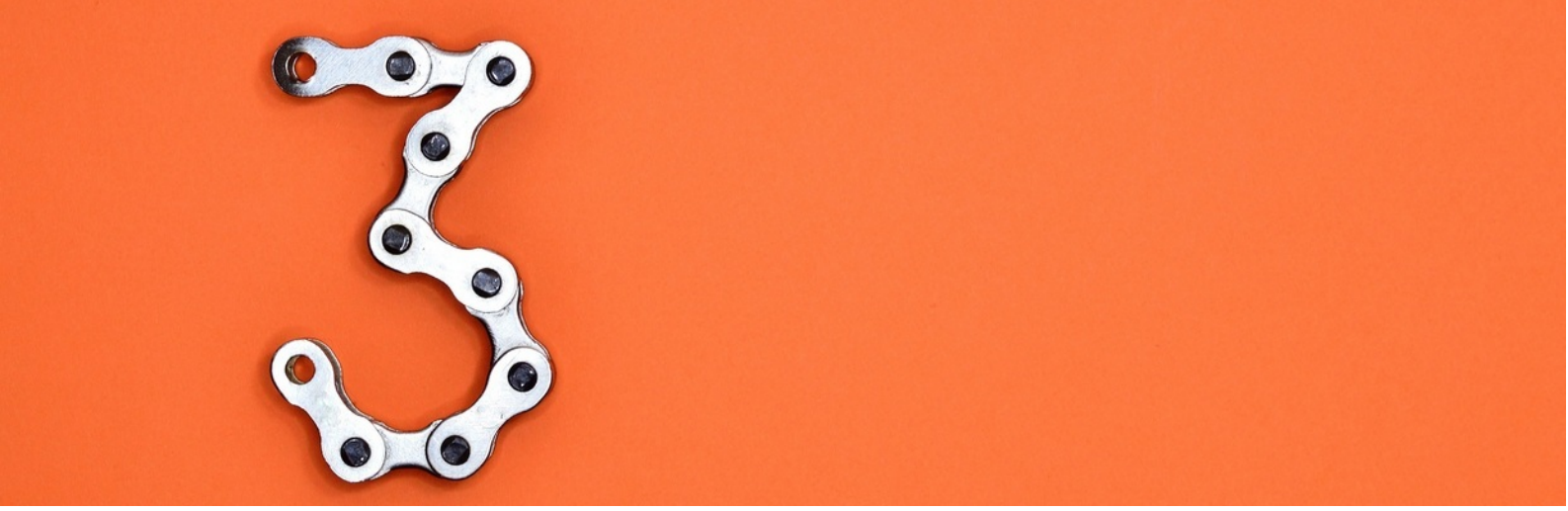

Three - Dimensional Culture For Continuous Long Term Live Cell Imaging

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Abstract:

Conventional cell culture systems are primarily two dimensional (2D). They are low cost and robust, and ensure homogeneous cell proliferation due to equal access to nutrients. However, they do not accurately represent in vivo conditions, with only horizontal spreading, in case of adherent cells. Three dimensional (3D) culture systems are now becoming increasingly popular since the conditions in these systems are similar to those in vivo, due to the presence of a matrix. 3D systems also make it easier to image and track individual cells over long periods of time, especially suspension cells. Continuous long term live cell imaging using a 3D culture system can aid in the detection of minute morphological changes in cells which can serve as indicators of complex cellular processes. In our study, we assessed the suitability of a collagen-based 3D culture system for continuous long term live cell imaging with respect to the collagen concentrations, cell seeding numbers for adherent and suspension cells and the longest duration for imaging. A primary test for diffusion of dye through the gel was also conducted. We found collagen concentrations of 1.5 and 2.0 mg/ml seeded with cell numbers from 1× 105 and 3 × 105 cells/ml

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UNIVERSITÄTS KLINIKUM FREIBURG

HOCHSCHULE FURTWANGEN UNIVERSITY HFU

Fakultät Medical and Life Sciences

Master-Thesis

Three - Dimensional Culture For Continuous Long Term Live Cell Imaging

Avani Sapre

Medical Diagnostic Technologies (MDT)

SoSe 2020

Supervisors

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Thema :

Three - Dimensional Culture For Continuous Long Term Live Cell Imaging

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Semester: SoSe 2020

Kurzfassung:

Konventionelle Zellkultursysteme sind in primär zweidimensional (2D). Sie sind kostengünstig und robust und sorgen durch den gleichberechtigten Zugang zu Nährstoffen für eine homogene Zellproliferation. Allerdings stellen sie bei adhärenten Zellen die in vivo-Bedingungen mit nur horizontaler Ausbreitung nicht genau dar. Dreidimensionale (3D) Kultursysteme werden jetzt immer populärer, da die Bedingungen in diesen Systemen aufgrund des Vorhandenseins einer Matrix denen in vivo ähnlich sind. 3D-Systeme erleichtern auch die Abbildung und Verfolgung einzelner Zellen über lange Zeiträume, insbesondere von Suspensionszellen. Die kontinuierliche Langzeit-Bildgebung lebender Zellen mit einem 3D-Kultursystem kann dazu beitragen, kleinste morphologische Veränderungen in Zellen zu erkennen, die als Anzeichen für komplexe zelluläre Prozesse dienen können. In unserer Studie untersuchten wir die Eignung eines kollagenbasierten 3D-Kultursystems für die kontinuierliche Langzeit-Lebendzell-Bildgebung im Hinblick auf die Kollagenkonzentrationen, die Zellaussaatzahlen für adhärente und Suspensionszellen und die längste Dauer für die Bildgebung. Ein Primärtest zur Diffusion des Farbstoffs durch das Gel wurde ebenfalls durchgeführt. Wir fanden Kollagenkonzentrationen von 1,5 und 2,0 mg/ml, die mit Zellzahlen von 1×10^5 und 3×10^5 Zellen/ml (je nach Zelltyp) besiedelt wurden, als optimal für die Bildgebung bis zu 120 Stunden. Der primäre Farbstoffdiffusionstest zeigte eine erfolgreiche Färbung der Zellen durch die Matrix hindurch. Damit demonstrieren wir die Anwendbarkeit der 3D-Kultur für die kontinuierliche Langzeit-Lebendzellabbildung von adhärenten und Suspensionszellen.

Schlüsselwörter: Three - Dimensional Culture, Collagen I, Live Cell Imaging

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Keywords: Three - Dimensional Culture, Collagen I, Live Cell Imaging

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Fakultät Medical and Life Sciences

Statutory Declaration

I hereby declare that I have produced the present work independently and without unauthorized outside assistance.

All sources used (literature, internet) are cited completely in the bibliography.

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October, 2020 **Avani Sapre**

TABLE OF CONTENTS

NO.	TITLE	PAGE NO.
	List of Abbreviations	i
	List of Figures and Tables	ii
1	Introduction	1-10
1.1	Cancer as a disease	1
1.2	Challenges in chemotherapy	2
1.3	Personalised chemotherapy	2
1.4.	Cell culture systems	3-
1.4.1	2D culture systems	3
1.4.2	3D culture systems	4
1.5	Long term live cell imaging	8
2.	Objectives and Hypothesis	10
3.	Materials and Methods	11-14
3.1	Cell lines	11
3.1.1	Adherent cell lines (JIMT-1 and MCF-7)	
3.1.2	Suspension Cell Lines (Jurkat and K-562)	
3.2	ibidi rat tail collagen type I	12
3.3	Long term live cell imaging	13
3.4	Primary testing of diffusion in Collagen type I gel	13
4.	Results	15-22
4.1	Rat Tail Collagen Type I Gel Fabrication	15
4.2	Long Term Live Cell Imaging	15
4.3	Primary testing of diffusion in collagen type I gel	21
5.	Discussion	23-25
5.1	Rat Tail Collagen Type I Gel Fabrication	23
5.2	Long Term Live Cell Imaging	24
5.3	Diffusion testing in collagen type I gel	24

6.	Conclusion	26
7.	Bibliography	26-31

LIST OF ABBREVIATIONS

ABBREVIATIONS	FULL FORM
µm	Micrometer
mm	Millimeter
nm	Nanometer
ml	Milliliter
µl	Microliter
^{o}C	Degree Celcius
ECM	Extracellular Matrix
2D	Two-dimensional
3D	Three-dimensional
PI	Propidium iodide

LIST OF FIGURES AND TABLES

NUMBER	TITLE	PAGE NUMBER
FIGURES		
1	Two Dimensional Versus Three Dimensional Cell Culture	3
2	3D Culture Systems (a)Hanging Drop Culture in PDMS-Based Hanging Drop Array (Kuo et al., 2017); (b) Hydrogels and Fiber Scaffolds (Kular, Basu and Sharma, 2014); (c) Paper (Li et al., 2012)	5
3	Augmented Microscopy: Integration of Microscopy with Machine Learning	9
4	Rat Tail Collagen Type I Gel Fabrication Protocol	12
5	Graph of Average Pore Size (µm) vs Collagen I Concentration (mg/ml)	13
6	MCF-7 Cells In Collagen I Matrix (a) 0h, (b) 24h, (c) 48h, (d) 72h	16
7	Jurkat Cells In Collagen I Matrix (a) 0h, (b) 24h, (c) 48h, (d) 72h	16
8	JIMT-1 Cells In Collagen I Matrix (a) 0h, (b) 24h, (c) 48h, (d)72h, (e) 96h, (f) 120h	17
9	Live/Dead Cell Count of Propidium Iodide (PI) Stained Cells Using IncuCyte® S3 Cell-By-Cell Analysis Software Module (a) and (b) JIMT-1; (c) Graph of Live Cell Count (Per Image) versus Time (Hours)	18
10	K562 Cells In Collagen I Matrix (a) 0h, (b) 24h, (c) 48h, (d) 72h, (e) 96h, (f) 120h	19
11	Live/Dead Cell Count of Propidium Iodide (PI) Stained Cells Using IncuCyte® S3 Cell-By-Cell Analysis Software Module (a) and (b) K562; (c) Graph of Live Cell Count (Per Image) versus Time (Hours)	20
12	K562 in Two Dimensional Culture (a) 0h, (b) 24h, (c) 48h, (d) 72h, (e) 96h	21
13	Three Dimensional Reconstruction Of Collagen Type I Gel	22
TABLE		
1	Cell Suspension Concentrations	15

1. INTRODUCTION

1.1 Cancer as a disease

Cancer is a complex set of diseases which results from the development of tumours. A tumour is an abnormal proliferation of cells (Cooper, 2000; Pardee & Stein, 2008). Cancer cells acquire eight biological capabilities – the hallmarks of cancer - during tumour development, consisting of sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, deregulating cellular energetics and metabolism, and avoiding immune destruction (Hanahan & Weinberg, 2011). Integration of these hallmarks involves interaction of a variety of cells, soluble factors, signaling molecules, extracellular matrix, and mechanical cues which form the tumour microenvironment (Pardee & Stein, 2008; Swartz et al., 2012).

