified, cell viability analysis was performed. This will ensure that the cells within the microfluidic droplets are not adversely affected by the new conditions. The optimisation of cell number within each droplet is investigated here, with the most effective method found for consistent cell numbers in each droplet. To validate this system for real time analysis the optical methods for light absorbance measurements were tested. This will allow each droplet to be analysed independently. From chapter three the internal circulations within the droplets are established and the effect of flow rate on these is investigated to observe if they

influence the cells orientation within a droplet. The optimisation of cells within the droplets dictates the parameters used for future testing on the instrument. This validation will ensure that the system will be sufficient for cellular analysis.

4.2 Temperature Optimisation

As it is necessary to test the cell samples at body temperature, temperature

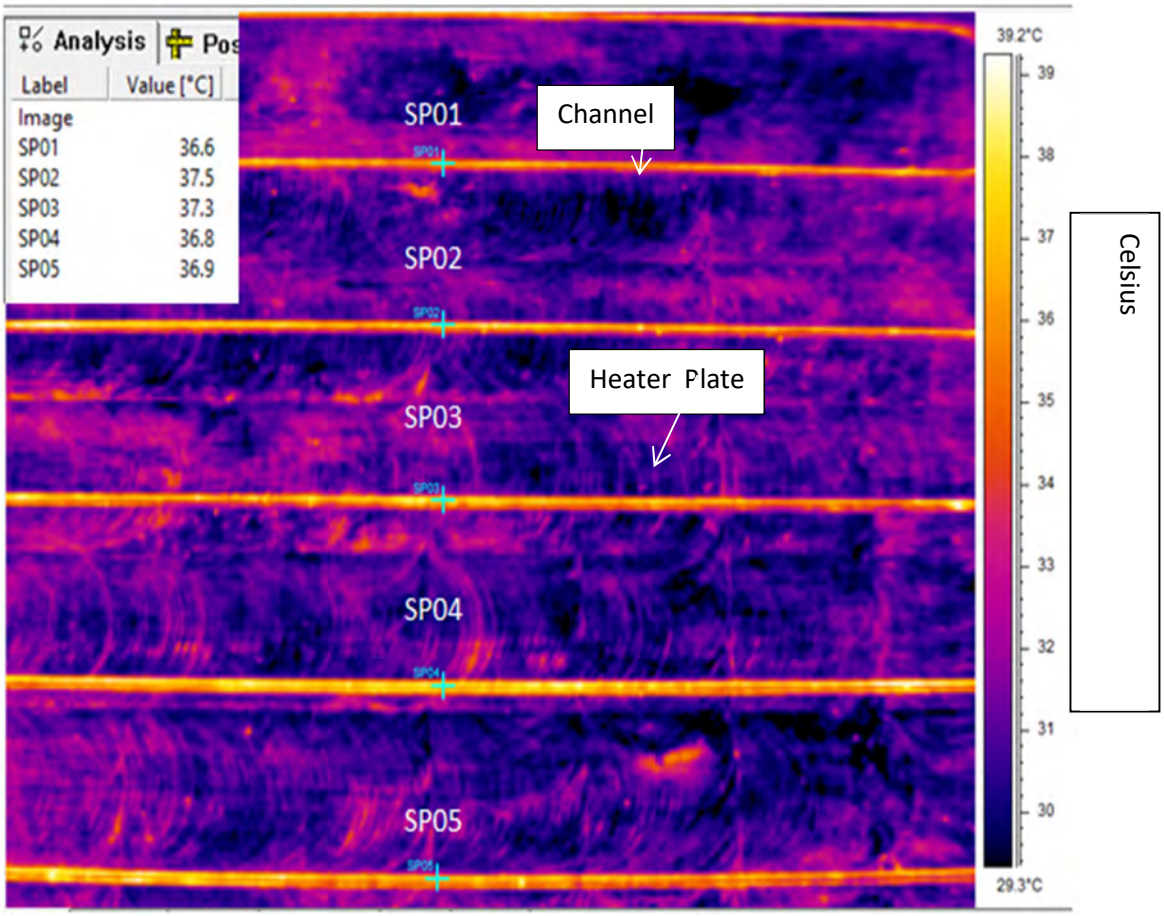


Figure 4.1 InfraRed temperature validation of the heater plate .Point measurements along each section of the plate where the tubing is placed indicates a constant temperature of 37°C

measurements of the heater plate were required. The consistent temperature of 37°C throughout the plate was confirmed by infra-red thermal imaging (Figure 4.1), where points along the plate and in the channels demonstrate isothermal conditions of the plate. The temperature was measured using a Flir thermal camera. The heater plate

was switched on and allowed to settle for 1 hour. 100 images were taken and average temperature was determined. Figure 4.1 shows a consistent temperature over the channels within the plate. With a desired temperature from the channel being 37°C, the average point temperature was found to be 37.05°C. This indicates that the conditions are stable and at the required temperature for cell proliferation.

4.3 Cell Viability

To examine the cells viability within the droplets a live/dead assay was used. Cell viability is the first indication in verifying if the droplets are possible bioreactors.

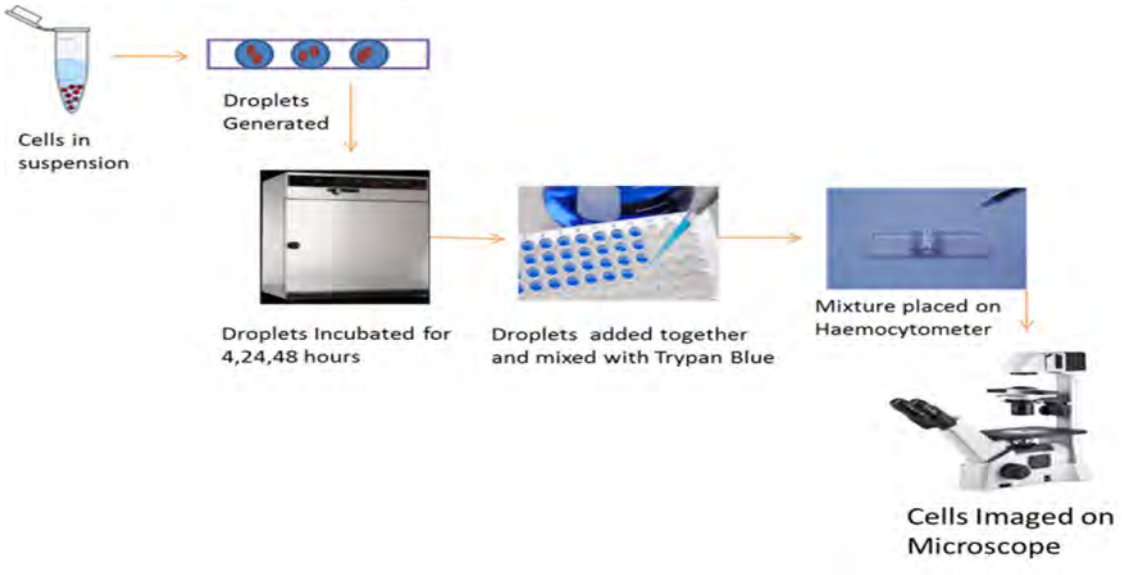


Figure 4.2 Flow chart of process of generating droplets to sampling the cells for live/dead analysis . Cells are prepared in suspension and used to generate droplets. Once droplets are generated they are incubated as droplets within the tubing in a standard culture incubator. After the droplets are incubated for the desired amount of time they are taken from the tube and trypan blue is added within a well plate. Cells are counted on a haemocytometer with a 10x microscope.

Trypan blue is a live/ dead assay used to determine cell viability by infiltrating the membrane of dead cells (procedure chapter two).This is a standard dye used for the establishment of cell concentration. It makes the dead cells appear as blue while viable cells remain unchanged. The flow chart of the procedure (Figure 4.2) shows that the cells are prepared into droplets and incubated as droplets within the tubing, in a standard incubator. A standard incubator was used to keep atmospheric

conditions the same for the cells at 5% CO_2 and 37°C. Once incubated for the required period of time, these droplets were then taken from the incubator and the cells were mixed with trypan blue. The cells were placed on a haemocytometer for counting and imaged using an Image Source camera attached to the Olympus microscope. To test the droplet conditions, 500,000 cells/ml concentrations of MCF-7 cells were generated into 100 droplets. The cells were then incubated for 4, 24 and 48 hours. After incubation the droplets were dispensed into a 96 well plate. 20µl of the cell samples were mixed with 20µl of trypan blue and 10µl of the resulting samples were placed on a haemocytometer and imaged. Figure 4.3 shows the images of the cells on the haemocytometer. It was clear that after 0 and 4 hours that the cells remain separate and are healthy as there are no blue cells visible. After 24 hours some of the cells accumulate together (Figure4.3). These accumulations of cells impede the process of counting as it can be difficult to determine the exact number in an accumulation. This makes cell counting inaccurate as it is not possible to precisely count the number of cells in each structure individually. However, it is clear that the cells remain healthy as they remain unaffected by the trypan blue. Trypan blue is toxic to cells so it was not possible to process the cells further after this is used. Figure 4.3 shows that at 48 hours there are some dead (blue) cells visible and these show the point at which the droplets have reached their limits to remain as viable bioreactors. The overall result from this verifies that counting cells using a haemocytometer will not be sufficient in providing exact cell number for the droplets. This test shows that cells are not adversely affected by the cell environment within the droplets as it demonstrates the viability of the cells up to 48 hours.

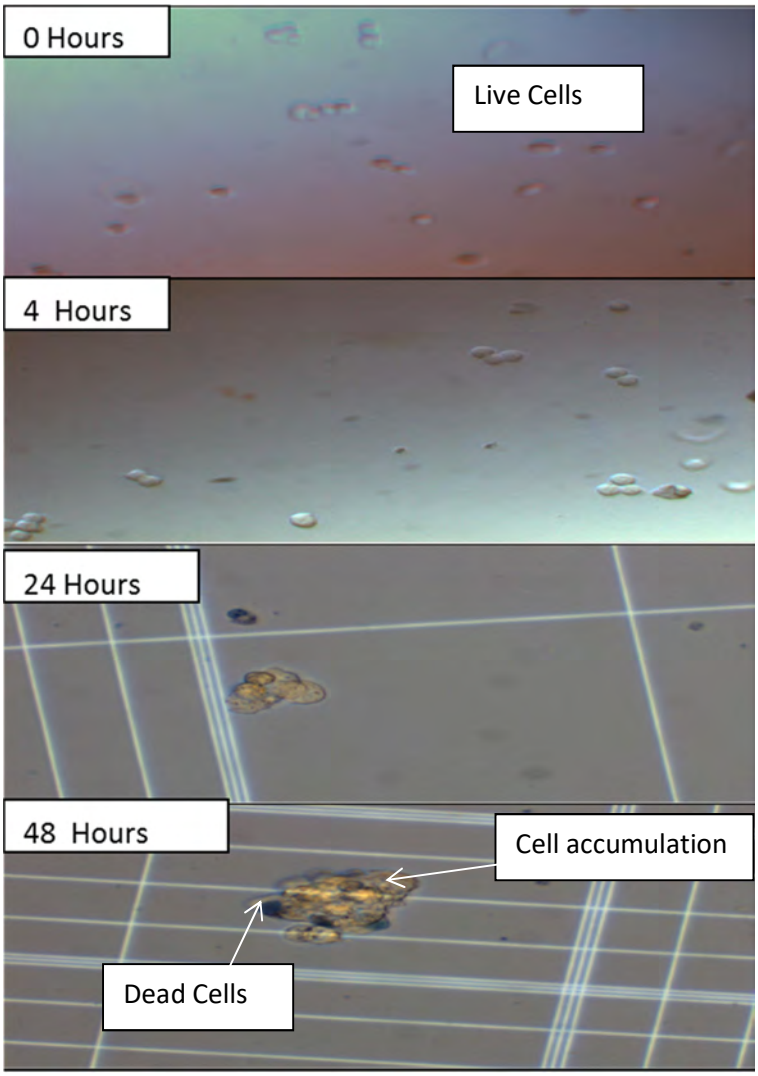


Figure 4.3 Images of cells on a haemocytometer grid with a 10x microscope with trypan blue exclusion assay over 0 hours shows all cells are alive at 4 hours the cells are still alive at 24 hours the cells have accumulated together and from a cell accumulation where all the cells are alive and at 48 hours cells shows indication of live and dead cells within the cell accumulation

4.4 Cell Counting

Over time when generating droplets it was noticed that the cells had were falling from the suspension and formed sedimentation at the bottom of the well plate. To

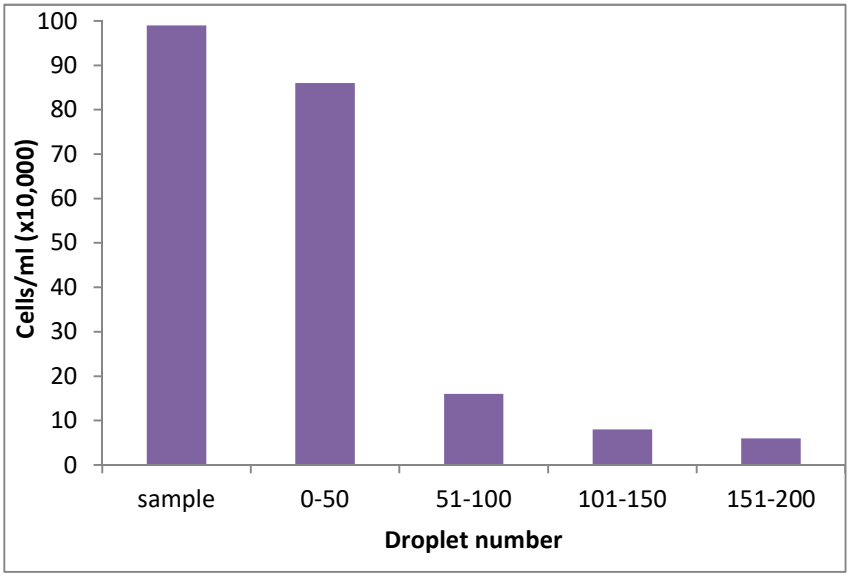


Figure 4.4 Variation in cell concentration measured using trypan blue exclusion assay using a haemocytometer. After 50 droplets there is a decrease in concentration due to cells sedimentation at the bottom of the sample container on the microfluidic instrument

address this, a count was conducted. As the cells are essentially a mixture with the growth media it is a requirement to know when it was necessary to remix the samples. A test that counts the cells at selected intervals was done to optimise the amount of cells in a droplet. To look at the consistency 1,000,000 cells/ml suspension was made of MCF-7 cells. Two hundred droplets were made and the droplets were then sampled into four sections. The first 50 droplets were sampled where 25µl of cell suspension was mixed with 25µl of trypan blue. The cells were evaluated on a haemocytometer. Figure 4.4 shows the initial sample measurement of just under 1,000,000 cells, at 990,000 cells/ml showing variation in counting. This shows a reliability issue when using a haemocytometer. As this system is highly manual human error can affect the count. If one cell is missed, the value is off by

10,000 cells/ml and this is the case in the very first reading of the sample. The first 50 droplets (Figure 4.4) show that there is a slight drop in the number of cells with a reading of 860,000 cells/ml counted. The drop in cell concentration is evident from droplet 51 onwards. The fall in cell number is apparent for all droplet samples after 51 and this suggests that the sample is no longer homogenous. This shows that variations can occur in the cell concentration within the droplets, if they are not stimulated during testing. This also shows that it necessary to have samples that were continuously mixed to provide equal distribution of the cells.

4.4.1 Sample Count Variation

As it was apparent that there is a difference in the amount of cells being counted using a haemocytometer, it was necessary to investigate further the cell count variation due to the haemocytometer. To demonstrate the variation in counting a

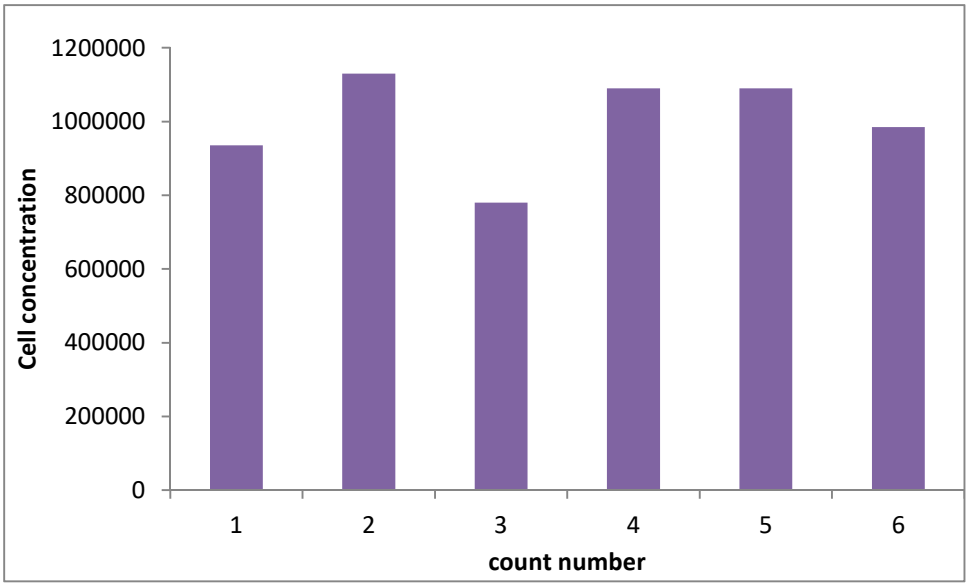


Figure 4.5 Graph of the cell count variation in in one sample counting it six times using a haemocytometer.

single sample, a test was carried out where the one sample was mixed and counted multiple times. A sample of MCF-7 cells of 1,000,000 cells/ml was made. A 50µl sample was taken for each count and mixed with 50µl of trypan blue. The haemocytometer was used for each count and 10µl of the mixture was displaced to the haemocytometer. Six samples were taken and counted individually. Figure 4.5 shows the variation in the counting. Over the six counts there is a clear difference in the amount of cells counted over the samples. The issue with these counts is the standard deviation within the value of 131,098 cells. This is a 13% deviation in cell count alone. Once the accuracy of the haemocytometer counting was found, it was clear that this, although standard lab practice, it is not entirely accurate. Although the variation found in Figure 4.4 over the droplets is high it is considerately higher than the 13% found in Figure 4.5 and this proves that the change in the amount of cells in the droplets is not solely due to the accuracy in the haemocytometer method but also due to the cells not staying in suspension.

4.4.2 Magnet mixing

To test if continuous stimulation of the cells will create a mix that will maintain a homogenous cell suspension, a magnetic mixing system was incorporated into the cell sample. A magnet was rotated continuously in a micro centrifuge tube containing the cell sample. The magnet was 1mm in diameter and was rotated for the duration of droplet generation. MCF-7 cells at a suspension of 430,000 cells/ml was used. This concentration was used as it was a trial test and this was the amount of cells available at the time of the testing. The sample was counted using a haemocytometer. 120 droplets were generated and each was 500nl in volume. Once all the droplets were made, they were distributed into 4 wells with 30 droplets in each well. 10µl of each sample was mixed with 10µl of trypan blue. 10µl of sample

was then displaced into the haemocytometer. To increase accuracy, four grids on the haemocytometer were counted and divided by two. Figure 4.6 demonstrates that the rotation of the mixture does not adversely affect the cells as over the 120 droplets

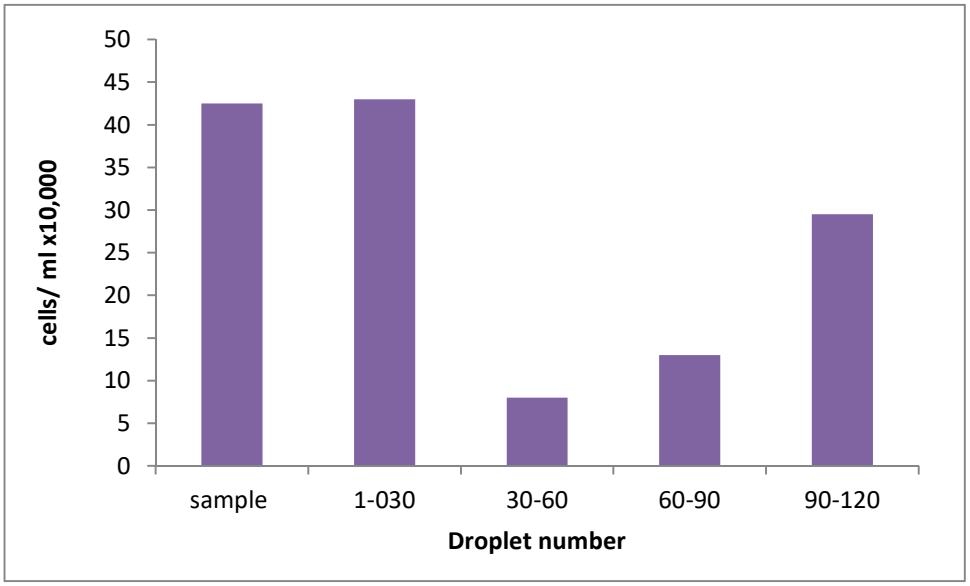


Figure 4.6 Cell concentration variation over 120 droplets using a magnetic bead to constantly rotate the cells.

that were generated and no dead cells were detected. Figure 4.6 shows that the first 30 droplets has the same amount of cells as the initial sample and this shows a similar result as with no mixing (see Figure 4.4). It is clear that the cells do not stay in suspension with magnetic mixer as the cell concentration reduces from droplet 30. Mixing does change the concentration over all the droplets, as Figure 4.6 shows an increase in concentration above droplet sixty however, it does not form consistent suspensions of cells. This method was not further investigated but highlighted the problem of equal distribution of cells in the droplets when using a spherical magnet mixer.

