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Nanoscale Mechanotransduction as a Tool for the Differentiation of Glioblastoma-Derived Stem Cells in a 2D and 3D Model

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Thesis submitted for the degree of

Doctor of Philosophy

THE UNIVERSITY OF EDINBURGH

College of Science and Engineering

The University of Edinburgh

2023
I declare that the thesis has been entirely composed by myself, and the work was undertaken by myself at the University of Edinburgh, or Strathclyde University.

This thesis has not been submitted for any other degree, entirely or in part. Except where stated otherwise by reference or acknowledgement, the work presented here is entirely my own.

Signed:

Date: 1.11.23
Abstract

Glioblastoma multiforme (GBM) is one of the most common and devastating primary brain tumours. The median survival rate for this tumour is around 15 months, using the gold standard of treatment: maximal surgical resection of the tumour, followed by concomitant chemotherapy and radiotherapy. However, GBM will often return even after these treatments, and this is the case with many other types of cancer. This is believed to be due in part to the existence of cancer stem cells (CSCs) which are unaffected by chemotherapeutic agents. These stem cells can be the source of another tumour which grows after the treatment. Therefore, the question arises: how can we remove the cancer stem cells? One method could be to encourage the stem cells to differentiate, thereby reducing the likelihood of a tumour growing back.

Differentiation describes the process by which stem cells become more specialised, since stem cells maintain their capacity for self-renewal, which is lost upon differentiation into normal cancer cells. There are many signals which a cell receives from its environment, and research has shown that cells can and do respond to changes in their environment. Whether chemical or physical messages are received, the cell may respond in a variety of ways—such as migration, differentiation, hormone secretion, protein production etc. and this includes changes in the extracellular matrix (ECM) to which the cells are connected.

The aim of this project is to establish whether nanoscale mechanotransduction may be a useful tool for the differentiation of stem cells derived from GBM. This approach was used first on stem cells cultured on a flat surface i.e. 2D cell culture, and then a variety of hydrogels were examined to establish a potential model for 3D culture. This culture of GBM
stem cells in neurospheres and hydrogels was used as a 3D model to imitate a more realistic environment for the stem cells within the brain.

The 2D-cultured cells or 3D-cultured neurospheres were exposed to a variety of experimental conditions, each with or without the administration of vibrations from a bioreactor, and the response of the cells to these conditions was monitored. These tests required multiple optimisation steps, and highlighted the variety of reactions cells can have in response to changes in their environments. While it is difficult to establish to what extent cells respond to this treatment, the findings of this project hint at the potential in this approach.
Lay Summary

Glioblastoma multiforme (GBM) is one of the most common and devastating brain tumours, which does not typically respond to aggressive treatment. GBM will often return even after treatment, and this is believed to be due in part to the existence of a small population of cells known as cancer stem cells. Treating the tumour by removing the cancer stem cell population may be a viable approach to finding a cure for GBM. This can be achieved by encouraging the stem cells to undergo a process known as differentiation, which results in the loss of stem cell characteristics.

Although there are drugs which can be used to achieve this, these are difficult to administer into a tumour; particularly within the brain, which is protected by the Blood Brain Barrier. The mechanisms which induce cellular differentiation can also be activated by physical changes to the cell. Therefore, in this study, the cancer stem cells were exposed to a variety of chemical alterations to their environment, as well as physical changes through the administration of tiny (nanoscale) vibrations. Responses were measured by examining the morphology of the cells, as well as genetic changes and protein expression.

Cells are commonly cultured in a monolayer, but this does not accurately reflect the environment in which cells persist in the brain. Therefore, the cells were cultured in a 3D ball known as a neurosphere to examine the effect of the nanoscale vibrations on this model. Changes in the morphology and protein expression of these cells were measured to determine responses. The results showed that the cells did respond to nanoscale vibrations, as well as some chemical cues in their environment, resulting in differentiation and morphological changes.
Acknowledgements

I extend my heartfelt gratitude to several individuals and groups whose unwavering support and guidance have been instrumental in my PhD journey.

I am deeply indebted to my supervisor, Dr Stewart Smith, whose cheery guidance and direction in the realm of engineering were indispensable, especially in the early stages of my project when my background in biology left me feeling somewhat clueless.

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In the midst of the pressures and challenges of my PhD, I found friendship and recreation through my involvement with the Caledonian Netball Club. Playing netball and participating in tournaments and events across Scotland helped me to unwind, and keep fit! I am deeply appreciative of my teammates and friends, whose companionship provided me with much-needed distractions and motivation. Their understanding of my late arrivals due to experiments running over time was a testament to their unwavering support.
A few friends must be mentioned here: firstly, my sister Beth who is my rock and biggest, most unrelenting cheerleader! My netball family: Eadaoin, Sophie, Rachael, Nat, Emily. My housemates through the years including Prats, Henri and Eva. My pub quiz team: Jed, Claire, Duncan and Lewis. There are far too many names to mention here in appreciation.

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<th>Description</th>
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<tr>
<td>Arg-Gly-Asp</td>
<td>Arginylglycylaspartic acid</td>
</tr>
<tr>
<td>β-Catenin</td>
<td>(beta) catenin</td>
</tr>
<tr>
<td>BCNU</td>
<td>Carmustine, a chemotherapeutic agent</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenic Proteins</td>
</tr>
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<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
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<td>Central Nervous System</td>
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<tr>
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<tr>
<td>ESC</td>
<td>Embryonic Stem Cells</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal Adhesion Kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fibroblast Growth Factor-2</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma Multiforme</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>HCMV</td>
<td>Human Cytomegalovirus</td>
</tr>
<tr>
<td>hESC</td>
<td>Human Embryonic Stem Cells</td>
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<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cells</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner Cell Mass</td>
</tr>
<tr>
<td>IE-1</td>
<td>Immediate Early-1 Protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>ISS</td>
<td>International Space Station</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KPS</td>
<td>Karnofsky Performance Score</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukaemia-Inhibitory Factor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>mESC</td>
<td>Mouse Embryonic Stem Cells</td>
</tr>
<tr>
<td>MGMT</td>
<td>Methylguanine-DNA methyltransferase</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cells</td>
</tr>
<tr>
<td>NF</td>
<td>Neurofilament</td>
</tr>
<tr>
<td>NFA</td>
<td>Neurosphere Formation Assay</td>
</tr>
<tr>
<td>NIH-3T3</td>
<td>Mouse Embryonic Fibroblast 3T3 cell line</td>
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<tr>
<td>NS</td>
<td>Neurosphere</td>
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<tr>
<td>NSC</td>
<td>Neural stem cells</td>
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<tr>
<td>PASMC</td>
<td>Pulmonary Artery Smooth Muscle Cell</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly (ethylene) Glycol</td>
</tr>
<tr>
<td>PGFR</td>
<td>Platelet-Derived Growth Factor Receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-Kinase</td>
</tr>
<tr>
<td>pRB</td>
<td>Retinoblastoma Protein</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homolog</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>R-Smads</td>
<td>Receptor-regulated Smads</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras Homolog Family Member A</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-Associated Protein Kinase</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immune-Deficient</td>
</tr>
<tr>
<td>SGZ</td>
<td>Subgranular Zone</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>Human Neuroblastoma Cell Line</td>
</tr>
<tr>
<td>SOX2</td>
<td>SRY-related HMG-box genes</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular Zone</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-binding protein</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>TERT</td>
<td>Telomerase Reverse Transcriptase</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolomide</td>
</tr>
<tr>
<td>Trypsin-EDTA</td>
<td>Trypsin- Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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Aims of the Investigation

Chapter 1: GENERAL INTRODUCTION

1.1 Aims of the Investigation

The aim of this work is to establish whether nanoscale mechanotransduction can be employed as a tool to induce the differentiation of glioblastoma-derived stem cells. The study focuses on two distinct culture environments: cancer stem cells grown as a monolayer on a two-dimensional (2D) surface and those cultured as neurospheres within hydrogels, thereby creating a three-dimensional (3D) model for experimentation. The objective is to determine if nanoscale mechanotransduction can effectively trigger the differentiation of these cancer stem cells, which could have significant implications for understanding and potentially treating glioblastoma. The next steps to follow on from these in vitro experiments, would be in vivo studies. As will be discussed later in this thesis, chemotherapy drugs including Temozolomide, do not target cancer stem cells and therefore inducing the differentiation of cancer stem cells may result in a more treatable tumour.

1.2 Background of Cancer

Cancer is a very commonly occurring disease, with over 19 million incidences diagnosed, and nearly 10 million deaths reported globally in 2020\(^1\). Cancer is characterised by the unregulated growth and reproduction of cells to produce a tumour, which can form in almost every tissue of the body\(^2\). Different cancers exhibit huge differences in their characterisation and features. In a typical human body, there are trillions of cells\(^3\) which exist in a complex, interrelated fashion. The survival, proliferation and senescence of these cells are regulated by endogenous cues. Some of the ways in which these messages can be delivered to cells are shown in Figure 1.1. When this balance of life- and death- signals is maintained, the healthy
Background of Cancer

body is maintained. However, cancer arises as a disease when these behaviours are unregulated: essentially, cancerous cells proliferate, persist, and even migrate in spite of the signals from healthy cells around them.

![Chemical signalling between cells](image)

**Figure 1.1: Chemical signalling between cells**

Different types of chemical signalling cells can send and receive. This is how cells receive information from around the body. Paracrine signalling describes signals received from nearby cells, and endocrine signalling travels from elsewhere in the body, usually via the circulatory system. Autocrine signalling describes a signal released from a cell, which affects the same cell, while Intracrine signalling involves signals which are both produced and detected inside the target cell.

Despite huge amounts of cancer research, costing $24.5bn per year globally\(^4\), the mechanisms which lead to the formation of tumours is not yet unified by one theory. The aberrant behaviour of the cells within a tumour is linked to genetic mutations\(^5\). The genes in which mutations are often found leading to cancer are called oncogenes, while mutations in tumour suppressing genes can also lead to cancer\(^6\). Hanahan and Weinberg authored an important review paper which narrowed cancer cell behaviour into six defined alterations of normal physiology (Figure 1.2). They suggested that most, if not all, human cancers shared these characteristics, and that they stimulate and support the malignant growth of tumours\(^7\).
Figure 1.2: Cancer cell behaviour

An illustration of the irregular cell behaviour most, if not all, tumours demonstrate. Adapted from Hanahan and Weinberg

Growth factors and the associated signalling are essential stimulatory signals received by a cell to induce proliferation\textsuperscript{8–10}. The self-sufficiency in growth signals describes the tumour cell’s ability to proliferate in the absence of these signals from outside sources – in fact, a number of oncogenes (genes which are known to cause cancer) mimic this normal growth signalling\textsuperscript{11–14}. Furthermore, growth factor receptors are commonly overexpressed in many types of cancer, which may lead to proliferation caused by detection of ambient levels of growth factors\textsuperscript{8}. There is evidence that tumour cells can also favour the expression of extracellular matrix receptor proteins which are involved in the relay of growth signals\textsuperscript{15}. Correspondingly, cancer cells may be insensitive to the antigrowth signals which work to
maintain quiescence and block proliferation. This is achieved in a couple of ways: cells may be forced into the $G_0$ phase of the cell cycle, and able to continue proliferating in the future when appropriate signals are received; or the cells may permanently enter a post-mitotic state. When cells are in the $G_1$ phase of the cell cycle, they monitor the external environment for cues to indicate their fate—for example, the retinoblastoma protein (pRB) plays an essential role in a pathway which regulates these anti-proliferative signals. Hypophosphorylated pRB protein blocks proliferation by sequestering E2F transcription factors, and therefore disruption of this pathway allows the cell to continue through the $G_1$ phase, into $S$ phase. Cells with disrupted pRB pathways are therefore insensitive to these antigrowth factors.

**Figure 1.3:** The cell cycle and some important regulation points

Cyclin/CDK activity move the cell through the cell cycle. At regular intervals and checkpoints, the activity of Cyclin/CDK can be halted by tumour suppressor
proteins such as P21, P27, P57 and P53. This halts the cell cycle, to allow DNA repair or apoptosis, which is also mediated by P53. TGF-β can also activate P27. The early phase of G1, before the restriction point, is mitogen-dependent, meaning the constant presence of mitogens and growth factors is required to sustain the cell cycle. After the restriction point, the cycle becomes mitogen-independent. At the restriction point, Hypophosphorylated pRB is hyperphosphorylated by the Cyclin D-CDK 4/6 complex, which releases E2F and allows it to move on to downstream activation.

Cancer cells are also able to evade programmed cell death- this is a tightly regulated series of steps known as apoptosis. Apoptosis is an essential element of the cell cycle, allowing the death of cells when they are no longer needed, or have become damaged. The steps of apoptosis includes a large number of components which regulate signals for cell death, as well as those pro-apoptotic components which bring about the cell death in response to the detection of abnormalities, such as DNA damage, oncogene action and hypoxia. P53, for example, is a key tumour-suppressor protein which activates Bax, a pro-apoptotic protein to induce cell death. The importance of cell-matrix and cell-cell adhesion survival signals is well-documented, and the fact that both soluble factors and immobilised regulatory signals play an essential role in apoptosis regulation, reflects the importance of a well-maintained cell and tissue architecture.

These elements of cancer cell behaviour, taken together, explain the behaviour of cancer cells as it pertains to a lack of control in the normal regulatory mechanisms. However, further studies have discovered that cancer cells also demonstrated limitless replicative potential. Normal cells in culture will only replicate a certain number of times before undergoing cell death, but cancer cells do not exhibit this behaviour. This innate ‘clock’ is related to the presence of telomeres, which are regions of DNA that sit on the ends of
Background of Cancer

chromosomes, shortening with each cell division. In cancer cells, an enzyme called telomerase is activated which allows the restoration of the telomeres lost during cell division

Tumours are recognised in their ability to recruit their own blood supply through angiogenesis, where new capillaries grow from existing blood vessels. In healthy tissue, the process of angiogenesis is tightly regulated; normal proliferating cells do not have an intrinsic ability to promote angiogenesis. There is an ongoing balance of signals to encourage or block angiogenesis - receptors sit on the surface of endothelial cells to detect soluble factors, while integrins and adhesion molecules mediate cell-matrix and cell-cell signalling. Integrins exist in many different forms, and sprouting capillaries have been found to express the integrin subtype \( \alpha_5 \beta_3 \), with later studies also implicating the \( \alpha_6 \) and \( \beta_1 \) subunits. These cell adhesion molecules will be discussed in more detail later (Section 1.5).

Tumours can also form which are not cancerous - these are called benign tumours, clumps of cells which over which the body has lost control but do not go on to invade the rest of the body and tend to grow more slowly than malignant tumours. Furthermore, it is possible for microscopic colonies of cancer cells to exist which do not grow and develop into the disease. This is believed to be related to the lack of blood supply to these particular cells.

Metastasis and tissue invasion is another acquired capability exhibited by cancer. This is a very complex process, usually involving many types of cells and signalling molecules. In cells which have come to possess invasive or metastatic abilities, the expression of many of the cell-cell adhesion molecules (CAMs) and integrins, are altered. As well as its role in tumour angiogenesis, the \( \alpha_5 \beta_3 \) integrin subunit mediates cellular adhesion with a huge number of extracellular matrix (ECM) proteins and in the case of cancer metastasis, it has been found to be expressed in migratory cells. E-cadherin is another cell-adhesion molecule
which operates by interaction with the catenin molecules (α, β, and γ catenin) however, this function can be lost during the development of many types of cancer\textsuperscript{41,42}. This decrease in E-cadherin function has been linked to infiltrative tumour growth and metastasis\textsuperscript{41,43}.

Finally, cancer cells frequently display alterations and mutations in their genomes. In healthy cells, a huge array of proteins and enzymes work to maintain the integrity of DNA through monitoring and repair. These systems operate in a hierarchical manner, with cascades of signalling to produce the correct proteins and induce the desired outcome, i.e. DNA repair or apoptosis. However, many mutations have been identified in the DNA of cancer cells. For example, the p53 tumour suppressor protein plays an important role in DNA maintenance, and aberrant functioning of this pathway has been confirmed in most cancers\textsuperscript{44}.

The cell cycle in Glioblastoma Multiforme has been reported to behave aberrantly in a number of ways, many of which are typical in many cancers. This includes P53 mutations, mutation or methylation of pRB, and the upregulation of anti-apoptotic pathways\textsuperscript{45}.

1.3 Glioblastoma Multiforme

Glioblastoma Multiforme (GBM) is the most aggressive and commonest primary brain tumour in humans\textsuperscript{46,47}. It is classified as high grade (grade 4) by the World Health Organisation (WHO)\textsuperscript{48} and is more specifically a type of astrocytoma, since it develops from an astrocytic lineage\textsuperscript{49}. GBM is a highly heterogeneous tumour, containing lots of different cell types, including: oligodendrocytes, macrophages, astrocytes, blood vessels and stem cells\textsuperscript{50}. It also frequently contains areas of necrosis and haemorrhage\textsuperscript{51}.

Glioblastoma tumours are commonly referred to as ‘primary’ or ‘secondary’, which is dependent on their clinical presentation. Secondary tumours show malignant progression
Glioblastoma Multiforme

from a pre-existing, lower-grade tumour: Around 10% of cases develop more slowly as a progression of low-grade gliomas, over the course of 4-5 years. While primary tumours present as advanced cancers without this history of progression- Primary glioblastomas are often termed de novo, referring to the lack of precursor lesions, and these tumours can form much faster, in <3 months. The vast majority of GBM cases are primary tumours- that is, they develop by multistep tumorigenesis from healthy cells, rather than arising from a metastatic cancer elsewhere in the body.

Like most cancers, there is no confirmed singular cause for GBM, but there is speculation that human cytomegalovirus (HCMV) – a common type of herpes virus- could play a role in the development of multiple types of cancer. Evidence of HCMV antigens have been detected in a variety of cancers, including Ovarian cancer, skin cancer and GBM. Proteins associated with the HCMV infection, such as the immediate early-1 protein (IE-1) can induce either proliferation or cell cycle arrest in GBM cells.

The theory of the role of HCMV in tumour formation stems from early reports by Rapp et al., who discovered that HCMV could transform human embryonic cell lines, causing them to exhibit enhanced tumorigenicity: accelerated growth patterns, and an altered morphology. Later, Cobbs et al., discovered HCMV proteins were present in 27 of 27 human GBM samples in their study, detected with Immunohistochemistry (IHC). However, subsequent reports have varied – with some studies confirming the presence of HCMV proteins in GBM samples, while others found no significant evidence of HCMV protein or genetic material in tumour or blood samples. Nevertheless, research into the link between HCMV and cancer continues, with papers commonly referring to HCMV as an ‘oncomodulator’,
suggesting that the virus can increase the malignancy of a tumour by infecting the tumour cells.  

Median survival rates remain low, with reports of between 6-21 months with the highest rates reported in patients selected for clinical trials. However, a recent report found that the average glioblastoma patient would survive just 9.2 months. The ‘gold standard’ of treatment for Glioblastoma is known as the 1regime- thanks to a clinical trial carried out in 2005 by a research group headed by Roger Stupp whereby glioblastoma multiforme is treated with maximal surgical resection- Unfortunately, glioblastoma exhibits a highly infiltrative characteristic, making total removal nearly impossible. Therefore the surgery is followed by the administration of concomitant radiotherapy and chemotherapy with the alkylating agent, Temozolomide (TMZ), which was found to lead to significantly improved rates of survival. Alkylating agents act by transferring an alkyl group to biological molecules, including DNA, which alters its structure and can therefore affect its function. Clinicians may also opt to implant biodegradable polymers known as Gliadel intra-operatively, during surgical resection. These polymers are impregnated with Carmustine (BCNU) which is another alkylating agent. The blood-brain barrier exists to protect the neural system, but it can be a problem when a therapeutic agent is required to penetrate the brain tissue. In this case, the physical proximity of the Carmustine-impregnated polymer to the brain tissue may play a role in its effectiveness in overcoming the blood-brain barrier.

Many different elements play a role in influencing the outcome of treatment and survival in GBM patients. For example, methylation of the promoter region of O-6-methylguanine-DNA methyltransferase (MGMT) DNA- repair gene has been linked to an increased efficacy of the chemotherapeutic agent, TMZ. Since MGMT is a DNA repair protein, it can repair the
alkylation damage which is done by the chemotherapeutic drugs, thus reducing the efficacy of these drugs\textsuperscript{86}.

One study found patients with methylated MGMT gene survived for a median of 18.2 months, as opposed to patients with unmethylated MGMT genes linked to a median survival of 12.2 months, when treated with TMZ + radiotherapy\textsuperscript{87}. Another study monitored the survival rates in 33 patients who underwent the Stupp regimen, along with the implantation of Carmustine (BCNU) biodegradable polymers and concluded that patients who were treated with this method had a median survival of 20.7 months, although this was also affected by the presence of methylated vs unmethylated MGMT\textsuperscript{82}. However, other studies have not decisively found a link between increased overall survival and use of BCNU polymers, compared to the Stupp regimen\textsuperscript{88}. Clearly, the approach to treating Glioblastoma is not straightforward due to the interplay of many different factors in the tumour environment and patient characteristics including age, overall health etc.