Cancers can be divided into three main types – carcinomas (cancers of the epithelial cells and include approximately 90% of human cancers), sarcomas (cancers of the connective tissues, rare), and lymphomas and leukaemias (cancers of the white blood cells and constitute about 7% of human malignancies). Most breast cancers are carcinomas. According to a 2018 study of 36 cancers in 135 countries worldwide, female breast cancer was found to be the second most commonly diagnosed type of cancer after lung cancer, accounting for 11.6% of all cancer sites, with a mortality of 6.6%. Incidences of breast cancer also occur in males, but they account for <1% of all breast cancer cases. Genetic predispositions are one of the major risk factors of breast cancer. Inheritance of a mutation in the BRCA1 or BRCA2 gene is a common cause of breast cancer. Women with BRCA1 mutation and males with BRCA2 mutation are at a higher lifetime risk of developing breast cancer. There is an increased risk of developing the disease in females with women with benign breast disease. Oestrogen also plays an important role in the development of the disease (Bray et al., 2018; Cooper, 2000; Cox et al., 2010; Feng et al., 2018; Miranda-Filho et al., 2018). Leukaemia is a heterogeneous group of haematopoetic cancers with an incidence of 2.4% of all cancer sites, and mortality of 3.2%. Leukaemia develops when blood cells fail to undergo terminal differentiation. The cells become arrested at early stages of maturation where they retain their capacity for proliferation and continue to reproduce (Bray et al., 2018; Miranda-Filho et al., 2018). The four major types of leukaemia are chronic lymphatic leukaemia (CLL), chronic myeloid leukaemia (CML), acute myeloid leukaemia (AML), and acute lymphatic leukaemia (ALL). Ionising radiations are the most common cause of leukaemia, except for CLL. Chemotherapy can also be a cause since chemotherapeutic agents such as alkylating agents and certain topoisomerase II inhibitors are leukaemogenic (Cox et al., 2010).

1.2 Challenges in chemotherapy

Treatment methods for cancer include surgery, radiation therapy and chemotherapy. Surgery is the primary means of curing most solid tumours i.e. when the cancer is confined to the primary site. Radiation therapy and chemotherapy are cytotoxic therapies, although they are not selective towards cancer cells. Both therapies target deoxyribonucleic acid (DNA) of tumour cells. Radiation generates free radicals in tumour cells, which introduce strand breaks in DNA. The DNA repair system in tumour cells is inefficient as compared to that of normal cells which provides a therapeutic window (Cox et al., 2010; Pardee & Stein, 2008). Tumour hypoxia can be targeted by treatment with radiosensitizers before radiation therapy (Chen & Kuo, 2017; Graham & Unger, 2018; Overgaard, 2011). Chemotherapy can also be used to target the actin cytoskeleton which is involved in cell motility, intracellular transport processes and cytokinesis (Foerster et al., 2014). Combination therapy involving radiation and chemotherapy has also been shown to have significant improvements in the treatment of cancers of the head and neck. Studies have also been conducted to test the efficacy of combination therapy targeting tumour vasculature, especially vascular-endothelial growth factor (VEGF) (Comunanza & Bussolino, 2017; Jain, 2001).

1.3 Personalised chemotherapy

The goal is to provide each patient with a specific most effective treatment rather than applying a one-fits-all general therapy. A personalized therapy could be guided by determining selected biomarker expressions in that patient. Prediction of what drugs could be effective against an individual patient's cancer are being developed from biomarkers that identify oncogenic pathways in subtypes of responding tumours (Bild et al., 2006). A set of biomarkers may be helpful in addition to the criteria of cancer stage and grade now used, especially for intermediate-grade (stage II) cancers. An initial aim could be to predict whether or not to apply chemotherapy following surgery. (Pardee & Stein, 2008)

1.4 Cell culture systems

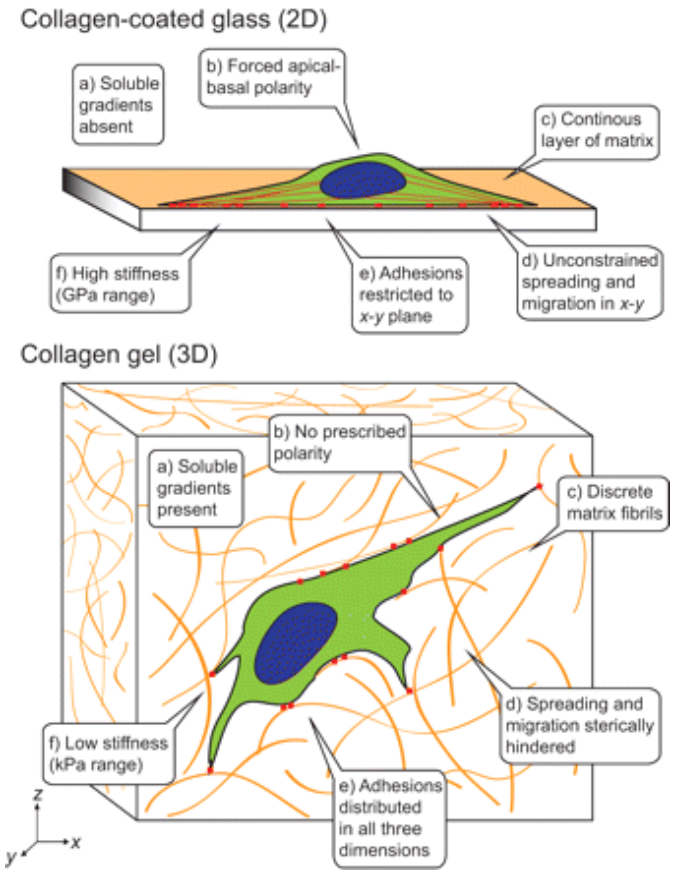


Figure 1: Two Dimensional Versus Three Dimensional Cell Culture (Baker and Chen, 2012)

1.4.1 2D culture systems

Conventionally, two-dimensional (2D) cell cultures have been used as *in vitro* models to study cellular responses to stimulations from biophysical and biochemical cues. 2D cell culture relies on adherence to a flat surface like petri dish, multiwell plate or flask, to provide mechanical support for the cells (Duval et al., 2017).

2D monolayer cell culture has several advantages. The cells have access to a similar amount of nutrients and growth factors present in the medium, which results in homogenous growth and proliferation. The culture vessel surfaces, especially polystyrene, can be easily modified with proteins or other surface functionalities to aid and enhance cell adhesion and cell health. The cost of maintaining cells in flasks is low. There exist robust and simple protocols which reduce the likelihood of contamination. Direct imaging can be done through these substrates, making it easy to monitor cell viability and morphology. It is also easy to add reagents and aliquot out samples for analysis. Thus, the simplicity of 2D culture has enabled reductionist approaches to understanding individual cellular phenomena (Baker and Chen, 2012; Castiaux, Spence and Martin, 2019; Duval et al., 2017).

These findings, however, come with the limitation that the 2D model might not dependably capture the physiological behavior of cells in vivo. 2D cell systems do not accurately represent the way cells grow and function in the body. Cells grown in a monolayer are flat, and can adhere and spread freely in the horizontal plane but have no support for spreading in the vertical dimension. One consequence of this is that cells that are cultured on 2D surfaces have a forced apical–basal polarity. These changes in cell geometry and organization can directly impact cell function. For example, changes in cell shape and size can result in local and global cell signalling pathways to be switched on or off (Baker and Chen, 2012; Meyers, Craig and Odde, 2006).

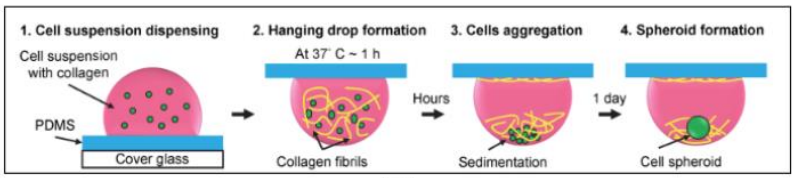
Another factor to be considered is that adherent cultures usually consist of a single type of cells. In *in vivo* conditions, however, heterotypic interactions occur. Mixed cell populations influence each other via cell-cell contacts and paracrine signals. This is particularly important when studying the effects of chemotherapy on tumours, where understanding the interaction between tumours and their microenvironment is key. The tumour microenvironment (TME) consists of different non-cancer cell types and their stroma, such as fibroblasts, immune cells (lymphocytes and macrophages), mesenchymal cells, and endothelial cells (EC), which all have a specific role in the physiology, structure, and function of the tumor (Hoarau-Véchot et al., 2018).