4.4.3 Surfactant Testing

As the cells will require an adhesion signal when in the mixture this may be contributing to the cells attaching together and falling to the bottom of the sample container. To investigate this, a surfactant was added to the mixture to form a barrier

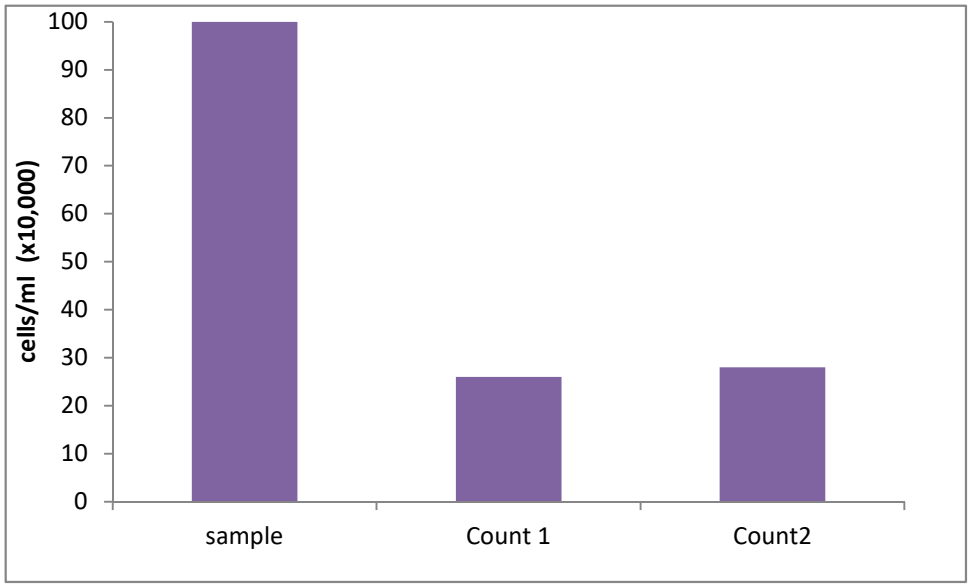


Figure 4.7 Graph of cell concentration after surfactant Pluronic F-68 mixing with cells shows a decrease in cell concentration.

around the cells. The surfactant selected was Pluronic F-68 which was added at 1% to the cells. Pluronic F-68 is nontoxic to the cells as it is used in large scale mammalian cell culture (Gigout *et al*., 2008). 1,000,000 cells/ml suspension was made and 60 droplets were generated. The consistency was counted using a haemocytometer. 10µl of cells mixture (droplets pooled together) were mixed with 10µl of trypan blue. Two counts were conducted using the droplet samples. Figure 4.7 shows the final concentration of 260,000 cells/ml and 280,000 cells/ml this is below the initial concentration. This shows that this type addition does not encourage sufficient distribution of cells within the droplets. This method was not further pursued as a method of cell suspension homogenisation.

4.4.4 Manual Mixing

A test was undertaken to determine if manual mixing would provide a more homogenous cell sample. Droplets of MCF-7 cells were generated, where the cell sample was manipulated manually by a pipette after every five droplets. This consisted of aspirating and depositing the cell sample ten times. The droplets were formed and then monitored using a haemocytometer. A cell suspension of 1,000,000 cells/ml was made and 100 droplets were generated. Figure 4.8 shows that both samples give the same cell concentration with a deviation of ±4% . This shows that an equivalent amount of cells are found in the sample and are within the limits of the haemocytometer of ±13%. The manual mixing of the cells is the best method for creating consistent cell concentrations within the droplets. This method was implemented for the remainder of the tests.

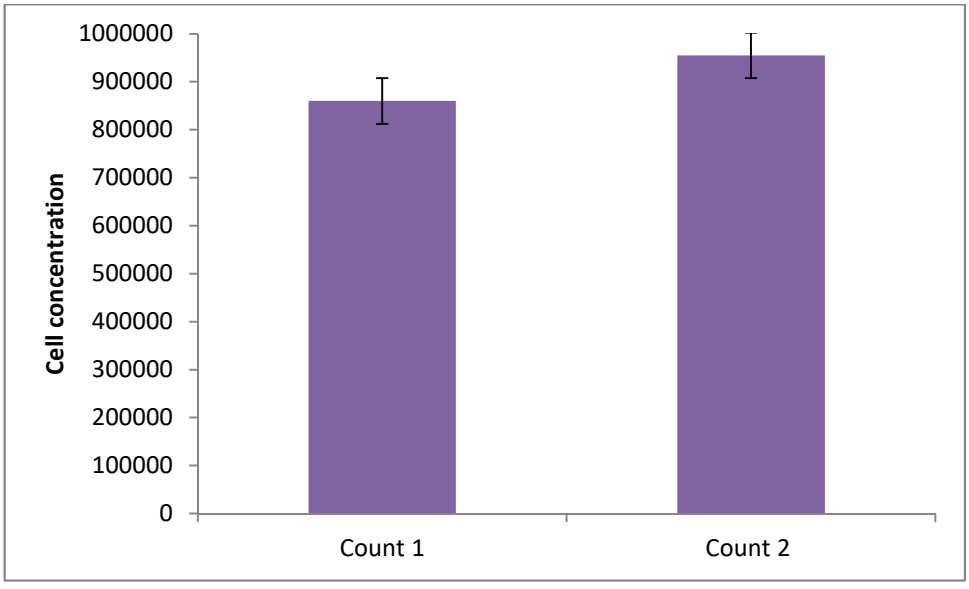


Figure 4.8 Cell count after mixing manually using a pipette after every 5 droplets were generated. This shows that the droplet concentration remains constant.

4.5 Optical Detection of Droplets on the instrument

To test if the instrument was capable of detecting droplets, droplets of green food dye were generated at 1400nl and moved past the photo diode system. Figure 4.9

shows the voltage reading corresponding to each part of the droplet. The graph shows initially the oil phase between the droplets. Figure 4.9 demonstrates the leading and trailing cap ends of the droplet as a small peak with the centre of the droplet being indicated as a larger peak. This shows that the instrument is sufficient in identifying droplets and was used in the bacterial tests. Due to this monitoring system being non-invasive, droplets can then be continuously monitored or used for further analysis.

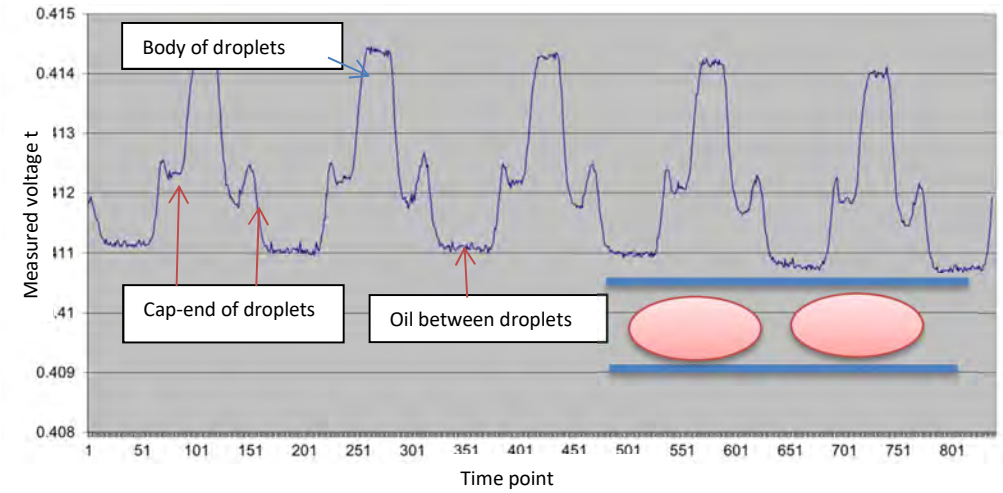


Figure 4.9 Measurements of optical density of droplets using the microfluidic system The optical density can show the difference in light going through the cap ends and the body of the droplets. Two measurements are taken each second.

4.6 Fluidic Conditions

The theoretical conditions within the microfluidic droplets are examined in Chapter 3. The flow regime dictated by the Reynolds number is displayed in Table 4.1. The flow rates are low for this instrument 15-60µl/min or 0.99-1.92mm/s. The Reynolds number indicates laminar flow which is highly dependent on inertial conditions and the capillary number indicates the interfacial tension dominates over the viscous

forces. This is indicative of the curvature at the cap ends of the flow. The Weber number indicates that surface tension effects are dominant.

Table 4.1 Fluidic properties of microfluidic instrument

Flow rate µl/min	Reynolds Number	Capillary number	Weber number
15	0.0902	4.60E-05	4.15E-06
30	0.180	9.2E-05	1.66E-05
60	0.360	1.84E-04	6.62E-05

4.6.1 Particles Within the Droplets

The internal circulations influences where the cells will reside within the droplets and this was outlined in chapter three. An investigation into if the internal circulations has an influence on where the cells reside is conducted here. Initially, silver powder 5-8µm in diameter (Sigma-Aldrich 327093-10g) were selected to aid clarity in visualisation and are approximately the same size as cells within a droplet. 0.05g of silver particles was suspended in 4.95g of H_2O. Droplets of 1500nl were created and imaged on the system using a microscope. The droplets were moved through the tubing approximately 300mm and the criteria for full mixing was satisfied. Three velocities that were used are 15µl/min 30µl/min and 60µl/min these three velocities are the equivalent to 0.48mm/s 0.96mm/s and 1.92mm/s respectively. Images were taken and analysed in Figure 4.10, it is clear from Figure 4.10 that the particles all move towards the rear of the droplet for all flow rates.

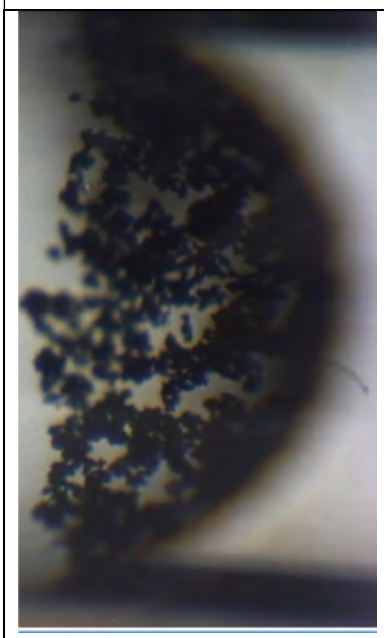
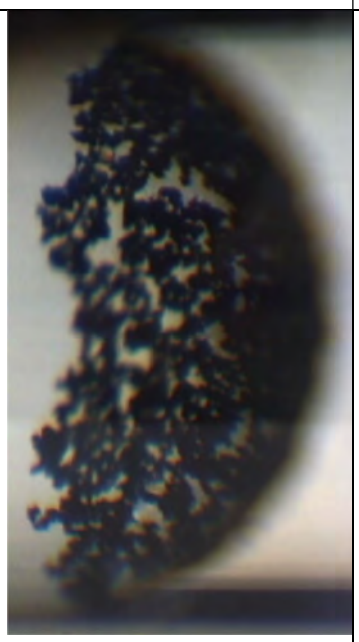
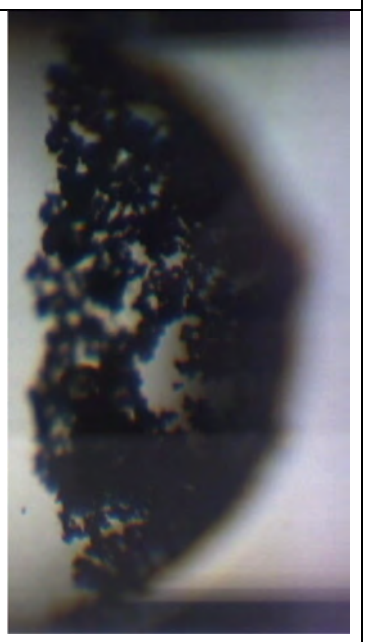
15ul/min	30ul/min	60ul/min
		

Figure 4.10 Images of sliver particles within the cap ends of droplets at varying velocities from 15-60ul/min

The cap end is only occupied at the back of the droplet indicating that the negative nature of the flow could be influencing the particle distribution. The second observation that was made was that the size of the area occupied at the cap end is reduced with increasing velocity. This demonstrates the same flow profile as the CFD prediction (Kurup and Basu 2010). The accumulation of particles at the back of the droplet could limit the droplets as bioreactors if the cells behave in the same way as the silver particles. As the bacterial cells are approximately 1µm in diameter and are less dense than the silver particles they may act differently. An investigation into *E.coli* where 1500nl droplets were generated was conducted. These droplets were imaged in the same way as the silver particles ensuring full mixing. Droplets were moved in the same fashion from 15µl/min to 60µl/min. The *E.coli* was in a dilution of 1:100 with LB. Figure 4.11 shows the lowest flow rate of the *E.coli*. Whilst these cells are difficult to image two regions are clear from Figure 4.11. The cells are distributed throughout the droplet and a clear line where the droplets begin to curve

shows a higher density of cells at the cap end. At the velocity of 30µl/min more cells reside in the cap end of the droplets than at 15µl/min and at the highest velocity of 60µl/min shows that most of the cells are within the cap end. The 60µl/min sample

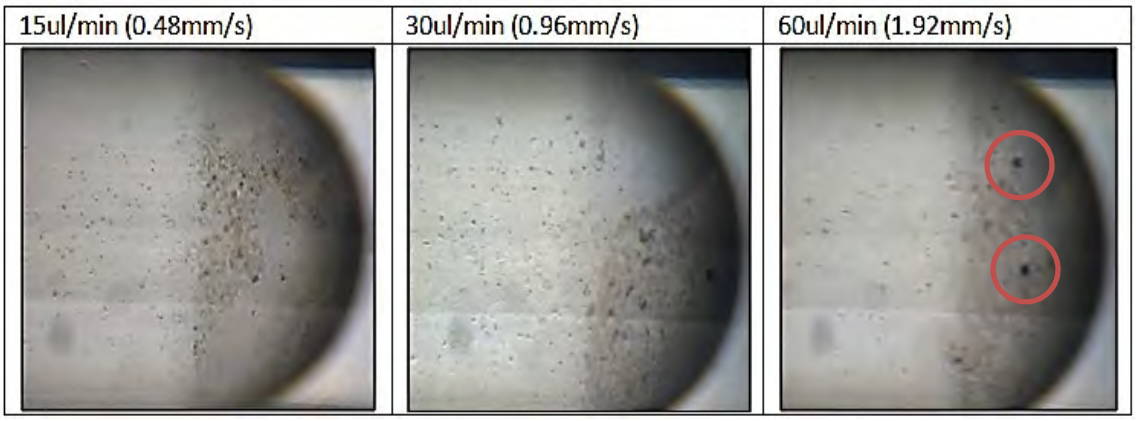


Figure 4.11 Images of E.coli within droplets at varying velocities . Red circles indicate the centre points of the vortex that formed at the highest velocity within the cap end of the droplet.

shows the cells are moving in two circulation regions within the cap end. These form accumulations of cells shown in the red circles Figure 4.11. These accumulations of cells are the centre of a circulation within the droplets. There are two rotations within the cap ends agreeing with numerical simulations (Kurup and Basu 2010).

As mammalian cells will be used for analysis within this system it is necessary to know how these cells will reside within a droplet As the cell accumulations in the bacterial droplets at high velocities could limit the nutrients reaching the cells, it was decided to also investigate the behaviour of mammalian cells. Cell to cell communications exist within any cell environment and it was necessary to understand how this could influence the cells within the droplets. As well as the velocities, the concentrations of cells were varied to examine if this would influence the location of the cells within the droplets. Mammalian MCF-7 breast cancer cells mixed with DMEM media were used to generate droplets. The flow rate remained the same as used for silver particles and bacterial cells 15-60µl/min. Each droplet

was generated and allowed to flow past the microscope. The same droplet was used for each flow rate to maintain consistency. Figure 4.12 shows at 100,000 cells/ml suspension there are approximately 100 cells in each droplet. It is clear that the cells do not act like particles as they are distributed around the droplet. The requirement for the cells to find an adhesion signal may cause the cells to be attracted to each other and this may dictate where the cell will reside. The cells are also less dense than the silver particles which shows that they may not be as influenced by the circulations within the flow.

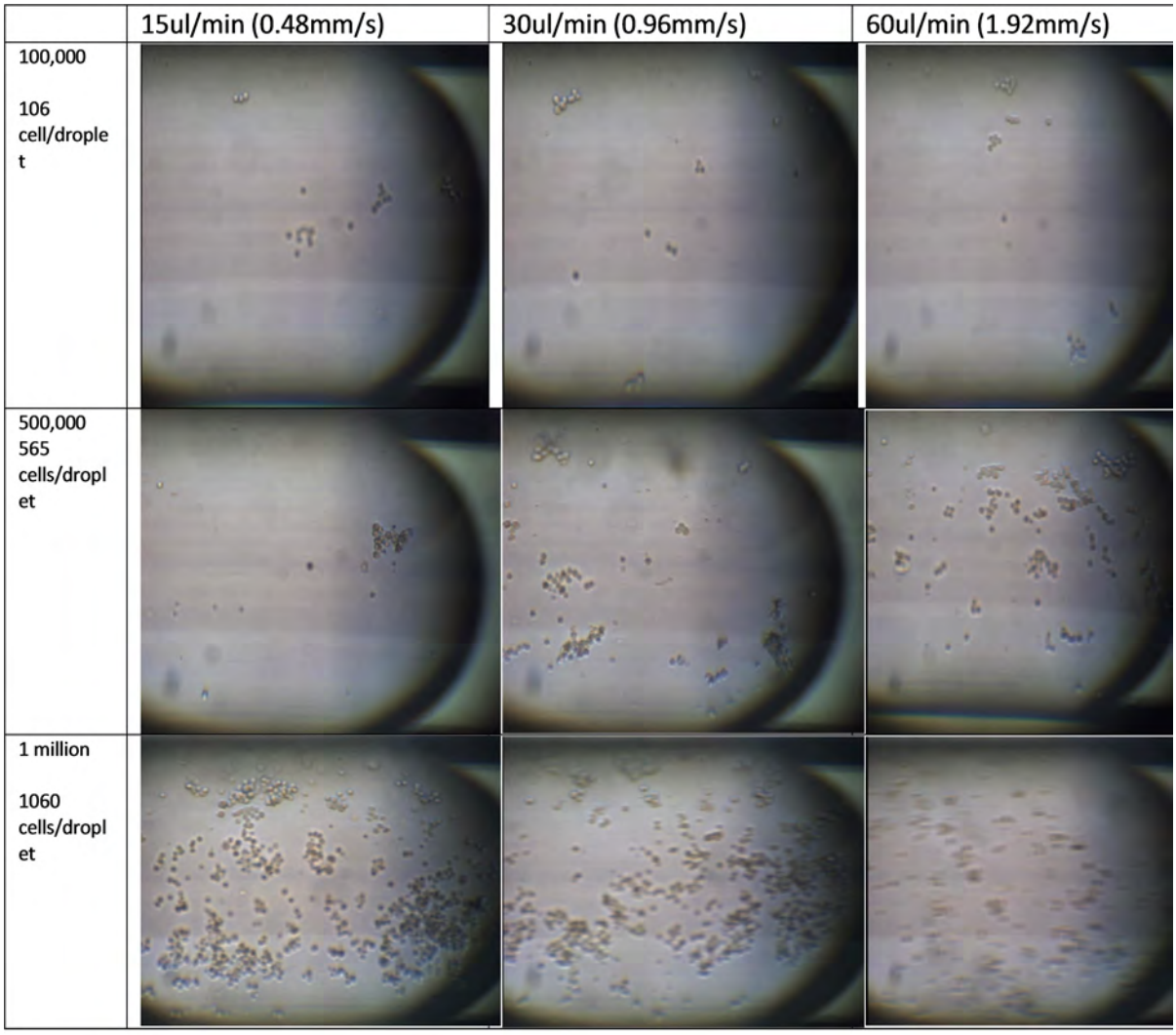


Figure 4.12 Images of MCF-7 cells in droplets at varying concentrations of cells and flow rates taken at 10X on the microfluidic system. Cells remain spread throughout the droplet at all concentrations and flow rates.