\textit{Table 1.1: A selection of factors which influence the survivability of patients with GBM}

Results are from broad studies following patients from diagnosis to death, or from survival probabilities using the Kaplan- Meier model. Mo = Months. Degree of necrosis was established by MR image analysis, where the grade corresponds to the percentage of necrotic tissue in tumour e.g. Grade 0 = no necrosis, grade I = less than 25\% of tumour volume, grade II = 25-50\%, grade III = >50\%.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Description</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Age</td>
<td>&lt;65 yrs (13.4 mo median survival)</td>
<td>\textsuperscript{89,90}</td>
</tr>
<tr>
<td></td>
<td>&gt;65 years (8.3 mo median survival)</td>
<td></td>
</tr>
<tr>
<td>Karnofsky Performance</td>
<td>&gt;90 (14.5 mo median survival) (^{89,90})</td>
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<td>----------------------------------</td>
<td></td>
</tr>
<tr>
<td>Score (KPS Score) (^{91})</td>
<td>&lt;90 (10.1 mo median survival)</td>
<td></td>
</tr>
<tr>
<td>Extent of Resection</td>
<td>&gt;98% (13 mo median survival) (^{89})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;98% (10.1 mo median survival)</td>
<td></td>
</tr>
<tr>
<td>Degree of Necrosis</td>
<td>Grade 0 (21 mo median survival) (^{89})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grade I-III (10.1 mo median survival)</td>
<td></td>
</tr>
<tr>
<td>MGMT Methylation</td>
<td>Methylated MGMT + Stupp Regime (^{82,87})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(18.2 mo median survival)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unmethylated MGMT + Stupp Regime</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(12.2 mo median survival)</td>
<td></td>
</tr>
<tr>
<td>Tumour location</td>
<td>Peripheral tumours had a more favourable outcome than centrally located tumours (8.9 mo vs 7.1 mo median survival) (^{90})</td>
<td></td>
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</table>

The amplification of epidermal growth factor receptor (EGFR) is recognised as a common marker of GBM\(^{92}\). Increased EGFR expression, along with the upregulation of key growth factors, can lead to an increased activation of certain pathways which allow GBM cells to flourish. For example, the receptor tyrosine kinase/Ras/phosphoinositide 3-kinase (RTK/RAS/PI3K) pathway can be activated, leading to a cascade of signalling and ligand binding which results in unregulated cell growth and proliferation\(^{93}\).

It is commonly reported that a hard lump can be indicative of the presence of a tumour. In fact, the presence of a stiff fibrotic lesion is associated with a poor prognosis\(^{94}\).
This is because the Extracellular matrix (ECM) is commonly dysregulated in diseases like cancer, and the matrix surrounding a tumour has been found to exhibit elevated levels of collagen, which are encouraged to form cross-linkages through the presence of soluble mediators\(^95\), thus increasing matrix stiffness\(^96,97\). This increase in stiffness is both a result of tumour formation, and a factor which can encourage tumour progression\(^98\). Studies have linked increased matrix stiffness to the reduced activity of tumour suppressing factors such as Phosphatase and Tensin homolog (PTEN), p15 and p21, as well as increased activation of integrins regulating β-catenin, Transforming Growth Factor-β (TGF-β) and other mediators of cell invasion\(^99\). While tumours exist in the context of a stiffened microenvironment, the individual cells themselves frequently exhibit lower elastic moduli than non-cancerous cells of the same type, which may aid in migration and invasion\(^100\)–\(^102\).

### 1.4 Stem Cells and Cancer Stem Cells

Stem cells are a particular type of cell which contain the potential to differentiate into different types of cell\(^103\). They exist in a structure which is described as hierarchical, whereby the cells with the most potential (at the top of the hierarchy) are the fertilised oocyte- these cells are described as ‘totipotent’ and they are able to give rise to all cells of the embryo and the trophoblasts of the placenta i.e. the cells which support the developing embryo\(^104,105\). These cells begin to specialise around 4 days after fertilisation, forming the blastocyst and the inner cell mass (ICM) which eventually forms the embryo. The cells of the ICM are described as ‘pluripotent’ as they can give rise to almost all cells of the three germ layers, but not the supporting tissues and placenta\(^106\). Embryonic stem cells (ESC) are examples of pluripotent stem cells. As an embryo develops, these cells become more specialised i.e. the range of cells
they can give rise to is smaller, and these are known as ‘multipotent’ stem cells – for example, mesenchymal stem cells (MSC) and Neural stem cells (NSC)\(^{107}\). At the bottom of this hierarchy are ‘unipotent’ stem cells, which can also be referred to as progenitor cells, at which point the cells are committed to producing a specific cell type\(^{103}\).

Stem cells are able to proliferate by dividing to produce two daughter cells. This may be symmetrical: producing two stem cells or two differentiated cells, or asymmetrical division: producing one stem cell and one differentiated cell (Figure 1.4). Symmetric cell division is particularly utilised during development, and after injury when a rapid increase in cell numbers is favoured, while asymmetric cell division allows stem cells to maintain an ideal number of progeny of the required type\(^{108}\).

![Figure 1.4: Symmetric and asymmetric cell division](image)
Stem cells may divide symmetrically, to produce two identical daughter cells which are either both stem cells or both differentiated cells. Stem cells may also divide asymmetrically, to produce one cell of each type.

A concept which was first postulated in 1978, and has since been studied extensively, is that of the stem cell niche\textsuperscript{109}. \textit{In vivo}, stem cells have been found to exist in a specific environment known as the niche, which is necessary for the persistence and differentiation of stem cells. The niche has been found to be essential for stem cell fate determination, where the cells receive stimuli from the external environment. This stimuli can come from signalling molecules in the matrix, or from the physical contact with the matrix itself, or from cell-cell interactions. Interestingly, the niche can exist independently of the presence of stem cells. For example, when radiation treatment results in the depletion of stem cells, the niche locale itself has been found to retain the properties and functions which define it as such. This may be a strategy which allows the recruitment of other stem cells from exogenous locations to replace the lost stem cells in the specific location.

Components of the stem cell niche include:

1- Extracellular matrix (ECM) proteins which function as a scaffold linking stem cells to each other and surrounding cells of the niche

2- Vascularisation which plays a role in cell signalling as well as recruitment of exogenous cells to the niche

3- Neural inputs which can relay signals from beyond the niche, and promote the mobilisation of stem cells out of their niche

4- Stromal cells are found here which support activity within the niche
There are several different types of stem cell found throughout the body: found in the skin, epithelial stem cells are involved in the regulation of hair follicles, hematopoietic stem cells reside in the bone marrow, and neural stem cells are found in the central nervous system\textsuperscript{110}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{brain_diagram.png}
\caption{The location of stem cell populations in the adult brain}
\end{figure}

GBM is understood to arise from cellular mutations in the brain, although the mechanism for this is not yet fully elucidated. It is known that neural stem cells exist in the adult brain, with distinct populations identified in two regions of the adult brain: In the subventricular zone (SVZ) of the lateral ventricles, there exists a population of neural stem cells which can differentiate into astrocytes, oligodendrocytes and neurons\textsuperscript{113}. Another population of NSC reside in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus\textsuperscript{114} (Figure 1.5).
Since gliomas often arise in locations which are contiguous to the SVZ, it has been postulated that these germinal regions could be the source of the tumour\cite{115,116} – this is further compounded by the fact that they often express stem-cell markers, such as Nestin\cite{117–119}. Studies carried out in animal models have confirmed that there is an increased susceptibility to chemically- or virally- induced oncogenesis in areas of the brain which contain stem cell populations\cite{120,121}. Furthermore, while there is evidence that glioma may develop from germinal cells in the SVZ, studies have shown that progressing tumours can lose this physical connection to their initial location, without clear evidence of their neoplastic germline history\cite{122}.

In solid tumours, including brain tumours\cite{123,124}, a different type of stem cell has been discovered- these are known as cancer stem cells (CSCs). The first modern study which evidenced a stem cell population inside a tumour was published in 1994, in which a number of tumour cells from human acute myeloid leukaemia were transplanted into severe combined immune deficient (SCID) mice. The cells proliferated extensively, and the mice presented with clinical signs comparable to the original cancer.\cite{125} This technique remains the preferred method for the isolation of cancer stem cells\cite{126}. The cancer stem cells can be identified by the expression of cell surface markers such as CD-24, CD44- and CD-133,\cite{127,128} although these can be unreliable markers, as they are not expressed equally and dependably even within different examples of the same cancer\cite{129}.

There are similarities between tumours and stem cells, as tumours can have the ability to continually renew and persist, in a similar manner to stem cells. This suggests that there could be similar mechanisms controlling the regulation of both of these cell types. For example, the Wnt signalling pathway has been shown to regulate both self-renewal and oncogenesis\cite{130},
while Notch and Sonic hedgehog (Shh) signalling pathways, which are essential signalling pathways associated with stem cell self-renewal, are also found to play a role in oncogenesis when their function is dysregulated.

It has been suggested that cancer stem cells are less dependent on the stem cell niche which, as previously discussed, constitutes a significant area of importance for healthy stem cells. This is based on the hypothesis that cancer stem cells may arise from a mutation of healthy stem cells, allowing them to develop a self-sufficient state of cell proliferation. In fact, several studies have linked the establishment of new glioma stem cells populations to therapeutic doses of TMZ and ionizing radiation, suggesting that the cancer treatment itself could encourage de-differentiation.

As previously described, the stem cell niche plays a significant role in determining the characteristics which are displayed by stem cells. Specific changes in this environment can alter stem cell fate, proliferation, gene expression and induce quiescence or apoptosis. Changes in this environment include chemical alterations such as signalling molecules, or changes in the molecular makeup or even stiffness of the surrounding tissue.

1.5 Stem Cell Differentiation

The differentiation of stem cells is an essential part of development, growth and wound healing. A huge number of signalling molecules are involved in the highly complex organisation of this system. When stem cells are cultured in vitro, a number of factors must be included in the environment in order to maintain their stemness, and these essential factors can vary depending on the origin and type of the stem cell. For example, mouse embryonic stem cells (mESC) may be maintained in their stem-state when cultured in media containing leukaemia-inhibitory factor (LIF), Foetal calf serum (FCS) or Bone Morphogenic
Proteins (BMP)\textsuperscript{134}. Conversely, human embryonic stem cells (hESC) do not depend on LIF\textsuperscript{135}, but can be maintained in serum-free media containing basic Fibroblast Growth Factor (FGF-2). Removing the stem cells from the environments which contain these factors, may then result in stem cell differentiation. Differentiation can also be actively induced by the inclusion of specific differentiation factors in the media, or by physically altering the cell culture environment.

Neural stem cells (NSC), a type of multipotent stem cell, can go on to produce neurons, astrocytes and oligodendrocytes\textsuperscript{136}. Isolating NSC from healthy brain tissue or a brain tumour such as GBM, can be carried out in a few different ways. A common method is the neurosphere formation assay (NFA) which was first described by Reynold and Weiss in 1992\textsuperscript{113}, whereby a population of cells from the central nervous system (CNS) were plated in serum-free culture media containing EGF and FGF-2 on a non-adherent surface, and a sphere of proliferating cells formed. This sphere can be disassociated into individual cells which can then be re-plated, and another neurosphere will form as the cells proliferate under these conditions\textsuperscript{113}.

There are a number of genetic and protein markers which can be specifically used to identify NSC and brain tumour CSC, such as Nestin\textsuperscript{137}, SOX2\textsuperscript{138} and CD133\textsuperscript{139}. However many tumours, including GBM, contain a heterogeneous population of cells- some cells contain multiple nuclei, and the tumours often demonstrate hypercellularity and areas of necrosis\textsuperscript{140}. This heterogeneity also applies to the stem cells within, and therefore identification of CSCs using markers alone is not a reliable method for stem cell isolation\textsuperscript{141} and the observation of cell behaviour in neurosphere (NS) formation is the preferred method of identification.
In order to maintain NSC and brain tumour CSC in vitro, the cells must be cultured in stem cell expansion media, which is serum-free and contains EGF and FGF-2. It may also contain additional nutrients such as B27 and N2 which aid cell expansion. The addition of serum or retinoic acid, or the withdrawal of growth factors from the media can result in differentiation\textsuperscript{142}. Cells may then express glial or neuronal markers, and the expression of stem cell markers will be restricted.

The regulation of differentiation signals is very complex, with the same signalling molecules playing different roles in different pathways to mediate control. These may encourage proliferation of stem cells in some conditions, while encouraging differentiation in others. Bone Morphogenetic Proteins (BMPs) are a member of the larger family of proteins known as transforming growth factor beta (TGF\(\beta\)), which function by binding to and activating type I and type II serine/threonine protein kinase subunits, and are embedded in the cell membrane. This is demonstrated in Figure 1.6 below. The formation of a BMP-type I/II receptor complex activates the type I receptor kinase domain, leading to the phosphorylation of Smad proteins\textsuperscript{143}. Different Smad proteins (of which eight types have been identified) are associated with a variety of downstream effects\textsuperscript{144}. For example, receptor-regulated Smads (R-Smads) interact with the type I receptor and are phosphorylated upon its activation. These phosphorylated R-Smads bind with Smad-4 which translocates to the nucleus to induce a target gene. Different members of the TGF-\(\beta\) superfamily can activate type I receptors, and each family member causes the activation of different R-Smads. It is also able to signal through Smad-independent pathways, using MAP kinases (MAPK) which can affect ERK, JNK, p38 and cause them to have a downstream effect on target genes.
Figure 1.6: The BMP-mediated SMAD/MAPK pathways

Activation of these pathways leads to increased expression of target genes. BMP forms a homodimer, and then activates the type I/II receptors. This can be inhibited by Noggin. The type II receptor is induced to phosphorylate the type I receptor, which in turn phosphorylates R-Smads. BMP4 is linked to the activation of R-Smads-1, -5 and -8\textsuperscript{145}. This activated R-Smad forms a heterodimer with another R-Smad and Smad4, which translocates to the nucleus to affect target gene expression. The MAPK pathway can also be activated by the phosphorylated type I receptor, which in turn activates ERK/ JNK/ p38 to affect target gene expression\textsuperscript{146}.

BMP4 has been implicated in a wide variety of cell types and stages of development, including the brain\textsuperscript{147}. It was first identified by its role in bone formation, and has since been confirmed as a mediator of hematopoietic stem cell lineage specification\textsuperscript{148–150} and neural
stem cell lineage specification\textsuperscript{151}. This is demonstrated in Figure 1.7 below. As a mediator of neural stem cell specification, BMP4 plays multiple roles; it can work alongside Smad, STAT and MAPK (Mitogen-activated Protein Kinase) pathways to encourage differentiation down a glial route, while inhibiting oligodendrocyte formation. Another commonly used chemical mediator of astrocytic differentiation is ciliary neurotrophic factor (CNTF). In fact, inhibition of the function of STAT3 has been shown to cause a total block on CNTF-mediated astrocytic differentiation\textsuperscript{152}. 

\textbf{Figure 1.7: The role of BMP4 in neural stem cell differentiation}

\textit{BMP4 has been found to play a role in astrocyte and neuron production, as well as inhibiting oligodendrocyte production.}

Stem cells isolated from the CNS have the potential to differentiate into three cell lineages including neurons, astrocytes and oligodendrocytes. These stem cells can be
maintained and expanded in cell culture media containing FGF-2\textsuperscript{153} and EGF; the removal of these factors from the cell culture media normally results in differentiation\textsuperscript{142,154}.

### 1.6 Interaction with the Cellular Environment

It is well established that the systems of the body must tolerate and adapt to mechanical forces acting upon them. For example, arteries have thicker and more stretchy walls than veins, since they transport blood of a higher pressure\textsuperscript{155}, while the bone density of astronauts living in space changes dramatically when they are living under earth’s gravity vs the ISS\textsuperscript{156}. On a microscopic level, embryonic development is dependent on sensing mechanical forces. The mechanisms which regulate and determine organ size and shape are not fully understood, but it is not solely encoded in our genes, with evidence that circulating factors as well as biomechanical signals dictate their potential\textsuperscript{157–160}. In fact, studies suggest that the physical stretching of the cells leads to translocation of transcription factors necessary for continued early development of embryos\textsuperscript{161,162}.

Regulation of cell growth and proliferation relies on the activation of the necessary signals within a cell, and this activity is regulated by the cues which are detected by the cell. A well-researched phenomenon known as mechanotransduction, explains that the physical environment in which a cell resides has a significant impact on the behaviour and characteristics of that cell\textsuperscript{163}. The cell is both responsive to, and responsible for, physical changes in its local environment. This applies to all cells, including stem cells and cancer stem cells.

Hematopoietic Stem Cells (HSC) can differentiate into blood cell types, as well as a number of solid tissues such as epithelial cells. A comparison of nuclear compliance between HSC and primary human fibroblasts found a 2.2 fold reduction in compliance (i.e. increased stiffening)
of the differentiated cell type\textsuperscript{164}. This complements findings from other studies which suggest that the intracellular tension of stem cells increases upon differentiation\textsuperscript{165–167}, since it is well established that there is extensive structural reorganisation within the nucleus during differentiation.

\textit{Figure 1.8: Diagram of the structures within a cell which contribute to the cytoskeleton}

The cell membrane surrounds the structures of the cell. The intermediate filaments support the structure of the cell, and attach to organelles such as mitochondria and endoplasmic reticulum, to maintain their location within the cell. Microtubules can be used to transport signalling molecules throughout the cell. Microfilaments (such as actin) allow the cell migrate, change shape and perform its function.

The way in which mechanical signals are relayed and received by cells through the intracellular and extracellular environment is complex and well understood. Cells typically experience forces from compressive pressure\textsuperscript{168}, mechanical stretch and shear flow\textsuperscript{155}. This is detected and regulated by the cytoskeleton, which is a dynamic array of proteins within the cell that play an essential role in cell mechanics (\textit{Figure 1.8}). The cytoskeleton is comprised of microtubules, intermediate filaments and actin filaments, and these proteins are essential in
orientating the cells, altering their architecture and linking the cell to the extracellular environment.

Intermediate filaments are so named because their diameter (about 10nm) is between that of microtubules (about 25nm) and actin filaments (about 7nm). They provide structural support to the cell and help it to resist mechanical stress by providing mechanical strength to the cells, and tissues as a whole\textsuperscript{169}.

\textit{Table 1.2: Intermediate proteins of eukaryotic cells}

\textit{Names and locations of examples of the six types of intermediate proteins (I – VI). GFAP is discussed in more detail in section 3.3, as a marker for astrocytes. Table adapted from\textsuperscript{169}}

<table>
<thead>
<tr>
<th>TYPE</th>
<th>PROTEIN</th>
<th>EXPRESSION LOCATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Acidic keratins</td>
<td>Epithelial Cells</td>
</tr>
<tr>
<td>II</td>
<td>Neutral or basic keratins</td>
<td>Epithelial Cells</td>
</tr>
<tr>
<td>III</td>
<td>Vimentin</td>
<td>e.g. Fibroblasts, white blood cells</td>
</tr>
<tr>
<td></td>
<td>Desmin</td>
<td>Muscle cells</td>
</tr>
<tr>
<td></td>
<td>Glial Fibrillary Acidic Protein (GFAP)</td>
<td>Glial cells</td>
</tr>
<tr>
<td></td>
<td>Peripherin</td>
<td>Peripheral neurons</td>
</tr>
<tr>
<td>IV</td>
<td>Neurofilament (NF) proteins</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NF-L (Light)</td>
<td>Neurons</td>
</tr>
<tr>
<td></td>
<td>NF-M (Medium)</td>
<td>Neurons</td>
</tr>
<tr>
<td></td>
<td>NF-H (Heavy)</td>
<td>Neurons</td>
</tr>
</tbody>
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What is Mechanotransduction?

<table>
<thead>
<tr>
<th></th>
<th>α-Internexin</th>
<th>Neurons</th>
<th>Nuclear Lamins</th>
<th>Nuclear lamina of all cell types</th>
<th>Nestin</th>
<th>Stem cells of central nervous system</th>
</tr>
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</table>

Microtubules are rigid and hollow, composed of a single type of protein called Tubulin, a dimer of α-tubulin and β-tubulin. They can rapidly shrink and grow with the addition and removal of dimers at the ends of the polymer, allowing the well-organized alteration of cell shape\textsuperscript{170,171}. Since microtubules stretch from one end of the cell to the other, they act like a system of railroads, creating an efficient platform for the transport of molecules around the cell\textsuperscript{172}. This is particularly important in neurons, which rely on these ‘railroads’ of microtubules to move molecules along the axon\textsuperscript{173}. They are also involved in spindle formation in mitosis\textsuperscript{174}, and they can extend outside the cell in the form of cilia- to move molecules outside the cell; and flagella- to move the cell itself, in the case of sperm cells\textsuperscript{175}.

Actin filaments work alongside a protein called myosin to create movement in cells- such as muscle cells to create movement\textsuperscript{176}, and they are also involved in mitosis, aiding in the physical splitting of the daughter cells\textsuperscript{177}. Actin and microtubules are both key players in molecular signalling within the cell, including the transduction of signals from outside the cell. Actin couples with cell adhesion molecules (CAMs) such as cadherin via the anchor proteins α-catenin and β-catenin, to coordinate with the extracellular environment\textsuperscript{178}.

1.7 What is Mechanotransduction?

CAMs bind to a variety of ligands in the ECM, or on other cells to form a cell-cell adhesion\textsuperscript{37}. There are five main ‘families’ of CAMs which include many different types of
What is Mechanotransduction?

receptors allowing the cell to interact with its extracellular environment, and neighbouring cells. These families are: integrins, selectins, cadherins, Immunoglobulin Superfamily CAMs and Mucins. Additionally, vascular adhesion proteins are enzymes which are involved in cell adhesion\textsuperscript{37}. The translation of mechanical forces from the ECM into intracellular messages is known as mechanotransduction.

Mechanoreciprocity is the term used to describe the cell’s response to the detection of mechanical signals: it maintains homeostatic tension within the cell through the exertion of a reciprocal force generated by the cell\textsuperscript{96}. Cells can adhere to surrounding cells using intercellular adhesion complexes such as the cadherin/ catenin complexes, which are reliant on the actomyosin cytoskeleton.

Early research into mechanotransduction focused on sensory cells, but as the understanding of this mechanism expanded, it became clear that mechanotransduction signalling plays a crucial role in a huge range of cellular functions throughout the body\textsuperscript{179}. Forces in the extracellular environment have been found to influence cell proliferation, gene expression and cytoskeletal organisation.
What is Mechanotransduction?