The extracellular matrix (ECM) also plays a role in the transport of effectors. Concentration gradients for oxygen, pH and soluble components such as nutrients and effector molecules as well as cellular metabolites exist within a tissue. These gradients are influenced by various factors including, but not limited to, the diffusion of molecules through the ECM, and thus, the composition of the ECM, and by the extent of cellular metabolism. Molecular concentration gradients affect a variety of cellular processes including cell motility, cell migration, and cell signaling and are important in chemotaxis and morphogenesis in normal development (Langhans, 2018). Under 2D monolayer culture conditions, cell-secreted or exogenously added soluble factors convectively mix and diffuse freely throughout the medium, and equilibrate rapidly. Temporary gradients can be created to study short-lived chemotactic events on 2D surfaces, but longer-term morphogenetic events require sustained gradients that can last from hours to days (Baker and Chen, 2012).

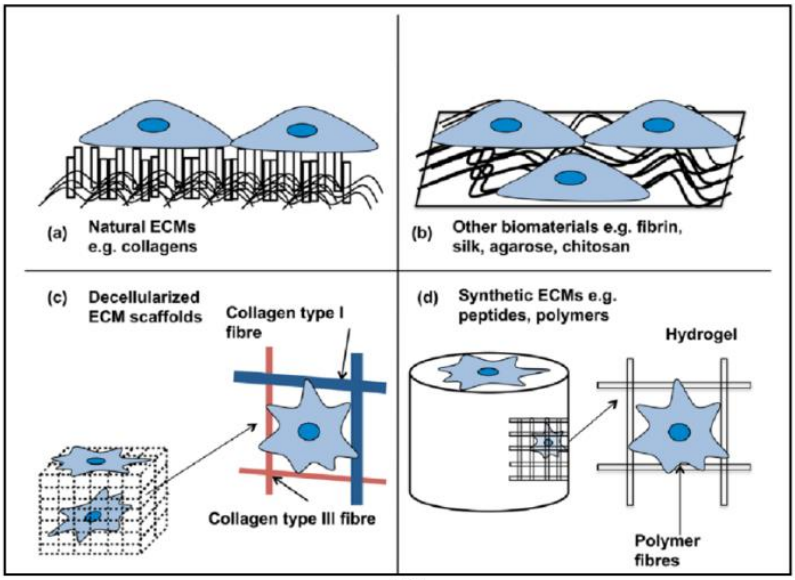
A switch to three-dimensional (3D) cell cultures can be a promising solution to a majority of the limitations of 2D cell culture.

1.4.2 3D culture systems

3D cell cultures closely mimic in vivo conditions and hence give a more realistic overview of cellular processes and interactions as compared to 2D systems. A wide selection of 3D culture systems exists, each with its benefits and limitations (Figure 2).



(a)



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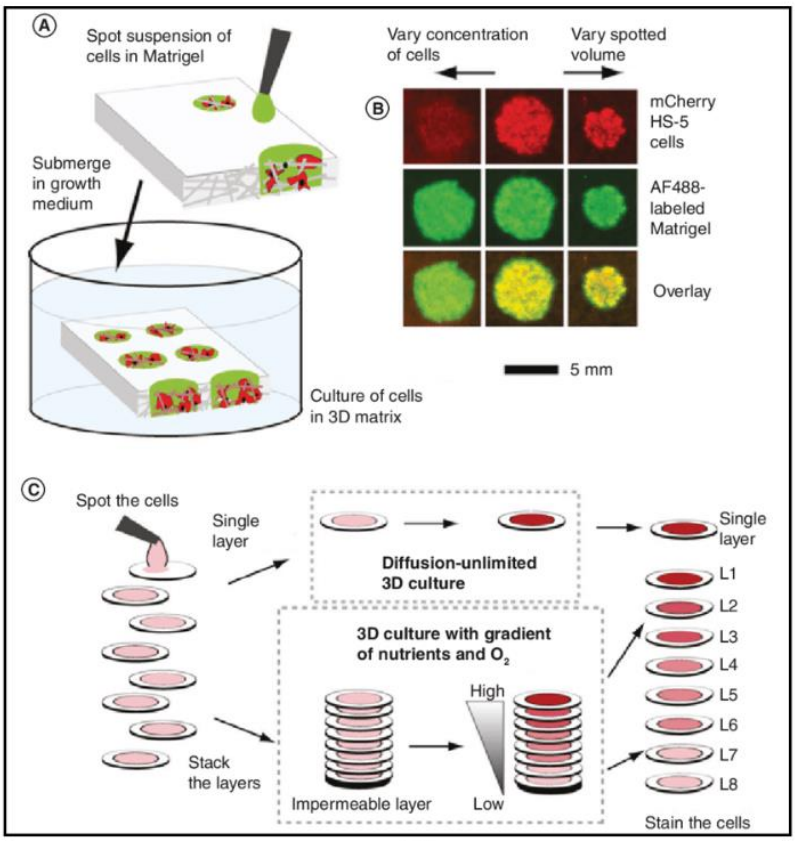

(c)

Figure 2: 3D Culture Systems
(a)Hanging Drop Culture in PDMS-Based Hanging Drop Array (Kuo et al., 2017);
(b) Hydrogels and Fiber Scaffolds (Kular, Basu and Sharma, 2014); (c) Paper (Li et al., 2012)

(a) Hanging drop culture – It is a sub-technique of suspension culture. This technique takes advantage of self-aggregation of cells into spheroids in the absence of a surface for attachment. Specialized plates with open, bottomless wells are used to create small droplets of media. The droplet is big enough for cells suspended in the medium to aggregate but small enough to prevent it from being dislodged during manipulation. Cells in hanging drop plates (HDP) can form spheres that may be representative of tumor layers in the vicinity of a capillary—a peripheral layer with proliferating cells due to the closeness to the supply of oxygen and nutrients, underlaid by an intermediate layer with quiescent cells and an inner necrotic core. Multi-cellular spheres may be created by co-suspending several cell types or by consecutive addition of different cell types to promote the formation of separate cell layers. Embedding of the formed spheres into ECM- like scaffolds allows for the modeling of cell adhesion of the outer layers of the spheres with ECM components surrounding tumor tissues. (Langhans, 2018)

(b) Hydrogels – Most mammalian cells *in vivo* proliferate on a complex scaffold known as the extracellular matrix. It exists as a hydrated protein matrix that allows cell adhesion and acts as a template for 3D cell growth. Hydrogels, composed of one or many different hydrophilic polymers having a polymerization pattern that enables cells and molecules to move in and out of the pores, can be used to mimic these *in vivo* conditions. They can be made from a wide range of synthetic (polyacrylamide, polyethylene glycol, poly(acrylic acid)) and natural polymers (collagen, fibrin, hyaluronic acid). This variety of materials allows fine tuning of properties like porosity, stiffness, and degradation of the matrix, which are important to cell health and function. However, flexibility of parameters comes at the cost of time, and complications related to production and stability. Hydrogels have thermal and mechanical limitations that limit the working conditions and the longevity of the material. Large scale production of hydrogels poses issues of uniformity and batch-to-batch consistency. Cells can be introduced to hydrogel matrices either during or after polymerization depending on the cell line used or experimental factors being explored (Castiaux, Spence and Martin, 2019). Hydrogels can have natural or synthetic origins, with the prospect of producing hybrid hydrogels with unique physical and biological properties. Such hybrid hydrogels can be customised to various applications. Examples of such hybrids include a collagen I coated poly(l-lactide-co-ε-caprolactone) (PLCL) scaffold for tissue engineering and alginate-collagen matrix for culturing human epiphyseal chondroprogenitor cells (He et al., 2018; Studer et al., 2017). Hydrogels from natural sources such as collagen, fibrin or Matrigel are

biocompatible, have natural adhesive properties and sustain many physiological cell functions— resulting in high cell viability, controlled proliferation or controlled differentiation, and often a cell phenotype typically observed *in vivo*. Collagen, with type I collagen being the most abundant form, is the most widely used ECM protein for 3D cell culture (Di Lullo et al., 2002; Langhans, 2018). Collagen type I gels are often used to provide a 3D microenvironment for cancer cell growth because it contains the tripeptide Arg-Gly-Asp (RGD), a short amino acid sequence found in several ECM components, such as fibronectin, which binds to integrin receptors on the cell surface. Collagen gel cultures have been employed to study tumor drug sensitivity, replicate the pre-vascularized stages of *in vivo* solid tumor progression, and construct an *in vitro* organotypic model of normal and malignant breast to investigate the role of cell–cell interactions in breast cancer progression (Fong et al., 2017).

(c) Fiber scaffolds – Fiber scaffolds combine the freedom of material provided by hydrogels with the scaffold of paper-based cell culture. There are several methods for producing nano-fibers that include solution blow spinning, melt spinning, and electrospinning. These techniques can be used to produce both natural (collagen, gelatin, silk fibroin) and synthetic fibers (polycaprolactone, polyurethane, polystyrene). The fiber sizes range from 10's of microns down to 10 nanometer diameters similar to the ECM. A blend of materials can be used to tune the structural properties of the material, making the fiber mat stronger or preventing degradation. Fiber scaffolds are stable over a wide range of temperatures and find applications in static cell culture as well as under flow conditions. Synthetic fibers are more stable in solution over long periods of time whereas natural fibers tend to degrade rapidly. Fiber scaffolds have biocompatibility to the point of being used in wound healing applications. However, production of electro-spun fibers often requires toxic volatile organic solvents, which have the potential to leach into cell cultures. Benign aqueous based solvents have been explored in limited applications and have been shown to minimize effects on protein structure. (Castiaux, Spence and Martin, 2019)

(d) Paper – While hydrogels require the synthesis of material as needed, paper-based cell culture provides similar scaffolding that is less labor intensive. The cellulose matrix of paper provides a rigid, but porous scaffold that can be folded into complex geometries, and serves as a mimic to monitor cell migration. Paper as a scaffold material is stable making it amenable to a wide range of surface modifications including chemical treatment, plasma treatments, and sterilization techniques. In addition, well-established patterning techniques allow for the fabrication of defined microfluidic networks on a single sheet of paper or

between multiple, stacked paper sheets. However, paper-based techniques do have limitations when considered as an *in vivo* mimic. Since the material is mostly cellulose-based, it requires modification with ECM proteins. Additionally, the fiber diameter for most paper-based systems is relatively large (>1 mm), compared to the ~500 nm size of most fibrils in the body (Castiaux, Spence and Martin, 2019). Nevertheless, paper has been successfully used as a scaffold for 3D culture. A prime example of this is the microfabricated paper based 3D cell culture system for tissue-based bioassays developed by Derda et al in 2009 (Derda et al., 2009).