The flow rate does not affect the location of the cells at any velocity and is deemed adequate for cell therapeutic coverage. The second concentration was used to access if the increased concentration will influence where the cells will move to, within a droplet. The droplet containing 500,000 cells/ml was tested at the same velocities (Figure 4.12). From the initial droplet it is unclear that there were 565 cells/ droplet however, the same droplet was used for all velocity analysis. More cells were apparent at the higher velocities which may be due to the location of cells at the time of imaging. This infers that it is not reliable to visually count cells within a droplet to

determine cell concentration. The increased concentration of cells within a droplet does not affect the location of cells within the droplets (Figure 4.12). At the highest velocity the cells do not appear to be influenced by the same internal circulations as the bacterial or particle flow. At 60µl/min the cells remain spread around the droplet and are more influenced by where the other cells are. Accumulations of cells binding together dictate where the cells will reside within a droplet. The accumulation of particles at the rear of the droplets is due to the interplay between the cap vortices and the internal vortices. Particles at the cap end of the droplet will remain there because it is an independent, co-rotating vortex. As more particles collect in the cap, the effective viscosity will increase, forming a stagnant region which, in turn, will collect additional particles (Kurup and Basu, 2012). This does not occur with the mammalian cells as the cells will remain within the main body of the droplet. This makes the droplets ideal culture reactors as the cells are allowed to communicate with each other.

4.7 Chapter closure

This chapter shows the optimisation of the microfluidic droplets. The physical conditions of the droplets as bioreactors show that the droplets are capable of creating a healthy environment for the cells. To have equal amounts of cells it is necessary to mix the cell sample using a pipette. Consistent temperature is verified for the heater plate using an IR camera. The optical capabilities of the instrument achieved droplet identification as well as indicating the cap ends of each droplet and also indicating the oil phase of the flow. The flow dictating the location of the cells within the sample was demonstrated here where bacterial cells act like particles and remain at the trailing cap end of the droplets and form rotations at higher flow rates. The mammalian cells do not act like the particles or bacterial cells and remain spread

out within the droplets for all flow rates. This chapter has found the optimum conditions for generating droplets in terms of having consistent cell concentrations and flow rates. The next chapter will discuss the bacterial reactions within the droplets.

Chapter 5

Bacterial Testing

5.1 Introduction

Bacterial infections that are resistant to antibiotics have become a worldwide problem. The constant mutation of bacteria causes new strains to develop. These strains mutate to become resistant to antibiotics (Andersson and Hughes, 2010). At the moment, it is not possible to keep up with the mutating bacteria in terms of developing new drugs. The issue in determining bacterial resistance is to identify the bacterial strain. Broad spectrum drugs are prescribed as a 'one size fits all' treatment when the specific bacteria are unknown. The bacteria then become resistant to these drugs causing a need for more precise identification and treatment (Woodford and Ellington, 2007). Currently, methods to culture bacteria include streaking of agar plates with bacteria, incubating overnight and visually monitoring cells growth (Sarkar *et al*., 2015). Figure 5.1A shows an empty agar plate with no bacteria and Figure 5.1B shows an agar plate with bacterial growth. This is the method used to

grow bacterial strains are and involves an overnight incubation with manual counting of the colonies.

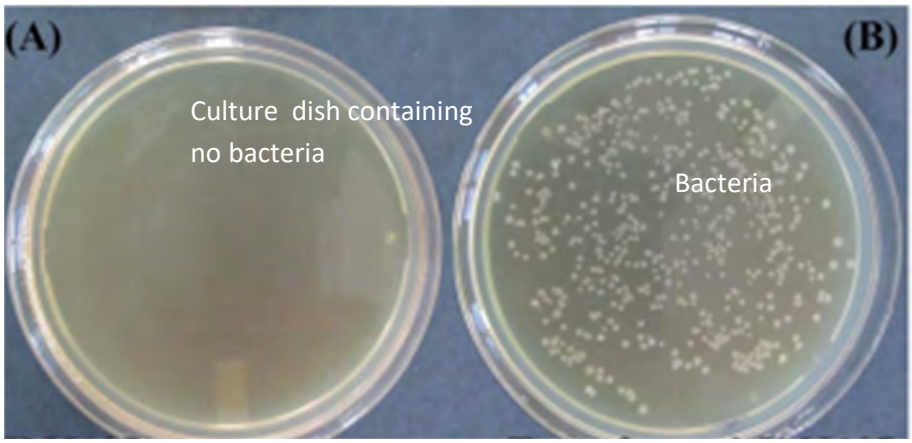


Figure 5.1 *E.coli* growth in a Petri dish (Sarkar et al., 2015) Where A is a agar plate with no bacteria and B. demonstrates the colony growth of *E.coli.*

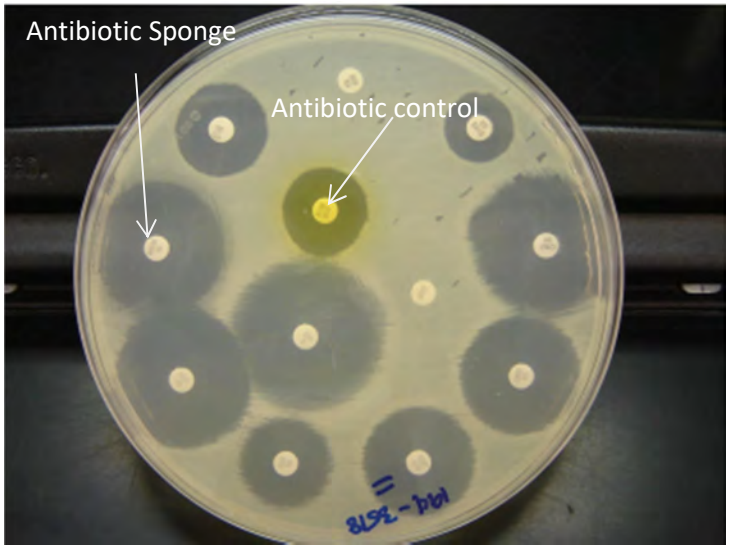


Figure 5.2 Disk dilution of antibiotics using a disk dilution method (Reller et al., 2009) Where each sponge contains an antibiotic. The inhibition of bacterial growth is evaluated by size of the diameter around the sponge where the bacteria have not grown, this inhibition is measured against a control highlighted in yellow.

The disk dilution method comprises of adding an antibiotic at a controlled concentration (Reller *et al*., 2009). Figure 5.2 displays a plate that has full growth of bacteria where each disk has a concentration of antibiotics with one control containing no antibiotics. It is clear from Figure 5.2 that some concentrations of the antibiotics are more effective in treatment of the bacteria. Other methods include

dilutions of broth with drugs (Mohan *et al*., 2013) and colony counts (Kiefer *et al*., 2010). These methods are visual based and are highly user dependant (Kiefer *et al*., 2010). A quantifiable bacteria method, that is based on absorbance of light is known as optical detection (Reller *et al*., 2009). The bacteria grow at such a fast pace that the media they grow in changes appearance from translucent to cloudy. This allows the quantification by measuring the light that can go through the sample. Finding a way of rapidly identifying a bacterial strain and subsequently a treatment for the patient in a time effective way is an urgent requirement. Currently, the most common method to culture bacteria is on agar plates and this can take over 18 hours to identify a strain of bacteria (Reller *et al*., 2009) This chapter introduces the initial testing on the microfluidic instrument for bacterial cultures. This chapter presents the results of the bacterial experimental work in three main areas:

1. The initial section will form a validation comparing the droplet cultures with current suspension orbital shaker cultures. This shows the suitability of the droplets as a new environment for bacterial analysis.
2. The optimisation of the bacteria within the droplets is discussed in the second section. This section characterises the fluid mechanisms and their impact on the bacteria. This includes the effect of the size of the droplet and increasing volume and its impact on the growth of the *E.coli.* The effect of the internal circulations within the droplets on the bacteria is shown here.
3. The final section in this chapter focuses on the application of treatment on the *E.coli*. Being able to identify and treat the bacteria effectively will validate this system as a practical alternative to current methods. The identification of antibiotic resistance within droplets is demonstrated here. This study

implements the application of antibiotics to the cultures and monitors their response on the instrument.

5.2 Droplet Culture Vs. Current Suspension Methods.

To create bacterial cultures a seeding culture is required to be diluted with Luria-

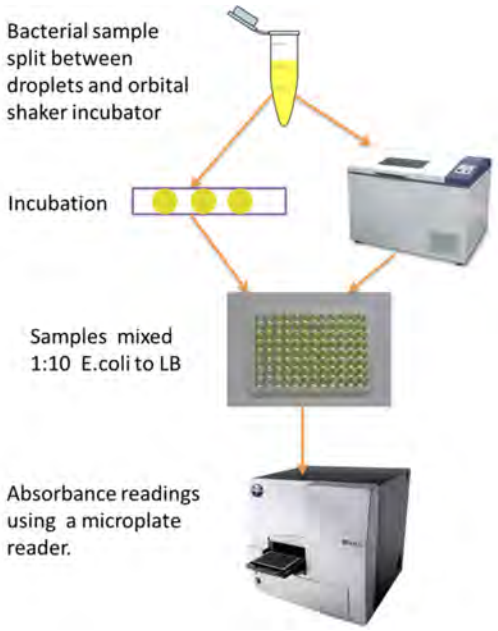


Figure 5.3 Flow chart of bacterial culture measurements where a solution of bacterial culture was made and split between droplet culture and orbital shaker incubator culture To measure the optical density a mixture of the *E.coli* was mixed with LB and a 100µl sample was measured in a 96 wellplate on a microplate reader.

Bertani (LB) and the bacteria will grow over time. This method can be used to test various antibiotics and determine if they are effective in treating the bacteria. The microfluidic droplets are generated using the instrument described in chapter three. A colony from *E.coli* cell line BL-21 is taken from an agar plate and suspended in LB and incubated at 37^{o}C for 16 hours in an orbital shaker incubator and rotated at 200 rpm. Figure 5.3 displays the flow chart of the experiment where a dilution of *E.coli*:LB sample is split in two to make the droplets and the suspension culture. 500µl of sample mixture was used for the production of droplets and placed in a micro-centrifuge tube with a PD5 oil overlay. 5ml of the initial sample was used for

incubation in suspension in the orbital shaker incubator. The initial validation test consisted of forming 615 droplets of 600nl volume in the microfluidic tubing and incubating for 10 hours. The droplet culture is incubated at 37^{o}C statically and they were agitated once every two hours while they are being removed from the system. 100 droplets were sampled every two hours and three 10µl samples were mixed with 90µl LB for reading. In parallel the shaker flask samples are continuously agitated at 200 rpm throughout incubation at 37^{o}C and also incubated for 10 hours. To monitor the cultures absorbance readings were taken every two hour using a microplate reader. To read the absorbance a 10µl sample from the shaker flask was mixed with 90µl LB and this was repeated three times, making three 100µl samples and each was placed in an individual well of a 96 well plate. After incubation the droplets were dispensed from the tubing and were pooled together. 10µl was added to each well and mixed with 90µl LB. The samples optical density (OD) was measured using a microplate reader at 600nm wavelength. A control sample of 100µl of LB was measured each time to compare growth rates with culture media that contained no bacteria. The optical density readings over 10 hours, of both cultures and the LB control are demonstrated in Figure 5.4. All the cultures start at the same density of 0.02 at the initial reading and this shows that the dilution of the *E.coli* is undetectably low for both shaker flask and droplet samples. The rate of growth is slow, with it taking up to 6 hours before the cultures begin to go from their lag phase to log phase growth (Figure 5.4). However, it is clear here that both bacterial cultures grow over 10 hours. After six hours the difference in growth rate between the droplets and the shaker flask culture are apparent. Figure 5.4 indicates that not only are the bacterial cultures thriving within the droplets but actually there is a higher rate of growth evident in the droplet cultures. The higher growth rates have been

seen in other microfluidic systems (Churski *et al*., 2012) and this led to the investigation of the optimum conditions for the bacterial cultures in microfluidic droplets discussed later in this chapter.

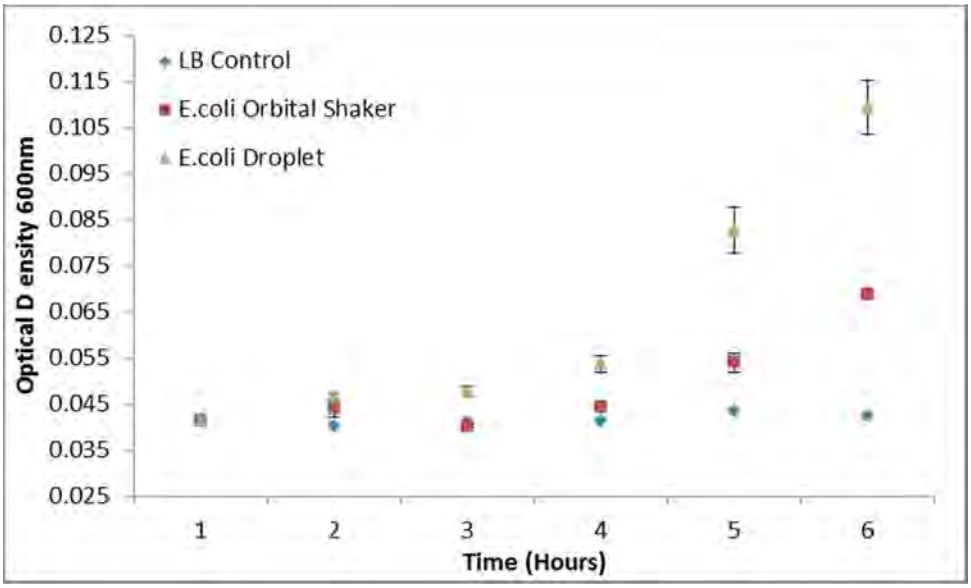


Figure 5.4 A graph comparing growth rates of traditional *E.coli* shaker flask culture with *E.coli* droplet cultures measured on a microplate reader. Droplet cultures show a higher growth rate than orbital shaker cultures.

The error analysis was standard error of the mean and this was used for all cultures investigated. The shaker flasks culture uses a larger volume than the droplets and this was for two reasons, the micro-volumes would evaporate in the shaker flask conditions due to the low volume and secondly to compare a typical volume used in current culture methods. To test the viability of the droplet cultures after incubation the bacteria was taken from theses droplets and re-incubated in 5ml of LB in suspension culture. This culture was incubated for 16 hours at 200 rpm, these cultures continued to grow demonstrating that the cultures remain viable after incubation and were used for subsequent testing if required. The constant agitation within the shaker flask culture encourages diffusion throughout incubation. The agitation within the droplets is in the form of the internal circulations, Figure 5.4

shows that these droplets are sufficient for providing growth of the *E.coli*. Minimum amounts of agitation are required as full mixing will be achieved once the droplet moves the equivalent distance of 4.5 times the droplet length (Tice *et al*., 2003). This will be achieved many times by all the droplets throughout the testing as the droplets are 1.6mm in length and move over 20cm within the tubing. Figure 5.4 presents two outcomes, that the droplets grow at a higher rate than shaker flask cultures and that it is not necessary to continuously agitate the cultures for the duration of incubation.

To combat the initial low growth rate in all subsequent tests, concentrations of 1:10 instead of 1:100 *E.coli*:LB were used and pre-incubated for two hours before droplet formation.

5.3 Optical System For Monitoring Cultures

Once it was established that the conditions for growth of the bacteria were sufficient within the droplet mechanism, the on-system optics were designed. These optics measure the absorbance of light or optical density (OD), through the tubing and this allows for the monitoring of the cultures throughout incubation without any additional fluid handling steps. All culture tests were measured by the on instrument optics minimising the need for additional microplate reading and additional fluid handling steps. A limitation of the current methods is that to take to measure of the optical density it is necessary to lose some of the sample for reading on the microplate reader. The optical density unit allows non-invasive monitoring of absorbance of light to each of the cultures in real time. Once the absorbance optics were incorporated an initial test to verify if the photo diode system could read the difference in optical density of droplets.

5.3.1 Absorbance Detection in Droplets

To verify that the optical system is capable of distinguishing between the *E.coli* and LB, a 9 hour test with 10 droplets of *E.coli* and 10 droplets of LB was conducted. Droplets of 600nl were made of each sample and 10 droplets were chosen to give more accurate statistical analysis. Figure 5.5 shows the optical density readings from the *E.coli* droplets and the LB droplets incubated on the system. The bacteria sample was mixed 1:10 *E.coli* to LB and pre incubated for two hours and a seeding density of 0.02 was measured before droplets were generated. The reason for pre-incubation was to have cells within the log phase growth so that analysis of growth could be

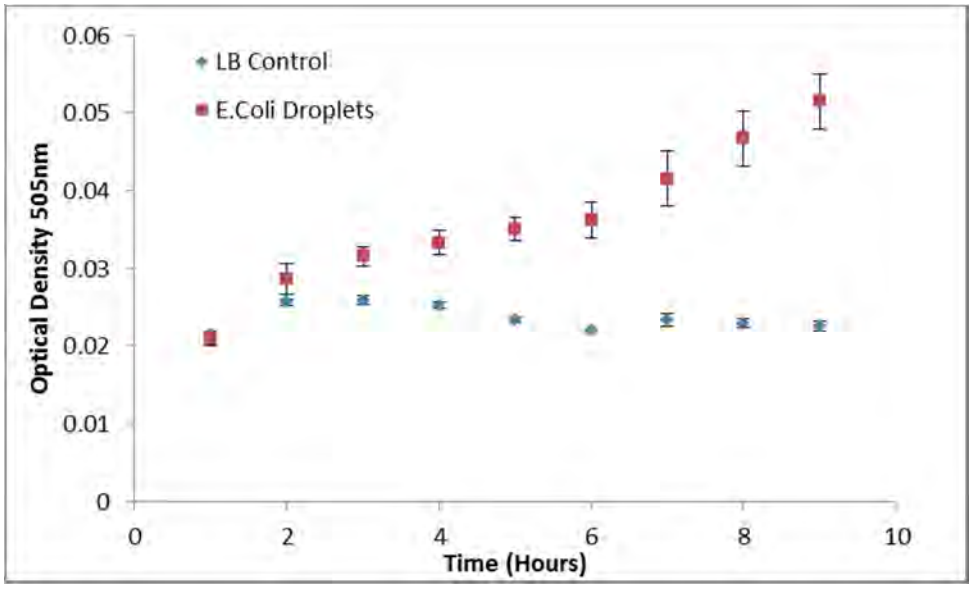


Figure 5.5 The photodiode system readings of *E.coli* droplets and LB droplets over 9 hours.

pursued rapidly. To measure the optical density, the droplets were moved past the photodiode system each hour and remained at rest in between readings. The droplets move forward and backwards in the tubing for monitoring and maintained at 37°C which keeps the cultures isothermal while full mixing is also achieved. The average optical density value of each droplet was found and error analysis of standard error of the mean was evaluated. Figure 5.5 demonstrates that the LB has constant

absorbance over the nine hours with very little error over 10 droplets. The measurement reference blank was taken as the OD of the oil between the droplets. The difference in optical density between the *E.coli* and LB is apparent after two hours of incubation (Figure 5.5). The *E.coli* growth increases over the nine hours. This increase in growth rate shows the same trend as found using the microplate reader in Figure 5.4 verifying the ability of this instrument to sufficiently measure the growth of bacteria. The obvious growth was measured here without sampling of a bulk culture, which is required with microplate readings. As the readings were taken every hour and can be taken as often as required proving this method as a real time method of measuring OD non-invasively. The photodiode provides a novel mechanism for real time analysis of the cultures. The results from the photodiode system verify that this is a sufficient method for determining the growth of bacteria non-invasively. Optimisation of the droplets is conducted to find the best conditions of bacterial growth.