Figure 1.9: A representation of the alpha- and beta- subunits of the Integrin family

Arg-Gly-Asp (RGD) describes the cell attachment site for many proteins found in the ECM, blood and cell surface\textsuperscript{180,181}. RGD receptors also found in fibrin. Image from \textsuperscript{180}

While the specific signalling pathways involved in this process of mechanotransduction are not yet fully understood, it is widely accepted that the transmembrane proteins called integrins are involved in the transduction of signals from the extracellular matrix (ECM) to the intracellular environment, as they are the site of focal adhesions. The ECM comprises tissue-specific proteins, such as laminin, collagen and fibronectin\textsuperscript{15}. Integrins play a crucial role in the relay of signals from the ECM to the cell. They consist of an alpha and beta subunit- the integrin family contains 18 alpha subunits and 8 beta subunits which combine to form 24 heterodimers, which bind specifically to particular ligands (Figure 1.9)\textsuperscript{180}. 

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As integrins bind to the ECM, they promote the reorganization of actin filaments which co-localize with the integrin. This clustering of actin filaments leads to the formation of large stress fibres, which encourages further integrin binding to the matrix in a positive feedback system. This results in an aggregation of ECM proteins, integrins and cytoskeletal proteins collectively known as focal adhesions\textsuperscript{182}. Integrins activate a multitude of intracellular protein tyrosine kinases, including focal adhesion kinase (FAK) and Src-family kinases\textsuperscript{183}. Activation of FAK leads to a cascade of signalling linked to increased cell migration, and regulation of cell proliferation and survival. Multiple studies have demonstrated an increase in Wnt/\(\beta\)-catenin signalling which is associated with the normal response of cells to mechanical stimuli\textsuperscript{184,185}.

![Signalling pathways associated with focal adhesions and chemical signals](image)

\textit{Figure 1.10: Signalling pathways associated with focal adhesions and chemical signals}
A simplified diagram of the interaction and overlap of signals which are received from mechanical signals in the focal adhesion via the membrane-bound integrin receptor (α and β subunits in yellow/green), and chemical signalling relayed through receptors, such as EGFR and PGFR. ERK and JNK can also be activated by the MAPK pathway, which itself can be activated via several different receptors, including BMP type I receptor (See Fig. 1.6)

1.8 Culturing Cells on Different Surfaces

The important role of mechanical cues on cell behaviour has been investigated using different types of biomaterials for in vitro studies. Since it has been established that mechanotransduction can alter cellular signalling, it follows that mechanotransduction can induce cells to migrate, change shape or undergo differentiation. The physical properties of the ECM, tissue and individual cells varies depending on location around the body, and this has been exploited experimentally to explore the effect of changing this environment on cell behaviour\textsuperscript{186–189}.

The ’stiffness’ of materials used in cell culture is a broad term, and therefore the particular characteristic which is being discussed must be made clear. Generally, stiffness describes the resistance of a material to deformation when force is applied\textsuperscript{190}. These deformations may be caused by different types of pressure- for example, tensile, compressive, shear and torsion. Young’s Modulus is commonly used to characterise stiffness, this is calculated using a stress-strain curve of the elastic modulus measured at low strain values\textsuperscript{191}. Where tissue stiffness is discussed in this paper, this refers to the Young’s modulus, unless otherwise stated. This topic is further described in Chapter 4.

A variety of surfaces have been employed experimentally to investigate the effect of the stiffness of the microenvironment on cell behaviour, including fibronectin\textsuperscript{192}, laminin\textsuperscript{193}, Poly
Culturing Cells on Different Surfaces

(ethylene) Glycol (PEG)\textsuperscript{194} and Matrigel\textsuperscript{195}, to name just a few. The stiffness of these and other surfaces can be altered by adjusting the concentration or cross-linking within the substrate.

Basement Membrane is a thin but dense layer of ECM which surrounds most human tissues including epithelial tissue, nerves, fat and muscle cells, and it forms a barrier to separate different types of cells\textsuperscript{196,197}. Basement membrane can be detected soon after embryogenesis, and its main role is to provide structural support to cells, helping them to attach to other tissue components\textsuperscript{198}. This also highlights its essential role in regulating cell behaviour, as it relates to the formation of focal adhesions and mechanotransduction. Furthermore, the basement membrane acts as a barrier between tissues to prevent tumour cell invasion\textsuperscript{199}. When culturing cells \textit{in vitro}, plastic or glass cultureware may require the addition of a basement membrane coating in order for the cells to survive and proliferate.

Fibroblasts are a type of cell which are particularly known for secreting ECM proteins\textsuperscript{200}. Studies have demonstrated that mouse fibroblasts (NIH-3T3 cell line) cultured on a surface of varying stiffness, will favourably migrate towards the stiffer substrate (30 kPa vs 14kPa), where they showed increased spreading\textsuperscript{201}. Furthermore, fibroblasts which were cultured on a soft substrate exhibited increased rates of motility, and irregularly shaped and dynamic focal adhesions\textsuperscript{202}. Protein tyrosine phosphorylation is an essential aspect of cytoskeletal reorganisation and cell spreading mechanisms\textsuperscript{203}, and the administration of a tyrosine phosphatase inhibitor to these fibroblasts cultured on soft substrates resulted in the formation of normal focal adhesions\textsuperscript{202}.
It has been observed that cells exhibit altered morphology, lineage specification and rate of proliferation when they are cultured on a surface of tuneable mechanical properties. The range of stiffnesses of some cell types are described in Figure 1.11 above. Engler et al. cultured MSCs on substrates of varying stiffnesses designed to mimic specific regions of the body: brain (0.1 – 1kPa), muscle (8 - 17kPa) and bone (25 - 40kPa). Their study found that the cells were morphologically comparable to the native tissue after one week in culture\textsuperscript{186}. MSCs will typically differentiate into fat, muscle, cartilage and bone\textsuperscript{207,208}. Several studies have gone on to report that MSCs can begin to express neural phenotypes, when they are transplanted into a neural environment\textsuperscript{209–211}, or cultured on surfaces which are similar to brains in their mechanical property (\textasciitilde 0.1 – 1kPa elasticity)\textsuperscript{186}. However, these conclusions have been disputed and refuted in some other studies, which have failed to replicate these results, and
it is widely accepted that the expression of neural markers was due to a stress response, rather than true neural differentiation\textsuperscript{212-215}.

\textit{In vitro}, cells cultured on top of soft gels which are not highly cross-linked (~1kPa) exhibit dynamic and diffuse adhesion complexes, while cells cultured on stiff substrates (>30kPa), exhibit very stable and static focal adhesions\textsuperscript{202}. Since the formation of Focal Adhesions requires the cluster of integrins to stably bind to the ECM, this is difficult in soft ECMs as the integrins are more easily internalised and therefore struggle to bind with proteins of the ECM\textsuperscript{165}.

Several studies have examined the effect of cell shape on lineage commitment, based on the signals induced by forces that arise inside a cell due to the activity of the cytoskeleton\textsuperscript{165,166,216}. For example, studies found that MSCs which were cultured on a flat surface and allowed to adhere to islands of fibronectin, forming a flattened shape, underwent osteogenesis. Conversely, cells which were allowed to form a round shape, became adipocytes. This was found to be regulated by RhoA/ ROCK signalling, whereby RhoA-mediated signalling was dependent on cell-shape, while ROCK signalling induced osteogenesis regardless of the cell shape\textsuperscript{217}. RhoA activation leads to an increase in myosin, and thus an increase in acto-myosin contractility, increasing intracellular tension\textsuperscript{218}.

Another particularly interesting study looked at culturing MSC on ‘islands’ of fibronectin, which were prepared in different shapes of the same area. These fibronectin shapes were pentagonal and resembled either normal pentagons, flowers, or stars with softer or sharper points at the tip of each shape, and straight or more curved lines between the points. No chemical cues for differentiation were included in the cell culture media. The study found that MSC cultured on the fibronectin in a ‘flower’ shape- with softer edges- led to an increased
percentage of differentiated adipocytes, while the ‘star’ shape – with pointier edges - led to an increased percentage of differentiated osteoblasts. The same number of cells cultured on the normal pentagon shape differentiated into an approximately equal number of each cell type\textsuperscript{166}. The study also discovered an increase in cytoskeletal organisation in cells cultured on star-shaped fibronectin as opposed to the flower-shaped fibronectin, confirming the link between cell shape and lineage commitment with the addition of chemical blockers which allowed the cell to spread outside the specified shape, and found that the cells overwhelmingly differentiated towards an osteogenic lineage\textsuperscript{166}.

### 1.9 The Effect of Physical Stimulation on Cells

In previous sections, the effect of culturing cells on surfaces of varying mechanical properties have been discussed, and the cellular mechanisms which are involved in their response have been explored. Therefore this section will investigate the effect of physical stimulation on cells, including but not limited to, nanoscale vibrations.

One study carried out at the University of Strathclyde found that, using a mesenchymal stem cell model, cells have been found to differentiate towards osteoblasts following exposure of these cells to physical stimulation on the nanoscale. In this study, a piezoelectric actuator was used to administer specific vertical displacement to an attached petri dish containing the MSC. The actuator was tuned to 10 or 20 V driving potential, at 1 kHz frequency, to give a displacement of between 5-30 nm. This stimulation has been found to activate ROCK/Rho\textsuperscript{219} and the canonical Wnt pathway, involving downstream activation of β-catenin, and it also increases the expression of other mechano-dependent markers such as integrins, vinculin and paxillin\textsuperscript{184,185,220}. The increased expression of several markers of osteoblasts was associated with this mechanical stimulation\textsuperscript{221}. The purpose-built ‘bioreactor’
which was used in these experiments, was designed and built at the University of Strathclyde. This ‘bioreactor’ featured a tuneable frequency of vibration on the surface, and is an earlier iteration of the equipment used in this project. Further information about this equipment can be found in Chapter 4.

Like other adhesion-dependent cells, NSC, neuronal and glial cells, are responsive to changes in their local microenvironment. In a normal human brain, the ECM has a variety of stiffnesses, ranging from around 0.1-1.2 kPa\(^2\). The stiffness of brain tumours is similarly heterogeneous, within a tumour and between types of tumour\(^2\), with a reported modulus of around 0.17 – 16 kPa\(^2\). Human glioma was found to be between 1.35 – 4.15 kPa\(^2\).

Comparisons of the behaviour of neural cells when they are cultured on surfaces of different stiffnesses, paints a complex picture of cell fate and organisation. For example, neurons which were cultured on a softer (~230 Pa) vs stiffer (~550 Pa) substrate, exhibited a higher rate of branching\(^2\). Neurons also exhibit increased neurite length when cultured on softer substrates (50 Pa vs 550 Pa) while glial cells cultured on this substrate failed to thrive\(^2\). It has been established that astrocytes are preferentially cultured on stiffer substrates\(^2\). Glioma Cells which were cultured on a stiff substrate (119 kPa) proliferated 5x faster than those cultured on a softer substrate (0.08 kPa)\(^2\). Interestingly, co-cultures of neurons and astrocytes can successfully proliferate together on substrates which are stiffer than that which normally allow the successful growth of neurons alone. In this case, the mechanical and chemical relationship between the neurons and astrocytes in the culture appears to be essential in the maintenance of the healthy neuronal culture. Glial cells are known to release soluble factors which support healthy neuronal growth\(^2\), and it has been established that cell-cell interaction also plays an important role in cell maintenance.
Mechanically-induced differentiation of cells can be achieved by physically stretching cells, which is typically achieved by inducing a slight bend in a scaffold to which a cell is attached. This displacement results in a ‘tightening’ of the cell membrane, and osteogenic differentiation of MSCs has been achieved using this method\textsuperscript{229}. While the physical environment is clearly influential, the chemical environment can also play a role in these interactions- for example, the osteogenic commitment of bone marrow cells can be strictly mediated by the presence of dexamethasone, with concentrations of 100nM found to be sufficient to suppress osteogenic lineage commitment entirely\textsuperscript{230}.

The effect of electromagnetic fields (EMF) is also an interesting and relevant area of research, which considers the effect of EMF fields, which can arise \textit{in vivo} as a natural consequence to the physical displacement of bone tissue associated with movement, and purpose-built EMF bioreactors, which aim to investigate the impact of these effects on cells \textit{in vitro}\textsuperscript{231}.

Historically, the majority of research looking into the effect of surface stiffness and nanoscale vibrations on cell behaviour has been directed at MSC. In recent years however, some groups have considered the effects of these applications on NSC. Recently, a study compared the effect of two different vibrational frequencies (40 Hz vs 100 Hz) on cells from an established Neuroblastoma cell line, SH-SYSY, which can differentiate into neurons. Using a purpose-built vibrational bioreactor, the cells were exposed to these frequencies for 8hrs/day for 5 days. Their results suggest that 40 Hz was the optimal frequency to encourage neurite length, while the neurite length was comparable to unstimulated cells when exposed to the 100 Hz frequency. Neurite density was unaffected by the change in vibrations\textsuperscript{220}. These findings implicate specific frequencies in having distinct impacts on cells.
As was briefly described previously, the native cellular environment exposes cells to a variety of stresses and strains, including shear flow from the movement of fluids, and physical strain on bones, muscles and blood vessels. Therefore, a multitude of approaches must be employed to examine the effect of these environments on cell behaviour. Fluid flow has been found to induce cellular differentiation using rotating bioreactors, in which cells are seeded inside a rotating tube, which allows a flow to be produced through the media surrounding the cells. This has been found to induce osteogenic differentiation in MSCs\textsuperscript{232}. A similar concept is the idea of a perfusion bioreactor, which physically pushes the media around the cells, which has also been found to induce osteogenic differentiation\textsuperscript{233}.

**1.10 Thesis Summary and Aims**

**1.10.1 Summary**

This thesis is structured into six chapters, each serving a specific purpose within the research framework. Here's a brief overview of the content in each chapter:

**Chapter 1 – Introduction**

This chapter introduces cancer with a focus on Glioblastoma, it delves into various aspects of the disease, its treatments, and the challenges associated with managing, treating, and improving survival rates.

The cancer stem cell theory is introduced, which posits the existence of a distinct population of stem cells within tumours. These stem cells are often not effectively targeted by conventional cancer treatments, contributing to the persistence and recurrence of the disease. Furthermore, the chapter explores the intricate interplay of chemical and mechanical signalling within tissues, cells, and the tumour microenvironment. It highlights how these
signalling mechanisms influence the behaviour of cells, including cancer cells, shedding light on the complex factors that govern tumour development and progression.

**Chapter 2 – General Methodology and Materials**

A full description of the materials used, pertaining to common techniques across chapters, the methodology employed for each technique and the experimental design.

**Chapter 3 – Nanoscale mechanotransduction in a 2D model**

The experimental design in this study begins with an initial optimization phase. The primary focus is to explore the influence of mechanotransduction on cells cultured in substrate-coated culture-ware. Data is collected through qPCR and microscopy analysis to comprehensively investigate cellular responses and behaviours, providing crucial insights into the effects of mechanical signalling on cultured cells.

**Chapter 4 – Establishing a 3D microenvironment**

This chapter explores the versatility of hydrogels for cell culture. It begins by introducing different types of hydrogels and then delves into their optimization for the experimental setup. Utilising rheological measurements and laser interferometry, these techniques ensure that there is efficient signal transduction within these materials. Moreover, the study investigates methods for cell retrieval from the hydrogels, ultimately determining the most suitable material for conducting 3D cell culture experiments.
Chapter 5 - Nanoscale mechanotransduction in a 3D model

This chapter provides a comparative analysis of 2D and 3D cell culture environments, examining their influence on mechanotransduction, drawing from prior experiments. It includes the culture of neurospheres within hydrogel matrices and investigates their viability in this unique environment. Furthermore, the research explores the impact of subjecting these embedded neurospheres to nanoscale mechanotransduction. Data is obtained through histological stains, immunohistochemistry, and confocal microscopy.

Chapter 6 – General Discussion

A summary of findings across each chapter, outcomes and the potential applications for this work in the future

1.10.2 Aims

The overall aim of the work is to establish whether nanoscale mechanotransduction can induce the differentiation of glioblastoma-derived cancer stem cells cultured on a 2D surface or as neurospheres cultured in hydrogels to provide a 3D model. In the case of MSCs, previous research has examined how variations in surface stiffness and topography influence stem cell fate. This study builds upon that foundation by investigating the impact of nanoscale vibrations on lineage specification. In contrast, the initial research on NSCs has primarily focused on how different surface properties, such as stiffness and topography, affect these cells. However, the specific influence of nanoscale vibrations on lineage specification within Glioblastoma-derived stem cells remains uncharted territory.
The overarching goal of this thesis is to address this knowledge gap by exploring the effects of nanoscale vibrations on the differentiation and fate of Glioblastoma-derived stem cells. By doing so, this research aims to provide valuable insights into the potential applications of mechanotransduction in the context of Glioblastoma research and its implications for cancer treatment.
Chapter 2: GENERAL METHODS AND MATERIALS

2.1 Cell Culture

Cancer Stem Cells extracted from Glioblastoma multiforme were isolated to produce an established stem cell line, known as M-cells (Provided by Dr Paul Brennan), received at Passage 10 (P10). These cells exhibit typical neural stem cell morphology, with stem cell markers such as Nestin and SOX2. Over the length of the entire study, the phenotype was confirmed by morphological assessment, as well as staining for stem cell markers. M-cells were used between Passage 15 (P15) up to a maximum Passage 28 (P28). All cell culture work was performed in a class II laminar flow biological safety cabinet to ensure sterility. Surfaces and equipment was decontaminated before use with 70% industrial methylated spirit (IMS) (Genta Medical, York, UK). Cell culture media was prepared in this sterile environment, and the added regents were sterile-filtered using a 0.2µm syringe filter (Sartorius Stedim Biotech, Goettingen, Germany). DPBS (Dulbecco’s Phosphate buffered saline; Gibco, Thermo Fisher Scientific, Cramlington, UK) was only used in the laminar flow cabinet.

For cell culture, T25cm³ or T75cm³ vented cell culture-treated polystyrene flasks (Greiner Bio-One Cellstar, Fisher Scientific) were coated with Growth factor-reduced Matrigel® (Corning Life Sciences, Amsterdam, Netherlands) diluted 1:80 in refrigerated DMEM-F12 (Gibco), as directed by the manufacturer. 100 µl Matrigel® was thawed at room temperature (RT) for 1-2 minutes before mixing with 7.9ml DMEM-F12, in accordance with the manufacturer’s instructions. This was added to the flask (5 ml in T75cm³, or 2.5 ml in T25cm³) which was gently tilted to fully coat the bottom of the flask, avoiding the formation of bubbles. The flask was then incubated at 37°C in 5% CO₂ [v/v] for at least 30 minutes. After
this time, the media was aspirated and the flask was washed twice with Phosphate Buffered Saline (PBS; Gibco).

2.1.1 Cell Culture Media

2.1.1.1 Full Expansion Media

Stem cell culture media (henceforth referred to as Full Expansion Media) was prepared using Advanced DMEM-F12 (Gibco), supplemented with 0.5% L-glutamine (Gibco), 1% 200mM Amphotericin B (Gibco), 1% B27 (Gibco), 0.5% N2 (Gibco), 1% Penicillin-Streptomycin (Sigma-Aldrich) and 0.5% Heparin (Sigma-Aldrich) with the addition of the following growth factors: Recombinant Human FGF-basic (Peprotech, Thermo Fisher Scientific, Cramlington, UK) and Recombinant Human EGF (Peprotech) at 10ng/ml each. When culturing cells in flasks for expansion, 50% of the full expansion media was changed every 2-3 days, and cells were passaged at confluence, approximately 5-7 days. Full Expansion Media was stored in the fridge (4°C) for up to two weeks, or in the freezer (-20°C) for up to 4 weeks. This was prepared in a class II laminar flow biological safety cabinet to ensure sterility.

2.1.1.2 Full Expansion Media without growth factors

Stem cell culture media without growth factors was prepared using Advanced DMEM-F12 (Gibco), supplemented with 0.5% L-glutamine (Gibco), 1% 200mM Amphotericin B (Gibco), 1% B27 (Gibco), 0.5% N2 (Gibco), 1% Penicillin-Streptomycin (Sigma-Aldrich) and 0.5% Heparin (Sigma-Aldrich). Media was stored in the fridge (4°C) for up to two weeks, or in the freezer (-20°C) for up to 4 weeks. This was prepared in a class II laminar flow biological safety cabinet to ensure sterility.
2.1.1.3 DMEM/F-12 +BMP4

Advanced DMEM-F12 (Gibco) was prepared with the addition of BMP4 (Gibco) to produce a final concentration of 20ng/ml, 1% 200mM Amphotericin B (Gibco) and 1% Penicillin-Streptomycin (Sigma-Aldrich). Media was stored in the fridge (4°C) for up to two weeks, or in the freezer (-20°C) for up to 4 weeks. This was prepared in a class II laminar flow biological safety cabinet to ensure sterility.

2.1.1.4 DMEM/F-12 +FBS

Advanced DMEM-F12 (Gibco) was prepared with the addition of 10% Foetal Bovine Serum (FBS; Labtech, Heathfield, UK), 1% 200mM Amphotericin B (Gibco) and 1% Penicillin-Streptomycin (Sigma-Aldrich). Media was stored in the fridge (4°C) for up to two weeks, or in the freezer (-20°C) for up to 4 weeks. This was prepared in a class II laminar flow biological safety cabinet to ensure sterility.

2.1.2 Passaging Cells

To passage, media was removed and cells were gently washed twice in DPBS (Gibco) before adding 3ml Accutase (Sigma-Aldrich) to the flask. This flask was then incubated at 37°C until the cells detached after around 5 minutes, this detachment was confirmed visually and using a light microscope (Leica). Cells were then collected in 10ml DMEM-F12 and added to a 15ml centrifuge tube.

2.1.3 Centrifuging

The 15ml centrifuge tube containing disassociated cells was centrifuged (Heraeus Stratos Biofuge) at 330 xg for 3 mins.
After centrifuging, the supernatant was removed from the centrifuge tube, without disturbing the cell pellet. This pellet was resuspended in 2ml fresh DMEM-F12, and the cells could then be counted using a haemocytometer (described in Section 2.1.4) and then pipetted into a freshly prepared flask at around 1/5th of total cell count, or used in experiments.