1.5 Long term live cell imaging

Organisms are composed of vast numbers of different molecules and cells that interact spatially and temporally. A wide array of molecular and cellular assays is available to reveal these interactions. However, most techniques look at cells or molecules at single time points, usually the endpoints. Thus, the complexity of highly dynamic processes cannot be entirely comprehended by such assays. The development of photography, microcinematography, fluorescence microscopy, cell and tissue culture and other techniques has however made it possible to observe living cells continuously over long periods. This has led to an almost exponential rise in studies using time-lapse imaging in most of the natural science fields.

The most critical parameter for long-term time-lapse imaging of mammalian cells is to keep cells alive and normal, which can be achieved by recreating their native environment as closely as possible. One important caveat of *in vitro* imaging is the near impossibility to recreate the *in vivo* microenvironment of the cell type studied. Cell dynamics, including migration, cell division and cell-cell interaction are fundamental processes in development, tissue repair and disease. These processes are specifically modulated by properties of the extracellular microenvironment. Moreover, cells usually exist *in vivo* as heterotypic populations. Many cell types do not behave normally when sorted to homogeneity and require interactions with other cell types (Coutu and Schroeder, 2013; Sapudom et al., 2017). In essence, even the smallest of events can have an impact on the final state of the cell; a phenomenon often known as the 'butterfly effect'. Thus, as a means to closely mimic *in vivo* conditions, one of the 3D culture techniques described in section 1.4.2 can be used. Other major factors that need to be considered during time-lapse microscopy are using an appropriate culture medium, temperature and pH control, oxygen and CO_2 supply, as well as maintenance of osmolarity. For long term imaging, it is also important to reduce evaporation and phototoxicity to a minimum. Microscopic images acquired during long term live cell imaging experiments are data-rich. These image datasets are lucrative for applications using the deep learning approach of machine learning.

While most applications involve fluorescently labelled molecules or cells for easy visualisation, there is always the hazard of phototoxicity associated with fluorescence imaging. Label-free approaches are hence gaining impetus in imaging. Machine learning can be used to train models based on existing data to predict characteristics of the cells. Machine-learning models trained on one data type are now also able to predict on another data type with very little retraining (transfer learning), making the models more generalised (Sullivan and Lundberg, 2018; Christiansen et al., 2018; Figure 3).

This approach can find applications in preclinical cancer research. Long term live cell imaging datasets of cells treated with permutations and combinations of chemotherapeutic agents can be used to train machine learning agents to detect subtle morphological changes as an indicator of the drug's efficacy. With these findings, further models can be developed to determine suitable doses and treatment schedules, before actual administration to the patients. Thus, *in silico* testing holds promise in the field of personalised medicine.

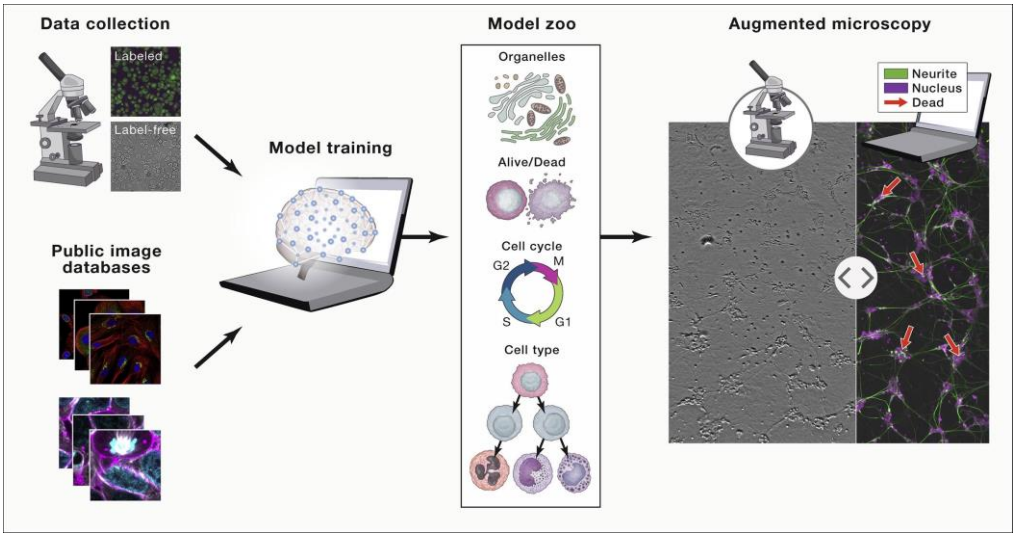

Figure 3: Augmented Microscopy: Integration of Microscopy with Machine Learning (Sullivan and Lundberg, 2018)

2. OBJECTIVES

(a) Determining optimal rat tail collagen type I concentration for hydrogel fabrication and cell number for visualisation

(b) Immobilising cells in collagen I matrix and imaging with IncuCyte® S3 (20X) and confocal microscope (Zeiss LSM 710)

(c) Determining longest duration for continuous long term live cell imaging

(d) Primary testing of diffusion of small molecules to investigate cell function

3. MATERIALS AND METHODS

3.1 Cell Lines

Two breast cancer (JIMT-1 and MCF-7) and two leukaemia (Jurkat and K562) cell lines were tested in this work. Of the four cell lines, two were narrowed down upon for further tests.

3.1.1 Adherent cell lines (JIMT-1 and MCF-7)

JIMT-1 is a carcinoma cell line from a pleural metastasis of a 62-year old patient with breast cancer who was clinically resistant to trastuzumab. JIMT-1 cells grow as an adherent monolayer. The cells overexpress HER-2 mRNA and protein and lack expression of estrogen and progesterone receptors (Tanner et al., 2004; Leibniz Institute German Collection of Microorganisms and Cell Cultures, Germany)

The MCF-7 cell line was established in 1973 by Dr. Soule and colleagues at the Michigan Cancer Foundation, where the name is derived. The MCF-7 cells were isolated from the pleural effusion of a 69- year-old woman with metastatic disease. It is positive for estrogen and progesterone receptors and belongs to the luminal A molecular subtype. (Soule et al., 1973)

The adherent cell lines were cultivated using Dulbecco's Modified Eagle Medium (DMEM; Gibco Thermo Fisher Scientific, USA). The medium was supplemented with 10% fetal bovine serum (Gibco Thermo Fisher Scientific, USA) and 1% Penicillin-Streptomycin 10,000 Uml-1 (Gibco Thermo Fisher Scientific, USA). The cells were incubated at 37°C and 5% CO2 in Heracell 150i (Thermo Fisher Scientific, USA), and passaged every three to four days, depending on their concentration.

3.1.2 Suspension Cell Lines (Jurkat and K562)

Jurkat cell line, previously known as JM, was derived from the peripheral blood of a 14-year-old boy with acute lymphoblastic leukemia (ALL). The line was established from cells obtained during the first relapse. (Schneider, Schwenk, and Bornkamm, 1977)

The continuous cell line K562 was established by Lozzio and Lozzio (1975) from the pleural effusion of a patient with chronic myeloid leukemia (CLM) in terminal blast crisis. K562 has been reported to carry the Philadelphia chromosome marker and is of the erythroid cell lineage. (Andersson, Nilsson and Gahmberg, 1979; Lozzio and Lozzio, 1975)

The suspension cell lines were cultivated using Roswell Park Memorial Institute (RPMI) 1640 (Gibco Thermo Fisher Scientific, USA). The medium was supplemented with 10% fetal bovine serum (Gibco Thermo Fisher Scientific, USA) and 1% Penicillin-Streptomycin 10,000 Uml-1 (Gibco Thermo Fisher Scientific, USA). The cells were incubated at 37°C and 5% CO2 in Heracell

150i (Thermo Fisher Scientific, USA), and passaged every three to four days, depending on their concentration.

3.2 Rat Tail Collagen type I Gel Fabrication

Rat tail collagen type I gel was fabricated using rat tail collagen type I solution (ibidi GmbH, Germany) following the order in the fabrication protocol provided by ibidi GmbH, with 10X DMEM replaced with 1X DMEM (Figure 4). For the suspension cell lines, RPMI 1640 was used instead of DMEM. Collagen I concentrations of 3 mg/ml, 2 mg/ml, 1.5 mg/ml, 1 mg/ml and 0.5 mg/ml were tested. Apart from the recommended 1.5 mg/ml, a range of concentrations was tested. The pore size of the resulting gel is inversely proportional to the concentration (Fraley et al., 2015; Sapudom et al., 2015; Figure 5). Pore sizes play an essential role in nutrient and oxygen diffusion and waste removal, and have an influence on cell adhesion and cell–cell interaction (Bružauskaitė et al., 2016). All the components, except the cell suspension, as well as sterile 1.5 ml Eppendorf tubes, were held on ice. Cell numbers of 1×10^4, 1×10^5, 2×10^5, 3×10^5, 1×10^6 and 2×10^6 cells/ml were tested. The concentration of the cell suspension was set to 6 times the required concentration, as recommended. (Application Note 26: Fabrication of Collagen I Gels (Version 2.4) - ibidi GmbH, Germany; Table 1).