5.4 Contamination of Droplets

As with all experiments performed, it is necessary to provide conditions that will not directly affect the cultures. Contamination of samples will render the results unusable for analysis. As this is a new method of bacterial culture it was necessary to show that the conditions within the microfluidic tubing are not adversely impacting the cultures. If the droplets merge or wet the tubing wall this would cause contamination and would make these droplets unreliable as bioreactors. As the optical unit has sufficiently distinguished between bacterial and LB droplets this system was used to verify if the droplets were contaminated or not. Keeping the same method as the previous 10 droplets of LB, containing no bacteria, and 9 droplets containing *E.coli* were formed in the tubing. Optical density readings were

taken each hour when the droplets were moved past the optical system. The droplets were then moved backwards through the tubing again to their original point. This was repeated each hour and from Figure 5.5 shows that there is no increase in OD of the LB verifying that no contamination is apparent. Figure 5.6 shows typical hourly voltage readings that are directly coming from the optical circuit after 6 hours of incubation.

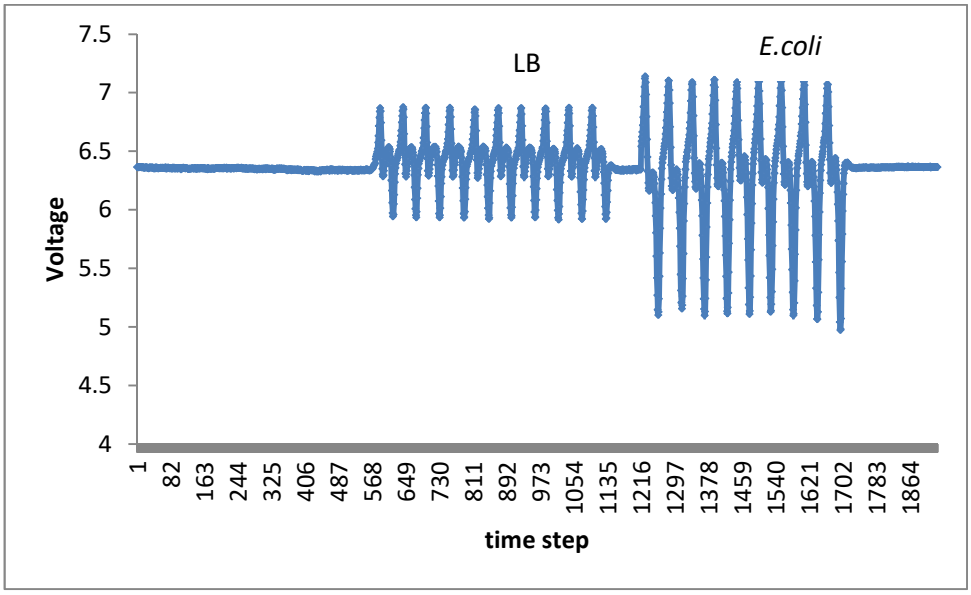


Figure 5.6 An image of the voltage readings from the photodiode system. Each peak represents a droplet where the first 10 droplets are LB followed by 10 droplets of *E.coli* readings taken after 6 hours of incubation.

The oil phase is indicated by a flat line of approximate reading 6.36v and this is used as the blank measurement point for all tests using the photodiode optical system. To quantitate the optical density the change in voltage between the peak of the droplet and the baseline is used. To visually demonstrate the difference in peaks of the LB and the *E.coli* Figure 5.6 was generated. The first set of peaks in Figure 5.6 is the control LB droplets where each peak is an individual droplet. The second set of peaks are the *E.coli* droplets incubated for 6 hours and it is clear from Figure 5.6 that

the peak is higher than the LB. The measurement of droplets after 6 hours was chosen as it shows the obvious difference between LB and *E.coli*. The increased peak is consistent over the 10 droplets which allow statistical analysis of multiple samples at small volumes. All the readings here were taken at the same time and each droplet can be monitored individually at any point during incubation. As the droplets are moved past the optical monitoring system and then back again through the tubing each hour, the LB droplets will have passed multiple times where the bacterial droplets have passed. This shows that the conditions set up in this system do not adversely impact the bacteria in terms of droplet to droplet contamination. This also proves that the droplets are fully wrapped in oil. This means that there are no bacteria available to the LB droplets and shows that each individual droplet is an individual bioreactor. This verification allows all future tests to use the droplets as robust bioreactors and can be used for mammalian cells.

5.5 Droplet Volume Effect on Bacterial Growth

Table 5.2 volume length properties of the droplets

Droplet Volume nl	Droplet Length mm
400	1.043095
600	1.429309
800	1.815523
1000	2.201737
1500	3.167272
2500	5.098342

The conditions within the droplets influence the growth of the cultures. To find the optimum conditions the volume of the droplet was examined. It was found in the initial test (Figure 5.4) of bacterial suspension culture and the droplet cultures, that there was a higher growth rate at a smaller droplet volume of bacteria. A test to investigate if the volume at a microscale level will demonstrate the same trend is shown here. To study this effect on the instrument, a range of droplet volumes of 400-2500nl were tested. The length and volume of each droplet within the tubing is shown in Table 5.1. Each test was conducted individually with control droplets of LB tested each time. Ten droplets of each volume were tested and optical readings were taken every two hours over a period eight hours. The samples were 1:10 *E.coli* to LB and these were pre-incubated for two hours before droplets were generated and formed a seeding density between 0.06 and 0.08 OD.

Table 5.3 Droplet volume to slope

Droplet volume nl	slope
400	0.006555
600	0.006975
800	0.007818
1000	0.011024
1500	0.01199
2500	0.008728

Figure 5.7 displays the log of optical density for all volumes studied over 8 hours.

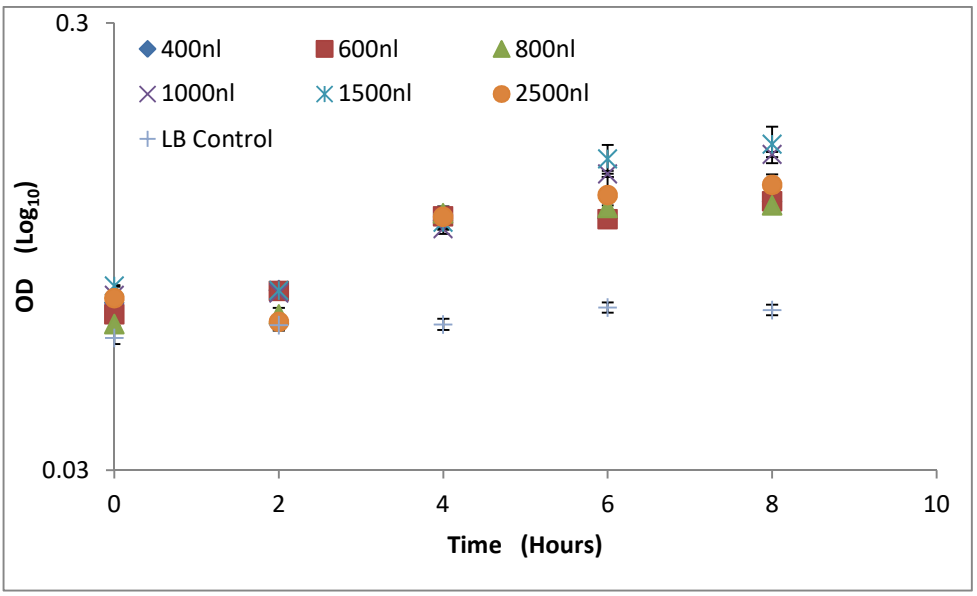


Figure 5.7 Monitoring the effect of variation of the droplets volume (600nl to 2500nl) on bacterial growth over time. Results indicated that the optimum growth rate is achieved in 1500nl droplet volume

The control in Figure 5.7 is the value for a 400nl LB droplet but as all LB controls remained constant it was not necessary to graph each one for each droplet size. All the *E.coli* growth patterns follow the same trend in Figure 5.7 and as they were all conducted independently this is an example of the repeatability of the instrument. Initially, similar optical densities for all droplets are observed, however the lowest being the LB control. After two hours of incubation an increase in OD is noticed in the droplets containing bacteria. This shows the consistency of rapid identification by growth as previously demonstrated in Figure 5.6. At the 4 hour mark (Figure 5.7) all cultures are distinguishable against the control and all have higher optical densities. It is at the six hour mark that the difference in growth due to volume becomes apparent. The droplets at 400nl, 600nl and 800nl are stagnated in growth and this may be due to the lack of nutrients in smaller droplets. At 1000nl and 1500nl the growth reaches the highest OD. Above 1500nl the effect on the growth

of bacteria droplets are sub-optimal. The optimum volume is found at 1500nl as this shows the highest growth rates over eight hours. The rate of each growth is demonstrated by the slope of each line (Table5.2). The values of slope for each sample also show that 1500nl had the highest growth rate. The higher growth rate will allow more apparent recognition of the effects of antibiotic treatment when examined later in this chapter. The increased length also means that the droplets will take longer to mix as they will have to travel longer to achieve this (Tice *et al*., 2003). The decrease in growth above 1500nl could be attributed to the increased volume effect as seen in the initial test (Figure 5.4). The optimum volume was found to be 1500nl and above this would not be recommended for growth of the bacteria.

5.6 Velocity effect on *E.coli* growth

The second condition that can be defined is the velocity of the droplet. The velocity of the flow will determine the amount of internal circulations and also where the cells will reside within the droplet. The velocity is dictated by the flow rate on the pump and can be altered from test to test if required. The internal circulations within the droplets can influence the diffusion of the nutrients into and out of the cells. A test was conducted to look at the growth rate effects with increasing velocity. Ten droplets of each *E.coli* velocity were made and each test was done individually. The *E.coli* was pre-incubated for two hours before testing and a seeding density of 0.06-0.08OD was established for each culture. The control in this test again was LB without any cells and the measurements shown in Figure 5.8 are for the static culture. All controls remained stable for the duration of the tests and therefore were not demonstrated on Figure 5.8. Measurements were taken every two hours using the photodiode system within the instrument. The flow rates examined were 0-60ul/min which is equivalent to 0mm/s-1.93mm/s. Figure 5.8 shows that growth is apparent

for all the cultures containing bacteria at all velocities however, after two hours it becomes evident that the velocity influences the growth trend as at 0ul/min the growth rate lags behind all other velocities and this gap remains throughout all testing in Figure 5.8. Here, it shows that the internal circulations have a more evident influence on the growth of bacteria than the volume of the droplet as it is seen after two hours that the difference in growth rate between the static culture and all the rotated cultures is evident. The static culture grows but the internal circulations within the droplet provide an increased growth rate. The growth rate for each culture is demonstrated by the slope in Table 5.3. The increased growth rate is apparent in all moving cultures however, this growth is impeded in flow rates above 30µl/min. At 30µl/min it is apparent that cells go through lag, log and plateau phase within 8 hours. At 60µl/min the growth rate tapers after four hours of incubation and at this point the optical density reading remains relatively static over hours 4-8 showing that the cultures are still alive but are not growing as the cultures have reached their plateau phase. The flow at 30µl/min indicates optimum growth as the *E.coli* growth rate shows that the plateau phase is not reached until six hours.

Table 4 Slope of the velocity of the bacterial cultures

Flow rate ul/min	**Slope**
0	0.016019
15	0.019411
30	0.021614
60	0.014766

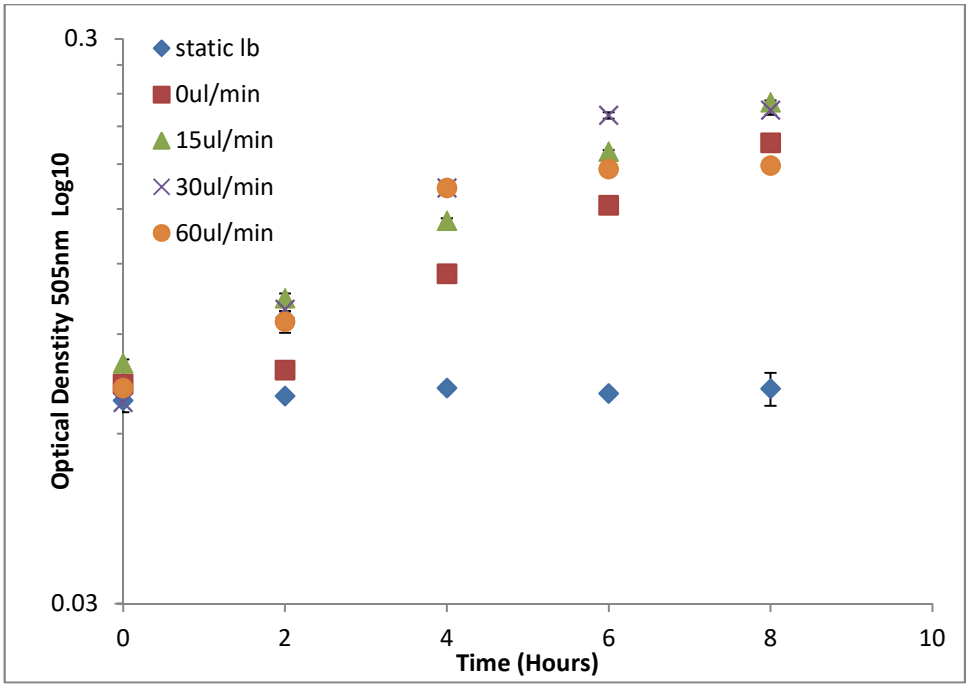


Figure 5.8 Monitoring the effect of dynamic conditions on *E.coli* growth within a droplet at varying flow rates from 0-60ul/min. Increasing the flow rate increases the growth rate up to 30ul/min.

As shown previously in chapter 4 *E.coli* acts like particles in the flow and will remain in the cap end of the droplet. If the length of the droplet is increased the amount of cells within the droplet will increase. So cells at 1500nl although having the best growth rate statically, will have more cells within the droplet than a 500nl droplet. This means that more particles or bacteria will be available to occupy the cap end of the droplet. The increasing velocity reduces the area at the cap ends inhabited by the bacteria. Due to the cells being more compacted at the back of the droplets there is a lack of availability of nutrients to the cells. This could be the reason why the growth rate is decreased above 15µl/min. The rotations present in at 60µl/min (see Chapter 4) also coincide with the reduced growth within the droplets. The rotations are only visually present at 60µl/min this increased rotational activity may be causing shear effects on the cells. Shear has a negative effect on the cells (Lange *et al*., 2001) by causing the rupturing of the cells and it has also been shown that at higher concentration there is a decrease in cell viability when shear is

introduced. This study shows that at 30µl/min and 1500nl droplets these are the optimum conditions for bacteria proliferation within the droplet structure to give the most effective reaction when tested against drugs at these conditions as they have the highest growth rates over eight hours.

5.7 Antibiotic effect on *E.coli.*

The validation of the system as a practical alternative to current bacterial identification methods will come in the reaction of the cultures to antibiotics. With a worldwide increase in resistance to antibiotics it is necessary to easily identify the bacterial reactions to specific antibiotics. Three tests were conducted where antibiotics were introduced to the droplets:

1. *E.coli* mixed with two different antibiotics.
2. Bacteria grown to log phase and then mixed with a drug to show a response to actively growing cultures
3. Identification of antibiotic resistance

These three tests give an insight into the potential of the instrument as a novel method of bacterial treatment identification.

5.7.1 Antibiotics and *E.coli*

The initial test adds two antibiotics, Ampicillin and Kanamycin to the *E.coli.* 10 *E.coli* droplets mixed with Kanamycin, 10 *E.coli* droplets with Ampicillin and 10 droplets with *E.coli* only were made. Mixing of the droplets took place on the instrument using the drop-off junction where one 1000nl droplet containing the *E.coli* was mixed with 500nl droplet of antibiotic forming a 1500nl droplet. The final concentration of the antibiotics was 0.05mg/ml for Ampicillin and 0.15mg/ml Kanamycin. The droplets were real time monitored using the photodiode system and

measurements were taken each hour. These droplets were made in series and were monitored at the same time. Figure 5.9 shows the cultures over nine hours. The initial seeding density of 0.02 for the mixed droplets shows consistency as the *E.coli* sample is the same for all three cultures. The only difference is that there are antibiotics mixed with some droplets while the other droplets are just mixed with LB. The LB was mixed with the *E.coli* only droplets to maintain the same concentration in each droplet. Figure 5.9 demonstrates that the *E.coli* containing the Kanamycin and Ampicillin were prevented from growing while the *E.coli* mixed with LB continued to grow. The error analysis is standard error of the mean and ten droplets are analysed at each time point. The antibiotics effectiveness in prevention

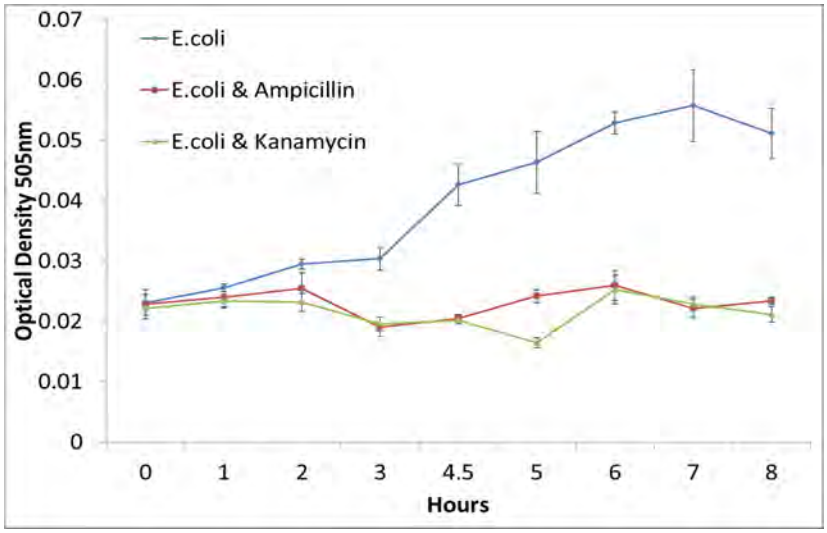


Figure 5.9 The treatment of bacterial cultures within the droplets. Droplets of *E.coli* mixed with ampicillin and *E.coli* mixed with kanamycin were monitored over time. Antibiotic treated droplets are prevented from growing while the non-treated *E.coli* continue to grow

of growth being identified using the photodiode system shows that this instrument is capable of sufficiently detecting a treatment for this type of bacteria. In this case two options for treatment were identified. This in a clinical setting could also allow for multiplexing the experiment by incorporating multiple combinations of drugs in the system and looking for multiple targets. A concentration gradient of these antibiotics

could also be mixed with the cultures to find the most effective amount of drug required for the treatment of the bacteria.

The continuation of growth of the *E.coli* shows that the antibiotics are preventing the other cultures from proliferation. A difference in growth was apparent after three hours making this a rapid way of identifying if the treatment is sufficient to target the bacteria.

5.7.2 Adding Antibiotics after the Establishment of Growth

The second investigation incorporating antibiotics allows the establishment of

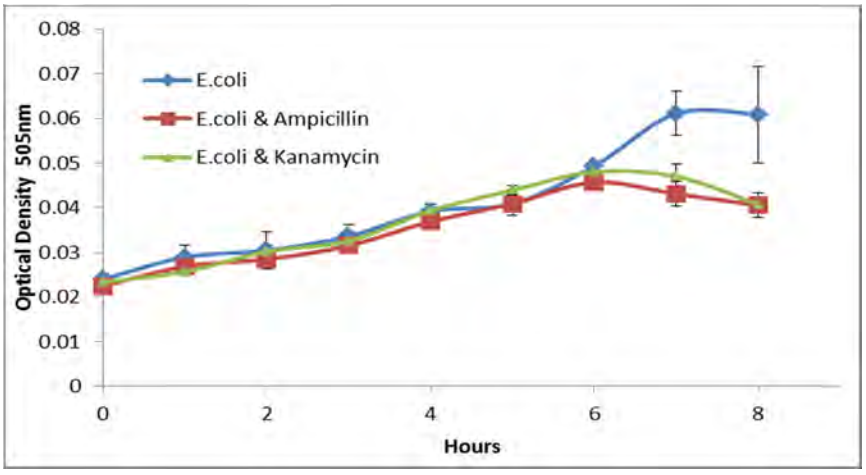


Figure 5.10 *E.coli* droplets cultured for 6 hours with ampicillin and kanamycin mixed at 6 hours using the drop-off junction. The reaction is rapid as the reduction in cell growth is evident after one hour of adding the antibiotics while the cells without antibiotics continue to grow

growth of the bacteria before adding the antibiotics. Thirty 1000nl droplets of *E.coli* cultures were generated and allowed to proliferate for 6 hours. After 6 hours the first 10 droplets were mixed with 500nl Ampicillin and 10 were mixed with 500nl Kanamycin while the final 10 were mixed with 500nl LB in the same concentrations as the previous test. The droplets were incubated on the system and monitored each hour. The seeding density is the same for all samples and the same growth trend is apparent for all three samples showing consistency over six hours. Figure 5.8 shows the 6 hour mark where the antibiotics are added to two of the cultures. After the

drugs were incorporated, it was clear that the cultures treated with drugs have an instant reaction. After one hour of treatment, the optical density has reduced showing a direct effect on the cultures (Figure 5.10) while the culture that was mixed with LB only continued to grow. The reaction within one hour of application of a drug shows that the system is capable of treating the cells within the droplets and also that the reaction is rapid. This has potential in a clinical setting as it allows the introduction of any assay once the culture is in its log phase of growth and identification of treatments on a personalised level. For example where a patient may present with an unknown bacterial infection, a clinician could let the bacteria grow in droplet detect it's growth rate over a number of hours and then add drugs to the culture. If there is a reduction in the growth after the drug has been introduced the bacteria is responding indicating a potential treatment for a patient.