2.1.4 Cell Counting

![Figure 2.1: The Neubauer Haemocytometer used for cell counting](image)

A glass coverslip is placed on the top of the counting grid, and the 10μl sample is pipetted into the sample and overflow channel. The sample fills the space below the coverslip, distributing the suspended cells throughout the grid. Using a microscope, the number of cells in each of the four corners of the grid are counted. If cells are touching the side of the measuring grid, only those touching the top and left sides are included, as represented by the green lines on the diagram. Cells touching the bottom and right sides are not counted, as represented by the red lines on the diagram. Image created using BioRender.com

Cells were detached from the flask and suspended in fresh Full Expansion Media [as described in Section 2.1.1] in a 15ml centrifuge tube they were then gently pipetted up and down to encourage the solution to form a single cell suspension. In order to maintain a
uniform suspension of cells, the tube was constantly swirled gently. From this suspension solution, a 10μl sample was extracted and pipetted into the haemocytometer (Hawksley, Lancing, UK). This allowed the cells to be viewed under a microscope, and manually counted as they appeared in the grid of the haemocytometer. The number of cells in each of the four corners of the grid was counted, and the average was calculated. This was then multiplied by the dilution factor and the total volume, which gave an estimate of the total number of cells in the suspension.

### 2.1.5 Cell Storage

The expanded cell population could be cryopreserved for longer term storage. Freezing medium was prepared: 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) in full expansion media containing growth factors as described in Section 2.1.1.1, and stored at 4°C until ready to use. Serum was not included in the media\textsuperscript{234}. Cells were dissociated and counted as described in Sections 2.1.2 - 2.1.4, before they were centrifuged at 200 xg for 3 minutes. The supernatant was aspirated and the pellet was then resuspended in the freezing medium at 1 million cells/ml. 1ml cell suspension in cryopreservation vials (Cryo.s, Greiner Bio-One, Stonehouse, UK) were transferred into a Mr Frosty isopropanol freezing container (Thermo Scientific, Cramlington, UK) which was stored in the freezer (-80°C) overnight, until they were transferred to liquid nitrogen storage (-196°C).

### 2.1.6 Cell Thawing

Cryopreservation vials were retrieved from liquid nitrogen storage and rapidly thawed in a 37°C water bath. The 1ml cell suspension was added to 4ml of DMEM, and this was centrifuged at 200 xg for 3 minutes. The supernatant was aspirated from the pellet, and the
cells were resuspended in full expansion media (Section 2.1.1.1) before they were transferred to a T25cm³ flask, coated with Matrigel. Cells were expanded for at least one passage after thawing, and transferred to a T75cm³ before they were used in experiments.

2.1.7 Imaging and Photography

Cell cultures and histology sections were studied with a Leica DMi1 light microscope (Milton Keynes, UK) and digital images captured with a fitted Leica MC170 HD microscope camera. Sterile cultures were photographed in the laminar flow cabinet with culture plate lids removed, unless otherwise stated.

2.2 RT-qPCR

2.2.1 Sample Collection

The media was aspirated from the well, and then the sample in the well was washed twice with PBS. The following protocol was carried out using ReliaPrep™ RNA Cell Miniprep System (Promega, Hampshire, UK). Samples were collected in 200μl BL/TG buffer (BL Buffer + 1% 1-Thioglycerol) then stored at -80°C or used directly. RNA was extracted and the yield was calculated using a Thermo Scientific™ NanoDrop™. The RNA was reverse transcribed to produce cDNA and this was used in qPCR analysis. Housekeeping genes used were HPRT1 and TBP.

2.2.2 RNA Preparation

RNA was extracted according to the following protocol: ReliaPrep™ RNA Cell Miniprep System (Promega). Briefly, cells were collected in BL/T buffer, then 100% isopropanol was added to this solution before vortexing. A multi-step process follows to isolate RNA and remove DNA. The final step was the elution of the RNA into nuclease-free water, which was
then stored on ice before analysing the samples with the NanoDrop (NanoDrop™ 2000 Spectrophotometer).

Using the NanoDrop, 1-2 μl of an RNA sample can be analysed to check for DNA contamination, and to quantify the concentration of RNA in the sample. As well as measuring the concentration of RNA in the sample, the NanoDrop also conveys a 260/280 ratio. This indicates the presence of DNA and RNA- briefly, a 260/280 ratio of 1.8 indicates DNA, while 2.0 or above indicates RNA. RNA samples were kept on ice for the duration of this step, and they could then be stored at -80°C until required for cDNA preparation.

Figure 2.2: The Nanodrop machine is used to assess RNA quality before cDNA formation

The NanoDrop is used to detect RNA in a sample. 1-2 μl of the sample is pipetted onto the NanoDrop machine, then the lid is lowered to make contact with the liquid sample. The bottom part of the machine with the sample on it lowers slightly, creating this ‘channel’ of sample (left) through which the light is transferred. The data is shown on the laptop software (right), indicating RNA concentration in the sample, and the 260/280 ratio, indicating the presence of DNA or RNA.

2.2.3 cDNA Preparation

cDNA was prepared according to the following protocol: RT² Easy First Strand Handbook (Quiagen, UK). Briefly, genomic DNA was first eliminated from the RNA sample. Then the RNA samples were diluted appropriately with nuclease-free water, using the
Nanodrop data to ensure each sample contains the same concentration of RNA. Then the reverse transcriptase mix is added. This mix is then incubated to anneal the reverse transcriptase, and the cDNA can then be used immediately for PCR, or stored at -20°C.

### 2.2.4 qPCR Procedure

cDNA was first diluted in nuclease-free water, resulting in a concentration of 4ng/μl, then a master mix was prepared for each primer being used. In each well of the qPCR plate, there was: 10μl SYBR Green, 2μl Sample, 1μl Primer and 7μl nuclease-free H₂O.

#### Table 2.1 Primers used for qPCR

<table>
<thead>
<tr>
<th>TARGET</th>
<th>FORWARD PRIMER</th>
<th>REVERSE PRIMER</th>
<th>COMPANY</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT1</td>
<td>CATTATGCTGAGGATTTGGAAAGG</td>
<td>CTTGAGCCACAGACAGGAGGGCTACA</td>
<td>BioRad</td>
</tr>
<tr>
<td>TBP</td>
<td>TGTATCCAGACGTGATCTTGGTTG</td>
<td>GGTTCGTGCTCTCTTATCCTC</td>
<td>BioRad</td>
</tr>
<tr>
<td>NESTIN</td>
<td>TCAAGATGTCCCTCAGCCTGGA</td>
<td>AAGCTGAGGGAAGTTCGAAAGC</td>
<td>Origene</td>
</tr>
<tr>
<td>GFAP</td>
<td>CTGGAGAGGAAGATGGAGTGC</td>
<td>ACGTCAAGCCACATGGACCT</td>
<td>Origene</td>
</tr>
</tbody>
</table>

The plate was designed on the software to correspond to the samples loaded into the wells. The holding stage was 95°C for 10 minutes, followed by the cycling stage which was 95°C for 15 seconds, followed by reducing the temperature to 60°C for 1 minute – these steps were repeated for 40 cycles. The Melt Curve stage raised the temperature to 95°C for 15 seconds, followed by a reduction of the temperature to 60°C for 1 minute, with the temperature raising by 0.3°C up to 95°C which was held for 15 seconds.
2.2.5 qPCR Statistical Analysis

The raw data obtained from RT-qPCR is given in the form of CT cycles, which are then normalised to the housekeeping genes, and transformed using the $2^{-\Delta\Delta CT}$ method, as described by Litvak and Schmittgen\textsuperscript{235}. This gives the relative change in gene expression, and fold change in gene expression.

$\Delta$Ct calculations:

Ct values for control and experimental conditions were first normalised to the housekeeping genes by subtracting the housekeeping gene value from the target gene value in each case, to give the $\Delta$Ct values. The $\Delta\Delta$Ct value was calculated by subtracting the $\Delta$Ct of control values from the $\Delta$Ct of experimental values. $2^{-\Delta\Delta CT}$ transformed the $\Delta\Delta$Ct values into fold changes.

The normalised change in gene expression ($\Delta\Delta$Ct) was used to perform statistical analysis, including ANOVA and Student’s T-Test.

2.3 Western Blot

2.3.1 Sample Collection

The media was aspirated from the well, and then the sample in the well was washed twice with PBS. The sample was collected in lysis buffer consisting of RIPA buffer (ThermoFisher) and protease inhibitor (ThermoFisher) in a 500:1 ratio. The samples were then spun in a centrifuge at 4°C for 20 minutes at 5960 xg to separate the cell membranes from the rest of the cell content. The liquid portion was then retrieved, being careful not to disturb the pellet, and decanted into a new, labelled Eppendorf. The sample was stored on wet ice for immediate use, or stored at -80°C for later analysis.
2.3.2 BCA Assay

A BCA assay was then carried out (Pierce™ BCA Protein Assay Kit, ThermoFisher) to calculate the protein content of each sample. The BCA mix was prepared by combining component A with component B in a 50:1 ratio. The amount required was determined by calculating the number of required samples, with the protein standards (BSA) added in triplicate, and the working samples added in duplicate. For example, if making 8 protein standards plus 12 working samples, the calculation would be:

\[(8 \times 3) + (12 \times 2) = 48 \text{ wells}\]

This would be rounded up to 50 wells to account for spillage or pipetting errors.

\[200\mu l \times 50 = 10,000\mu l \text{ total}\]

\[9800\mu l \text{ Reagent A} + 200\mu l \text{ Reagent B}\]
Western Blot

A clear-bottomed 96-well plate for optical density measurements. Shows the addition of BSA standards in triplicate, and samples in duplicate.

These calculated volumes were pipetted into a clear-bottomed 96-well plate, with 1μl of protein sample added to each of the protein wells in duplicate, and a range of the BSA protein standard pipetted in triplicate to the appropriate wells. The plate was then transferred to a heat block, and heated at 60°C for 10 minutes. There was a visible change in reagent colour, as the light green of the initial reagent turned to a dark purple in relation with an increased protein content. Finally, the plate was then transferred to the plate reader (Glowmax) and, with wavelength absorbance set to 560nm, the light absorbance was measured to produce a comparative table from which protein content would be calculated.

To calculate the total protein content, the average optical density of the blank protein wells was averaged, and this was subtracted from the other wells. A scatter graph was produced from the BSA protein standards, and a linear trendline was added, with the equation displayed. The average optical density of the samples was divided by the slope value of the trendline, which gave the protein concentration in μg/μl and then dilutions were calculated to give each sample an equal concentration for analysis.

2.3.3 Gel and Transfer

Front and back plates for the gel scaffold were thoroughly cleaned with methanol, then assembled in the clamps, resting on rubber to create a seal. A 12% acrylamide gel was prepared using the following recipe:

APS and TEMED were added at the final step, as these are the crosslinking agents which create the gel. The separating gel was added first, and left for 15 minutes to form the
Western Blot

gel, before the stacking gel was added on top, with the addition of a 1mm comb and again left to form a gel for 15 minutes. Once the gel formed, the scaffold containing the gel was transferred to an electrophoresis box with the front plate facing inwards. The comb was removed, and the inner chamber was filled with 1x SDS Running Buffer (Recipe in Appendix 1) and allowed to overflow to cover the bottom wires outside the plates.

The prepared samples were then added to the wells of the gel, with 5μl of protein ladder added to the first well followed by 20ng/ well per sample. The gel was left to run at 130V for 5 minutes, which allowed the proteins to move into the separating gel. The voltage is then increased to 170V for 45-60 minutes, to separate the proteins by molecular weight.

The transfer membrane (Immobilon®-FL PVDF Membrane, Millipore) was cut to the size of the gel, and activated in 100% methanol with the target protein written in pencil. Materials required for the membrane transfer stack were soaked in Transfer (TB) buffer (Recipe in Appendix 2) for 5 minutes, and then the stack was assembled with a fibre pad and filter paper on either side of the stack, with the gel and membrane sandwiched inside. This stack was then transferred into a box filled with TB buffer and the current was run through again to allow the proteins to transfer onto the membrane. This was run at 20V overnight with an ice pack to prevent the gel from melting with heat, or 100V for 60 minutes when thoroughly packed around with ice.

2.3.4 Quantification and Addition of Antibodies

2.3.4.1 Quantification with Ponceau Stain

Following protein transfer, the membrane was retrieved from the stack, and rolled up with the transferred proteins inside the roll. This was placed inside a 50ml Centrifuge tube,
Western Blot

with 5ml ddH$_2$O added and placed on a roller for 30 seconds. The ddH$_2$O was discarded, and
5ml of Ponceau stain was added (0.2% Ponceau in 1% acetic acid). This was placed back on
the roller for 5 minutes to allow protein to cover all areas. Ponceau stain was discarded, and
the membrane was rinsed with ddH$_2$O. Background staining was removed by washing the
membrane in 5% acetic acid. The membrane was rinsed with dH$_2$O and imaged using a
lightbox.

The membrane was then fully destained with several washes of 5% acetic acid, and
rinsed in dH$_2$O before proceeding to immunostaining.

2.3.4.2 Quantification with Coomassie Blue Stain

Following protein transfer, the membrane was retrieved from the stack, and rolled up
with the transferred proteins inside the roll. This was placed inside a 50ml Centrifuge tube,
with 5ml dH$_2$O added and placed on a roller for 30 seconds. The dH$_2$O was discarded from the
tube, and then the membrane was briefly rinsed with 100% methanol. Then 5ml of 0.1%
Coomassie blue stain was added to the tube. This was placed back on the roller for 5 minutes
to allow protein to cover all areas. Coomassie stain was discarded, and the membrane was
rinsed with ddH$_2$O. Background staining was removed by washing the membrane in 50%
methanol + 1% acetic acid. The membrane was rinsed with ddH$_2$O and imaged using a
lightbox.

The membrane was then fully destained by placing the membrane in 25% acetic acid
for 15 minutes, and rinsed in PBS before proceeding to immunostaining.
2.3.4.3 Quantification with Revert Total Protein Stain

Following protein transfer, the membrane was retrieved from the stack, and rolled up with the transferred proteins inside the roll. This was placed inside a 50ml Centrifuge tube, with 5ml ddH$_2$O added and placed on a roller for 30 seconds. The ddH$_2$O was discarded, and 5ml of REVERT Total Protein Stain (LI-COR Biosciences™, Nebraska, USA) was added. This was placed back on the roller for 5 minutes to allow protein to cover all areas. Protein stain was discarded, and the membrane was washed twice with Wash Buffer (LI-COR Biosciences™), followed by a final wash with ddH$_2$O. The membrane was then imaged using Licor Odyssey M (LI-COR Biosciences™) to determine total protein quantification.

After protein quantification, the membrane was then stained with antibodies to detect the relevant proteins. 5ml Odyssey Blocking Buffer (Odyssey™ Blocking Buffer (PBS), LI-COR Biosciences™) was added to the Centrifuge tube containing the membrane, and this was put on rollers for an hour.

The primary antibody:

- GFAP (Rabbit anti-GFAP; Invitrogen, Thermofisher Scientific) diluted 1:5000
- Nestin (Mouse anti- Nestin; Invitrogen) diluted 1:500
- Olig2 (Rabbit anti- Olig2; Abcam, Cambridge, UK) diluted 1:500
- SOX2 (Rabbit anti- SOX2; Abcam) diluted 1:500

was added directly to the blocking buffer, and returned to rollers for another hour, or overnight at 4°C. The antibody solution was then removed, and the membrane was thoroughly washed with PBS to remove excess antibodies. 5ml of Odyssey Blocking Buffer was added to the Centrifuge tube, along with a secondary antibody (Mouse anti-rabbit or
Goat anti-mouse; Alexafluor 647, Abcam) with fluorescence at 700nm, which was diluted 1:10,000. This was put back on rollers for an hour, before the antibody solution was removed and the membrane was washed again with PBS.

The membrane was then dried, and imaged again using the Licor Odyssey M to measure the fluorescent intensities of each protein band. This fluorescence was then normalised to the total protein quantification levels which were previously measured.

2.3.5 Quantification

Images retrieved from the Licor Odyssey M were analysed in Image Studio (Licor). For total protein quantification using the data collected from the initial scan at 700nm, a rectangle of equal size was drawn inside each of the columns of detected proteins. The data from inside these columns was exported to an excel spreadsheet for analysis. For protein band analysis using the data collected from the antibody stains at 800nm, the bands were highlighted inside a box which excluded any background fluorescence. This data was exported to the same excel spreadsheet as the 700nm data. The fluorescence values for the antibody stains are then normalised to the fluorescence of the total protein stains, to allow an accurate comparison of the change in protein expression.

2.4 Immunocytochemistry

16mm Sterile glass coverslips (Fisherbrand, Fisher Scientific) were coated with 200μl Matrigel, diluted 1:80 in DMEM, and cells suspended in media were allowed to adhere for 5-6 hours in an incubator. Cells were not cultured on coverslips for NMT experiments because the coverslips may have interfered with the transduction of the vibration, and were therefore not used in control experiments to maintain the same conditions in both. Furthermore, the
addition of magnets to the bottom of the cell culture plate meant that imaging the plate directly was impossible, as the magnets were in the way and could not be reliably removed. Therefore, once the experiment ended, the cells were transferred onto the coverslips for imaging. Once the cells had adhered to the coverslip, the cell culture media was removed. The coverslip was gently washed twice for 5 seconds in PBS, before adding 4% Paraformaldehyde (PFA; Sigma-Aldrich) for 20 minutes at RT. Then the PFA was removed, and the coverslip was washed three times for 5 seconds with PBS. The cells were blocked with PBS+ 0.05% Tween + 3% BSA for an hour at RT (Tween; BSA; Sigma-aldrich). This was removed, and the cells were washed three times for 5 seconds with PBS. Primary antibodies were prepared using recommended concentrations, with dilution in PBS. The primary antibodies used as follows:

- GFAP (Rabbit anti-GFAP; Invitrogen; #PA1-10019) diluted 1:1000
- Nestin (Mouse anti- Nestin; Invitrogen; #14-9843-80) diluted 1:500
- SOX2 (Rabbit anti- SOX2; Abcam; #ab97959) diluted 1:1000

A strip of Parafilm (PARAFILM® M SEALING FILM) was secured flat to the workbench, and a small dot of 100μl of each antibody solution was dotted onto the Parafilm. The coverslips were dropped face-down onto this drop of liquid, and then covered for an hour at RT or overnight at 4°C.

The coverslips were retrieved after the Primary antibody step, and gently washed three times for 5 seconds in PBS before repeating the same procedure using the Parafilm for the secondary antibodies. The secondary antibodies were as follows:

- Goat anti-Rabbit Alexa Fluor™ 488 (Invitrogen, # A-11008)
Donkey anti-Mouse Alexa Fluor™ 680 (Invitrogen, # A10038)

This was incubated for an hour at RT and then washed three times with PBS. To stain the nucleus with DAPI (Tocris; Oxford, UK), this was diluted 1:3000 in PBS and left on the coverslip for 1 minute before washing off three times for 5 seconds with PBS, and finally dipped into ddH₂O. The coverslip was left to dry slightly before mounting on a slide.

To mount the coverslip on a slide, 10μl of MOWIOL (Mowiol® 4-88, Sigma-Aldrich) was dotted onto a slide; a single slide could mount two coverslips. The dry coverslip was then placed face-down onto the MOWIOL and left to dry before imaging. This was carried out in the dark due to the photosensitive nature of the secondary antibodies. The slides were imaged using a Leica TCS SP8 confocal microscope (Milton Keynes, UK)

2.5 Statistics

Independent experiments were carried out a minimum of three times for statistical tests to be performed. Statistics were carried out using GraphPad Prism (GraphPad Software Inc, San Diego, USA), and XLSTAT (Lumivero, Colorado, USA). The statistical tests used were ANOVA, followed by a Dunnett’s post-Hoc test if required, for more than 2 sets of data. A Students t-test was used to determine difference between two sets of data.
Chapter 3: MECHANICAL STIMULATION OF CANCER STEM CELLS IN A 2D MODEL

3.1 Chapter Overview

This chapter provides insights into the foundational aspects of 2D monolayer stem cell culture. It details the creation of initial culture conditions for stem cells, including the incorporation of specific additives aimed at either maintaining stemness or promoting differentiation. The chapter goes on to describe the effect of the physical environment on cell behaviour, and outlines experiments conducted to examine how the introduction of vibrations affects stem cells when cultured on a 2D surface. These experiments serve as a starting point for investigating the impact of mechanical stimuli on neural stem cell behaviour, laying the groundwork for further research in the next chapters.

3.2 Introduction

Cell culture can be considered as much an art form as a science: the composition of glioma cell culture media and conditions can vary greatly between different research publications and laboratories, and the behaviour of different cells and cell lines can add to the differences. This lack of consistency in the treatment of cultured glioma stem cells can make it difficult to compare studies, and replicate their findings. Therefore some of this chapter is dedicated to establishing an optimal environment for GBM CSC culture.

Alterations to the topography and stiffness of the surface to which cells adhere have been found to encourage differentiation of MSC and NSC by activating pathways associated with cellular mechanotransduction\textsuperscript{186,236,237}. In MSC, this mechanism has been exploited through
the application of nanoscale vibrations to induce differentiation\textsuperscript{186,238}. This phenomenon has not been well explored in NSC\textsuperscript{239}. Therefore in this chapter, initial investigations were carried out to establish the effect of nanoscale vibrations on GBM CSC cultured in a 2D environment.

Understanding the effect of these conditions on CSC may represent a novel therapeutic approach for the treatment of cancer\textsuperscript{240,241}.

3.2.1 Structure of GBM Stem cells compared to Astrocytes

Astrocytomas including GBM, are believed to originate from astrocytes within the brain. In the case of GBM, this is often near to areas of neural stem cell populations. The cells comprising the GBM tumour are typically heterogeneous, and include a small population of cancer stem cells\textsuperscript{54,80}. Distinguishing these stem cells from the typical cells of the brain or GBM tumour, relies on phenotypic and genotypic markers. Once a population of stem cells has been isolated, it can be challenging to maintain their stemness. This requires a specific formulation of cell culture media and an environment which mimics their niche \textit{in vitro}\textsuperscript{154,242,243}.