	DMEM				
Final Collagen I concentration in gel (mg/ml)	**3**	**2**	**1.5**	**1**	**0.5**
10 x DMEM	21	20	20	20	20
NaOH 1M	7	6	5	5	4
H_2O	-	49	81	112	141
$NaHCO_3$ 7.5 %	3	5	4	3	5
1 x DMEM	39	50	50	50	50
Collagen I, 5 mg/ml	180	120	90	60	30
Cell suspension	50	50	50	50	50
Σ	300	300	300	300	300

Figure 4: Rat Tail Collagen Type I Gel Fabrication Protocol
(Source: Application Note 26: Fabrication of Collagen I Gels (Version 2.4) - ibidi GmBH, Germany)

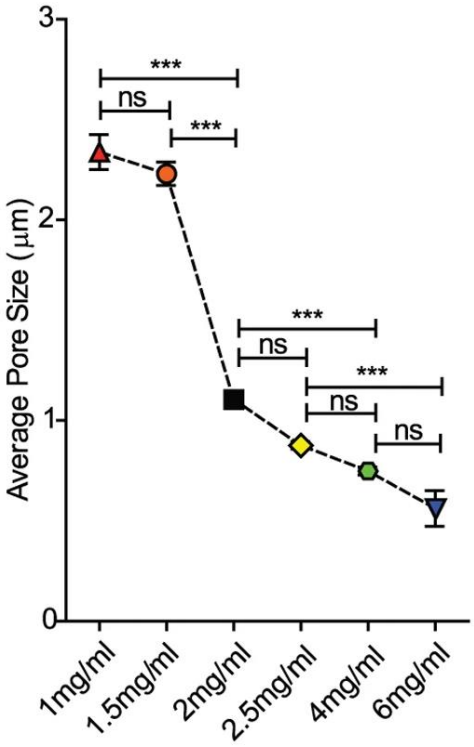

Figure 5: Graph of Average Pore Size (µm) vs Collagen I Concentration (mg/ml) (Source: Fraley et al., 2015)

The resulting mixture was dispensed as follows:

(a) For imaging with IncuCyte® S3 - 100 µl per well in 96-well, clear flat bottom plate (Greiner Bio-One GmbH, Germany)

(b) For imaging with Zeiss LSM 710 - 50 µl per well in ibidi 15-well µ-slide Angiogenesis (ibidi GmbH, Germany)

In case of live/dead imaging with, an equal volume of a 0.25 mg/ml propidium iodide (PI) solution (100 µl and 50 µl, respectively) was overlaid after gelation.

3.3 Long term live cell imaging

The above experimental setups were incubated under the standard conditions of 37°C and 5% CO_2. Images were acquired either every hour or every 30 minutes with IncuCyte® S3. Cell counts were done using IncuCyte® Cell-By-Cell Analysis Software Module which employs an image segmentation algorithm for the purpose.

3.4 Diffusion testing in collagen type I gel

The collagen I gel was fabricated and dispensed as described in section 3.3 and 3.3 (b), respectively. 50 µl of a 0.25 mg/ml PI solution was overlaid after gelation. Z-stack imaging of the gel was done

with Zeiss LSM 710. A 3D reconstruction of the imaged collagen I gel was obtained using Imaris software (v. 9.6.0).

4. Results

4.1 Rat Tail Collagen Type I Gel Fabrication

Of the five concentrations of rat tail collagen type I tested, 1.5 mg/ml and 2 mg/ml were found to be suitable for imaging with IncuCyte® S3 and Zeiss LSM 710, respectively. The higher concentrations were prone to bubble formation during mixing for homogeneous distribution of the cell suspension in the gel, which caused focussing problems during imaging.

Apart from rat tail collagen type I, a range of cell numbers was also tested. The cell numbers that were suited for microscopy for each cell line are summarised in Table 1.

Table 1: Cell Suspension Concentrations

Cell Line	Required Cell Density (cells/ml)	Set Concentration of Cell Suspension (cells/ml)
JIMT-1	1×10^5	6×10^5
MCF-7	1×10^5	6×10^5
Jurkat	2×10^5	12×10^5
K562	3×10^5	18×10^5

4.2 Long Term Live Cell Imaging

Acquisition of images every 30 minutes was observed to cause drying of the gel, and cell death due to phototoxicity, in case of fluorescently stained cells. Hence, a one hour interval was set for image acquisition during further experiments with IncuCyte® S3.

The cells were imaged for a minimum of 72 hours and a maximum of 120 hours. MCF-7 cells remained as clusters, even in a trypsinized suspension. As a result, the seeded cells grew as a single mass, making it difficult for image analysis. The Jurkat cells, on the other hand, showed poor growth in the gel (Figures 6 and 7). Hence, the imaging was limited to 72 hours and only JIMT-1 and K562 cells were used for further experimentation.