5.7.3 Identification of Antibiotic resistance

One potential issue with identification of treatment is the increasing incidence of

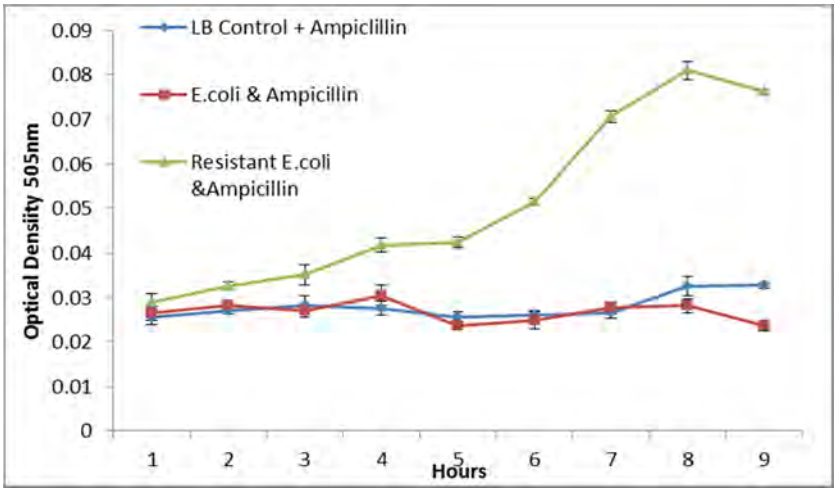


Figure 5.11 Identification of Ampicillin resistant *E.coli* droplets and ampicillin resistant *E.coli* droplets were monitored over time with ampicillin mixed at time 0. The continued growth of the resistant *E.coli* is evident while the non-resistant strain were prevented from growing

antibiotic resistance. Antibiotic resistance is becoming an increasing problem and hence, a rapid detection of a bacterial culture reaction to a treatment could identify in

a clinical setting the most appropriate treatment for a patient. The final bacterial investigation uses Ampicillin resistant *E.coli*. For this test 30 droplets were made, 10 contained the same *E.coli* strain as used in all previous tests the next 10 contained Ampicillin resistant *E.coli* and the final 10 contained LB. The droplets had a final volume of 1500nl and Ampicillin concentration of 0.05mg/ml. All droplets were mixed with ampicillin using the drop-off junction and optical density readings were taken each hour using the photodiode system. Figure 5.11 demonstrates the cultures over nine hours. It is clear from Figure 5.11 that the *E.coli* that is ampicillin resistant continues to grow even though a drug has been applied to the culture. The *E.coli* that is non-resistant is directly affected by the ampicillin as it does not proliferate. Between hours one and two the distinction between resistant and non-resistant bacteria is apparent. This graph shows the identification of a resistant bacteria and the effective treatment of bacteria on the instrument at the same time. This test shows that essentially two different bacteria are identified on the system at the same time and that a treatment for one strain was identified while the resistance to treatment was also identified. This method shows that after four hours the difference in the resistant and non-resistant bacteria reaction to an antibiotic is apparent, clear validation of this instrument as a new method of identifying antibiotic resistant bacteria.

The identification of successful treatment of *E.coli* while identifying resistance to ampicillin was done at the same time on the instrument. This gives the potential to have an unknown bacteria identified as resistant to a drug and a potential treatment identified within a number of hours with a quantitative evaluation compared to visual petri dish methods.

5.8 Chapter closure

This chapter has outlined the experiments into *E.coli* bacterial culture which will act as a pre-test to ensure that the system is capable of maintaining biological samples. The use of droplets as bioreactors for bacterial cultures was validated here. Initial growth of the *E.coli* within droplets was compared to suspension methods and this proved that the growth rate is higher in the droplets over conventional methods. This shows that the droplets are biocompatible and provide a new environment for bacterial cell culture. The incorporation of optical density absorbance capability transformed this into a non-invasive instrument. Real time monitoring of all the cultures individually without any additional fluid handling steps was performed. This allowed each individual droplet to be monitored without being taken out of incubation. The droplets were created, incubated and monitored in the tubing and the difference between the LB and the *E.coli* prove that this system is capable of recognising bacterial growth.

The optimising of the droplets to find the most progressive droplet conditions for maximum cell growth was presented. To optimise the instrument the ideal volume was found at 1500nl giving the best growth rates. The system also recognised that above 1500nl droplets that there was a decrease in growth rate. A range of velocities were investigated and found the best proliferation to be at 30µl/min. The velocity will stimulate the internal circulations within the droplets and this can aid in the diffusion of the media in and out of the cells. It can also sort the cells into distinct patterns within the droplet and at higher flow rates than 30µl/min this reduces the growth rate of the bacteria.

The final validation of the instrument was the introduction of antibiotics to the bacteria. Mixing within the drop-off junction allows for the addition of antibiotics

Ampicillin and Kanamycin to the cultures. Initially, the two antibiotics were mixed with *E.coli* and it was clear that the cultures containing drugs were prevented from growing. This was followed by establishing cultures in the droplets and adding antibiotics after six hours of growth. The reaction of the cultures to the drugs was instant with the decrease in growth evident one hour after addition of the antibiotics. The final test was using the system to detect if antibiotics resistance is present. Ampicillin resistant *E.coli* was mixed with Ampicillin, whilst the same non-resistant *E.coli* was also mixed with Ampicillin and tested. The identification of the *E.coli* resistance was established showing the multiplexing of the instrument.

This chapter shows the reduction of reactions to 1500nl with reduction of liquid handling steps meaning that it is less user dependant than current methods. There rapid identification of growth and reaction to treatment in a number of hours compared with overnight incubation of using agar plate. This instrument is validated as an alternative to current methods, and promotes the introduction of this instrument in a clinical setting. This novel method can produce micro cultures of bacteria, add antibiotics to the cultures and identify a reaction non-invasively allowing any bacteria and any potential drug to be evaluated in a preclinical setting. With this validation the system is deemed sufficient to test mammalian cells and this will be discussed in the following chapter.

Chapter 6

Mammalian Cells

6.1 Introduction

To challenge this system as a viable preclinical method, mammalian breast cancer cells were analysed. Breast cancer is one of the most prevalent types of cancer affecting 2600 people each year in Ireland (Society', 2016) This is caused by cells mutating and forming masses within the breast. Uncontrolled growth of cells causes abnormal changes to gene expression. One of the main issues with breast cancer is the metastasis of cancer from one site in the body to another (Chambers *et al*., 2002). Cells break off from a primary cancer site and move to a secondary site through the blood stream or through the lymphatic system. This causes cancer in other sites of the body for example, the bones and lungs (Aceto *et al*., 2014). At the moment the

amount of information about why cancer cells travel is limited. A better understanding of this could enhance how cancer is treated. Breast cancer is divided up into four sub groups each indicative of receptors that are used to identify the type of cancer Luminal A, Luminal B, Her2 positive and triple negative (Senkus *et al*., 2013). The triple negative breast cancer has no known receptor used for the diagnosis of the disease. It is for this reason that it is important to have methods of investigating and diagnosing cancer. Currently in breast cancer diagnosis, if a patient presents with an abnormal mammogram scan where a mass is detected, a biopsy is taken and used to diagnose if cells are cancerous or benign. A course of treatment is prescribed and regular scans are used to determine if the mass has changed size (Senkus *et al*., 2013). Included in the initial work up is the pathology review of a biopsy and this will indicate if the cancer is positive for oestrogen or progesterone receptors. This will point to the best treatment for the patient. The staging is a clinical level of the progression of the cancer (Senkus *et al*., 2013). The only indication of successful treatment of disease is the reduction in size of a tumour. The cost of treatment of cancers is also high (Liu *et al*., 2010) and if the treatment is ineffective, this can lead to the additional cost of a new treatment and also the cancer progressing in the meantime. Personalised medicine where samples from a patient are tested could provide a way of using the original patient biopsy and assessing if a treatment is effective on a person by person basis.

This chapter introduces microfluidic droplets as bioreactors for mammalian cells. The conditions within the droplets are analysed and the cell's reactions to these conditions will ultimately determine if the droplets are adequate as a new method for preclinical cell analysis in terms of viability and health.

There are four sections to this chapter, the first section focuses on how the cells react within the droplets and this will include the quantification of the cells. The second section discusses the health of the cells over longer periods. Cell concentrations are small compared to current culture methods and initially standard lab methods such as live/dead staining are used to evaluate the cells. Florescence based systems are used to quantify where cells were below a detectable level using standard methods. The third section will concentrate on the development of three dimensional cell cultures within the droplets. As discussed in chapter one, three dimensional models give more accurate reactions to drugs compared with two dimensional cell models. With each individual droplet containing an individual culture, this means that it would be possible to create many 3D cultures of breast cancer and have them examined on the system at the same time. The 3D cultures are developed in the droplets and their structure is analysed.

The final section in this chapter, demonstrates the treatment of the cells within the droplets. A previous study by O'Neill *et al* (2012), where a gene signature for the treatment of breast cancer carcinoma was found, was replicated in microfluidic droplets. The novel aspect here is that the tests were conducted with considerably less cells than current methods. The comparison of treated to untreated cells is used to generate a gene signature. The comparison to O'Neill *et al* 2012 is discussed here to show how the droplet bioreactors are capable mimicking larger cultures.

The novel aspect of this whole system is demonstrated in this chapter by the miniaturisation of the whole process from the generation of mammalian droplet cell cultures, creating 3D cultures to treating the cells within the droplets. The use of continuous droplets allows many cultures to be examined and minimised the amount of reagents required to analyse precious samples.

6.2 Cell Health

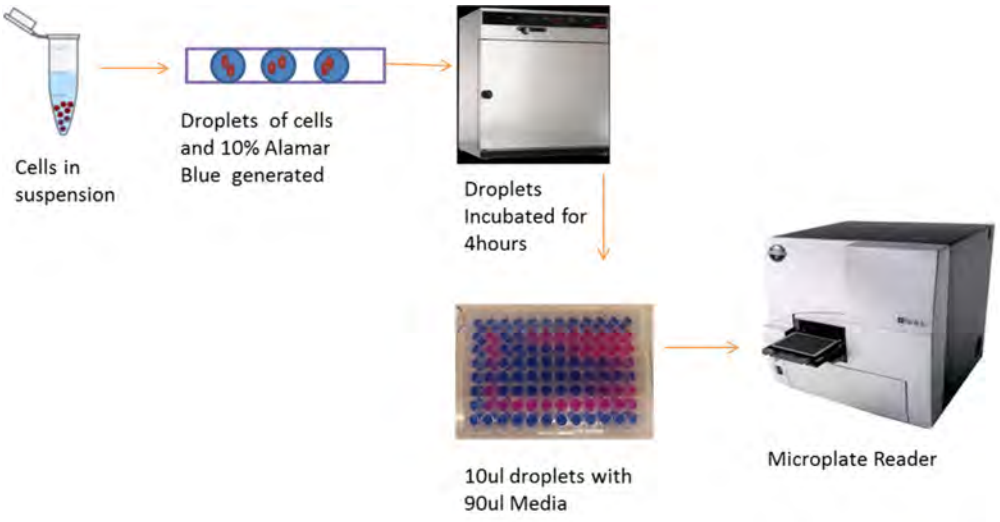


Figure 6.1 Process flow chart of Alamar blue assay from creating a cell sample from which droplets are generated with 10% alamar blue . The droplets are incubated in a standard culture incubator and after which are mixed with media and florescence detected on a microplate reader to florescence detection

The health of the cells for prolonged periods of time within the droplets was analysed. This consists of monitoring the cells after incubation within droplets. This method used a metabolic activity assay to measures the amount of cells in a sample as well as determining the cells health over a 48 hours. Cell quantification and health can also be obtained by measuring the viability of the cells. Here, Alamar blue is a non-toxic assay used to quantify the cells using fluorescence of resazurin. Alamar blue is metabolised by cells and changes the assay from blue to pink in colour when the cells are viable. This process does not happen if the cell is not viable as it will not be metabolised by the cell. As it was not possible to accurately quantify the cells using a haemocytometer after incubation of the cells due to the cells accumulating together (Chapter 4 Figure 4.3) the Alamar blue method was applied. The Alamar blue analysis was used to determine if low concentrations of cells are quantifiable within the droplets. The process of application of this assay was described in Chapter

2. Figure 6.1 shows a flow chart of the process. MCF-7 cells were prepared at various suspensions depending on the test and 10% Alamar blue was added to the cells sample before the droplets were generated. There were two separate tests conducted on this, initially to quantify the cell concentration of the droplets and secondly to determine the health of the cells within the droplets over 48 hours. To quantify cell concentration, droplets were generated from the cell sample containing 10% Alamar blue and incubated for 4 hours. To quantify the lower amount of cells a range from 10,000 to 300,000 cells/ml concentration was selected. Figure 6.2 shows the fluorescence readings over increasing concentrations. Figure 6.2 demonstrates that there is a linear increase in florescence with increased cell concentrations. This linear relationship shows that cell concentrations as low as 50,000 cells/ml up to 300,000 within the droplets can be detected. As larger cell concentrations are necessary for further cellular analysis and cell sedimentation could have a larger affect at higher cell numbers, it was essential to quantify larger cell concentrations. Cells concentrations of 500,000 to 1,000,000 cells/ml were made with 10% Alamar

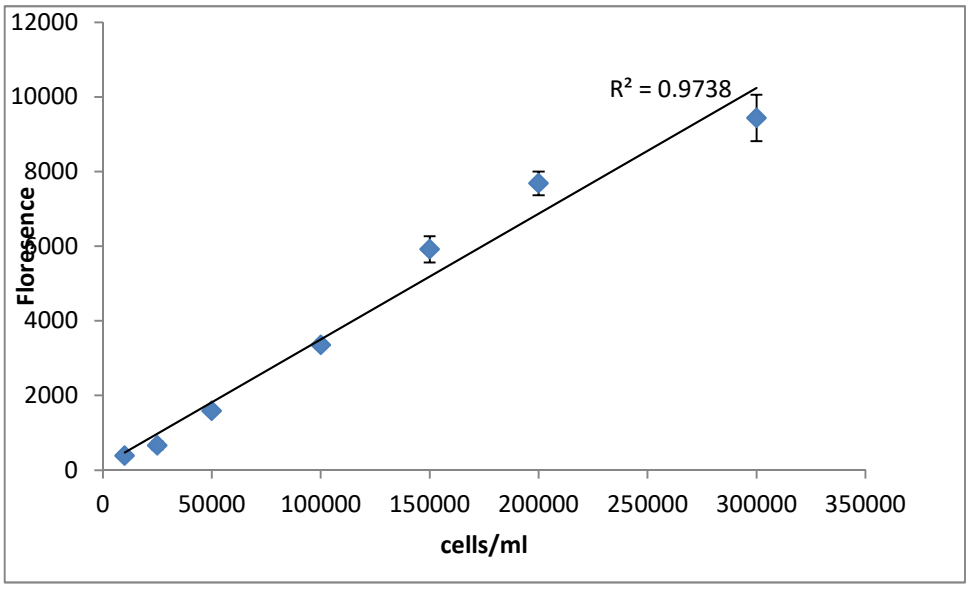


Figure 6.2 Alamar blue florescence readings in low cell concentrations from 10,000 to 300,000 cells/ml.

blue. These droplet cultures were incubated for four hours and florescence readings were taken on the microplate reader. Each concentration was made separately with individual counts on the haemocytometer to insure discrete results were found. Figure 6.3 also shows the linearity of the cell concentration. It is shown here in a separate graph as the concentrations are much higher than with the lower concentrations. From both figures (6.2 and 6.3) it is clear that cell concentration gives an increased cell florescence reading. This method therefore shows that it is capable of determining cell number within a sample as low as 50,000 and cells up to

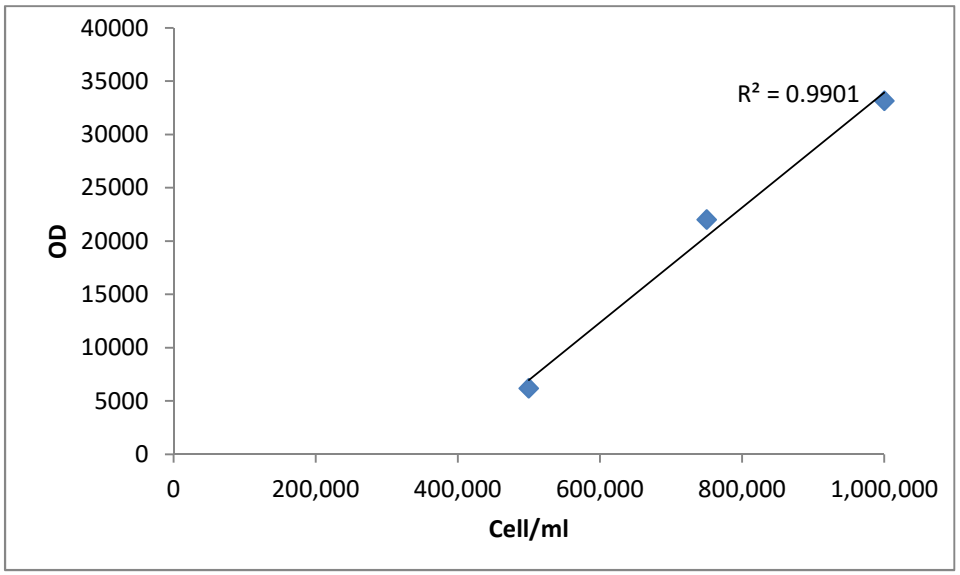


Figure 6.3 Alamar blue concentrations for higher cell concentration

1,000,000 cells/ml concentrations within droplets.

The second investigation was the analysis of the cells health over 48 hours using Alamar blue. From the trypan blue tests it was shown that the cells remain healthy up to 48 hours (Chapter 4) and it was necessary to evaluate this quantitatively as it was not possible to accurately count the number of cells in each concentration. Two tests were conducted, one with low concentration of cells where the Alamar blue was

added to the cells when the initial sampled, the second test with

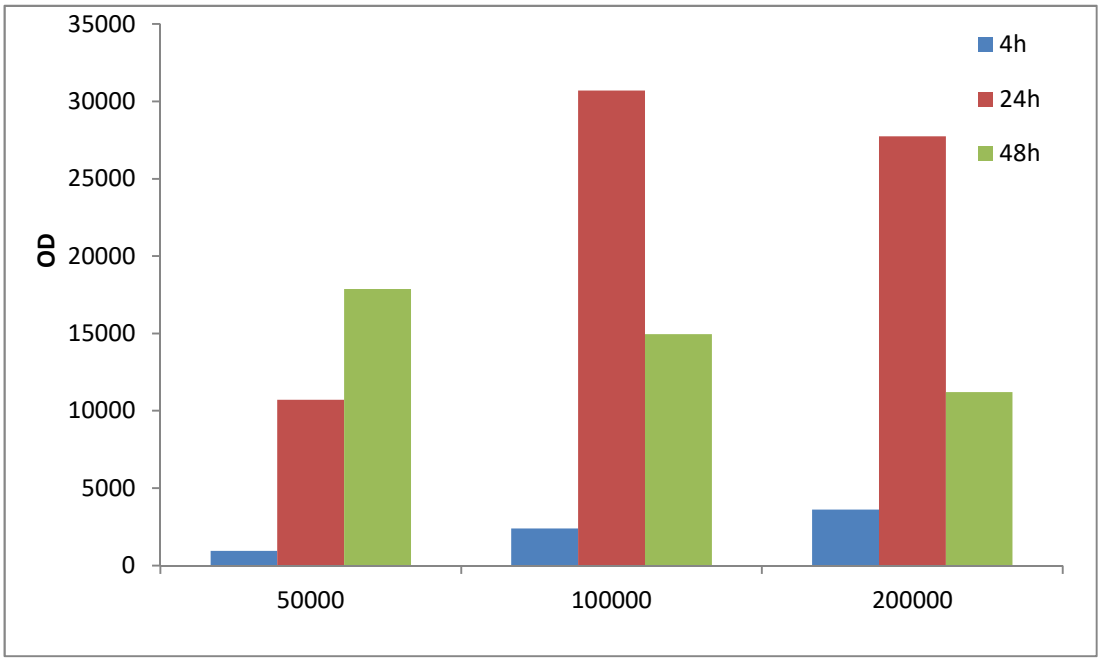


Figure 6.4 Cell viability over 48 hours for low cell concentrations demonstrating that there is an increase in florescence at 24 hours for all concentrations while at 48 hours there is a decrease for higher cell concentrations.

larger cell concentrations where Alamar blue was added to the cell concentrations after incubation at 4, 24 and 48 hours.