Glial cells reside in the brain in a ratio with neurons which varies in reports from an equal expression of both\textsuperscript{244}, to a 1.4x greater instance of glial cells\textsuperscript{245}, to a 5x greater instance\textsuperscript{246,247}. This discrepancy can depend on factors such as the species being investigated, and diverse cell populations at different locations within the brain. Astrocytes themselves comprise, on average, 50% of the glial cell population\textsuperscript{248}.

Mature astrocytes can be recognised through their genetic and phenotypic markers- the name ‘Astrocyte’ originates from the cell’s shape, which resembles a star. The most widely accepted marker of mature astrocytes is Glial Fibrillary Acidic Protein (GFAP) which is an
Intermediate filamentary protein. Markers of neural stem cells include Nestin\(^{249}\), SOX2\(^{110}\) and OLIG2\(^{250}\). Nestin is another intermediate filamentary protein, and the expression of this protein can persist until the cell becomes an astrocyte\(^{251}\).

Table 3.1: A comparison of neural stem cells, progenitors and astrocytes identification

Included is a number of common protein markers associated with the cell types. GLAST = Glutamate Aspartate Transporter.

<table>
<thead>
<tr>
<th>NEURAL STEM CELLS</th>
<th>ASTROCYTE PROGENITORS</th>
<th>TYPICAL ASTROCYTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>DESCRIPTION</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Round cell body</td>
<td>More elongated</td>
<td>‘Star’ shaped</td>
</tr>
<tr>
<td>May be slightly</td>
<td>May have a couple of</td>
<td>Large cell body</td>
</tr>
<tr>
<td>elongated</td>
<td>processes</td>
<td>Several neurites</td>
</tr>
</tbody>
</table>

**CELL SHAPE**

<table>
<thead>
<tr>
<th>COMMON MARKERS</th>
<th>SOX2</th>
<th>SOX2</th>
<th>GFAP</th>
<th>Vimentin</th>
<th>GLAST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nestin</td>
<td></td>
<td>Nestin</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Olig2</td>
<td></td>
<td>Olig2</td>
<td></td>
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</table>

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3.2.2 2D Cell Culture

When cancer cells are grown in vitro, they will continue to divide even when the surface of the culture dish or flask becomes densely populated, a point known as confluence. At this point, healthy cells would respond to signals to stop dividing\textsuperscript{252}.

This uncontrolled and relentless division in cancer cells is often referred to as 'replicative immortality.' This phenomenon underscores one of the fundamental distinctions between cancer cells and their healthy counterparts. While normal cells have a limited number of divisions due to the shortening of their telomeres\textsuperscript{253,254} (the protective caps at the ends of chromosomes), cancer cells have evolved strategies to bypass this limit\textsuperscript{255}.

Replicative immortality in cancer cells arises from a complex interplay of genetic mutations, particularly in genes that regulate cell death and proliferation. One pivotal player in this process is Telomerase Reverse Transcriptase (TERT), a protein that helps maintain the length of telomeres. In cancer cells, the activation of TERT prevents telomeres from shortening, thus averting the cellular senescence that typically constrains the lifespan of normal cells\textsuperscript{256}.

Cells grown in a laboratory flask or petri dish experience distinct physical stresses that diverge from the complex, tissue-specific environments found in living organisms\textsuperscript{257}. The materials used in these containers, typically plastic or glass, markedly contrast with the intricate composition of brain tissue, muscles, or bone. Moreover, many cell types are "anchorage-dependent," necessitating a specific surface to adhere to for proper growth and proliferation. To replicate this condition in vitro, researchers will often introduce a basement membrane layer onto the culture ware\textsuperscript{197,198}. This added layer mimics the cellular
microenvironment more closely, facilitating the attachment, growth, and proliferation of cells

3.2.3 Additives in Stem Cell Culture

3.2.3.1 Growth Factors

First characterised by Rita Levi-Monetelcini and Stanley Cohen in the 1950s, growth factors play important roles in several aspects of cell maintenance and growth. In healthy tissue, growth factors are secreted from surrounding cells, but when grown in vitro, healthy cells require growth factors in order to expand. However, cancer cells can proliferate in vitro without additional growth factors in the media due to the aberrant function of growth factor receptors.

3.2.3.1.1 Epidermal Growth Factor

Epidermal Growth Factor Receptor (EGFR) is an Actin-binding protein, and its upregulation is a hallmark of glioblastoma. Epidermal growth factor (EGF) binds to the EGFR and this initiates a cascade of signals which can activate the MAPK pathway, thus increasing proliferation. Furthermore, the binding of EGF to EGFR has been demonstrated to cause a rapid depolymerisation of actin, and polymerisation of some Myosin proteins, which forms ‘ruffles’ in the membrane, aiding in cell migration. The withdrawal of growth factors from the media has been found to reduce the expression of several genes (Glut1, Hexokinase 2 and phosphofructokinase 1) which are involved in the regulation of glucose uptake and metabolism, suggesting that growth factors play an important and complex role in cell maintenance and homeostasis. EGFR can also be activated by Transforming Growth Factors (TGF), which aid cell growth on soft substrates.
Cancer cells often have aberrant growth factor pathways, in which they can make their own growth factors, for example, virally transformed cells produce EGF-like factors, and TGF\textsuperscript{13} \textit{in vitro}, and some growth factor pathways are constantly activated. Meanwhile, some cancer cells have been found to trick surrounding cells into producing the necessary growth factors. Growth factors have been found to play a big role in documented cancer cell behaviour, aiding in the evasion of apoptosis, tumour cell migration and angiogenesis, amongst other effects\textsuperscript{264}.

3.2.3.1.2 Fibroblast Growth Factor

Human Fibroblast Growth Factor (FGF) can be secreted by astrocytes\textsuperscript{265}, and it exists in 22 forms, although the most commonly recognised are acidic FGF (FGF-1) and basic FGF (FGF-2/ bFGF). They bind to Fibroblast Growth Factor Receptors (FGFR) and activate some of the same pathways as EGFR, such as MAPK. The activation of FGFR by FGF-2 pathways in neural stem cells can promote cell proliferation\textsuperscript{266} and migration. Heparin also plays a role in aiding FGF signalling\textsuperscript{267,268}.

An early study by Cattaneo and McKay (1990) demonstrated that the removal of bFGF from serum-free media halted the proliferation of Nestin- expressing rat striatum cells, and lead to differentiation\textsuperscript{269}. In fact, FGF-2 has been implicated in the de-differentiation of GBM cells, inducing stem cell characteristics\textsuperscript{270}.

3.2.3.2 Foetal Bovine Serum (FBS)

Stem cells must be cultured in serum-free conditions in order to maintain their stem cell characteristics\textsuperscript{269}. CSCs cultured in serum-containing media have been found to exhibit vastly different characteristics from their parent cells\textsuperscript{271}. It has been established that the addition of serum to the cell culture media can result in differentiation of NSC and Glioma-
derived CSC\textsuperscript{272–274}. Studies have shown that culturing cells in FBS leads to a decrease in SOX2 expression\textsuperscript{271} which is indicative of differentiation.

Interestingly, some studies have found that differentiation of GBM CSC with serum can result in increased invasive potential of the cells, which is associated with enhanced expression of Matrix Metalloprotease-9 (MMP9)\textsuperscript{275}. MMP9 and other members of the matrix metalloprotease family play a role in degradation of the ECM, which aids in migration and tumour invasion\textsuperscript{276}. Furthermore, there is indication that serum-induced differentiation may be reversible, with some serum-differentiated cells reacquiring neurosphere-forming potential after transplantation into serum-free media\textsuperscript{275}. However, this has been disputed with other studies finding no change in differentiation markers after transplantation to serum-free media\textsuperscript{142,271}.

3.2.3.3 Bone Morphogenetic Protein -4 (BMP4)

BMP4 can be added to cell culture media in order to induce the differentiation of NSC towards an astrocytic lineage. Bone morphogenetic proteins, and in particular BMP4, have a powerful effect in triggering the Smad pathways in glioblastoma-derived stem cells, resulting in an increased expression of differentiation markers. Experimentally, \textit{in vivo} administration of BMP4 in mice effectively blocked tumour growth and reduced mortality\textsuperscript{277}. The interaction of BMP4 and its inhibitor, Noggin, have been found to regulate the astroglial cell lineages in the central nervous system (CNS)\textsuperscript{278}.

BMP4 has also been found to directly inhibit cell proliferation, causing the cell cycle to arrest in the G1 phase by downregulating the expression of cyclin D1\textsuperscript{279}. BMP4 has also been found to induce apoptosis in in some models including cells of the developing mouse limb\textsuperscript{280}, human Pulmonary Artery Smooth Muscle Cell (PASMC)\textsuperscript{281} and Glioma-derived stem cells\textsuperscript{279}. 
BMP4 not only reduces the growth rate of GBM CSC, but it appears to be a specific modulator of differentiation in GBMs. The addition of BMP4 to GBM CSC cultured in vitro has been found to elicit a marked decrease in the number of CD133+ cells (CD133+ is a marker used to identify a stem cell subpopulation in glioblastoma)\textsuperscript{282}. This specificity is underscored by the fact that TGF\(\beta\), which participates in signalling mechanisms which overlap with BMP4, did not have the same effect on stem cell fate\textsuperscript{283}.

Generally, astrocytes have been found to preferentially grow on stiffer substrates (1kPa - 9kPa), relative to the stiffness of surfaces which encourage neuronal cell growth (100-500 Pa)\textsuperscript{225,226}. The behaviour of GBM cells in matrices of varying stiffness has been investigated, with cells cultured in PEG-based hydrogels tuned to 1kPa and 26kPa, finding that a stiffer environment in this case led to reduced cell proliferation, and extended protrusions from the encapsulated cells. The increased matrix stiffness also stimulated the upregulation of genes related to mechanosensing pathways, such as Rhoa and ROCK\textsuperscript{284}.

This suggests that the differentiation of GBM stem cells may be encouraged by culture on a stiffer substrate, and the related increase in mechanosensing pathways. However, there are conflicting reports regarding the sensitivity of GBM stem cells to mechanical cues, with some studies reporting reduced cell invasion in softer matrices\textsuperscript{227,285}. Contrasting, some primary GBM cell lines have been found to be less sensitive, or rigidity-independent\textsuperscript{286–288}. This is hypothesised to be an advantageous trait which allows cells to undergo invasive migration in spite of the mechanical cues which might otherwise reduce this potential\textsuperscript{288}. Interestingly, a 2020 study found that BMP4 can increase the sensitivity of GBM CSC to the stiffness of its culture surface, and associated mechanotransduction within the cell\textsuperscript{289}. This can reduce invasiveness while also decreasing the tumour-initiating potential of the cells.
3.2.4 Nanoscale Mechanotransduction Studies

The significance of the physical environment and mechanical cues on cell behaviour was introduced in Chapter 1. Building on the findings of cellular response to mechanical cues, studies have been carried out using a purpose-built piezo-electric, nanovibrational bioreactor, to investigate the effect of physical stimulation on stem cell behaviour290. This nanovibrational bioreactor was designed to induce displacement from piezo ceramics through the application of a specific voltage and frequency. A metal plate sits on top of the piezo actuators, allowing cell culture plates to be mounted, with magnets attached to their bottom.

Initial investigations carried out on Mesenchymal stem cells (MSC), where a measured 10-14nm surface displacement which was found to result in increased osteogenesis, compared to MSC which were not stimulated221. These studies first considered the effect of these vibrations on MSC in 2D culture, receiving variable frequencies of stimulation to ascertain optimal conditions for osteogenesis. Their work concluded that 1kHz was the optimal frequency for osteogenic differentiation of MSC219.

Studies using this vibrational bioreactor have found that the mechanical stimulation of MSC resulted in an increased number of focal adhesion sites, with a well-organised actin cytoskeleton219,221. This echoes the findings of studies which look at the effect of increasing substrate stiffness on cell behaviour, resulting in an increased number of focal adhesions186,291 and cell spreading.
3.2.5 Aims & Hypotheses of this Chapter

Aims:

1. To optimise experimental design to ensure the cultivation of a healthy population of stem cells with appropriate additives and conditions, based on existing information and observed behaviour.
2. To establish the effect of culturing GBM CSC in different media on their proliferation and differentiation
3. To establish the effect of culturing GBM CSC under the influence of vibrations on their proliferation and differentiation

Hypotheses:

1. Cells cultured in full expansion media will maintain stem-cell characteristics, and the associated rate of proliferation.
2. Stem cells cultured in media without growth factors, and with the addition of BMP4 or FBS, will proliferate more slowly and will exhibit astrocytic characteristics.
3. Culturing cells on vibrating surfaces will lead to increased stress fibres within the cells, increasing intracellular tension and inducing astrocytic differentiation.

3.2.6 Summary of Methodology

The Flowchart shows the goals for this chapter, and the methodologies employed to achieve them. The methods are expanded on in section 3.3.
3.3 Methods

3.3.1 Stem Cell Culture

A full description of the methodology is described in Section 2.1. Cells were cultured in T75cm³ vented cell culture-treated polystyrene flasks, coated with Matrigel (diluted 1:80 in DMEM) in full expansion media. Full expansion media was prepared with the inclusion of additives to promote cell proliferation [as described in Section 2.1.1.1], including EGF and FGF at 10ng/ml.
3.3.2 Variable Growth Factors Study

Cells were passaged [as described in Section 2.1], and counted then seeded into 6-well cell culture plates (Greiner) at 20,000 cells per well. Cells were cultured for seven days in full expansion media, but with the addition of varying concentrations of the growth factors, EGF and FGF. Concentrations were as follows: 0ng/ml; 2.5ng/ml; 5ng/ml; 7.5ng/ml and 10ng/ml. 50% of the media was changed every 2-3 days. After 7 and 14 days of culture, the cells were imaged using a Leica DMI1 light microscope (Milton Keynes, UK) and digital images were captured with a fitted Leica MC170 HD microscope camera.

3.3.3 2D Nanoscale Mechanotransduction Experiment

3.3.3.1 Media preparation for different experimental conditions

*Table 3.2: Cell culture media preparations used for stem cell maintenance and experimental protocols*

A full description of additives and preparation is described in Section 2.1

<table>
<thead>
<tr>
<th>Condition</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Stimulated</td>
</tr>
<tr>
<td>CONT +GF</td>
<td>NMT +GF</td>
</tr>
<tr>
<td>CONT -GF</td>
<td>NMT -GF</td>
</tr>
</tbody>
</table>

‘Control’ plate is not attached to the vibrational bioreactor, while ‘Stimulated’ cells are attached to the vibrational bioreactor with a magnet, and are therefore receiving vibrational stimulation.

Full expansion media (See Section 2.1): Contains growth factors EGF and FGF.

Full expansion media: Does not contain EGF or FGF.
Throughout this thesis, ‘Control’ refers to cell culture plates not attached to the bioreactor, and therefore not receiving any vibrational stimulation. ‘NMT’ refers to cell culture plates which are stuck to the vibrational bioreactor via a magnet. This means the cells are receiving vibrational stimulation, since this is designed to encourage nanoscale mechanotransduction within the cell. In every case, both experimental and control, the individual wells of the cell culture plates were prepared with Matrigel to encourage cell adhesion.

3.3.3.2 Nanomechanical Stimulation Experiment

Experiments were designed to investigate the effect of nanoscale mechanotransduction (NM) on the isolated CSCs. Firstly, the wells of two 24-well cell culture plates were prepared with Matrigel, prepared according to the manufacturer’s instructions, as described in section 2.1. In some earlier experiments, solid magnets were attached to the bottom of the cell culture plate using epoxy (Results not included). In later work, a sheet of sticky-back, flexible magnet was cut to the size of the cell culture plate, and attached to the bottom of one of the plates. Cells were then passaged [as described in Section 2.1] and after centrifuging, the cells were resuspended in fresh DMEM-F12 and counted [as described in
Methods

Section 2.2.1. Meanwhile, the cell culture plates were prepared; two 24-well plates (Greiner) were labelled with the conditions for the experiment [as described in Table 3.2].

The appropriate media was pipetted into each well (1 ml per well), and the cells were seeded at 20,000 cells per well. The plate with the magnet on the bottom was attached to the bioreactor inside the incubator. This experiment was carried out for 7 days, with media changed every 2-3 days. After 7 days, samples were collected for analysis.

**Figure 3.1**: Arrangements of experimental conditions in a 24-well plate

A 24-well plate was labelled with the conditions [as described in Table 3.2], and a sheet of magnet was then attached to the bottom of one plate, which could then be attached to the bioreactor.
3.3.4 Image Analysis for Variable Growth Factors Study

Protocol adapted from Venter & Niesler, 2019\textsuperscript{292}. To calculate the percentage area of cells within a well for the variable growth factors study (Section 3.4.1), ImageJ software was used. First, the image was opened with ImageJ, and converted to a grayscale image using Image > Type > 8-bit. Image noise was removed using Process > Noise > Despeckle. The brightness was adjusted to increase the distinction of the cells using Image > Adjust > Brightness/Contrast.

![ImageJ software interface for image analysis](image)

*Figure 3.2: Threshold set for highlighting cells*

The threshold is manually altered to encompass all of the individual cells.

A threshold was then set to ascertain areas of cells, compared to the background using Image > Adjust > Threshold. This was converted to binary using Process > Binary > Make Binary. Due to the lighting in the micrograph, setting the threshold often resulted in a nonspecific ‘shadow’ along the edges which could affect the cell count. This phenomenon has been highlighted with yellow arrows in the image above (Figure 3.2).
Since this shadowing was only seen on the edges of the images in each case, the cells were measured as a whole image, excluding those touching the edges (Highlighted in the image below). Therefore, the cells were measured using the Analyze > Analyze Particles function. The cells which were analysed by the programme are highlighted in red below (Figure 3.3).

![Analyze Particles](image)

*Figure 3.3: Selected cells for analysis*

Threshold was set, and the analyse particles function was used to quantify the highlighted area, excluding the cells touching the edges to avoid the nonspecific 'shadowed' area.

### 3.3.5 Image Analysis for Different Media Conditions Study

Protocol adapted from Venter & Niesler, 2019\(^2\). To calculate the percentage area of cells within a well for the different media conditions study (Section 3.4.2), ImageJ software was used. First, the image was opened with ImageJ, and converted to a grayscale image using Image > Type > 8-bit. Image noise was removed using Process > Noise > Despeckle. The brightness was adjusted to increase the distinction of the cells using Image > Adjust > Brightness/Contrast. A threshold was then set to ascertain areas of cells, compared to the
Methods

background using Image > Adjust > Threshold. This was converted to binary using Process > Binary > Make Binary.

![Image of thresholding](image1.png)

**Figure 3.4: Threshold set for highlighting cells**

The threshold is manually altered to encompass all of the individual cells. Some clumps of cells are highlighted with yellow arrows.

Due to the confluence of cells in this study, the threshold was manually set to individually encompass the vast majority of cells, while the edges, and some clumps of cells were nonspecifically highlighted by the programme (**Figure 3.4**). This made separate cells appear as one large cell (some examples highlighted with the yellow arrow).

![Image of measured cells](image2.png)

**Figure 3.5: Three equally sized boxes were drawn to measure the cells**

Due to the density of the cell population, the cells were measured in three equally sized boxes to avoid large cell clusters which the programme could not avoid.
Three equally sized boxes were drawn in the frame, without overlapping, avoiding the nonspecific shaded areas (Figure 3.5). These three measurements were then averaged. Statistics were performed using XLSTAT to calculate an ANOVA, followed with Dunnett’s post-hoc test.

A student’s t-test was then carried out between the Control and NMT conditions for each media type.

### 3.3.6 RT-qPCR Data Analysis

The full RT-qPCR methodology can be found in Section 2.2.

The data was compiled in an Excel spreadsheet, where ΔCt and ΔΔCt were calculated for each result. Data analysis was then carried out using GraphPad Prism software and XLSTAT.

2^−ΔΔCt denotes the fold change in gene expression, which is displayed in the graphs. ΔΔCt describes the linear relationship of gene expression, therefore this is used for data analysis. In this case, ANOVA was carried out to ascertain a difference across all data points, and a student’s t-test was used to determine differences between specific values.

ΔCt calculations:

Ct values for control and experimental conditions were first normalised to the housekeeping genes by subtracting the housekeeping gene value from the target gene value in each case, to give the ΔCt values. The ΔΔCt value was calculated by subtracting the ΔCt of control values from the ΔCt of experimental values. 2^−ΔΔCt transformed the ΔΔCt values into fold changes.

### 3.3.7 Immunofluorescence Analysis

The full immunofluorescence methodology can be found in Section 2.4.
Briefly, the media was aspirated from the experimental well, and the well was washed twice with 500μl PBS. 250μl of Accutase was added to the well, and this was incubated for 3-4 minutes to allow the cells to detach. The detached cells were resuspended in 1ml of fresh DMEM-F12 and transferred to 1.5ml reaction tube and micro-centrifuged at 200 xg for 1 minute. The supernatant was discarded, and the cell pellet was resuspended in 250μl of prepared media, corresponding to its experimental condition.

Sterile glass coverslips were placed in the wells of a new cell culture plate and coated with Matrigel, prepared according to the manufacturer’s instructions. The cells were pipetted onto the coverslip, and this was incubated at 37°C, 5% CO₂ [v/v] for 5-6 hours to allow the cells to adhere to the coverslip.

After this length of time, the media was aspirated and the coverslip was washed twice in PBS, before adding 4% Paraformaldehyde (PFA) for 20 minutes at RT. Then the PFA was removed, and the coverslip was washed three times with PBS. The staining protocol described in Section 2.4 was carried out. The cells were probed using SOX2 and GFAP primary antibodies, counterstained with DAPI. The cells were imaged using a Leica TCS SP8 confocal microscope (Milton Keynes, UK)

3.4 Results

3.4.1 Microscope Images of Cells in Variable Growth Factor Concentrations

In order to assess the cell growth response to different experimental conditions, the GBM stem cells were cultured in media containing different concentrations of growth factors across 7 or 14 days. This growth rate was measured as a % area of cell cover, and the results are presented in Figure 3.6.
Results

Day 7

Day 14

0 ng/ml

2.5 ng/ml

5 ng/ml

250 μm

250 μm

250 μm

250 μm

A

B

C

D

E

F

G

H
Figure 3.6: Cell count after 7 or 14 days in culture with varying concentration of growth factors.