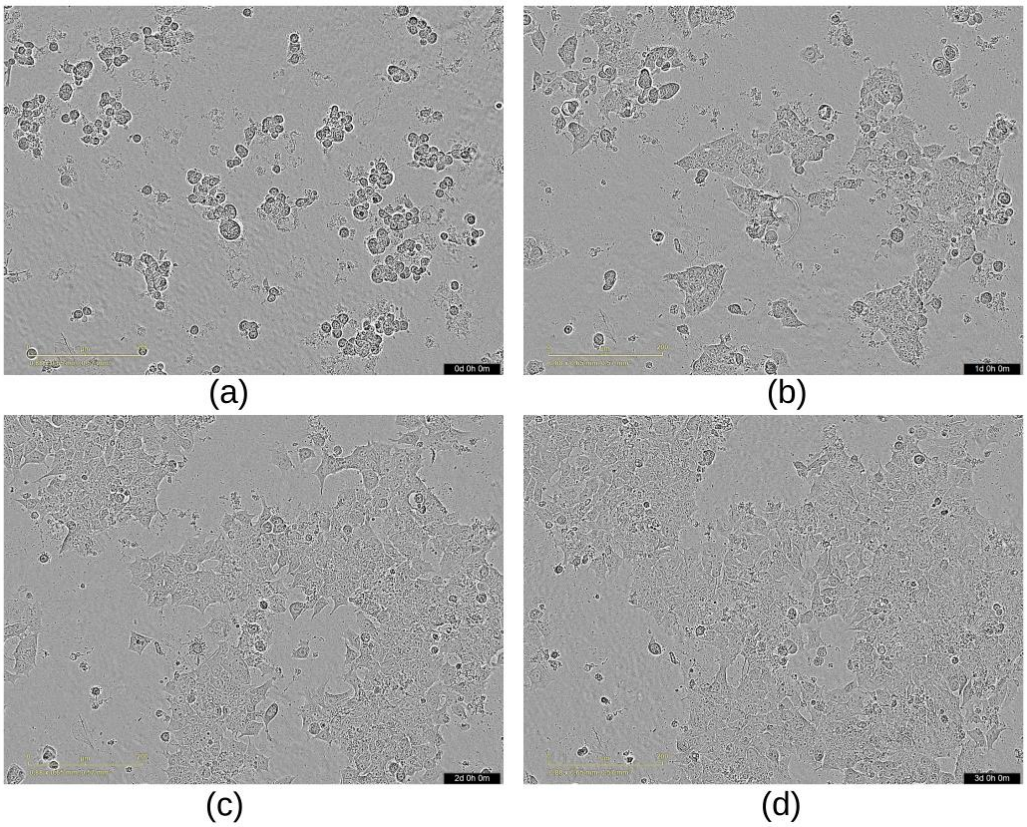

Figure 6: MCF-7 Cells In Collagen I Matrix (a) 0h, (b) 24h, (c) 48h, (d) 72h

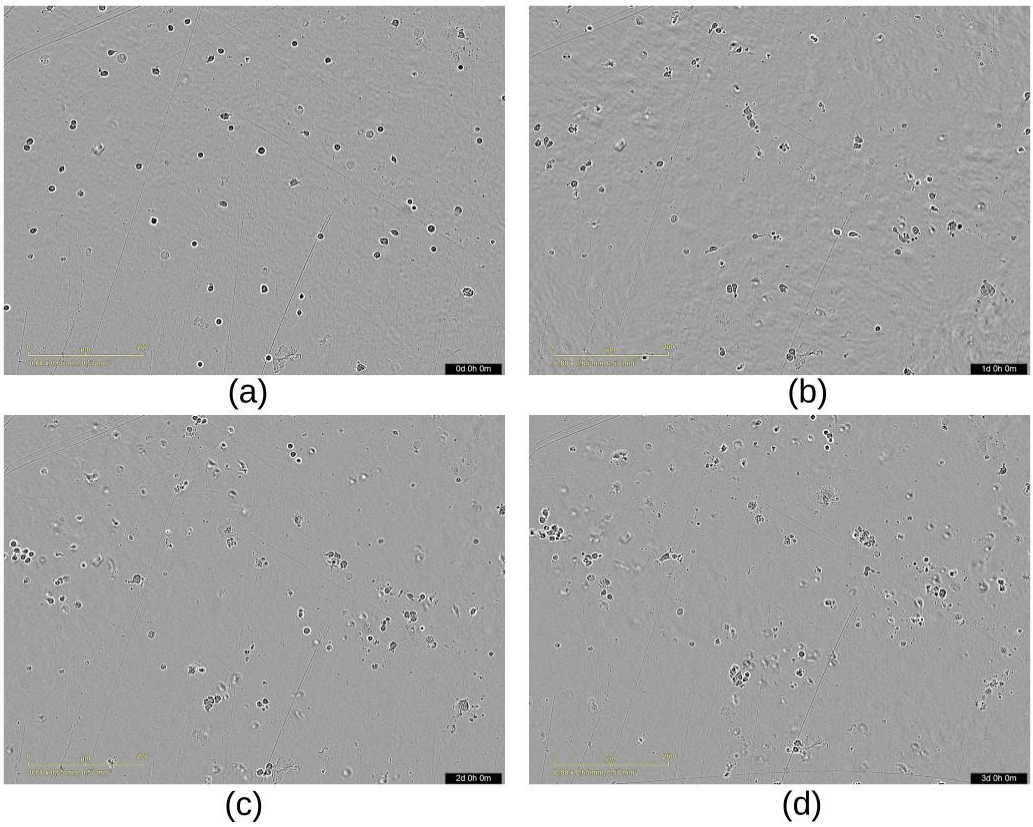

Figure 7: Jurkat Cells In Collagen I Matrix (a) 0h, (b) 24h, (c) 48h, (d) 72h

JIMT-1 and K562 cells were imaged with IncuCyte® S3 for 120 hours (Figures 8 and 10). The cells were stained with PI as described in section 3.3. The PI-stained dead cells were represented with a blue mask while the live cells were represented in yellow (Figures 9a and 9b, 11a and 11b). The output was the number of cells per image over time (Figures 9c and 11c). As seen in Figures 9c and 11c, consistent cell growth was observed for both cell types.

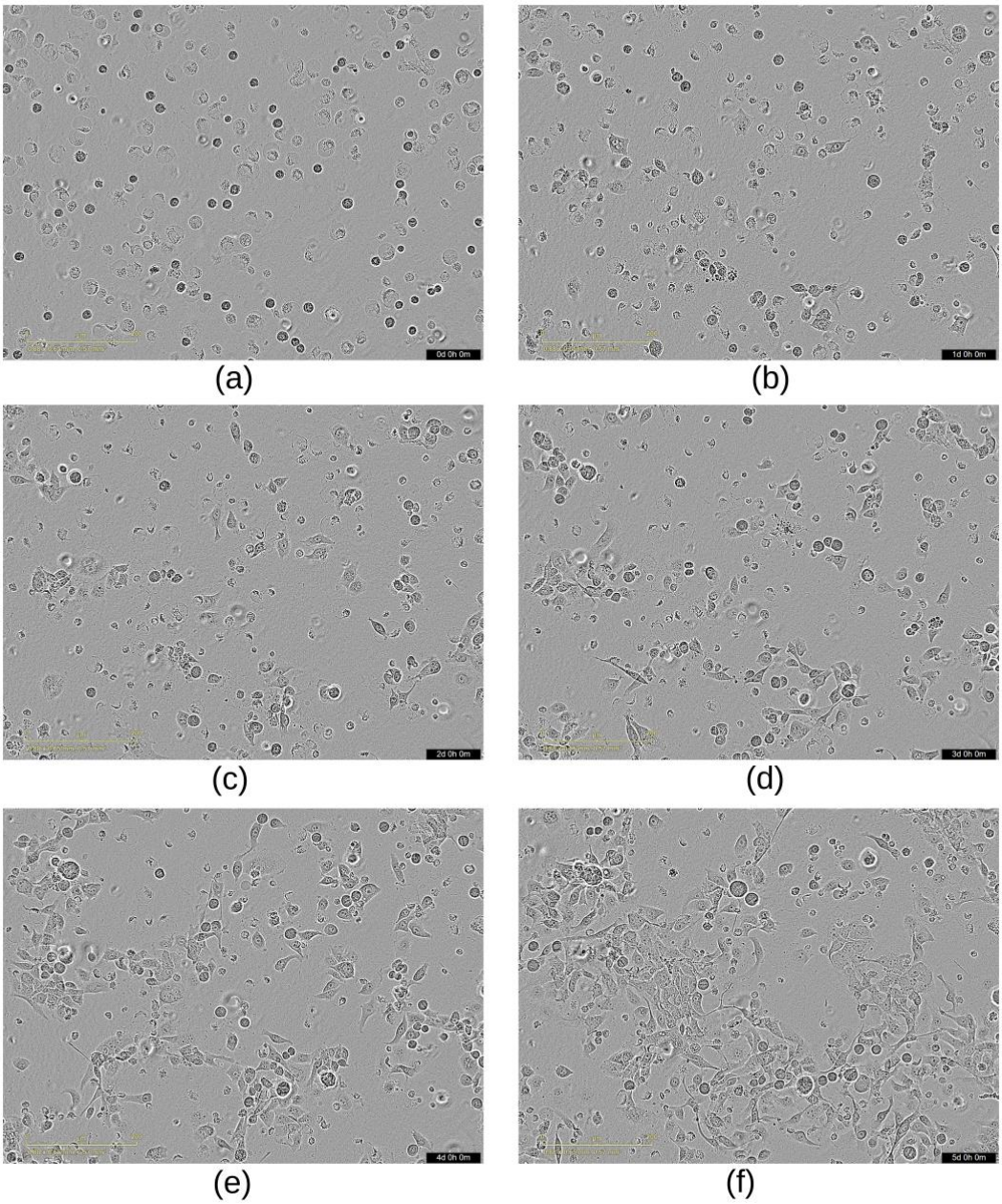

Figure 8: JIMT-1 Cells In Collagen I Matrix (a) 0h, (b) 24h, (c) 48h, (d) 72h, (e) 96h, (f) 120h

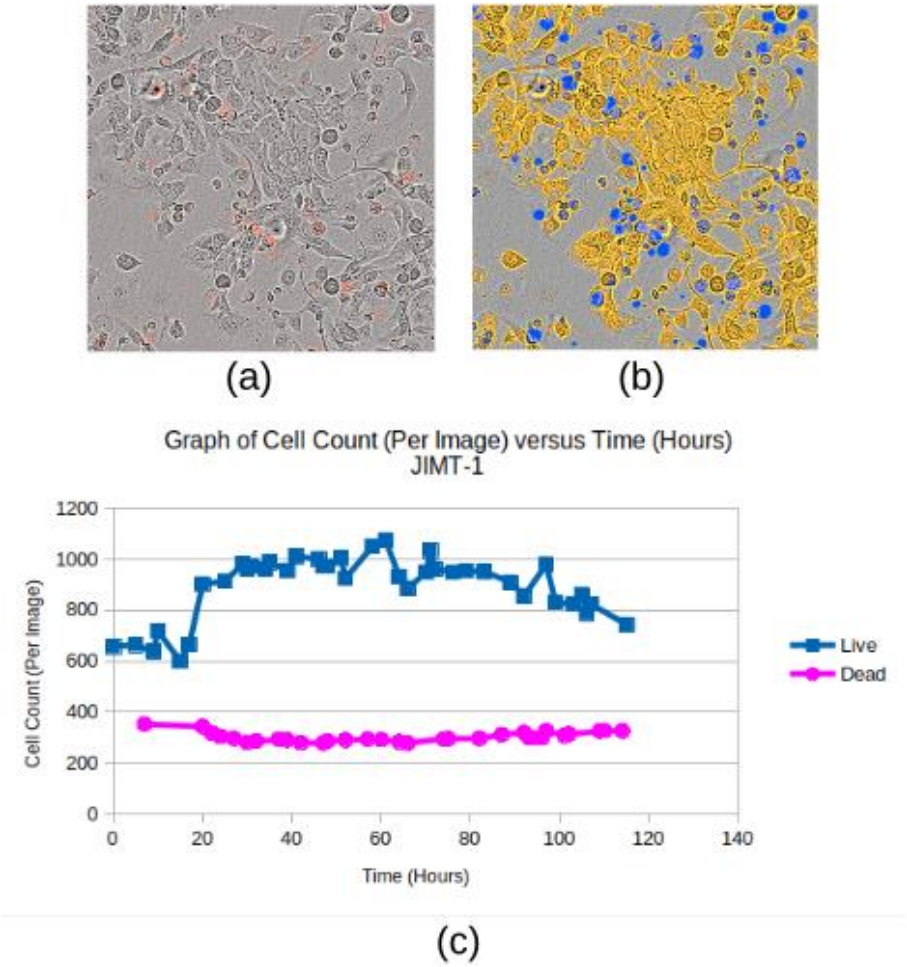

Figure 9: Live/Dead Cell Count of Propidium Iodide (PI) Stained Cells Using IncuCyte® S3 Cell-By-Cell Analysis Software Module (a) and (b) JIMT-1; (c) Graph of Live Cell Count (Per Image) versus Time (Hours)