Figure 6.4 shows the optical density for the lower concentrations of cells. The concentrations were made of MCF-7 cells, where 10% Alamar blue was added to the sample before droplets were made. The droplets were incubated for 4, 24 and 48 hours where a sample of the droplets was taken and the fluorescence was measured in a well plate. Three samples were measured each time to ensure consistency. From Figure 6.4 it is clear that there is an increase in fluorescence over 24 hours for all samples. This shows that the cells are viable up to 24 hours as they are still alive. At 48 hours the only increase in optical density is clear from the 50,000 cells/ml sample. There is a decrease in optical density for both the 100,000 and 200,000 cells/ml concentrations shown in Figure 6.4 at 48 hours. This indicates a low amount of

viable cells, however as the Alamar blue has been added to the cell sample from which the droplets are made all of the Alamar blue may have been metabolised by the cells previously. This shows that the cells are healthy cells up to 48 hours but further analysis is required to determine if the cells are viable or if the Alamar blue has been depleted by the cells.

To evaluate if the Alamar blue was being depleted if mixed in the sample before incubation, a test was conducted where the droplets were incubated and Alamar blue was added after the incubation. This test was also used to find the limits to the amount of cells that can be held within the droplets where the cells remain healthy. Figure 6.5 shows the cell concentrations of 500,000cells/ml to 1,000,000cells/ml.

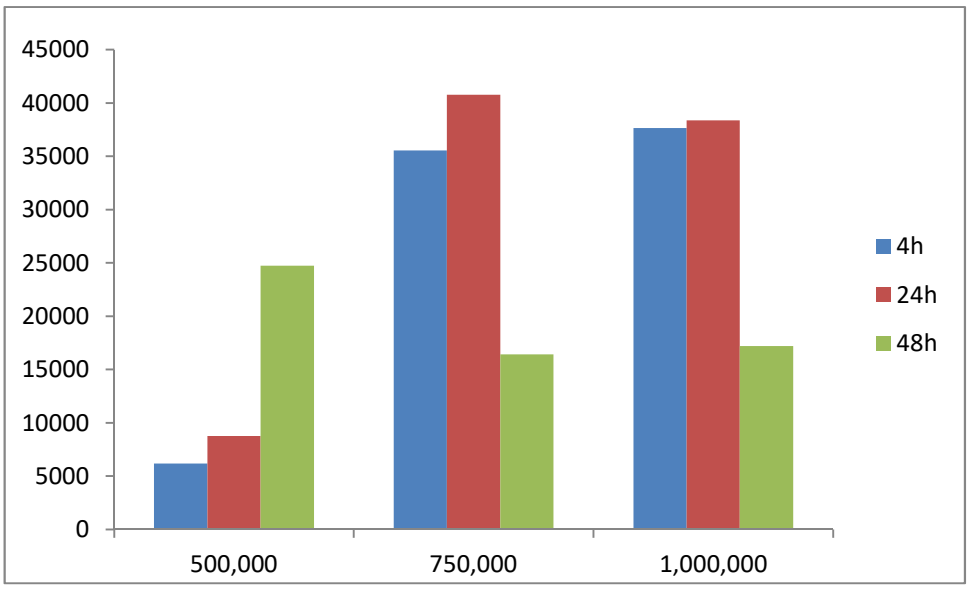


Figure 6.5 Cell viability measurements over 48 hours for cell concentrations (500,000-1,000,000) where Alamar blue is added at 4 hours before each time point reading,

Alamar blue at 10% (10ul) was added to the 90ul cell sample and incubated for 4 hours within a well plate. This was done to ensure that there was sufficient Alamar blue available for the cells. Three samples were then measured on the microplate reader. As with Figure 6.4, Figure 6.5 shows that for all the cultures there is an increase in fluorescence over 24 hours and shows that all cells remain viable and

healthy. As these concentrations are higher than the previous test this proves that it was depletion in the Alamar blue in the previous tests that caused a decline in fluorescence. Figure 6.5 shows that for cultures above 750,000cells/ml that the viability begins to declines at 48 hours. The cells health is suboptimum beyond 24 hours. The only culture that remain fully viable up to 48 hours are at concentrations of 500,000cells/ml.

This test shows that the cells are healthy and viable within the droplet up to 24 hours and 48 hours for lower cell concentrations. All cell cultures up to 1,000,000 cells/ml remain viable within the droplets for up to 24 hours. It was concluded that the Alamar blue should be added to the droplets after incubation to have the most accurate results.

6.3 3D cell culture

From finding the optimum conditions for the cells to thrive within the droplets 500,000cells/ml concentration was used to understand the cells structure within the droplets. To evaluate the structure, MCF-7 cells were imaged using an optical light microscope on the imaging block through the tubing integrated into the microfluidic instrument. The droplets were held in an incubator and taken to the instrument for imaging. The images in Figure 6.6 are all from the same droplet imaged over 48 hours at 10x magnification . It is clear from the images in Figure 6.6 that the cells are initially dispersed throughout the droplet at time 0 hours. After 24 hours within the same droplet the cells have accumulated together and formed one structure. This structure circulates around the droplet while being moved through the tubing. This does not disrupt the cells structural form. After 48 hours (Figure 6.6) the cell

structure is closer together and appears tighter. However, to verify the true structure of the cells further analysis is required.

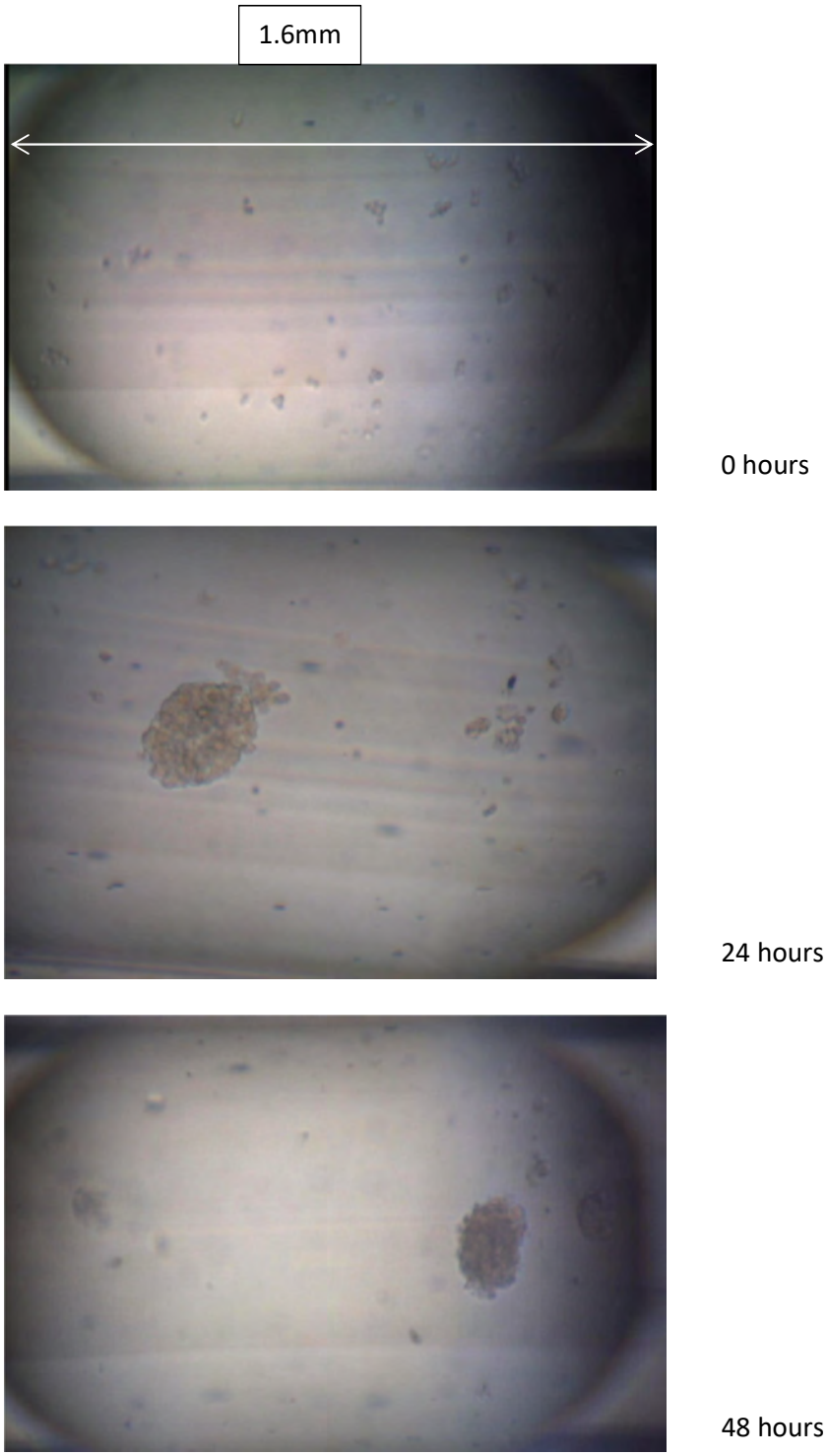


Figure 6.6 10x microscope images of MCF-7 cells at 0, 24 and 48 hours within the microfluidic droplets images taken on the system through the tubing.

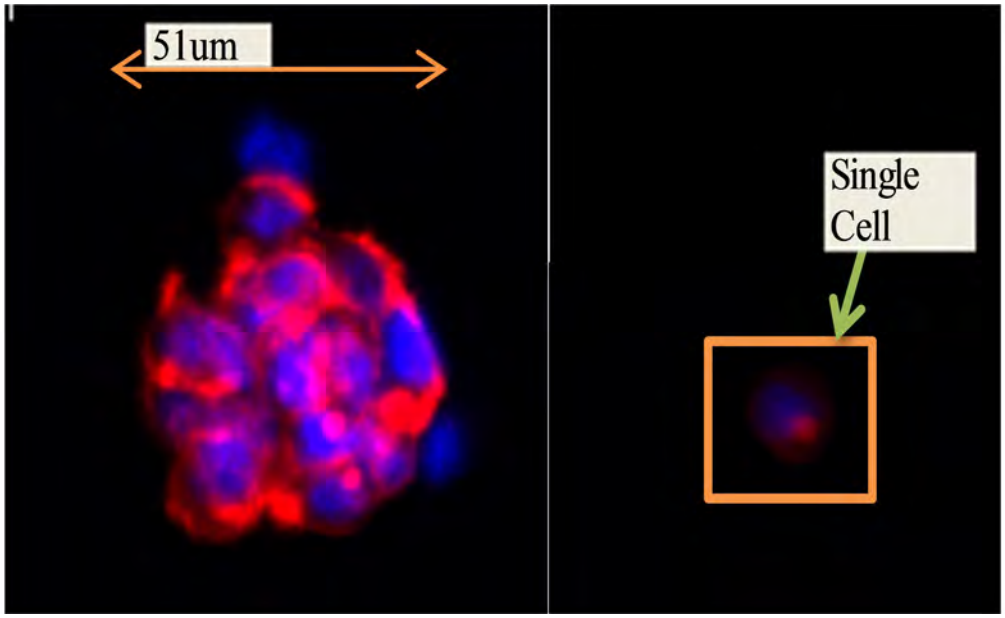


Figure 6.7 Confocal Image of MCF-7 cells after 24 hours of incubation within a microfluidic droplet Cells were stained with Hoechest and Phoilidin. A single cell is also represented imaged to contrast the size in structure.

To examine these cell structures and verify that the cells can form representative cancer tumour models, confocal microscopy with cell staining was used. MCF-7 cells were prepared in a sample at a concentration of 500,000 cells/ml. After it was found that the cells were at their healthiest at 24 hours, a sample of droplets were taken at this time point. The cells were then incubated within the droplets and as with Figure 6.7 cells formed a 3D structure. To examine this structure it was necessary to dye the cell's nucleus blue and the cytosol red. The dyes used were Hoechst and Pholilidin. The confocal procedure was carried out as outlined in Chapter 2. The droplets were placed on a glass slide and allowed to attach to the slide. One difficulty that was experienced when forming these cultures was that there was typically one culture per droplet, with multiple washing steps it was difficult to verify if any of the cultures had adhered to the glass slide. Careful preparation of the cultures is required. Once the cells adhered to the glass slide and the dye applied, the cells are examined using the confocal microscope. These dyes are excited by a laser and are fluorescent. The resulting image from the 100x magnification confocal is shown in

Figure 6.7. This is a composite image of the two dyes showing the relation of the cytosol to the nucleus within the cell structure. The image (Figure 5.7) shows the spherical nature of the structure and the cells have formed a single culture with a blue nucleus and a red cytosol. As these are breast cancer cells this structure is a replication of the mass like structure shown previously by Kenny *et al* 2007. This mass like structure is described as colonies formed in a round structure and have disorganised nuclei and filled colony centres. As these structures formed within the droplets are showing the same trend in cell structure formed as in the ECM study this verifies that this structure is a 3D spheroid and is equivalent to a tumour model.

The ability to form 3D structures that are biologically relevant, with a minimum amount of cells and within 24 hours demonstrates a novel aspect of these droplets. For a typical 650nl droplet there are 325 cells. This gives the opportunity in small volume reactions to establish spheroids that can be tested as individual bioreactors with any substance making this a high-throughput generator of 3D cancer tumour models.

6.4 Treatment of Breast Cancer

As the droplets have been established as bioreactors in generating stable three dimensional cultures the next verification is to treat the cells within the droplets. The application of drugs to the cancer cells within the droplets was investigated. Gene expression was used to evaluate these droplets as reactors for the analysis of cancer cells. The investigation here replicates a study performed by O'Neill *et al* (2012) where conventional cell culture methods (10cm petri dishes) were used. O'Neill *et al* investigated the treatment of BT474 cells with Lapatinib. Lapatinib is an approved treatment for breast cancer and is an inhibitor of the epidermal growth factor

receptor (EGFR) pathway. As BT474 cells are EGFR positive this treatment directly impacts the signalling pathway within the cancer cells and prevents them from proliferating further. O'Neill *et al* 2012 investigated a range of genes to evaluate if there was a change in gene expression by the treatment. It was found that a range of genes were differentially expressed when treated. The aim in this section is to replicate the treatment of the cells in microfluidic droplet platform. Here initially BT474 cells were mixed with Laptinib using the drop off junction on the microfluidic system. Droplets of 600nl were generated with a cell concentration of 1,000,000 cells/ml. 1,000,000 cells/ml concentration was selected as the treatment is only required for 12 hours and this concentration of cells will remain healthy up to 24 hours within the droplets. Laptainib of final concentration of 1μM was mixed with each droplet. One 400nl cell droplet was mixed with 200nl drug droplet. The control droplets contained a mixture of the cells from the same source of as the treated droplets where 400nl of cells mixed with 200nl of media onl . 150 droplets of Laptinib treated cells and 150 droplets of cells mixed with media only were made all at the same time using the same cell sample. The droplets were incubated in a standard incubator for 12 hours of treatment. After treatment, the droplets were pooled together and the oil overlay was removed from the cells. The cells were analysed for gene expression. It was necessary to pool the droplets together as 80μl was required to have enough DNA for the post treatment analysis at a detectable level. This was a requirement of the kits used and the detection capabilities of the instrumentation used for the analysis.

6.4.1 RNA quantification

In most PCR processes it is necessary to quantify the amount of cDNA in a sample. To quantify the amount of cDNA used in the process it is usually necessary to

quantify the amount of RNA in a sample. Due to the low number of cells required within the droplets the detection of small amounts of RNA was required. The extraction of the RNA was performed using the Quiagen RNEasy Mini kit. This kit allows for up to 100µg of RNA to be prepared. To examine this, BT474 cells were cultured in a flask and counted using a haemocytometer. As using 80µl of droplets will give a maximum amount of 53,000 cells a representative test on the Nanodrop system three concentrations 10,000, 50,000 and 100,000cells/ml were made. This

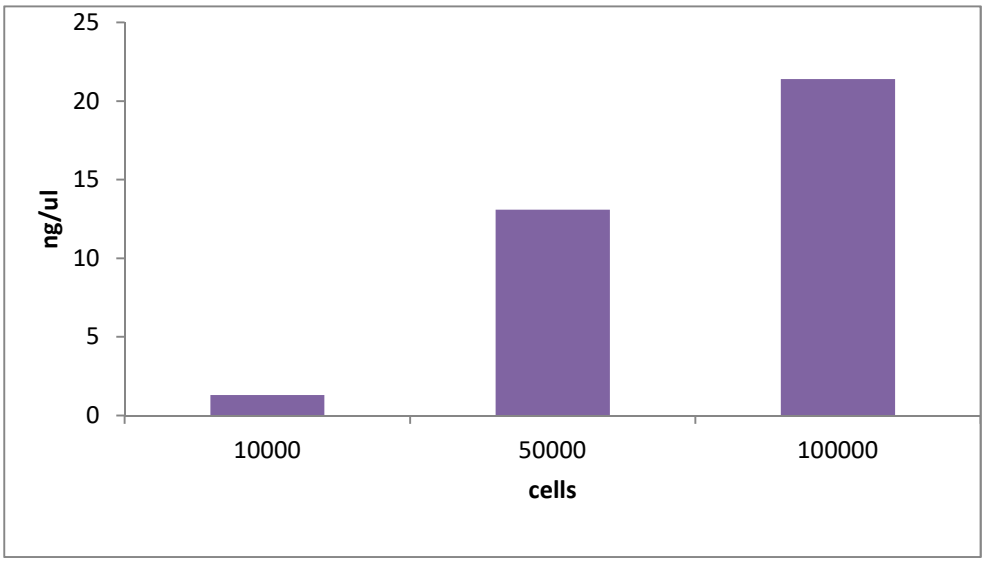


Figure 6.8 Nanodrop RNA concentrations of RNA

was done to test if the Nanodrop has the ability to detect low levels of RNA. The Quiagen protocol consists of many washing steps which is not desirable to maintain the levels of RNA from small sample volumes. The RNA was extracted and 1µl was placed on the Nanodrop for detection. The Nanodrop detects absorbance in 1µl of RNA at wavelengths of 230nm to 260nm. These results indicate that the Nanodrop is not sensitive enough to analyse the samples as no peak is present. More sensitive equipment is required to determine the integrity of the RNA. These peaks do not give definitive RNA quality for any of the concentrations however, figure 6.8shows a graph of the amount of RNA measured on this Nanodrop from the samples and there is an increase from 10,000 to 100,000 cells showing that increased cells have

increased RNA. As this method is not sensitive enough and as more sensitive equipment for RNA analysis was not available a second kit, cells-to cDNA ii kit was used.

6.4.2 Cells-to-cDNA

A second method for the synthesis of cDNA was required and the cells-to cDNA kit was selected. The cells-to-cDNAii kit does not use include an RNA step so this could not be quantified. Four genes were examined at three different cell concentrations in droplets. To determine if the amount of cells within a droplet sample will influence the critical threshold (ct) value found using this kit a qPCR experiment was undertaken. The ct value is the critical threshold where the fluorescence signal has reached above a selected level and in this case it is 0.2. The higher volume of cells should give a lower ct value if there is more DNA available to be amplified (Schmittgen and Livak, 2008). This means that if there is a variance in the amount of cDNA in a sample (i.e not the same number of cells) the ct value will be different. BT474 cells were cultured in droplets in increasing concentrations to make sure the same amounts of cells are in the sample. Table 6.1 shows the concentrations of cells used. 80µl of each sample was used and these droplets are mixed 2:1 cells to drug making final concentrations Table 6.1.