Photographs A-E show images of cells after 7 days in culture, and photographs F-J show images of cells after 14 days, under conditions of varying growth factor concentrations (A/F = 0ng/ml; B/G = 2.5ng/ml; C/H = 5ng/ml; D/I = 7.5ng/ml; E/J = 10ng/ml). Scale bars are shown for reference. Images taken at 5x magnification. Cell counting with ImageJ software. Graph shows the change in % area of cells in the above images, after 7 days (Blue) and 14 days (Yellow). N=1
Results

After 7 days, the phenotypes of the cells under each condition appear similar upon visual comparison, as shown in figure 3.6. After 14 days, the cells grown without any growth factors (F) appear to show a reduced quantity of neurites, and rounder cell bodies compared to the cells grown with the highest concentration of growth factors (J).

The % area of cell cover increased from day 7 to day 14 across all conditions - this was to be expected, as the cells were supplied with fresh media frequently to replace used nutrients. At day 7, there is a small trend of increasing % area along with an increase in growth factor concentration, although this is much clearer by day 14. This suggests that the rate of proliferation was similar across each of the conditions in the first 7 days. By day 14, there is a clear trend of increasing % area along with an increase in growth factor concentration, up to 7.5ng/ml, at which point the % area is very similar to the number seen in the 10ng/ml cells, suggesting that the highest proliferation rate could be achieved with the addition of at least 7.5ng/ml growth factors after 14 days of culture. While these studies are preliminary, and thus not repeated, the findings here are supported by the literature and are behaving as would be expected in this case.

3.4.2 Microscope Images of Cells in Different Media Conditions

The effect of the different experimental media conditions, i.e. vibrated or control cells cultured in full stem cell expansion media, expansion media without growth factors, or with the addition of BMP4 or FBS, was investigated by observing the morphology of cells under these conditions after 7 days of treatment. The results of this morphological comparison are presented in Figure 3.7, while the effect of these experimental conditions on cell growth was also measured, and these results are presented in Figure 3.8.
Figure 3.7: Photographs of cells taken after 7 days of culture in 4 different types of media, with or without nanovibrational stimulation.

Cells cultured without vibrational stimulation (Control; left) and with vibrational stimulation (NMT; right). Scale bars represent 100μm. 10x magnification. Astrocytes (RED arrows) and Progenitor cells (BLACK arrows) highlighted. N=1
Results

From a visual comparison of figure 3.7, the cells which were cultured in full expansion media containing growth factors as a control (CONT+GF) and vibrated (NMT+GF) show a different morphology. The cells which were exposed to vibrations (NMT+GF) show more defined edges of the cells, although cells in both pictures contain a heterogeneous population of rounded stem cells and more elongated progenitor cells.

Cells which were cultured without growth factors as control (CONT-GF) and vibrated (NMT-GF) show a similarly heterogeneous pattern of morphology to the cells cultured with growth factors. A number of cells of astrocytic morphology are visible in the CONT-GF image, while only one or two were easily visible in the NMT-GF image.

The cells which were cultured in DMEM containing BMP4 as control (CONT+BMP4) and vibrated (NMT+BMP4), showed a very different morphology to each other. Nearly all of the cells in the BMP4 control plate (CONT+BMP4) display typical astrocyte morphology, some of these are highlighted with red arrows. Only one or two cells remain which appear to be progenitor cells, based on their morphology; these are highlighted with a black arrow. The well containing vibrated cells (NMT+BMP4) still contained a large number of cells displaying stem cell or progenitor characteristics, with only one or two visible cells which have an astrocytic morphology- again, these are highlighted with a red arrow.

The cells cultured in DMEM containing 10% FBS as control (CONTROL+FBS) and vibrated (NMT+FBS) also contained a mix of cells displaying both stem cell (Black arrow) and astrocyte (Red arrow) morphology.
Figure 3.8: Cell count after 7 days in culture under different media conditions, cultured with or without vibrational stimulation.

Cell counting with ImageJ software. Graph shows the change in % area of cells in the above images, without vibrations (Blue) and with vibrations (Yellow). Bars show average cell number ±SEM. Kruskal Wallis statistical test showed a statistically significant difference between the cells in all the groups. * Denotes statistical significance (*p<0.05; **p<0.01; ***p<0.001) with Dunnett’s Post-Hoc Test for all conditions compared to control +GF. N=3

Due to the high confluence, and complex shape of the cells, the ImageJ software struggled to select and identify individual cells. Therefore while there is a ‘cell count’ programme available, an analysis of the % area covered by the cells was utilised instead, the results of which are shown in figure 3.8. This method was described in Section 3.3.5.

The graph shows that control cells, grown without the influence of vibrations, had a larger range of cell coverage %, ranging from 9.53% - 27.06% while cells grown under the
influence of vibrations had a much smaller range of values, between 20.3% - 25.13%. The data shows a generally higher area % for cells which were cultured in media containing growth factors under the control condition (CONTROL +GF). A Kruskal-Wallis statistical test was carried out to establish the statistical significance of the differences in cell coverage % for cells across all groups. This found there was a significant variation between at least one of the group averages.

Since the Kruskal-Wallis test showed a statistically significant difference between the groups, a post-hoc Dunnett’s test was carried out. This test found a statistically significant difference in the % area coverage of cells grown in control conditions in all media types i.e. media without growth factors (CONT –GF; p<0.05), media containing BMP4 (CONT +BMP4; p<0.001) and media containing FBS (CONT +FBS; p<0.001) compared to CONTROL +GF. There was also found to be a significant difference between the CONTROL +GF and NMT +BMP4 (p<0.05).

There is a measurable difference in the % area of cells which were receiving vibrations (NMT), versus those which were not (CONTROL). When growth factors are included in the cell culture media, the cells which were not exposed to NMT (CONTROL +GF) had a higher % of area coverage in the well compared to the cells which were cultured under the influence of vibrations (NMT +GF). Contrastingly, in the three other pairs of conditions, cells which were receiving vibrations had a higher average % area coverage. This difference was found to be statistically significant for the cells grown with BMP4 or FBS in the media, but not for the cells grown without growth factors in the media (-GF) using a student’s t-test (CONTROL –GF v NMT –GF, p>0.05; CONTROL +BMP4 v NMT +BMP4, p<0.001; CONTROL FBS v NMT FBS, p<0.01).
3.4.3 RT-qPCR data of 2D Nanoscale Mechanotransduction Experiments

In this section, qPCR analysis was carried out to detect the expression of differentiation markers from cells which were cultured under different media conditions, and vibrated or control conditions. The results of this analysis are presented in Figure 3.9 and 3.10.

3.4.3.1 GFAP Expression

![Relative GFAP Expression for Each Condition Compared to Control +GF](image)

<table>
<thead>
<tr>
<th>CONDITION</th>
<th>RELATIVE EXPRESSION</th>
<th>N=</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL +GF</td>
<td>1.00</td>
<td>9</td>
</tr>
<tr>
<td>NMT+GF</td>
<td>1.40</td>
<td>8</td>
</tr>
<tr>
<td>CONTROL -GF</td>
<td>21.43</td>
<td>7</td>
</tr>
<tr>
<td>NMT -GF</td>
<td>28.11</td>
<td>7</td>
</tr>
<tr>
<td>CONTROL +BMP4</td>
<td>52.97</td>
<td>3</td>
</tr>
<tr>
<td>NMT +BMP4</td>
<td>118.39</td>
<td>4</td>
</tr>
<tr>
<td>CONTROL +FBS</td>
<td>2.37</td>
<td>3</td>
</tr>
<tr>
<td>NMT +FBS</td>
<td>11.37</td>
<td>3</td>
</tr>
</tbody>
</table>

*Figure 3.9: RT-qPCR data of GFAP expression after 7 days of treatment.*

Graph of 2-ΔΔCT values of GFAP expression, which shows the fold change in relative gene expression – data normalised to the control sample (CONTROL +GF), which appears at 1 since gene expression is relative to the control. Mean values are included in the table, and marked on the graph. ANOVA showed a statistically significant difference between the cells across all conditions. * denotes statistical significance (*p<0.05) with Student’s t-test, comparing all conditions to CONTROL +GF. N= different for each group as some qPCR runs result in errors which mean data should not be included in analysis.
The fold change graph illustrates change in GFAP expression, relative to the control culture (CONTROL +GF; n=9). The level of GFAP was found to have increased in cells cultured in every condition, with a statistically significant change in expression in the cells exposed to vibrations, in media containing BMP4 (NMT +BMP4; n=4) and media without growth factors (NMT –GF; n=7), as confirmed by a student’s t-test. The cells grown in full expansion media containing growth factors which were exposed to vibrations (NMT +GF; n=8), showed a small average increase in GFAP expression compared to control. In each case, cells which were vibrated expressed a higher level of GFAP than the same media condition which was unstimulated. Cells cultured in media which did not contain growth factors and were exposed to vibrations (NMT –GF; n=7) expressed a relatively higher amount of GFAP compared to the unstimulated cells (CONTROL –GF; n=7). Cells cultured in DMEM containing BMP4 which were exposed to vibrations (NMT +BMP4; n=3) expressed a higher relative amount of GFAP compared to the unstimulated cells of the same condition (CONTROL +BMP4; n=3). And finally, the cells cultured in DMEM containing FBS which were exposed to vibrations (NMT +FBS; n=3) expressed a higher relative amount of GFAP compared to the unstimulated cells of the same condition (CONTROL +FBS; n=3).
3.4.3.2 Nestin Expression

Figure 3.10: RT-qPCR data of Nestin expression after 7 days of treatment

Graph of 2-ΔΔCT values of Nestin expression, which shows the change in relative gene expression – data normalised to the control sample (CONTROL +GF), which appears at 1 since gene expression is relative to the control. Mean values are included in the table, and marked on the graph. An ANOVA did not find a statistically significant difference between means. N= different for each group as some qPCR runs result in errors which mean data should not be included in analysis.
Results

A decreased expression of Nestin in comparison to \textbf{CONTROL +GF} was measured in the control wells containing media without growth factors (\textbf{CONTROL –GF}) and DMEM with FBS (\textbf{CONTROL +FBS}), as well as the cells which were exposed to vibrations, cultured in media without growth factors (\textbf{NMT –GF}), DMEM containing BMP4 (\textbf{NMT +BMP4}) and DMEM containing FBS (\textbf{NMT +FBS}). The vibrated cells cultured without growth factors (\textbf{NMT –GF}) exhibited a relatively small measured decrease of 2%.

The cells cultured in media containing BMP4 which were not exposed to vibrations (\textbf{CONTROL +BMP4}) and the cells which were vibrated in media containing growth factors (\textbf{NMT +GF}) expressed higher levels of Nestin compared to the control. In the case of the cells which were cultured in full expansion media containing growth factors and vibrated (\textbf{NMT +GF}), the average difference in expression was extremely small with a relative 0.02 fold increase.

The largest decrease in Nestin expression was seen in the control cells cultured in DMEM with FBS (\textbf{CONTROL +FBS}), with a relative expression of 0.48 which corresponds to a decrease of just over 50%. The cells which cultured in media containing FBS which were vibrated (\textbf{NMT +FBS}) had a 16% decrease in Nestin expression, relative to the control measurements, while the vibrated cells in DMEM with BMP4 (\textbf{NMT +BMP4}) expressed an 11% relative decrease.

\textbf{3.4.4 Immunofluorescence of 2D Nanoscale Mechanotransduction Experiment}

In the following section, immunostaining techniques were utilised in order to detect the expression of proteins indicative of differentiation.
Figure 3.11: Control +GF stained for the stem-cell marker, SOX2.

SOX2 (Red) and nucleus counter-stained with DAPI (Blue). Scale bar included for reference (100μm ABOVE and 50μm RIGHT). The image on the right is zoomed in to highlight the morphology of the cell. This cell (white arrow) exhibits a classic progenitor cell shape, and SOX2 which is a stem cell marker, is highly expressed.

In Figure 3.11, The cells cultured in full expansion media with growth factors (CONTROL +GF) express SOX2 in every visible cell, with many cells exhibiting the morphology associated with neural stem cells in expansion i.e. a cell body with neurites extending from two ends, as demonstrated in the picture on the right. These cells were not stained for GFAP. All of the stained nuclei are surrounded by SOX2 staining, suggesting there are no astrocytes present in this population.
Results

*SOX2* (Red) was used to stain for the stem cell marker, SOX2, and the astrocytic marker, *GFAP* (Green). The white arrow points to astrocytic cells, orange arrow points to SOX2 positive cells. Scale bar: 50 μm.

Figure 12. Immunohistochemistry for stem cell marker SOX2 and astrocytic marker GFAP.
Results

There was a visibly reduced number of cells in figure 3.12: the culture without growth factors (CONTROL –GF) than in the cells grown with growth factors (CONTROL +GF), shown in figure 3.11. Furthermore, the cells grown without growth factors (CONTROL –GF) contained a heterogeneous population of cells, with cells expressing both GFAP and SOX2. Some cells expressing GFAP exhibited the long shape of a neural stem cell (orange arrow), while others appeared more like astrocytes (white arrow).

![Figure 3.13: NMT +GF stained for the astrocytic marker, GFAP.](image)

GFAP (Green) and nucleus counter-stained with DAPI (Blue). Scale bar included for reference (100μm). Some cells express GFAP (white arrow), while others do not (red arrow).

Figure 3.13 shows cells cultured in full expansion media with vibrational stimulation (NMT +GF). There is a very small amount of GFAP staining visible, which surrounds the nucleus of some cells, but there are no visible neurites in the population.
The **NMT + FBS** cells have stained strongly for GFAP in figure 3.14, with every cell expressing some of the protein, although there is a small population of cells towards the centre of the image which express only a small amount of GFAP. The general cell morphology is rounded,
although some cells appear to be extending neurites, these are in many directions (star-shaped). This is indicative of astrocytic cells.

**Figure 3.15: NMT + BMP4 stained for the astrocytic marker, GFAP**

GFAP (Green) and nucleus counter-stained with DAPI (Blue). Scale bar included for reference (100µm LEFT and 50µm RIGHT). Astrocytic cells highlighted with white arrows, stem cells highlighted with orange arrows.

The cells in **figure 3.15** display a heterogenous population. Cells were exposed to nanoscale vibrations in media containing BMP4 (NMT + BMP4), exhibiting a range of different morphologies in the population. The majority of cells express some GFAP, highlighted with a white arrow, although there are a couple of instances of DAPI-stained nuclei which are not
associated with any GFAP, which are highlighted with an orange arrow. Most cells appear rounded, but some small sections (zoomed in above), contain cells with neurites and astrocytic morphology.

3.5 Discussion

3.5.1 Cells cultured with growth factors: Comparison of Control and NMT

The regulation of proliferation and differentiation of cells is a multi-faceted mechanism. As has been previously discussed, growth factors like EGF and FGF-2 play important roles in the cell cycle \cite{260,293}, and therefore it is logical, and also reported in previous studies, that if stem cells are dividing this would happen faster in media containing growth factors \cite{294}. This was demonstrated by De Jong et al., who associated the presence of five different growth factors including EGF and FGF-2, with increased mitosis and therefore proliferation \cite{295}. The data in this chapter supports these findings, as the cells cultured in media containing a greater concentration of growth factors cover a greater well surface area, as demonstrated in figure 3.6. This is further compounded by the results in figure 3.8, which shows the highest cell density in the control cells cultured with growth factors.

It is also recognised that CSC are able to produce growth factors endogenously \cite{296}, or have aberrantly active growth factor receptors on account of genetic abnormalities which can mitigate the need for additional growth factors in the media \cite{295}. Therefore this may allow the cells to proliferate more successfully than healthy (non-tumour-derived) cells in the absence of additional growth factors in the media. In this case, cells cultured in media which did not contain growth factors, were still able to proliferate. This was demonstrated in figure 3.6,
where there was an increase in cell coverage of cells cultured in 0ng/ml concentration on day 14, versus day 7. This indicates cell proliferation in the absence of additional growth factors.

Since studies have shown that tumour cells can produce their own growth factors, or growth factor-like mitogens\textsuperscript{297,298}, changing the cell culture media may remove some of the endogenously produced mitogens, thus reducing the growth rate for the cells which were cultured in growth factor-free media. Studies also show that differentiation could lead to a reduced rate of proliferation, since differentiated cells are usually not dividing\textsuperscript{269}. The immunofluorescence data (\textit{figures 3.11-3.15}) indicates that the cells cultured in media without growth factors express some markers of differentiation, and the data in \textit{figures 3.6-3.8} supports the proliferation of cells in the absence of growth factors.

The area of cell coverage in the well is indicative of cell numbers, and since stem cells including CSC are known to proliferate very successfully in the full expansion media\textsuperscript{10} (as was used for control +GF cells), these conditions are likely to result in the highest rate of cell proliferation. This was reflected in the results, which showed that the cells cultured in full expansion media had the greatest area of cell coverage in the well (\textit{Figure 3.8}).

The slower rate of proliferation seen in the lower concentrations of growth factors (\textit{Figure 3.6}) could also be attributed to differentiation of some cells in the population. This is because CSC can both proliferate and differentiate, at which point they may become quiescent (non-dividing) cells. There was a very small difference between the cell numbers when cells were cultured in media containing 7.5ng/ml and 10ng/ml either at 7 or 14 days. This suggests that 7.5 ng/ml may be the lowest concentration required to maintain the highest level of cell proliferation in these conditions i.e. with this cell type, and with the addition of Heparin, B27, N2 and other additives.
It has been established that stem cell maintenance typically requires the addition of growth factors in the media. Therefore, the cells which were cultured in the full expansion media would be expected to show high rates of proliferation, and the maintenance of stem cell characteristics. This was generally the case for both control (not vibrated) and vibrated cells (henceforth referred to as NMT cells), although there are small hints of differences between the two conditions, as shown in Figure 3.8. Firstly, the proliferation rate was high in comparison to most other conditions – however, there was evidence of a reduced rate of proliferation in the vibrated cells. Oddly, this is the opposite of the case in each of the other conditions, where NMT cells showed an increase in cell number in comparison to the control (Figure 3.8). This could indicate that there was some differentiation in the NMT cells, when taken together with the slight increase in GFAP expression (Figure 3.9), and evidence of a GFAP protein surrounding some nuclei in the immunofluorescent images (Figure 3.13).

The fact that established cell lines like CSC in the appropriate media can theoretically expand indefinitely while maintaining their stemness, suggests that the CSC population is usually proliferating through symmetrical cell division i.e. a single stem cell produces two new daughter cells (Figure 1.4) under these conditions. Therefore, a physical or chemical inducement to differentiate could result in promoting asymmetric cell division. This has been confirmed in BMP4-treated GBM stem cells in vitro, resulting in a population of stem cells growing alongside the differentiated cells- therefore the stem cell number would not increase, but this could explain the presence of a heterogeneous population of cells. Alternatively, the cells could be induced to differentiate, without also producing a stem cell, and therefore the stem cell population would be expected to dwindle while the number of differentiated cells increased. However, as was described in Chapter 1, cancer cells are able
Discussion
to undergo unregulated mitosis even when they are no longer stem cells. Therefore the
overall cell population can still increase even after cells have differentiated. Thus it can be
assumed that due to the cancerous nature of these cells, proliferation may be occurring, even
if the cells have differentiated.

Overall, there appear to be very slight differences between the cells cultured with and
without the application of nanoscale vibrations when cultured in full expansion media. The
vibrated cells express some GFAP, and proliferate more slowly, which are both indicators of
differentiation.

3.5.2 Cells cultured without growth factors: Comparison of Control and
NMT

When comparing the proliferation of the cells which were cultured without growth
factors in the media as control or vibrated (NMT) cells, the behaviour was generally similar.
Overall, there was a reduced rate of proliferation, as would be expected due to the absence
of growth factors, and there was not a significant difference between the numbers of cells in
either control or vibrated conditions (figure 3.8). There was a slightly higher coverage of cells
in the vibrated plate in this case, which is consistent with findings in studies which suggest
that the mechanical signals received by NMT cells may increase proliferation through the
activation of mechanosensing proteins, such as FAK300,301.

Since growth factors are included in the media to encourage proliferation, when this
is removed, the number of cells would be expected to remain the same, or increase more
slowly as the rate of proliferation decreases. As discussed previously, cancer cells are able to
proliferate in the absence of growth factors in the media, therefore when culturing a
population of cancer cells in media without growth factors, some proliferation would be expected. This was demonstrated in figure 3.6.

SOX2 is widely expressed in glioblastoma, and is a marker of CSC; generally, differentiated cells would not be expected to express SOX2. The CSC population which was maintained in full expansion media with growth factors included, expressed SOX2 in each visible cell (figure 3.11). Contrastingly, while cells grown in the same conditions and media but without growth factors added also expressed SOX2, they concurrently expressed GFAP (figure 3.12). This heterogeneous population suggests that differentiation may have been occurring in the absence of growth factors, although this did not appear to be uniform across the entire cell population. The cells cultured without growth factors were also found to express another stem cell marker, Nestin$, at a similar level to the CSC population (Figure 3.10). As a stem cell marker, the expression of Nestin usually correlates alongside the expression of SOX2. Remarkably, the control cells cultured without growth factors also showed an increase in GFAP expression (Figure 3.9) of over 21-fold compared to the control population, which is indicative of some differentiation. This was reflected in the immunocytochemistry findings, as the control cells without growth factors were stained for both SOX2 and GFAP, and exhibited the morphology of both stem/ progenitor cells, and differentiated astrocytes (Figure 3.12).

When considering the individual phenotypes of the cells cultured in expansion media without growth factors, cells which exhibited stem and progenitor cell- like morphology were visible, as well as cells which resembled astrocytes. Interestingly, each of these examples were found in populations of GFAP- and SOX2- stained cells, suggesting that some of the cells in
Discussion

this condition could be progenitor cells i.e. on their way towards differentiation, but not fully differentiated\(^3\).