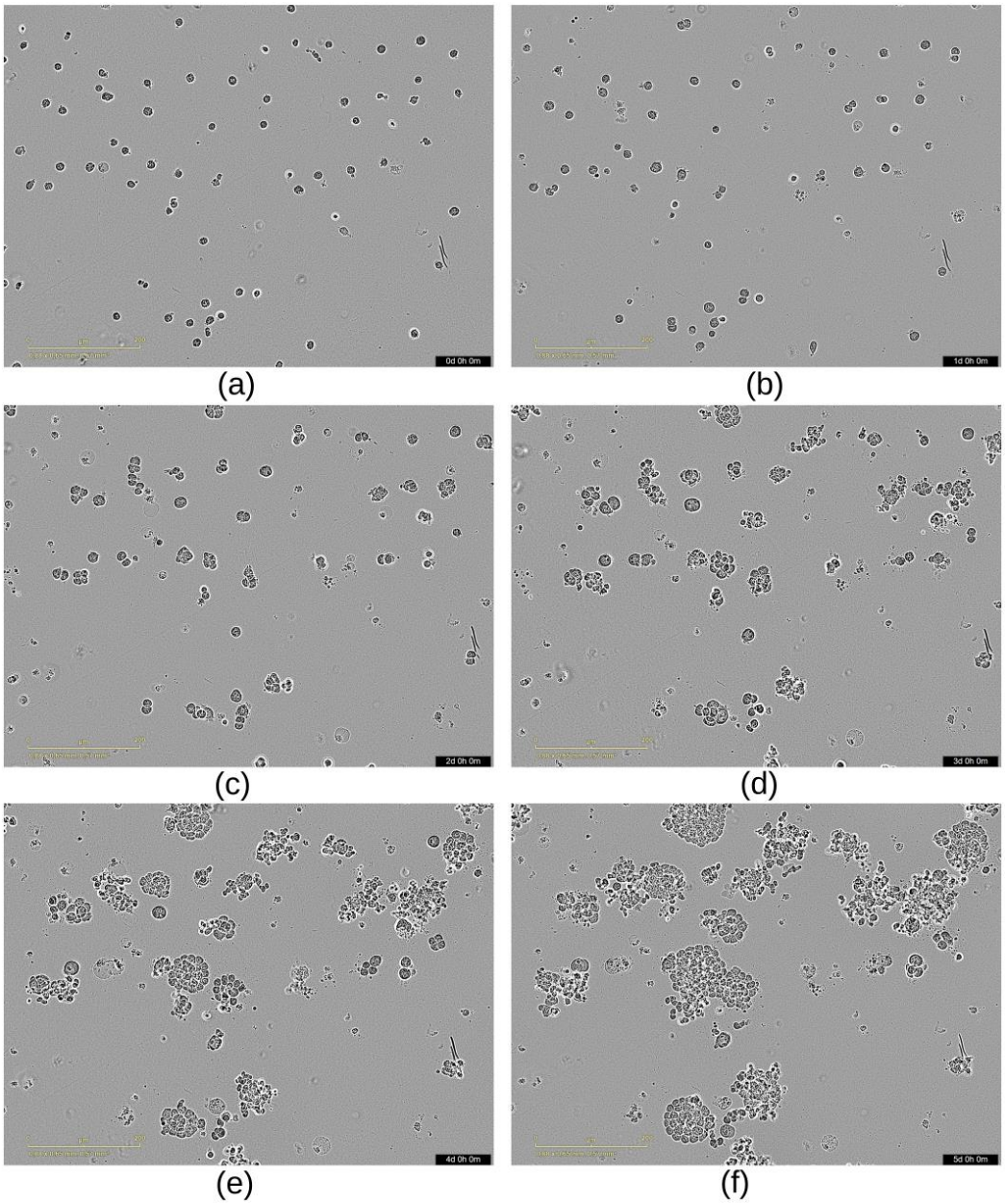

Figure 10: K562 Cells In Collagen I Matrix (a) 0h, (b) 24h, (c) 48h, (d) 72h, (e) 96h, (f) 120h

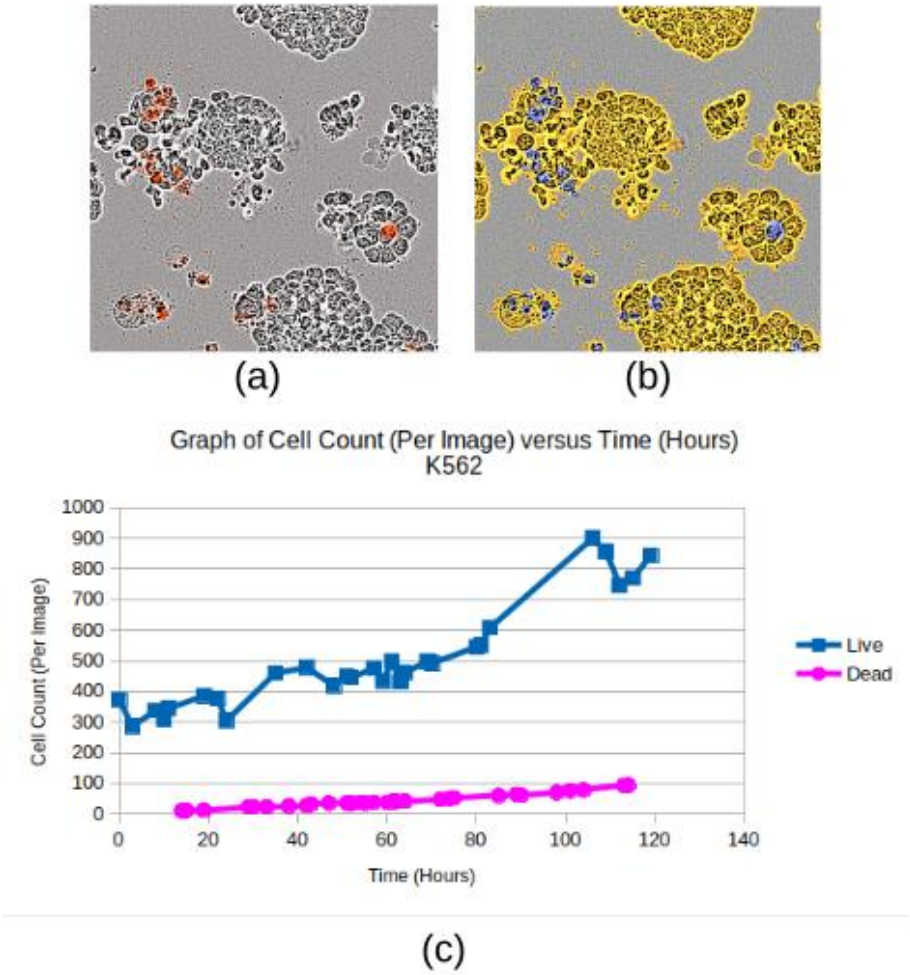

Figure 11: Live/Dead Cell Count of Propidium Iodide (PI) Stained Cells Using IncuCyte® S3 Cell-By-Cell Analysis Software Module (a) and (b) K562; (c) Graph of Live Cell Count (Per Image) versus Time (Hours)

It was also observed that the K562 cells grew in a 'flower-like' organised cluster in the gel as opposed to the freely growing cells in 2D culture (Figures 11a and 12).

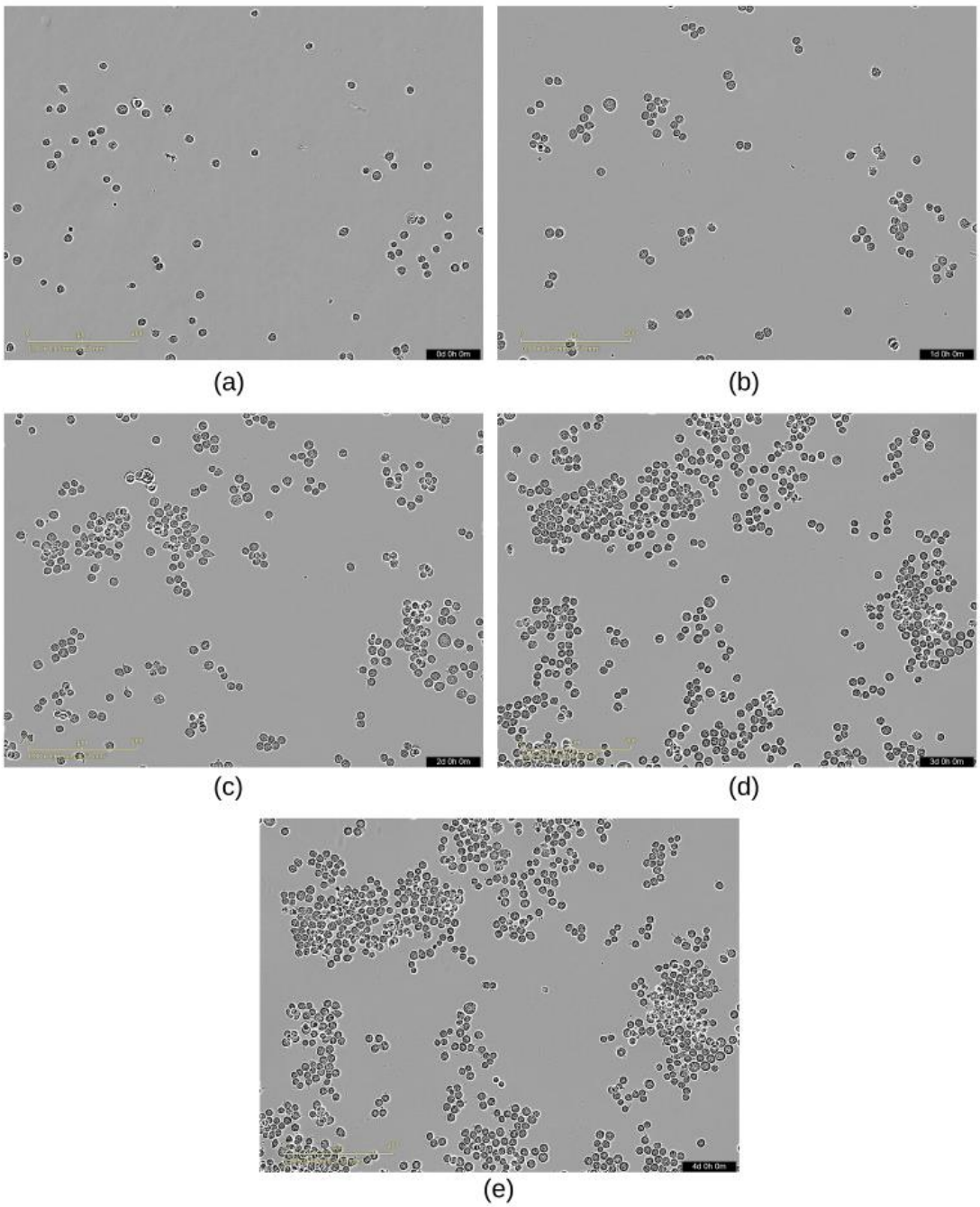

Figure 12: K562 in Two Dimensional Culture (a) 0h, (b) 24h, (c) 48h, (d) 72h, (e) 96h

4.3 Primary Testing Of Diffusion In Collagen Type I Gel

430 µm of the collagen I gel was imaged as a Z-stack with 44 slices at an interval of 10 µm with the Zeiss LSM 710 microscope. The 3D reconstruction of the gel section revealed a higher number of PI stained JIMT-1 cells i.e. dead cells in the matrix (Figure 13). A possible explanation for this would be the shock experienced by the cells when added to the collagen I solution which is held on ice. It is also worth noting that despite this shock, the cells survive and multiply over time after an initial growth lag as seen in Figures 9c and 11c.