Table 6.1 Cell concentration in 80ul mixed droplets

Cell concentration (cell/ml)	Cells in 80ul
500,000	26,000
1,000,000	52,000
4,000,000	240,000

To identify the ct value, four genes were investigated using qPCR. The genes B2M, PPIA, PGK1 and GAPDH were used for all samples. These genes were selected as they are housekeeper genes and remain stable when treated. 150 droplets of 600nl of each concentration were made. After the droplets were generated they were dispensed into a tube where they were centrifuged at 1000 RPM for 5 minutes and the cells remained as a pellet at the bottom of the tube. These cells were then used for the cells-cDNAii kit. Once the cDNA was synthesised PCR was performed on a Life Technologies ABI7900. Figure 6.9 shows the ct value for each concentration over the 4 genes. As expected, the lower ct values were found using higher numbers of cells this shows that higher amounts of cDNA are made from higher cells concentrations. This shows a direct correlation to cell number and ct value. It also shows that there is increased cell number in droplets with higher cell concentrations. This will also be sufficient for the quantification of fold change within treated and untreated cells. Figure 6.9 validates that if there are different cell numbers between the treated and untreated cells that the ct value will not be the same. All genes show a higher ct for the lowest amount of cells. GAPDH shows very little change between 500,000 and 1,000,000 cells. As all genes were examined from the same cell sample more than one endogenous control should be used to identify an increase or decrease in gene expression as there is such little change in GAPDH. This method shows that for cell numbers as low as 26,000 cells from 80µl are detectable without the requirement of using the Nanodrop method.

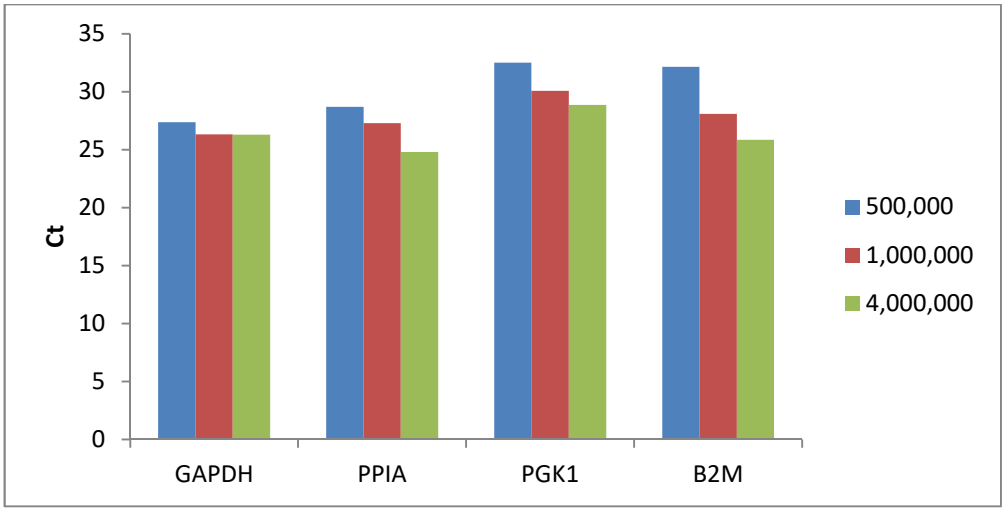


Figure 6.9 Gene expression ct of 500,000 to 1,000,000 cells over four genes . 6.4.3 Selection of housekeepers for gene expression

For any real time polymerase chain reaction (RT-PCR) endogenous controls or housekeeper genes are required to be used as a control within an experiment. Housekeeper genes are genes that will not change expression when treated compared to untreated cells. In the case of O'Neill *et al* (2012) it was found that a range of genes change over the course of the treatment and GAPDH was used as the housekeeper gene. As the Cells-cDNA kit was sufficient in detecting ct values for low concentrations of cells using qPCR this method was used for all subsequent tests. Although the aim is to replicate previous treatment of BT474 cells by O'Neill *et al* 2012 it is recommended that all q-PCR tests use stable housekeeper genes (Vandesompele *et al*., 2002). As the previous Figure 6.9 has shown it is important to have a gene that does not change expression when treated so that a stable control is found. In repeating the study by O'Neill *et al* only one housekeeper gene was used where it has been shown that the use of more than one endogenous control can give more accurate results (Liu *et al*., 2015). A range of genes that have previously been used as housekeeper genes were selected GAPDH (O'Neill *et al*., 2012), PPIA(Liu *et*

al., 2015) and PGK1(Obermayr *et al*., 2010) to test these genes 150 droplets of 600nl in volume were generated.

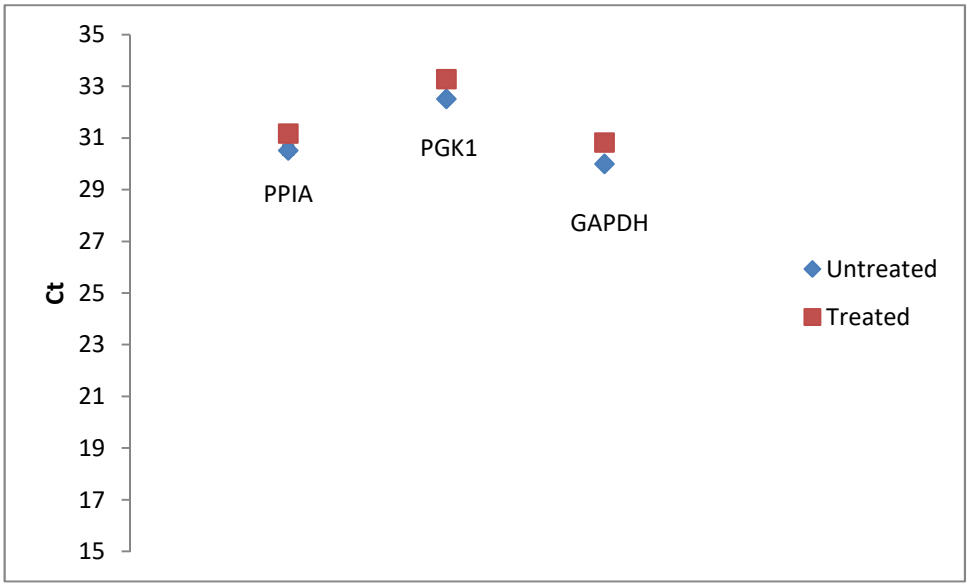


Figure 6.10 Gene expression of House keeper genes to demonstrate no variation in expression between untreated and untreated samples.

Table6. 5 Standard deviation of housekeeper genes

	GAPDH	**PPIA**	**PGK1**
Standard Deviation	0.304091	0.194623	0.34902

A concentration of 1,000,000 cells/ml of BT474 cells was mixed with Laptinib using the drop off junction. Once the reference genes were determined the target genes were examined for up regulation or down regulation as a result of Lapatinib treatment. Figure 6.10 shows that there is little variation between treated and untreated genes. It has identified that each of the genes can be categorised as housekeeper genes for this type of analysis. Figure 6.10 has also identified GAPDH as a housekeeper gene as found by O'Neill *et al* 2012. GAPDH is used as a single

reference gene in 40% of breast cancer studies (Liu *et al*., 2015) The standard deviation error bars are incorporated on the graph as they are so small the deviation is not apparent. Table 6.2 shows the values of the standard deviation for the genes shows little variation between treated and untreated. The ct values are less than 0.4 ct in standard deviation and this shows that the ct values are consistent for both treated and untreated samples. These genes are all deemed good controls and will be used for gene analysis.

Once suitable housekeeper control genes were found and the cells have been treated with Lapatinib further gene expression analysis was explored. A selection of genes were chosen from the O'Neill *et al* study, ERBB3, CCND1 AND FOXO3. ERBB3 is a member of the epidermal growth factor receptor group and hence is affected by Lapatinib treatment. CCND1 is from the cyclin family and this is associated with tumour suppressor proteins. FOXO3 is involved in tumour genesis. 1,000,000 cells/ml concentrations were used to evaluate the treatment of cells. 150 treated droplets and 150 untreated droplets were generated. 80µl of these droplets was used for post evaluation. Figure 6.11 shows the gene expression found using one house keeper gene as used by O'Neill *et al* 2012. Using the ΔΔCt method on REST software the fold change was found. REST is used to identify if the treated and untreated samples have a statistically significant gene expression. This calculates the variance of gene expression both within groups and between groups. (Jurcevic *et al*., 2013) This is a commercial tool that calculates a stability value for each gene. Figure 6.11 shows dysregulation in all three genes with ERBB3 and FOXO3 being upregulated and CCND1 being down regulated.

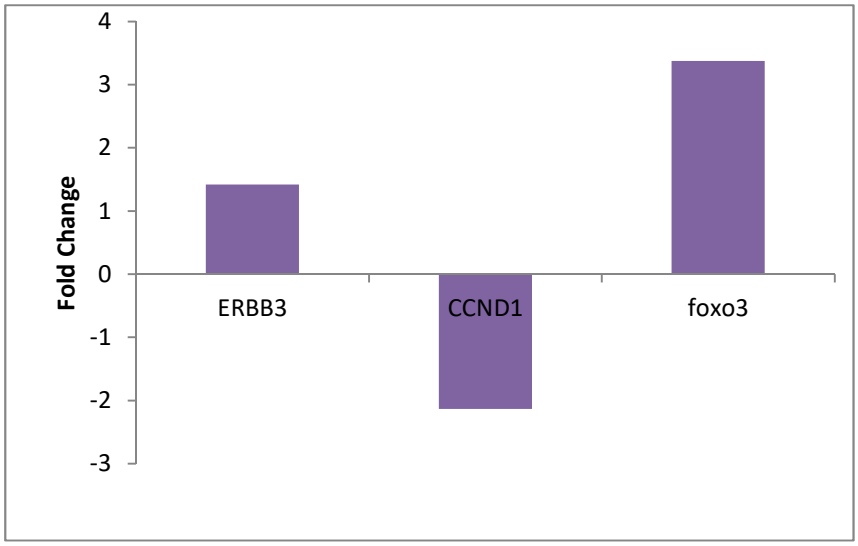


Figure 6.11 Gene expression lapatinib treated MCF-7 cells in droplets using one housekeeper gene.

A comparison of the results is found by using droplet culture which used traditional petri dish culture shown in Figure 6.12. It is clear that from a sample containing 52,000 cells that it shows the same expression signature as the conventional culture containing 8,000,000 cells. This trend shows that treating the cells within the droplets is sufficient in finding a gene expression equivalent to found by O'Neill *et* (2012).

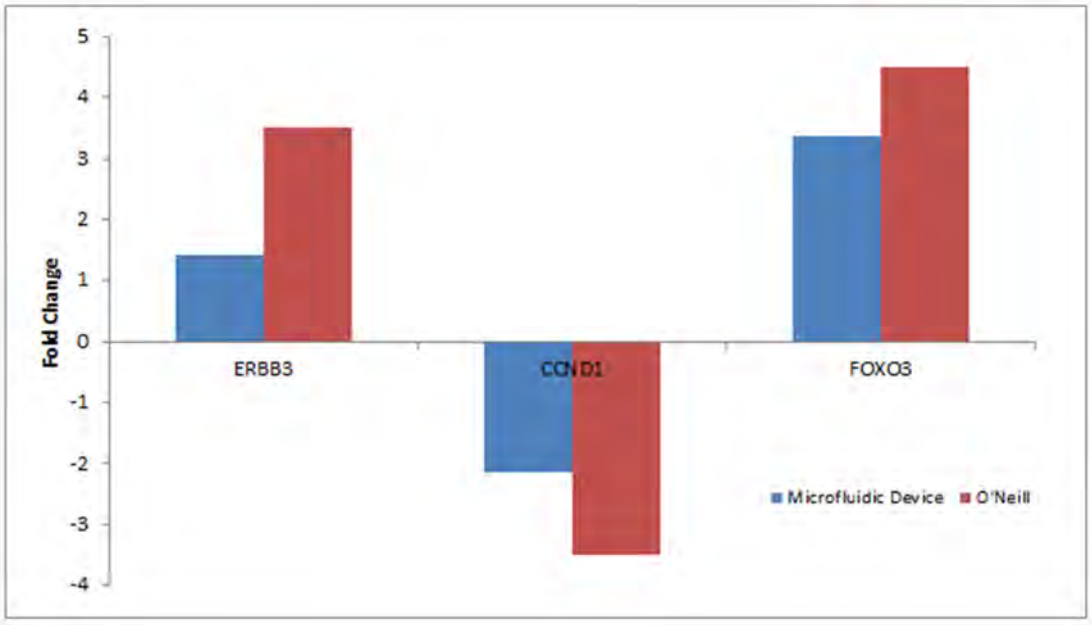


Figure 6.12 Comparing gene expression in O'Neill *et al* study to the microfluidic device method

As there is such small amount of cells required per droplet to do gene expression analysis, this gives the ability to do much more tests. One such test is looking at gene expression using all the housekeeper genes found from the analysis as a control for the genes of interest. Figure 6.13 shows that using all controls PGK1, PPIA and GAPDH, only FOXO3 is the only significant up regulated gene. This also reaffirms that it is necessary to have more than one control gene for analysis.

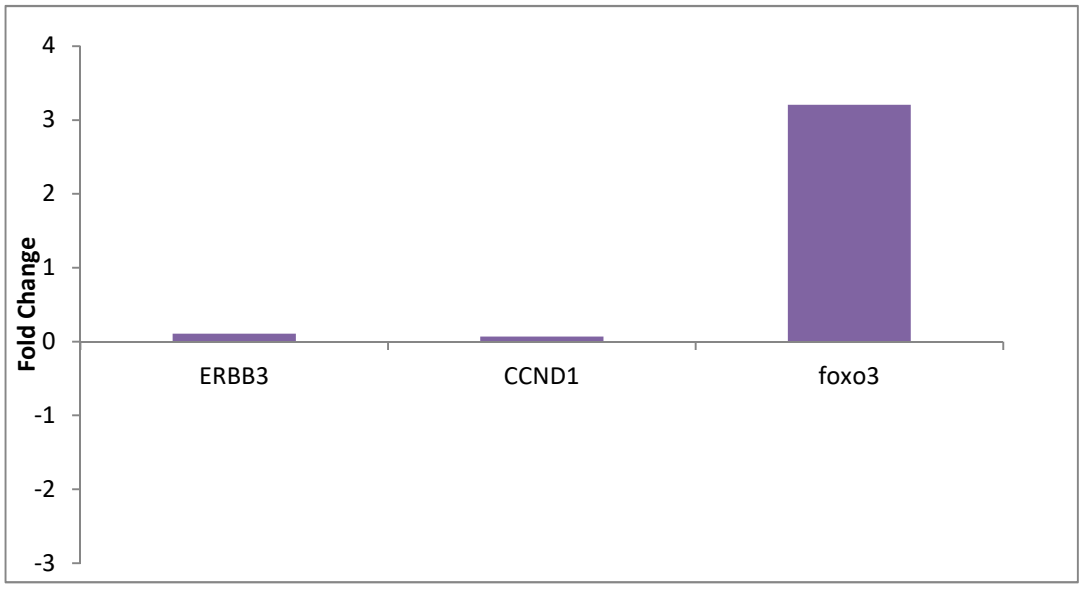


Figure 6.13 Gene expression of lapatinib treated mcf-7 cells using three housekeeper genes GAPDH PPIA and PGK1 showing low levels of expression for each of the genes.

From this section it is clear that the ct value is directly related to the amount of cells within a sample. It also shows the small amount of cells have the ability to identify, as well as current methods, the dysregulation of genes. This confirms that the droplets are capable bioreactors for the treatment of cancer cells to form a gene signature.

6.5 Chapter closure

This chapter has analysed mammalian cells within the microfluidic droplets. The capability of the droplets to act as miniaturised bioreactors is demonstrated here. The integrity of the droplets in shown as cells remain viable within the droplets for up to 48 hours. The optimum amount of cells within the droplets were found with 1,000,000cells/ml surviving up to 24 hours while 500,000cell/ml will remain viable up to 48 hours.

Three dimensional cultures are developed within the droplets. It gives the ability to create biologically relevant models within each droplet. 3D breast cancer models were generated in each droplet and structurally identified using confocal microscopy. This shows that individual 3D culture could be created with a low amount of cells and the structure verified. This proves that this system has the ability to create relevant cell models for preclinical analysis.

This droplet system has the ability in small volumes to do processes such as show that the ct value can be directly affected by the concentrations of the cells and used to identify housekeeping genes. It shows that the same gene signature can be found using 52,000 cells compared with 8,000,000 cells used in the comparison study. This could allow samples such as biopsy samples to be cultured and analysed as such few cells are necessary to give an indication of cell reaction. The ability within a 600nl droplet to treat, culture and generate a gene signature that agrees with literature is unique to these droplets. The capability of the droplets shows that a cell can be seeded and generated in a 3D culture and treated on a continuous microfluidic system giving the ability to do thousands of tests at any time on the system. This system sufficiently decreases the volume of each culture to create biologically

relevant models and due to this brings the capability for high-throughput experimentation.

Chapter 7

Conclusions and Recommendations

The main objective of this work is to contribute a new microfluidic method to address preclinical cell analysis. The aim is to provide a high-throughput, continuous system that will not affect the cells and allow them to proliferate as closely as they would in the body as possible. This chapter is in two sections the first draws conclusions form the body of work presented in the previous chapters and the second gives recommendations for future work.

7.1 Conclusions

- From reviewing the literature it was concluded that a system for preclinical bacterial and mammalian cell culture in one instrument that is high throughput is not currently available

- A microfluidic device for the generation of individual droplet bioreactors was developed

- Real time monitoring of the cultures was developed through visual microscopic imaging of the cultures and optical density absorbance measurements
- Mixing of individual droplets was facilitated using the drop-off junction. This allows any combination of drugs with any culture droplet
- Droplets can be retrieved from the system for further analysis if necessary

- The optimum conditions for droplet analysis found that visual methods of Trypan blue live/dead assay was not sufficient in counting cultures once they have formed accumulations of cells
- The most effective way of forming consistent cell numbers within the droplets is by using a pipette to distribute the cells after every 5 droplets generated
- The internal circulation velocity will determine where the cells will reside within a droplet. The internal circulations within the droplets shows that the bacterial cells act like particles and move to the cap end of the droplet while the mammalian cells are more evenly distributed around the droplets.
- The bacterial cells grow at a faster rate than the traditional method of an orbital shaker flask culture.
- Bacterial growth rate can be detected in real time by the photodoiode system incorporated into the instrument.
- The optimum conditions for bacterial cell growth within the droplets was found at a droplet volume of 1500nl and continuous circulation at 30µl/min

- The microfluidic droplet instrument is sufficient in identifying if an antibiotic for treatment has the ability to effectively stop the growth of the bacteria. The mixing system is sufficient in adding a treatment to the droplets individually and at any point during testing
- Antibiotic resistance can be detected using the microfluidic droplet instrument also showing that many experiments can be done at the same time on the system in series. This exhibits the instruments capability of being a multiplexed instrument with high throughput capability without impacting other culture
- The mammalian cell concentration can be verified using a metabolic activity assay showing mammalian cells can survive in droplets up to 48 hours where up to 24 hours is the optimum
- Biologically relevant 3D models are developed within the droplets and these are verified by confocal microscopy showing the same structure as 3D cultures formed using current methods. This shows that spheroid tumour models are formed within the droplets
- The instrument shows the ability to treat breast cancer models within 12 hours. These cells show the same trend in gene expression comparable to study by O'Neill *et al* 2012.
- This study shows that when more than one house keeper gene is applied as a control that the fold change in the genes of interest are not as significant as when using only one control house keeper gene
- This instrument has the ability to create cell models and detect a reaction to treatment making it a viable preclinical system of evaluating new drugs.

7.2 Recommendations for future work

- Design an optical system incorporating a multi wavelength LED or have the ability to measure florescence within the droplets to have the ability to real time monitor the amount of cells in each droplet
- Design a stage that can move in the x and y axis that will allow dipping head to be move to any position within a well plate precisely allowing any mixture of cells and drugs to be achieved
- Make co-cultures of more than one cell type in 3D structures within the droplets to investigate if these provide better examples of cell to cell interactions within the body
- Validate the bacterial concentration in terms of a multi-drug approach. As current treatment sometimes requires multiple antibiotics to target a particular bacterial strain. Multiple drugs could be mixed on the system with the implementation of either three way mixing or optimising the drop-off junction
- Identification of bacterial strains can be achieved using genetic analysis. A PCR evaluation of the droplets could be implemented after incubation and detect at a gene level the specific strain of bacteria.
- Add rare bacteria types to the droplets and find out if these will survive within the droplet environment
- Add more optical system photo diodes to have a continuous system of generating and monitoring cultures reducing need to move the droplets forward and back over the photo diode.