Based on the qPCR data, the alteration in GFAP expression showed a similar pattern in both the control and NMT cells when grown in growth factor-free media. Notably, the expression of GFAP (Figure 3.9) was substantially higher compared to the control +GF cells, with increases exceeding 21-fold and 28-fold, respectively. These results indicate that the absence of growth factors in the media stimulated a large amount of differentiation in the cells.

Furthermore, the statistically significant increase in GFAP expression, exceeding 28-fold, in the NMT -GF cells suggests a substantial discrepancy in the level of differentiation between the control and NMT cells in this condition. This contrast with the control cells cultured in growth factor-free media, implies that the vibrations induced a higher degree of astrocytic differentiation in the NMT cells compared to the control cells.

3.5.3 Cells Cultured with BMP4: Comparison of Control and NMT

BMP4 is commonly employed to induce astrocytic differentiation in these cells, typically resulting in a reduced cell count under these conditions\(^2\). As anticipated, our results demonstrated the highest proliferation rate in stem cells cultured in growth factor-rich expansion media. In contrast, cells cultured in BMP4-containing media displayed a significantly lower proliferation rate (Figure 3.8).

Of particular interest is the notable difference in the percentage of cell area between control and NMT cells when cultured with BMP4. The NMT group exhibited a larger cell area, suggesting that NMT exposure may facilitate proliferation or differentiation by signalling
through focal adhesions\textsuperscript{303}. This could explain the increased cell count compared to the control group under the same conditions (Figure 3.8).

Morphological examination in BMP4-containing media revealed a heterogeneous cell population, with some exhibiting classical stem cell/progenitor features and others displaying astrocyte-like shapes. Interestingly, the control group exhibited a strong response to differentiation cues, primarily adopting astrocytic morphology (Figure 3.7). In contrast, the NMT + BMP4 cell population contained both astrocytes and a significant number of stem/progenitor cells (Figure 3.15). This suggests that mechanical stimulation may reduce the cellular response to differentiation cues. However, given the differing cell quantities in each condition, it's possible that a similar number of astrocytes exists in the NMT population, alongside a stem/progenitor cell population. This is supported by the qPCR data (Figure 3.9), although the small sample size in this case will limit the findings.

GFAP expression substantially increased with the addition of BMP4 in both control and NMT cells, with the NMT group showing a significant increase (Figure 3.9). This was associated with decreased Nestin expression in the NMT cells and an unexpected increase in Nestin expression in the control cells (Figure 3.10). Notably, the reliability of Nestin as a stem cell marker has been debated, since it is broadly expressed in glioblastoma cells\textsuperscript{304–306}.

The data, and published work, suggests multiple mechanisms are at play when GBM stem cells are undergoing proliferation and differentiation\textsuperscript{116,282,307}. Firstly, BMP4 likely induces differentiation, leading to slower population proliferation, while some cells acquire an astrocytic morphology. Secondly, BMP4 has been known to suppress the growth of glioma-derived stem cells\textsuperscript{243}, explaining the significantly lower cell count in control BMP4 media.
Finally, BMP4 has also been linked to cell apoptosis, potentially reducing the number of viable cells capable of proliferation or differentiation.

Furthermore, as was described in Section 3.4.1, the addition of BMP4 to cell culture media has been linked to an overall increase in sensitivity to mechanical cues. Clearly, treatment with BMP4 may have a multi-faceted effect on the population of GBM CSCs, which could explain the range of behaviours seen in these cells in terms of their morphology and protein expression.

In summary, the indication that the NMT cells may have undergone more differentiation than the control cells could be expected, suggesting that the mechanical cues received by the cells upon vibration are sufficient to encourage differentiation, and this is enhanced by the sensitising and differentiating-inducing effect of BMP4.

3.5.4 Cells cultured in FBS: Comparison of Control and NMT

FBS is commonly included in cell growth media, however for stem cell maintenance it must be omitted. This condition was included to establish the effect of serum on these cells in monolayer culture, and determine the level of differentiation it may induce. The cell population in media containing FBS, like cells cultured with BMP4, exhibited heterogeneous morphology, as depicted in Figure 3.7. This variability is expected, as not all cells would necessarily undergo differentiation simultaneously or at the same rate. Moreover, cells may have been in different phases of the cell cycle before treatment.

The difference in area of cell coverage in the micrograph was found to be significant between the control +GF condition, and the control +FBS condition, but not the NMT +FBS condition. This suggests that the NMT condition had some effect on increasing
Conclusions

either size of the cell, or cell number. The micrograph in Figure 3.7 appears to suggest that there was a greater number of cells overall. This could suggest that the NMT had some effect on proliferative signals within the cell. It has been established that there are many crossover effects between pathways such as ROCK/Rho and JNK, which can be activated by physical stimulation, and are involved in both proliferation and differentiation.

The immunofluorescent image of the cells which were cultured in serum-containing media, and stimulated with vibrations (Figure 3.14), show a population of cells which are expressing GFAP and exhibiting an astrocytic morphology. There are also a small number of cells with which the nucleus is visible, but which show little to know GFAP expression, which are most likely stem/progenitor cells. Unfortunately, the control cells cultured in serum-containing media were not stained for GFAP.

Interestingly, the qPCR data did not show a particular change in GFAP expression, whereas the morphology of the cells in Figure 3.15 and the GFAP staining visible, are indicative of differentiation. Furthermore, there was a general decrease in Nestin expression which was detected by qPCR (Figure 3.9), although it was not found to be statistically significant, with a decrease of more than 50% for the control condition, and a 16% decrease for the NMT condition. While a decrease in Nestin is normally indicative of differentiation, it has been established that some glioblastoma cells may also express Nestin.

3.6 Conclusions

It is established that the behaviour of cells can be influenced by chemical mediators, as well as physical stimulation. While some elements of these processes are exceptionally well understood, there remain some unanswered questions regarding the regulation of some cellular mechanisms. It is clear that the inclusion or lack thereof, of growth factors in the
media affects proliferation and differentiation. This has been confirmed here through a reduction in cell numbers in growth factor-free media, alongside an upregulation of astrocytic cell markers and altered morphology, compared to the stem cell expansion population. The heterogeneous populations of cell types seen in each condition is indicative of a changing population of cells, which is undergoing some differentiation. An increased length of time for the cell culture may have resulted in a more homogeneously differentiated population.

The cells which were grown in full expansion media, designed to maintain a population of stem cells, did not show a significant change in behaviour when they were stimulated with vibrations vs control. Remarkably, the NMT cells appear to express a small amount of GFAP around the nucleus. This did not generally extend to the neurites, tending instead to remain localised to around the nucleus, but it could be indicative of some differentiation of the population.

Strikingly, cells which were vibrated covered a higher % area of the cell culture well, in comparison to their control counterparts. This was true in each condition, except for the cells cultured with growth factors in the media (+GF), in which case the control +GF had the highest rate of proliferation, as indicated by the largest surface area coverage of cells in the well. This could be expected, if the vibrated cells have indeed formed focal adhesions with the extracellular environment. As discussed in Section 1.5 and illustrated in Figure 1.10, there can be some overlap between signalling pathways which are activated by mitogens, and focal adhesions. Such as the ERK/ Rho signalling cascades, which are involved in both proliferation and differentiation. This means that cells which are cultured in media without growth factors, may be induced to produce the required signals to proliferate in the absence of mitogens.
Conclusions

To summarise, removing growth factors from the expansion media led to increased differentiation, and the addition of mechanical cues in this case resulted in a significant increase in differentiation markers. This also led to an overall reduction in cell number after 7 days of treatment, with no notable difference between stimulated and control cells.

The addition of BMP4 to the media also had a significant effect on the expression of differentiation markers, and in this case there was also a notable effect on cell number when the cells were stimulated with nanovibrations. The difference between the populations of BMP4-treated and FBS-treated cells could be explained by the fact that BMP4 has been found to increase the sensitivity of GBM CSC to mechanical cues\textsuperscript{289}. Therefore although BMP4 and FBS both act as differentiation-inducing agents, the cells which were treated with BMP4 were also able to respond more strongly to the mechanical cues from vibrations.
Chapter 4: HYDROGEL STUDIES FOR USE IN 3D CELL CULTURE

4.1 Chapter Overview

In this chapter, the use of hydrogels to generate a 3D model for GBM cell culture was explored. Three different hydrogels were produced, with optimisation of each hydrogel performed at each step. Using rheology, the Young’s modulus was examined for each hydrogel type to establish a physical environment which was comparable to the native brain tissue. Finally, laser interferometry was employed to investigate the propagation of vibrations through the hydrogels.

4.2 Introduction

There are clear differences between the native environment from which cells derive, and the traditional method of cell culture which is typically a 2D surface made of glass or plastic. There has therefore been a transition in recent years towards culturing cells in a more physiologically relevant environment. Differences in cell behaviour, including protein expression and motility have been reported when cells were cultured on 2D and 3D substrates. Furthermore, since many anti-cancer drugs which show success in the laboratory go on to fail clinical trials, 3D culture studies may represent a more accurate model for these initial studies. Hydrogels are commonly employed for this purpose.

Hydrogels describe a particular type of material consisting of an elastic network of cross-linked polymers which are highly absorbent. Hydrogels can contain as much as 100g/g or higher of water, in the spaces between the fibres. The material properties are highly tuneable, and together these properties make hydrogels an invaluable material for a wide
range of biomedical applications\textsuperscript{316,317}. Their use in glioblastoma cell culture is expanded on in Chapter 5 of this thesis.

### 4.2.1 Background of Hydrogels

There are a huge variety of natural and synthetic hydrogels available for different purposes, a selection of those used for biomedical applications will be discussed here. Table 4.1 contains a comparison of different hydrogels and their uses. Synthetic hydrogels including poly-(ethylene glycol) (PEG) and poly(hydroxyethyl methacrylate) (PHEMA) have been widely used as biomaterials in drug delivery\textsuperscript{318}, scaffolds in regenerative medicine\textsuperscript{319}, and even contact lenses\textsuperscript{320}.

<table>
<thead>
<tr>
<th>Table 4.1: Types of Hydrogel and Biomedical Applications</th>
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<tbody>
<tr>
<td>Poly-(ethylene glycol) (PEG); poly(hydroxyethyl methacrylate) (PHEMA); poly(methyl methacrylate) (PMMA)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Type of Hydrogel</th>
<th>Uses</th>
<th>Advantages/Disadvantages</th>
<th>Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrin</td>
<td>3D Cell culture, Medical Fibrin sealant</td>
<td>+ Tunable physical and chemical properties, + Can be derived from target species, - High batch-batch variability</td>
<td>\textsuperscript{321–323}</td>
</tr>
<tr>
<td>PEG</td>
<td>3D Cell culture, Surface coating of medical devices, Drug delivery</td>
<td>+ Tunable physical and chemical properties, - Requires addition of cell adhesive peptides</td>
<td>\textsuperscript{284,324,325}</td>
</tr>
<tr>
<td>PHEMA</td>
<td>Contact lenses</td>
<td>+ Biocompatible</td>
<td>\textsuperscript{326–328}</td>
</tr>
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<thead>
<tr>
<th></th>
<th>Drug delivery</th>
<th>+ Inexpensive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- May induce calcium deposits after an extended period in situ</td>
<td></td>
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**Chitosan**

<table>
<thead>
<tr>
<th>Drug Delivery</th>
<th>+ Biocompatible</th>
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<tbody>
<tr>
<td>Antimicrobial agent</td>
<td>- Not naturally abundant</td>
</tr>
<tr>
<td></td>
<td>- Forms large particles (potential problem for drug delivery)</td>
</tr>
</tbody>
</table>

**Agarose**

<table>
<thead>
<tr>
<th>DNA separation by molecular weight</th>
<th>+ Inexpensive</th>
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<tbody>
<tr>
<td>3D Cell culture</td>
<td>+ Tuneable physical and chemical properties</td>
</tr>
<tr>
<td></td>
<td>- Requires addition of cell adhesive peptides</td>
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**PMMA**

<table>
<thead>
<tr>
<th>Biomedical implants</th>
<th>+ Promotes cellular adhesion and expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug Delivery</td>
<td></td>
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</table>

Biological (natural) hydrogels can be formed from polymers like Chitosan, Fibrin, Collagen and Alginate, among many others. An advantage of natural hydrogels, and one of their main functions, is to mimic the ECM environment in cell culture and regenerative medicine. Combinations of natural and synthetic hydrogels can result in increased durability for the natural hydrogels, and increased biocompatibility for the synthetic hydrogels.

**4.2.2 Characterising the Mechanical Properties of Hydrogels**

Rheology describes the methodology used to ascertain the deformation and flow behaviour of materials. The scale of materials which can be measured with rheology ranges from liquids like water and oil, to solids like wood and metal, with materials like honey and hydrogels in between. Most materials will exhibit elements of behaviour which are typical of both solids and liquids, regardless of where they sit on this scale.
Introduction

When liquids are put into motion, the molecules and particles they contain are forced to slide along each other. Therefore the material will exhibit a certain amount of resistance cause by internal friction. Generally, elasticity is used to describe the behaviour of materials which are more solid, while viscosity describes the behaviour of materials which are more liquid. Materials which share the properties of viscous and elastic materials, such as hydrogels, are described as viscoelastic\textsuperscript{338,339}.

![Figure 4.1: Agarose gel Tested with the Rheometer](image)

The oscillating plate (A) was lowered to make contact with the hydrogel surface, contact confirmed by an increase in pressure measured by the oscillating plate. The hydrogel (B) sits between the oscillating plate and the fixed plate (C).
Introduction

When measuring the viscoelastic properties of materials using a rheometer, stress is applied to measure the deformation of a material. **Figure 4.1** demonstrates the setup of a rheometer, with gels on the bottom (fixed) plate which are contacted by the oscillating top plate to detect resistant shear force.

**Figure 4.1:** Demonstrates the setup of a rheometer, with gels on the bottom (fixed) plate which are contacted by the oscillating top plate to detect resistant shear force.

**Figure 4.2:** Different methods for measuring Elasticity using Stress/ Strain

Measuring the elastic moduli of a hydrogel example with the top area, $A$. When the gel is compressed, height decreases from $(H_0)$ to $(H)$ and width increases from $(W_0)$ to $(W)$. This is inverse to the behaviour of the hydrogel undergoing tensile testing. In these cases, $\sigma$ and $\varepsilon$ are calculated as shown. When the hydrogel is measured using shear testing with a rheometer, the bottom of the gel is fixed while the top is displaced by the value, $L$. If the gel is a linear elastic material, it will produce a straight stress/strain curve, thus the elastic modulus can be determined from the slope of the curve. If the material is viscoelastic, $G$ can be used to calculate the Young’s modulus using Poisson’s ratio. Stress is measured in Pascals (Pa). Image created using Biorender.com, adapted from Lee et al.\textsuperscript{340}
The Young’s modulus, a type of elastic modulus, is measured by examining the relationship between a force which is applied to a material (such as a hydrogel), and the resulting deformation of the material. When testing a material using a simple compression or tensile method (i.e. application of stress in a single direction e.g. push/pull), the stress/strain data allows the Young’s modulus to be calculated. When testing with rheology, (i.e. shear stress) the Young’s modulus can be calculated by considering the relationship between the obtained shear modulus and Poisson’s ratio.

In Figure 4.2, the modulus which is retrieved from compression, tensile or shear testing, Poisson’s ratio ($\nu$) describes the ratio between the lateral strain, and normal strain of the gel. For example, in the compression testing example, the normal strain is the change in height due to compression while the lateral strain is the change in width associated with this stress. This is written as:

$$\nu = \frac{(\Delta W/W_0)}{(\Delta H/H_0)}$$

If the volume does not change with applied stress because the material is incompressible, the value of its Poisson’s ratio is 0.5 (written as: $\nu = 0.5$)

In the case of most hydrogels, the material is considered to be incompressible since it is mainly composed of water, which is incompressible. Therefore, the Young’s modulus of the material can be calculated by measuring the stress-strain curve, and interconverting the measured elastic modulus and shear modulus using Poisson’s ratio. This is written as:

$$E = 2G (1 + \nu)$$

Where $E$ = Young’s modulus; $G$ = shear modulus; $\nu$ = Poisson’s ratio
4.2.3 Designing a biomimetic hydrogel

In their native tissues, cells may be exposed to a range of physical and chemical cues. The stiffness of tissues, measured by the Young’s modulus, varies within the body depending on the location and role of the tissue. Bone is much stiffer than brain tissue, for example\textsuperscript{186,216}. An illustration of this scale is demonstrated in Figure 4.3 below.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4_3.png}
\caption{A range of stiffnesses of healthy tissue indicated by their Young’s modulus

Scale shows glass at the far end of the scale\textsuperscript{203,204}. The range of stiffnesses of tissue in brain cancer is also included, which ranges from 0.17 – 26 kPa. Image adapted from Barnes et al\textsuperscript{205}.}
\end{figure}

Therefore the successful culture of neural stem cells in a biomimetic environment required the design of a 3D model which was physiologically comparable to native brain tissue. Anatomy studies have determined that the stiffness of tissues in the brain is not uniform throughout the organ, with higher stiffnesses reported in the brainstem\textsuperscript{341}, and differences in stiffness between white and grey matter\textsuperscript{342,343}. Gliomas, like many solid tumours, exhibit a generally higher tissue stiffness then the healthy native tissue, with a reported modulus of up to 26kPa\textsuperscript{344} compared to a range of 200Pa – 1kPa for healthy brain tissue\textsuperscript{224,345}. 

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Some hydrogels already contain proteins which are also produced by the ECM of GBM cells, such as collagens and proteoglycans\textsuperscript{346,347}. These may be complex hydrogels containing multiple different materials, such as Agarose + Collagen\textsuperscript{348,349}, or a single type of hydrogel material such as Fibrin\textsuperscript{350}. The way in which cells interact with hydrogels is expanded on in Chapter 5.

### 4.2.4 Different Hydrogel Applications

#### 4.2.4.1 Agarose

Agarose is a polysaccharide which is obtained from marine red algae, a type of seaweed\textsuperscript{351}. Some hydrogels, such as Agarose, are naturally thermo-responsive and therefore their structure varies depending on the temperature they are exposed to. Agarose is classified as having an upper critical solution temperature (UCST) since it is a polymer solution which exists as a solution at high temperature, and forms a gel upon cooling. Conversely, polymer solutions which are gels at higher temperatures, and exist in solution at lower temperatures, are referred to as having a lower critical solution temperature (LCST)\textsuperscript{352,353}.

The most common application for Agarose is its use in gel electrophoresis, which allows the separation of DNA fragments by size\textsuperscript{332}. In order to prepare the hydrogel, Agarose powder is dissolved in hot water and forms a gel as it cools. The gelling temperature of standard Agarose is around 36-40 °C which limits its use as a 3D scaffold in live cell culture, since mammalian cells are maintained at around 37°C\textsuperscript{354}. This Agarose will henceforth be referred to as HT Agarose, due to its high gelling temperature. However, low gelling temperature (LT) Agarose forms a gel at around 26-30 °C, which allows it to more stably
contain live mammalian cells\textsuperscript{355}. \textbf{Figure 4.4} demonstrates the difference in gelling and melting temperatures of HT and LT Agarose.

\textbf{Figure 4.4: Temperature scale of HT Agarose and LT Agarose hydrogel formation}

LT Agarose melts at around 60°C and gels when it cools to around 26-30°C. HT Agarose melts at around 86.5 – 89.5°C and gels when it cools to around 36 - 40°C.

LT Agarose is not generally utilised as an environment for cell growth on its own, but may be used in conjunction with another hydrogel such as Matrigel\textsuperscript{333}. This is due to the fact that polysaccharide-based hydrogels, such as Agarose and Alginate, lack the proteins required for cell adhesion. Furthermore, their hydrophilic nature discourages protein adsorption\textsuperscript{356}. this means that cells which are usually adherent should grow in a spherical shape, regardless of their morphology in 2D culture\textsuperscript{357}. \textbf{Table 4.2} lists some differences between HT and LT Agarose.
Table 4.2: Differences between HT Agarose and LT Agarose

<table>
<thead>
<tr>
<th></th>
<th>HT Agarose</th>
<th>LT Agarose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical Compound</td>
<td>\textit{D-galactose and 3,6-anhydro-L-galactopyranose}</td>
<td></td>
</tr>
<tr>
<td>Gel Point</td>
<td>36-40 °C</td>
<td>26-30 °C</td>
</tr>
<tr>
<td>Melting Point</td>
<td>86.5-89.5 °C</td>
<td>60 °C</td>
</tr>
<tr>
<td>Uses</td>
<td>Gel electrophoresis\textsuperscript{358}</td>
<td>Cell culture\textsuperscript{361}</td>
</tr>
<tr>
<td></td>
<td>Embedding tissue samples for histological processing\textsuperscript{359}</td>
<td>Single cell gel electrophoresis\textsuperscript{362}</td>
</tr>
<tr>
<td></td>
<td>Drug delivery\textsuperscript{360}</td>
<td></td>
</tr>
</tbody>
</table>

The formation of Agarose gel is demonstrated in Figure 4.5, adapted from Zarrintaj et al\textsuperscript{363}. The diagram highlights the formation of filaments, made from single- or double- helices, occurring when the gel cools to a specific temperature, with water filling the pores between filaments. Strong hydrogen bonds exist between the single and double helices within the agarose compound\textsuperscript{364}, which contribute to its high gel strength.
Agarose can be modified to produce a compound which has a lower gelling temperature. This may be achieved in a number of ways including carboxylation\textsuperscript{365} (a chemical process involving treatment with carbon dioxide\textsuperscript{366}), acetylation\textsuperscript{367} (a chemical process involving treatment with acetic acid) or alkylation\textsuperscript{368}. These modified agarose compounds were reported to have a lower gelling temperature as well as higher transparency, believed to be related to the decline of hydrogen bonds between the individual agarose strands\textsuperscript{369}.