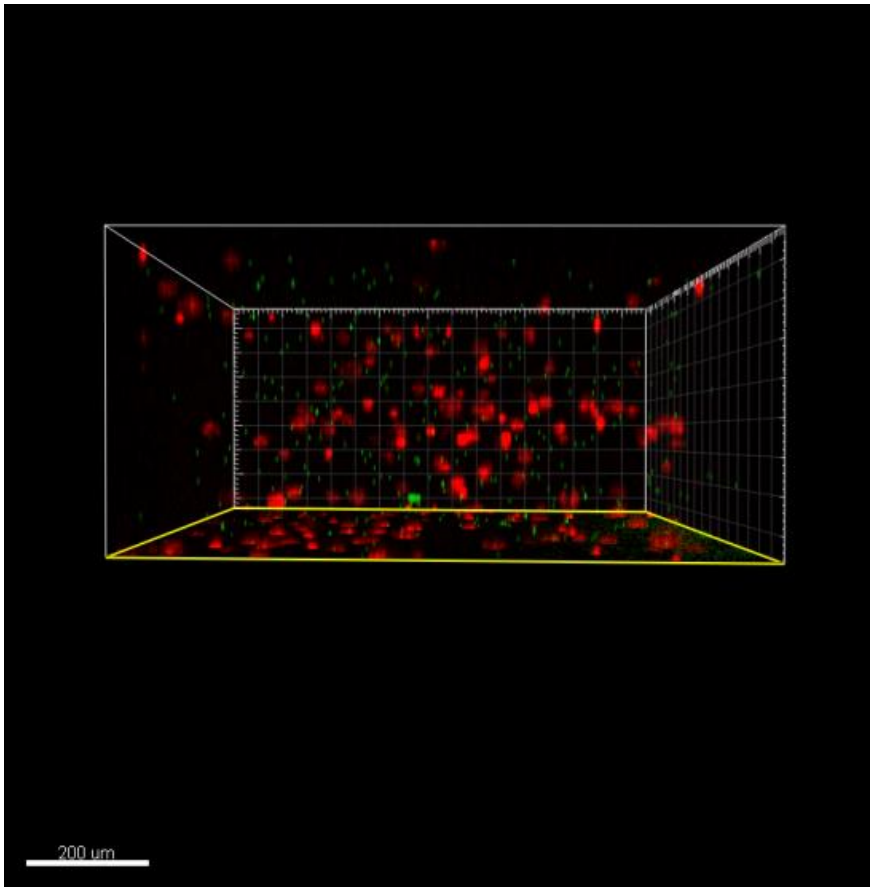

Figure 13: Three Dimensional Reconstruction Of Collagen Type I Gel With Imaris (v. 9.6.0)

5. Discussion

Conventionally, preclinical cancer research has relied on two-dimensional cell culture techniques. These involve growing cells in a monolayer or as a suspension culture. Although growing cells with these methods is cost effective and has a certain ease of handling, the conditions are not representative of the *in vivo* environment in which the disease develops. Moreover, it presents challenges for imaging single cells, especially in suspension. Cancer is a disease that develops as a combination of factors that constitute the tumor microenvironment. These factors include intercellular interactions amongst a heterotypic cell population, a cocktail of molecules, the ECM, and by extension, the mechanical properties of the matrix. 3D cell culture techniques provide a way to simulate these conditions *in vitro* and to image and track single cells over time, with relative ease. Furthermore, it has been reported that cells grown in 3D culture show morphological and physiological differences as to when grown in 2D culture (Baker and Chen, 2012; Meyers, Craig and Odde, 2006). A plethora of 3D culture systems exist, some of the popular ones being hanging drop culture, hydrogels, paper, and fiber scaffolds. In this study, we used a collagen I based hydrogel system. Collagen I is the predominant type of collagen found in the ECM.Various studies have shown that collagen also plays a role in progression and detection of the disease (Boyd et al., 2002; Ferruzzi et al., 2019; Nissen, Karsdal and Willumsen, 2019).

5.1 Rat Tail Collagen Type I Gel Fabrication

The concentration of the gel plays an important role in the behaviour of the cells. For example, the pore size of the resultant gel is inversely proportional to the concentration, and has been shown to have an effect on cancer cell morphology and invasion (Sapudom et al., 2015). Another study by Mah *et al* demonstrated that triple-negative breast cancer cells grown in lower concentrations of collagen showed a more oxidative phosphorylation (OXPHOS) signature of metabolism (Mah et al., 2018).

We tested various concentrations of rat tail collagen I for adherent and suspension cell lines. Concentrations of 1.5 mg/ml and 2 mg/ml were found to be optimal for long term live cell imaging. During these studies, we also came to the conclusion that since the short window of five minutes between the addition of the cell suspension to the collagen I solution and plating is critical, the handling techniques play a key role in the success of 3D culture. Moreover, testing a range of concentrations for both, the collagen I gel and the cell numbers, is also necessary to determine the optimum for the imaging system in use, because each system has its own focusing capability.

5.2 Long Term Live Cell Imaging

Cells are dynamic structures with cellular events occurring at any given point of time. Standard end-point assays provide a glimpse of these events, but are sometimes not sufficient to track minute changes. The evolution of time lapse imaging and therefore, long term live cell imaging has emerged to be the solution to this particular problem. This technique can be adapted to the application, a host of parameters and available resources, to non-invasively observe and document minor cellular events.

Olofsson et al developed a live cell imaging based migration assay that could be used to study natural killer (NK) cell migration and cytotoxicity. For the development of this assay, they imaged fluorescently labelled NK and target cells every two minutes for nine hours (Olofsson et al., 2019). However, the downside of imaging fluorescently labelled cells over longer periods of time is the risk of cell death due to phototoxicity. In 2017, Sapudom et al reported a label-free approach for quantitative single cell tracking. They demonstrated that the cell viability after label-free time lapse imaging experiments was comparable to cells in cell culture (Sapudom et al., 2017). In view of this, we decided on imaging the PI labelled as well as the unlabelled cells every hour.

As mentioned in section 5.1, the collagen matrix has an influence over the characteristics of the cells. We observed a flower-like growth pattern in the K-562 cell line cultured in the collagen I matrix as opposed to the dispersed growth in 2D culture. It has been previously demonstrated that the collagen matrix induces a mechanosensitive invasive epithelial phenotype in MCF-10A breast cancer cell line (Carey, Martin and Reinhart-King, 2017). Thus, it is possible that the flower-like growth pattern is due to the influence of the collagen matrix. The K-562 cell line can also be tested for the development or enhancement of chemoresistance when grown in 3D culture, since another leukaemic cell line, Jurkat, has been shown to have enhanced chemoresistance in 3D culture. This enhanced chemoresistance was attributed to the increased collagen I activated expression of the discoidin domain receptor 1 (DDR1) (Guo et al., 2019). This line of inquiry can be pursued in the future experiments.

5.3 Diffusion testing in collagen type I gel

Under physiological conditions, concentration gradients of biomolecules are formed by their varying diffusion rates through the ECM. The diffusion rates are usually governed by the molecular weight. These molecules play a role in regulating a variety of processes. (Hettiaratchi et al., 2018) A study of the diffusion of molecules through the matrix can be helpful in determining the effects of chemotherapeutic agents while treating cancer. We conducted a primary test of diffusion of PI through the collagen I matrix. With the help of confocal microscopy, we were able to observe a large number of PI-stained cells distributed through a section of the matrix.

The modelling of solute diffusion in hydrogels has been based on three main theories, namely, hydrodynamic, free volume, and obstruction theory. In 2019, Eneko et al described a multiscale diffusion model to predict the diffusion of solutes in hydrogels. Their model, a combination of the three theories, was able to predict solute diffusion better than the conventional models (Eneko et al., 2019). Further studies can be conducted using such models to predict the diffusion of chemotherapeutic agents in a collagen matrix and subsequently, their diffusion *in vivo*.

6. Conclusion

In this study we demonstrate the applicability of 3D culture coupled with continuous long term live cell imaging for documenting and tracking single cells. 3D culture proves to be a better system especially for imaging and tracking suspension cells. Integration of solute diffusion studies with 3D culture and imaging can be a starting point for testing chemotherapeutic agents. The morphological changes that occur over time might serve as cues to the efficacy of the treatment. Artificial Intelligence (AI) can be used to process the data derived from such experiments to optimise drug selection, dosage, and personalise treatment schedules.

While 3D culture is an attractive system, it has its limitations. A major hurdle is reproducibility, which can be overcome by trial-and-error experiments and extensive data collection. Another challenge would be to adapt the system to a fluidic system to facilitate AI based experiments.

In conclusion, long term live cell imaging of cells in 3D culture can be a useful tool in recognising subtle changes that may have larger impact on the cellular system as a whole. When used in concert with chemotherapeutic drug testing, it can prove to be beneficial in the field of personalised medicine.

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