- Design a system that could separate droplets where, once a droplet of interest is identified this could then be progressed for further testing while other droplets could be discarded.
- As it is desirable to have more sensitive detectable devices for RNA to ensure the same concentration is used for all gene expression tests. Design a more sensitive detection method for this.
- Add different cancer types to the droplets to analyse if the system is as consistent for other types of cancer

References

ALESSANDRI, K., SARANGI, B. R., GURCHENKOV, V. V., SINHA, B., KIEßLING, T. R., FETLER, L., RICO, F., SCHEURING, S., LAMAZE, C., SIMON, A., GERALDO, S., VIGNJEVIĆ, D., DOMÉJEAN, H., ROLLAND, L., FUNFAK, A., BIBETTE, J., BREMOND, N. & NASSOY, P. 2013. Cellular capsules as a tool for multicellular spheroid production and for investigating the mechanics of tumor progression in vitro. *Proceedings of the National Academy of Sciences,* 110**,** 14843-14848.

AMIN, J., RAMACHANDRAN, K., WILLIAMS, S. J., LEE, A., NOVIKOVA, L. & STEHNO-BITTEL, L. 2016. A simple, reliable method for high-throughput screening for diabetes drugs using 3D β-cell spheroids. *Journal of Pharmacological and Toxicological Methods,* 82**,** 83-89.

ANDERSSON, D. I. & HUGHES, D. 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Micro,* 8**,** 260-271.

ANTONI, D., BURCKEL, H., JOSSET, E. & NOEL, G. 2015. Three-Dimensional Cell Culture: A Breakthrough in Vivo. *International Journal of Molecular Sciences,* 16**,** 5517-5527.

BARBULOVIC-NAD, I., AU, S. H. & WHEELER, A. R. 2010. A microfluidic platform for complete mammalian cell culture. *Lab on a Chip,* 10**,** 1536-1542.

BAROUD, C. N., GALLAIRE, F. & DANGLA, R. 2010. Dynamics of microfluidic droplets. *Lab on a Chip,* 10**,** 2032-2045.

BERG JM, T. J., STRYER L. 2002. RNA Synthesis and Splicing. *Biochemistry.*

BHATIA, S. N. & INGBER, D. E. 2014. Microfluidic organs-on-chips. *Nat Biotech,* 32**,** 760-772.

BLAIR, J. M. A., WEBBER, M. A., BAYLAY, A. J., OGBOLU, D. O. & PIDDOCK, L. J. V. 2015. Molecular mechanisms of antibiotic resistance. *Nat Rev Micro,* 13**,** 42-51.

BOEDICKER, J. Q., LI, L., KLINE, T. R. & ISMAGILOV, R. F. 2008. Detecting bacteria and determining their susceptibility to antibiotics by stochastic confinement in nanoliter droplets using plug-based microfluidics. *Lab on a Chip,* 8**,** 1265-1272.

BRESLIN, S. & O'DRISCOLL, L. 2013. Three-dimensional cell culture: the missing link in drug discovery. *Drug Discovery Today,* 18**,** 240-249.

CHEN, M., ZAMORA, P. O., SOM, P., PENA, L. A. & OSAKI, S. 2003. Cell attachment and biocompatibility of polytetrafluoroethylene (PTFE) treated with glow-discharge plasma of mixed ammonia and oxygen. *J Biomater Sci Polym Ed,* 14**,** 917-35.

CHURSKI, K., KAMINSKI, T. S., JAKIELA, S., KAMYSZ, W., BARANSKA-RYBAK, W., WEIBEL, D. B. & GARSTECKI, P. 2012. Rapid screening of antibiotic toxicity in an automated microdroplet system. *Lab on a Chip,* 12**,** 1629-1637.

CLANCY, S. B., W. . 2008. *Translation: DNA to mRNA to Protein* [Online]. Nature Eduction. [Accessed 11/03/17 2017].

CUKIERMAN, E., PANKOV, R., STEVENS, D. R. & YAMADA, K. M. 2001. Taking Cell-Matrix Adhesions to the Third Dimension. *Science,* 294**,** 1708-1712.

DE GROOT, T. E., VESERAT, K. S., BERTHIER, E., BEEBE, D. J. & THEBERGE, A. B. 2016. Surface-tension driven open microfluidic platform for hanging droplet culture. *Lab on a Chip,* 16**,** 334-344.

DESBORDES, S. C. & STUDER, L. 2013. Adapting human pluripotent stem cells to high-throughput and high-content screening. *Nat. Protocols,* 8**,** 111-130.

DU, G., FANG, Q. & DEN TOONDER, J. M. J. 2016. Microfluidics for cell-based high throughput screening platforms—A review. *Analytica Chimica Acta,* 903**,** 36-50.

ERAMO, A., LOTTI, F., SETTE, G., PILOZZI, E., BIFFONI, M., DI VIRGILIO, A., CONTICELLO, C., RUCO, L., PESCHLE, C. & DE MARIA, R. 2007. Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ,* 15**,** 504-514.

FENNEMA, E., RIVRON, N., ROUWKEMA, J., VAN BLITTERSWIJK, C. & DE BOER, J. 2013. Spheroid culture as a tool for creating 3D complex tissues. *Trends in Biotechnology,* 31**,** 108-115.

FIORINI, G. S. & CHIU, D. T. 2005. Disposable microfluidic devices: fabrication, function, and application. *Biotechniques,* 38**,** 429-46.

FISCHBACH, C., CHEN, R., MATSUMOTO, T., SCHMELZLE, T., BRUGGE, J. S., POLVERINI, P. J. & MOONEY, D. J. 2007. Engineering tumors with 3D scaffolds. *Nat Meth,* 4**,** 855-860.

GONG, X., LIN, C., CHENG, J., SU, J., ZHAO, H., LIU, T., WEN, X. & ZHAO, P. 2015. Generation of Multicellular Tumor Spheroids with Microwell-Based Agarose Scaffolds for Drug Testing. *PLoS ONE,* 10**,** e0130348.

GU, H., DUITS, M. H. G. & MUGELE, F. 2011. Droplets Formation and Merging in Two-Phase Flow Microfluidics. *International Journal of Molecular Sciences,* 12**,** 2572-2597.

GUO, M. T., ROTEM, A., HEYMAN, J. A. & WEITZ, D. A. 2012. Droplet microfluidics for high-throughput biological assays. *Lab on a Chip,* 12**,** 2146-2155.

GUO YOU, H., LI HONG, Z., QIAN CHENG, Z., YONG MEI, C., WEI, S., FENG, X. & TIAN JIAN, L. 2011. Microfluidic hydrogels for tissue engineering. *Biofabrication,* 3**,** 012001.

GUPTA, P. B., ONDER, T. T., JIANG, G., TAO, K., KUPERWASSER, C., WEINBERG, R. A. & LANDER, E. S. 2009. Identification of Selective Inhibitors of Cancer Stem Cells by High-Throughput Screening. *Cell,* 138**,** 645-659.

HAYES, C. J. & DALTON, T. M. 2015. Microfluidic droplet-based PCR instrumentation for high-throughput gene expression profiling and biomarker discovery. *Biomolecular Detection and Quantification,* 4**,** 22-32.

HEATH, C. & KISS, R. 2007. Cell Culture Process Development: Advances in Process Engineering. *Biotechnology Progress,* 23**,** 46-51.

HOU, H. W., LI, Q. S., LEE, G. Y. H., KUMAR, A. P., ONG, C. N. & LIM, C. T. 2009. Deformability study of breast cancer cells using microfluidics. *Biomedical Microdevices,* 11**,** 557-564.

HUGHES, J. P., REES, S., KALINDJIAN, S. B. & PHILPOTT, K. L. 2011. Principles of early drug discovery. *British Journal of Pharmacology,* 162**,** 1239-1249.

HUTCHINSON, L. & KIRK, R. 2011. High drug attrition rates[mdash]where are we going wrong? *Nat Rev Clin Oncol,* 8**,** 189-190.

ILOKI ASSANGA, S. B., GIL-SALIDO, A. A., LEWIS LUJ&AN, L. M., ROSAS-DURAZO, A., ACOSTA-SILVA, A. L., RIVERA-CASTAÑEDA, E. G. AND RUBIO-PINO, J. L. 2013. Cell

growth curves for different cell lines and their relationship with biological activities. *Journal of Biotechnology and Molecular Biology Research* 4**,** 60-70.

KAMINSKI, T. S., SCHELER, O. & GARSTECKI, P. 2016. Droplet microfluidics for microbiology: techniques, applications and challenges. *Lab on a Chip*.

KANG, L., CHUNG, B. G., LANGER, R. & KHADEMHOSSEINI, A. 2008. Microfluidics for drug discovery and development: From target selection to product lifecycle management. *Drug Discovery Today,* 13**,** 1-13.

KASHID, M. N., GERLACH, I., GOETZ, S., FRANZKE, J., ACKER, J. F., PLATTE, F., AGAR, D. W. & TUREK, S. 2005. Internal Circulation within the Liquid Slugs of a Liquid−Liquid Slug-Flow Capillary Microreactor. *Industrial & Engineering Chemistry Research,* 44**,** 5003-5010.

KENNY, P. A., LEE, G. Y., MYERS, C. A., NEVE, R. M., SEMEIKS, J. R., SPELLMAN, P. T., LORENZ, K., LEE, E. H., BARCELLOS-HOFF, M. H., PETERSEN, O. W., GRAY, J. W. & BISSELL, M. J. 2007. The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Molecular Oncology,* 1**,** 84-96.

KIEFER, J., EBEL, N., SCHLUCKER, E. & LEIPERTZ, A. 2010. Characterization of Escherichia coli suspensions using UV/Vis/NIR absorption spectroscopy. *Analytical Methods,* 2**,** 123-128.

KURUP, G. K. & BASU, A. S. 2010. Hydrodynamic particle concentration inside a microfluidic plug. *14th International Conferene on Minaturized Systems for Chemsity and Life Sciences* 2010.

KURUP, G. K. & BASU, A. S. 2012. Field-free particle focusing in microfluidic plugs. *Biomicrofluidics,* 6**,** 22008-2200810.

LANGE, H., TAILLANDIER, P. & RIBA, J.-P. 2001. Effect of high shear stress on microbial viability. *Journal of Chemical Technology & Biotechnology,* 76**,** 501-505.

LIM, C. T., ZHOU, E. H. & QUEK, S. T. 2006. Mechanical models for living cells—a review. *Journal of Biomechanics,* 39**,** 195-216.

LIPSKY, M. S. & SHARP, L. K. 2001. From idea to market: the drug approval process. *The Journal of the American Board of Family Practice,* 14**,** 362-7.

MAC GIOLLA EAIN, M., EGAN, V. & PUNCH, J. 2013. Film thickness measurements in liquid–liquid slug flow regimes. *International Journal of Heat and Fluid Flow,* 44**,** 515-523.

MACKENZIE, C. G., MACKENZIE, J. B. & BECK, P. 1961. The effect of pH on growth, protein synthesis, and lipid-rich particles of cultured mammalian cells. *J Biophys Biochem Cytol,* 9**,** 141-56.

MALCHIODI-ALBEDI, F., MORGILLO, A., FORMISANO, G., PARADISI, S., PERILLI, R., SCALZO, G. C., SCORCIA, G. & CAIAZZA, S. 2002. Biocompatibility assessment of silicone oil and perfluorocarbon liquids used in retinal reattachment surgery in rat retinal cultures. *J Biomed Mater Res,* 60**,** 548-55.

MARTIN, K., HENKEL, T., BAIER, V., GRODRIAN, A., SCHON, T., ROTH, M., MICHAEL KOHLER, J. & METZE, J. 2003. Generation of larger numbers of separated microbial populations by cultivation in segmented-flow microdevices. *Lab on a Chip,* 3**,** 202-207.

MATHIS, D., VENCE, L. & BENOIST, C. 2001. [beta]-Cell death during progression to diabetes. *Nature,* 414**,** 792-798.

MCCARTHY, C. 2015. *On the Utilisation of Taylor flows for Automation Cell to Signal.* Ph.D PhD Thesis, University of Limerick.

MOHAN, R., MUKHERJEE, A., SEVGEN, S. E., SANPITAKSEREE, C., LEE, J., SCHROEDER, C. M. & KENIS, P. J. A. 2013. A multiplexed microfluidic platform for rapid antibiotic susceptibility testing. *Biosensors and Bioelectronics,* 49**,** 118-125.

NELSON, C. M. & CHEN, C. S. 2002. Cell-cell signaling by direct contact increases cell proliferation via a PI3K-dependent signal. *FEBS Letters,* 514**,** 238-242.

NEVE, R. M., CHIN, K., FRIDLYAND, J., YEH, J., BAEHNER, F. L., FEVR, T., CLARK, L., BAYANI, N., COPPE, J.-P., TONG, F., SPEED, T., SPELLMAN, P. T., DEVRIES, S., LAPUK, A., WANG, N. J., KUO, W.-L., STILWELL, J. L., PINKEL, D., ALBERTSON, D. G., WALDMAN, F. M., MCCORMICK, F., DICKSON, R. B., JOHNSON, M. D., LIPPMAN, M., ETHIER, S., GAZDAR, A. & GRAY, J. W. 2006. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell,* 10**,** 515-527.

NOMURA, K., KOSHIZUKA, S., OKA, Y. & OBATA, H. 2001. Numerical Analysis of Droplet Breakup Behavior using Particle Method. *Journal of Nuclear Science and Technology,* 38**,** 1057-1064.

O'NEILL, F., MADDEN, S. F., AHERNE, S. T., CLYNES, M., CROWN, J., DOOLAN, P. & O'CONNOR, R. 2012. Gene expression changes as markers of early lapatinib response in a panel of breast cancer cell lines. *Molecular Cancer,* 11**,** 1-14.

OLEKSOWICZ, L., MROWIEC, Z., SCHWARTZ, E., KHORSHIDI, M., DUTCHER, J. P. & PUSZKIN, E. 1995. Characterization of tumor-induced platelet aggregation: The role of immunorelated GPIb and GPIIbIIIa expression by MCF-7 breast cancer cells. *Thrombosis Research,* 79**,** 261-274.

ONG, S.-M., ZHANG, C., TOH, Y.-C., KIM, S. H., FOO, H. L., TAN, C. H., VAN NOORT, D., PARK, S. & YU, H. 2008. A gel-free 3D microfluidic cell culture system. *Biomaterials,* 29**,** 3237-3244.

PAUL, S. M., MYTELKA, D. S., DUNWIDDIE, C. T., PERSINGER, C. C., MUNOS, B. H., LINDBORG, S. R. & SCHACHT, A. L. 2010. How to improve R&D productivity: the pharmaceutical industry's grand challenge. *Nat Rev Drug Discov,* 9**,** 203-214.

PFAFFL, M. W., HORGAN, G. W. & DEMPFLE, L. 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research,* 30**,** e36-e36.

PICKL, M. & RIES, C. H. 2008. Comparison of 3D and 2D tumor models reveals enhanced HER2 activation in 3D associated with an increased response to trastuzumab. *Oncogene,* 28**,** 461-468.

RALF, S., MARTIN, B., THOMAS, P. & STEPHAN, H. 2012. Droplet based microfluidics. *Reports on Progress in Physics,* 75**,** 016601.

RELLER, L. B., WEINSTEIN, M., JORGENSEN, J. H. & FERRARO, M. J. 2009. Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. *Clinical Infectious Diseases,* 49**,** 1749-1755.

SAKAI, S., ITO, S., INAGAKI, H., HIROSE, K., MATSUYAMA, T., TAYA, M. & KAWAKAMI, K. 2011. Cell-enclosing gelatin-based microcapsule production for tissue engineering using a microfluidic flow-focusing system. *Biomicrofluidics,* 5**,** 013402.

SARKAR, M., ALAM, N., CHAUDHURI, B., CHATTOPADHYAY, B. & MANDAL, S. 2015. Development of an improved E. coli bacterial strain for green and sustainable concrete technology. *RSC Advances,* 5**,** 32175-32182.

SEZONOV, G., JOSELEAU-PETIT, D. & D'ARI, R. 2007. Escherichia coli Physiology in Luria-Bertani Broth. *Journal of Bacteriology,* 189**,** 8746-8749.

SHAH, P., VEDARETHINAM, I., KWASNY, D., ANDRESEN, L., DIMAKI, M., SKOV, S. & SVENDSEN, W. E. 2011. Microfluidic bioreactors for culture of non-adherent cells. *Sensors and Actuators B: Chemical,* 156**,** 1002-1008.

SQUIRES, T. M. & QUAKE, S. R. 2005. Microfluidics: Fluid physics at the nanoliter scale. *Reviews of Modern Physics,* 77**,** 977-1026.

STEINMETZ, K. L. & SPACK, E. G. 2009. The basics of preclinical drug development for neurodegenerative disease indications. *BMC Neurology,* 9**,** S2-S2.

STEWART, B. W., CP. 2014. World Cancer Report 2014. Lyon France: International Agency for Research on Cancer

STROBER, W. 2001. Trypan blue exclusion test of cell viability. *Curr Protoc Immunol,* Appendix 3**,** Appendix 3B.

TEH, S.-Y., LIN, R., HUNG, L.-H. & LEE, A. P. 2008. Droplet microfluidics. *Lab on a Chip,* 8**,** 198-220.

TICE, J. D., SONG, H., LYON, A. D. & ISMAGILOV, R. F. 2003. Formation of Droplets and Mixing in Multiphase Microfluidics at Low Values of the Reynolds and the Capillary Numbers. *Langmuir,* 19**,** 9127-9133.

TUNG, Y.-C., HSIAO, A. Y., ALLEN, S. G., TORISAWA, Y.-S., HO, M. & TAKAYAMA, S. 2011. High-throughput 3D spheroid culture and drug testing using a 384 hanging drop array. *Analyst,* 136**,** 473-478.

WANG, J., WANG, J., FENG, L. & LIN, T. 2015. Fluid mixing in droplet-based microfluidics with a serpentine microchannel. *RSC Advances,* 5**,** 104138-104144.

WANG, P., ROBERT, L., PELLETIER, J., DANG, W. L., TADDEI, F., WRIGHT, A. & JUN, S. 2010. Robust Growth of Escherichia coli. *Current Biology,* 20**,** 1099-1103.

WHITESIDES, G. M. 2006. The origins and the future of microfluidics. *Nature,* 442**,** 368-373.

WOODFORD, N. & ELLINGTON, M. J. 2007. The emergence of antibiotic resistance by mutation. *Clinical Microbiology and Infection,* 13**,** 5-18.

YOON, S., KIM, J. A., LEE, S. H., KIM, M. & PARK, T. H. 2013. Droplet-based microfluidic system to form and separate multicellular spheroids using magnetic nanoparticles. *Lab on a Chip,* 13**,** 1522-1528.

YU, I. F., YU, Y. H., CHEN, L. Y., FAN, S. K., CHOU, H. Y. E. & YANG, J. T. 2014. A portable microfluidic device for the rapid diagnosis of cancer metastatic potential which is programmable for temperature and CO2. *Lab on a Chip,* 14**,** 3621-3628.

ZHANG, J., LI, J. & HAN, Y. 2004. Superhydrophobic PTFE Surfaces by Extension. *Macromolecular Rapid Communications,* 25**,** 1105-1108.

ZHONG, J.-J., FUJIYAMA, K., SEKI, T. & YOSHIDA, T. 1994. A quantitative analysis of shear effects on cell suspension and cell culture of perilla frutescens in bioreactors. *Biotechnology and Bioengineering,* 44**,** 649-654.

ZIÓŁKOWSKA, K., STELMACHOWSKA, A., KWAPISZEWSKI, R., CHUDY, M., DYBKO, A. & BRZÓZKA, Z. 2013. Long-term three-dimensional cell culture and anticancer drug activity evaluation in a microfluidic chip. *Biosensors and Bioelectronics,* 40**,** 68-74.

Publications:

KEAYS, M. C., O'BRIEN, M., HUSSAIN, A., KIELY, P. A. & DALTON, T. 2016b. Rapid identification of antibiotic resistance using droplet microfluidics. *Bioengineered,* 7**,** 79-87.