\textit{Figure 4.5: Formation of Agarose hydrogel}

When the Agarose is heated and dissolved in solution, it exists in single strands. As the Agarose solution cools, the strands begin to intertwine to form the final gel structure. Water fills the pores between filaments. Image created using Biorender.com
4.2.4.2 Alginate

Alginate is a naturally occurring polysaccharide, derived from the cell walls of brown algae\(^3\). A variety of cations such as Calcium ions (Ca\(^{2+}\)), Barium (Ba\(^{2+}\)) and Copper ion (Cu\(^{2+}\)) enable the cross-linking between alginate chains\(^4\), replacing sodium ions (Na\(^+\)) – this is usually achieved through the addition of CaCl\(_2\), although CaCO\(_3\) can also be used as a Ca\(^{2+}\) donor. With CaCl\(_2\) as the cross-linker, Ca\(^{2+}\) replaces Na\(^+\) to join alginate chains together. Adapted from Erdal et al., 2019\(^5\). The gelation mechanism is termed the ‘egg-box model’, owing to its shape, a term coined by Grant et al.\(^6\).

\[ \text{Figure 4.6: Formation of Alginate hydrogel} \]

\(\text{With CaCl}_2 \text{ as the cross-linker. Ca}^{2+} \text{ replaces Na}^{+} \text{ to join alginate chains together. Adapted from Erdal et al., 2019. The gelation mechanism is termed the ‘egg-box model’, owing to its shape, a term coined by Grant et al.}\)

CaCl\(_2\) or CaCO\(_3\) can both be utilised as a source of Ca\(^{2+}\) ions for crosslinking. The main difference between these two methods is the speed of the reaction\(^7\): CaCl\(_2\) dissolves easily in water, and therefore the Calcium ions rapidly become available when added to the alginate solution. Conversely, CaCO\(_3\) dissolves slowly in water, resulting in a slower release of ions, and thus a slower rate of gelation. Hydrolysed lactones such as (D-glucono-δ-lactone) GDL precipitates acid-induced alginate hydrogel formation\(^8\). When GDL is added in conjunction
with CaCO₃, it triggers the release of the Ca²⁺ ions²⁷⁶. Non-conventional approaches to inducing slower gelation include cryogelation i.e. freezing the solution²⁷⁷,²⁷⁸.

Alginate is widely used in food, chemical and pharmaceutical industries- there are many benefits to its use, as it is relatively simple to produce, nontoxic and biocompatible²⁷⁹. Therefore it can be used in specific applications. One of the most common applications involves the use of hydrogel particles, which can be used to encapsulate cells, or even small molecules such as drugs²⁷¹,²⁸⁰. In studies where alginate hydrogels were used to encapsulate drugs in wound dressings, the bioavailability of the drugs was increased, thus improving the efficacy of the drugs used in wound healing²⁸¹,²⁸².

4.2.4.3 Fibrin

Fibrin is a naturally occurring protein associated with wound healing and thrombosis²⁸³,²⁸⁴. Its production comes from the enzymatic cleavage of Fibrinogen by Thrombin, allowing binding sites to become available for multiple monomers to combine to produce the Fibrin polymer²⁸⁵.
The first step of Fibrin production is the cleavage of fibrinopeptides A & B which also makes ready the αC regions for the next step\textsuperscript{385,386}.

The next step of fibrin production. Protofibrils form from the combination of multiple monomers (forming oligomers as an intermediary step) including the formation of αC-polymers. The protofibrils pack together to form Fibrin fibres, which can then go on to branch into the final gel structure.

\textbf{Figure 4.7: Formation of Fibrin Hydrogel}
The αC regions are not necessary for lateral aggregation of the fibres, but they have been found to enhance it\textsuperscript{387}. Furthermore, fibrinogen which is missing the αC region leads to thinner, denser fibre formation which is more susceptible to fibrinolysis\textsuperscript{388}.

Fibrinogen exhibits an affinity for Calcium ions (Ca\textsuperscript{2+}) with several binding sites for Ca\textsuperscript{2+} which are related to the stability of the protein, and promote polymerisation\textsuperscript{389,390}. Furthermore, Ca\textsuperscript{2+} plays a role in fibrinopeptide release\textsuperscript{391} and influences downstream polymerisation steps\textsuperscript{392,393} such that the binding of Ca\textsuperscript{2+} to key high-affinity sites leads to an increased rate and greater extent of lateral aggregation of Fibrin monomers. This means that increasing Ca\textsuperscript{2+} concentrations present during Fibrin formation can result in the formation of thicker Fibrin fibres, which can functionally increase the rigidity of the fibre\textsuperscript{385,394,394}. This thickening of the individual fibrin fibres can also cause the gel to appear more opaque\textsuperscript{395}.

Fibrin is a hydrogel which has many functions and applications particularly in tissue engineering and research- including as a scaffold for tissue engineering\textsuperscript{322,396,397}. It is particularly useful for the clinical production of tissue for transplants, since it can be produced using the patient’s own blood, thus reducing the risk of an immune response to transplanted tissue\textsuperscript{396}.

Fibrin is also widely used as a scaffold for cell culture, and more recently for ‘bio-printing’ cells, especially for applications in neural tissue engineering\textsuperscript{398,399}. It has been particularly utilised in neural cell culture as it allows neurite growth to be observed\textsuperscript{400}, Furthermore, embryoid bodies (EBs) which contain neural progenitor cells have been found to differentiate when they are embedded in Fibrin hydrogels\textsuperscript{401}.
4.2.4.4 Laser Interferometry

Laser Interferometry is a technique used to measure distances with extreme accuracy. Using this method (Figure 4.8), a single beam of light is emitted from a laser which travels through the interferometer and is split into two beams: a reference beam, and a measurement beam. The reference beam travels a known distance, while the measurement beam travels to the measurement point. Both signals are received at a detector, and the difference in the distance travelled by both light beams results in a phase differential which is processed at the detector, to give highly specific information about the distance travelled.

![Figure 4.8: Laser interferometry setup](image)

*Light emitted from source (1), travels to the interferometer, where it is split into two beams – a reference beam (2) and a measurement beam (3). These are reflected back to recombine at the interferometer, and then travel to the detector (4). Image created using Biorender.com. Adapted from Loughridge and Abramovitch*
Introduction

Laser interferometry is a technique which can be used to give specific measurements of the amount of displacement of a surface under vibration, as in this case. The effect of vibrations in different materials can be complicated. For example, materials can be susceptible to resonance upon the application of a certain vibrational frequency, resulting in a sensitivity of the material to this specific frequency input. The effect of resonance on cell culture plates has been examined in relation to cell culture plates containing cells and media, and with the application of ultrasound waves\(^{403}\). While in this study, the plates have a magnet attached to the bottom, and contain hydrogels, this may result in a slightly different resonance effect.

![Photograph of the setup for laser interferometry](image)

*Figure 4.9: Photograph of the setup for laser interferometry*

The laser was suspended above the (circled) bioreactor, to which the culture ware was attached.
4.2.4.5 Aims & Hypotheses of this Chapter

Aims:

1. To optimise experimental design to ensure the preparation of a biomimetic hydrogel which accurately reflect the physical properties of the GBM tumour microenvironment
2. To ensure the faithful propagation of a specific vibrational frequency through the hydrogel
3. To establish the most appropriate type of hydrogel for this purpose overall

Hypotheses:

1. Increasing concentration of each hydrogel material will result in a stiffer material
2. The addition of CaCl2 to Fibrin gel formulation will result in a stiffer hydrogel
3. The combination of rheology data and interferometry data will give an ideal formulation for each hydrogel for use in neurosphere experiments

4.2.5 Summary of Methodology

Flowchart shows the goals for this chapter, and the methodologies employed to achieve them. The methods are expanded on in section 4.3.
4.3 Methods

4.3.1 CaCl₂ Formulation

Preparation of 1M Calcium Chloride (CaCl₂) : 147.02 g anhydrous Calcium Chloride (Sigma) was dissolved in 800ml deionized water (diH₂O), before adding more deionized water to adjust the volume to 1L.
Preparation of 30mM CaCl₂: 0.45 g CaCl₂ was dissolved in 80ml deionized water, before adding more deionized water to adjust the volume to 100ml. This was autoclaved before use in tissue culture or hydrogel formation.

4.3.2 Agarose Hydrogel & Low Temp Agarose Hydrogel

A 12-well cell culture plate was labelled with the concentrations used for each hydrogel. Agarose (Sigma-Aldrich) or low-temperature gelling agarose (LT Agarose; Sigma-Aldrich), was weighed and mixed with diH₂O at a ratio of 1:100, then heated and agitated until fully dissolved. Concentrations listed in Table 4.3

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Dry Weight Agarose (g)</th>
<th>diH₂O Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>0.3</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td>0.7</td>
<td>0.7</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

Each dilution was added in triplicate to the wells, at 1 ml per well. The plate was left to gelate at room temperature for 1 hour.
4.3.3 Alginate Hydrogel

4.3.3.1 CaCl₂ Method

A 12-well plate was labelled with the concentrations used for each hydrogel. Alginic acid sodium salt (Sigma-Aldrich) was weighed as described below in Table 4.4 and mixed with de-ionised water (diH₂O) at a ratio of 1:100, then heated and agitated until fully dissolved.

Table 4.4: Alginate Concentrations

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Alginic Acid Sodium Salt (g)</th>
<th>diH₂O (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.2</td>
<td>40</td>
</tr>
<tr>
<td>1</td>
<td>0.4</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>0.8</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>40</td>
</tr>
</tbody>
</table>

1 ml of each dilution was added in triplicate to the wells. A circle of filter paper that would fit just inside the well was carefully cut out, soaked it in 100 mM CaCl₂ and placed gently on top of the alginate liquid using forceps. The plate was allowed to gelate at room temperature (RT) for ~5 minutes.
4.3.3.2 CaCO₃ Method

Adapted from Grownay Kalaf et al.³⁷⁴

Dried alginate (Alginic acid sodium salt) was weighed and mixed with de-ionised water at a ratio of 1:100, then heated and agitated until fully dissolved. Concentrations are described in Table 4.4 above.

Prior to gelation, the prepared alginate solution was placed in an incubator to bring the temperature up to 37°C. CaCO₃ (0.03g) and glucono-δ-lactone (GDL; 0.1068g) were weighed out and mixed together per 5 ml of alginate solution.

Tests (shown in Figure 4.11) were carried out in 7ml tubes (Thermo scientific) to test methods of fully dissolving the powdered CaCO₃ and GDL.
Figure 4.11: Alginate hydrogel formed with the addition of powdered GDL/CaCO₃

(A) The left bottle shows hydrogel formation with constant, gentle rolling of the mixture, resulting in some large bubbles. The bottle on the right was constantly shaken vigorously until the gel formed, resulting in a large number of small bubbles.

(B) The bottles were upturned for 10 seconds and returned to standing to confirm gel formation (not shown).

The left bottle shows hydrogel formation with constant, gentle rolling of the mixture, resulting in some large bubbles.

The middle bottle was rolled once every 10 seconds, resulting in some undissolved CaCO₃.

The right hand side bottle shows the hydrogel formation with no agitation of the bottle. The CaCO₃ did not fully dissolve, forming a pellet, and the gel formed was very viscous. When the bottle was upturned for 10 seconds, the pellet moved slowly from the bottom of the bottle, to the middle of the gel.
4.3.4 Fibrin Hydrogel

4.3.4.1 Reagents Preparation

4.3.4.1.1 Fibrinogen

An initial 2% stock solution (20mg/ml) was prepared. 1g of bovine plasma fibrinogen powder (Sigma-Aldrich) was dissolved in 50ml F-12K nutrient mixture (Kaighn’s modification) medium (Gibco). This solution was filter sterilised by 0.22µm bottle-top vacuum filtration (Corning Life Sciences, Amsterdam, Netherlands), and split into 15ml Falcon tube (Fisher Scientific, Loughborough, UK) aliquots for storage at -20°C.

4.3.4.1.2 Thrombin

An initial stock solution of 200nM was prepared. Bovine Serum Albumen (BSA; Sigma-Aldrich) was dissolved in F-12K (Kaighn’s modification) nutrient mixture medium to produce a 0.1% solution. A 1000U vial of powdered bovine thrombin (Merck, Darmstadt, Germany) was dissolved in 5ml of the BSA solution. These aliquots were stored at -20°C.

4.3.4.1.3 Aprotinin

A 1% (10mg/ml) stock solution was prepared. 10mg of aprotinin powder (Sigma-Aldrich) was dissolved in 1ml PBS. This solution was sterilised in ultraviolet (UV) light in the laminar flow cabinet, for 20 minutes. Aliquots of 50µl were stored at 20°C.

4.3.4.1.4 Aminohexanoic Acid

A 200mM stock solution was prepared. 0.262g of 6aminohexanoic acid powder (Sigma-Aldrich) was dissolved in 10ml PBS and sterile filtered using a 0.2µm syringe filter. Aliquots of 5ml were stored at 4°C.
4.3.4.2 Standard Fibrin Method

A 12-well plate was labelled with the final concentrations of Fibrinogen found in each hydrogel.

**Table 4.5: Fibrinogen Concentration for standard Fibrin protocol**

<table>
<thead>
<tr>
<th>Solution Concentration (mg/ml)</th>
<th>Fibrinogen (ml)</th>
<th>DMEM- F12K (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
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<tr>
<td>12</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>14</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>16</td>
<td>16</td>
<td>4</td>
</tr>
</tbody>
</table>

Thrombin was diluted to a 10 U/ml solution in F12K before adding Aminohexanoic acid at 2 µl/ml, and Aprotinin at 2 µl/ml. Fibrinogen was diluted in DMEM to double the required final concentration. The Thrombin and Fibrinogen were then mixed at a 1:1 ratio in the wells, then the plate was transferred to the cell culture incubator at 37°C.

4.3.4.3 CaCl₂ Method

A 12-well plate was labelled with the final concentrations of Fibrinogen found in each hydrogel.
Table 4.6: Fibrinogen Concentration for CaCl₂ Fibrin protocol

<table>
<thead>
<tr>
<th>Solution Concentration (mg/ml)</th>
<th>Fibrinogen (ml)</th>
<th>DMEM- F12K (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>14</td>
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<tr>
<td>8</td>
<td>8</td>
<td>12</td>
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<tr>
<td>10</td>
<td>10</td>
<td>10</td>
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<tr>
<td>12</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>14</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>16</td>
<td>16</td>
<td>4</td>
</tr>
</tbody>
</table>

A 30mM solution of CaCl₂ was prepared [Described in Section 4.3.1] and Thrombin was diluted to a 10 U/ml solution in CaCl₂ before adding Aminohexanoic acid at 2 μl/ml, and Aprotinin at 2 μl/ml. Fibrinogen was diluted in DMEM to double the required final concentration. The Thrombin and Fibrinogen were then mixed at a 1:1 ratio in the wells, then the plate was transferred to the cell culture incubator at 37°C.

4.3.5 Rheology Tests

Hydrogels were prepared [as described in Sections 4.3.2 – 4.3.4] and following gelation, were transferred to the bottom plate of the rheometer (Kinexus pro+, Malvern Instruments) to measure the composition of the material using a plate-plate geometry. An amplitude sweep was applied, with a starting shear strain of 0.01% and ending at 0.4% at a
Methods

frequency of 1Hz and a constant temperature of 25°C. Humidity was maintained by the addition of a cover to reduce evaporation.

This shear elasticity modulus data obtained was then used to calculate the Young’s modulus using the equation $E=3G$, as describe in Section 4.2.2.

Rheology for each hydrogel was completed at least in triplicate to allow an average to be calculated. The goal was to establish the optimal hydrogel formation to give a Young’s modulus as close as possible to 1kPa.

4.3.6 Laser Interferometry

First, a 12-well plate was prepared containing samples of each hydrogel at varying concentrations, and a magnet was attached to the underneath of the plate. An empty 24-well cell culture plate was also prepared by attaching a magnet to the underneath of the plate, this was used for calibration. Using the method established by project collaborators at the University of Strathclyde, a small piece of reflective tape was cut to fit within the well. This was placed on top of each sample of hydrogel, or in the empty well of the calibration plate.

The plate was then placed on top of the bioreactor at a specified height beneath the laser interferometer (SIOS). The behaviour (Frequency or Amplitude) of the bioreactor was altered using an Arbitrary Function Generator (GW INSTEK, AFG-2005) and connected to an Oscilloscope (Keysight, EDUX 1002A). The displacement of the surface of the well was measured using the linked software (INFAS Vibro) according to the inputted Frequency and Amplitude. First, the displacement of the bioreactor was calibrated using an empty cell culture plate, without hydrogels. This was attached to the bioreactor using a sheet of magnet,
with small pieces of reflective tape within the well. Five readings were taken at randomly selected points inside each well for the calibration plate, as well as the hydrogel plates.

**Figure 4.12: Demonstration of the setup for measuring hydrogels with laser interferometry**

Photograph shows Fibrin hydrogels in cell culture plate, with different concentrations in columns in triplicate. A small piece of red reflective tape is added to the surface of each hydrogel to reflect the laser, for detection by the interferometer. The same technique was used for the Alginate and Agarose hydrogels, as well as for calibration by placing the tape in empty wells, without any hydrogels present.

### 4.3.7 Histology of hydrogels

1ml of each hydrogel (LT Agarose, Alginate and Fibrin) was produced in a 12-well plate using the methods described in Sections 4.3.2, 4.3.3.3 and 4.3.4.3. An hour after gelation in the incubator, the gels were covered in 1ml PFA, and left at room temperature for 15 minutes. The PFA was aspirated, and was washed twice in 500μl PBS. The gel was then removed from the well, and transferred to a labelled tissue cassette in a jar of 70% ethanol (industrial methylated spirits) (Fisher Scientific) and then stored at 4°C for wax processing.

The cassette was processed using a VIP E300 Tissue-Tek processor (Sakura, Alphen aan den Rijn, Netherlands). During this process, the sample was dehydrated through a series of
increasing ethanol concentration from 70% to 100%, followed by 100% xylene, and finally submerged in paraffin wax maintained at 60°C and placed in a vacuum to allow the paraffin to infiltrate the tissue.

Samples were then embedded in paraffin blocks and sectioned using a Leica RM 2245 microtome (Milton Keynes, UK) at 10μm intervals. This was carried out by Viv Allison and Louise Dunn from the University of Edinburgh. Sections were floated onto standard glass slides (Thermo Scientific, Cramlington, UK) and transferred to a 37°C oven to dry overnight before storing at room temperature.

4.4 Results

4.4.1 Rheology Measurements

The mechanical properties of the gels were measured using rheology, in order to establish the formulation of each gel which most accurately represents the tumour microenvironment. The results of these measurements are presented in Sections 4.4.1.1-4.4.1.3.

4.4.1.1 Rheology of Agarose Hydrogels
Results

A comparison of the Young’s modulus (Pa, Pascal) of HT Agarose (ORANGE) and LT Agarose (BLUE) at varying concentrations (n ≥3). Error bars show SEM.

HT Agarose hydrogels were prepared and rheology was carried out to determine the Young’s modulus. LT Agarose hydrogels were produced of the same concentration as the HT Agarose - however, these hydrogels were extremely soft and rheology was not possible (Data not shown). Therefore, LT Agarose hydrogels were prepared at higher concentrations than the standard HT Agarose to achieve a Young’s modulus of around 1kPa. The HT Agarose followed a close linear trend in its increasing Young’s modulus alongside an increase in concentration.

The 0.2% HT Agarose had the lowest modulus (522Pa, n=5; SEM= 7.8), with a steady increase to 673Pa (n=3; SEM= 1.2) for the 0.22% gel. The 0.25% HT Agarose hydrogel had an average Young’s modulus of 999Pa (n=4; SEM= 51.2) which was the closest to the target of 1kPa. The 0.3% gel was stiffer than this with a mean modulus of 1356Pa (n=3; SEM=74.5)
The rheology of the LT Agarose hydrogels did not follow such a close linear trend, with the lowest modulus of 555Pa in the 0.4% gel (n=3; SEM= 31.3). A hydrogel of 0.45% was found to be the closest obtainable Young’s modulus to the target of 1kPA, with a mean modulus of 664 Pa (n=3, SEM= 85.2), since a hydrogel of 0.5% had a significantly higher Young’s modulus of 4874 Pa (n=3; SEM= 16.6).

4.4.1.2 Rheology of Alginate Hydrogels

Rheology was carried out on gels produced with the addition of CaCO3/GDL, as described in Section 4.3.3.2.

**Figure 4.14 : Measured elasticity of Alginate hydrogel**

The graph shows the Young’s modulus (Pa, Pascal) of Alginate hydrogels prepared with CaCO3 with varying concentrations of alginate solution (n ≥3). Error bars show SEM.
There is a clear pattern of increasing Young’s modulus alongside the increase in alginate concentration. The lowest modulus was recorded in the 0.4% gel (463 Pa; n=3; SEM=33), with an increase to 846 Pa for the 0.45% gel (n=4; SEM=21.8).

The hydrogel with a concentration of 0.5% had an average stiffness of 1019 Pa (n=6; SEM=3.5), which was the closes average value to the target of 1kPa. When the concentration was increased to 0.75%, the modulus was 1360Pa (n=4; SEM=58.6) and the highest modulus was recorded in the 1% gel (3866 Pa; n=3; SEM=680). The 1% gel had a large SEM, since there was a large variation in the measured stiffnesses (Range=6548.02 Pa), which may indicate that the materials within the hydrogel have not dissolved homogenously.

### 4.4.1.3 Rheology of Fibrin Hydrogels

Rheology was carried out on gels produced with Thrombin diluted in either F12K or CaCl₂, as described in Sections 4.3.4.2 & 4.3.4.3.
Figure 4.15: Measured elasticity of Fibrin hydrogels.

A comparison of the Young’s modulus of Fibrin hydrogels at varying concentrations of Fibrinogen. These hydrogels were prepared with Thrombin that was diluted in either F12K nutrient mix, or 30mM CaCl₂ (n ≥3). Error bars show SEM.

Fibrin hydrogels which were prepared with Thrombin diluted in F12K had a much lower Young’s modulus than those diluted in CaCl₂, even when the same concentration of Fibrinogen was used to make the hydrogel. This was also clear in the morphology of the hydrogel (data not shown) in which the Fibrin hydrogel prepared with only F12K was very soft, and appeared more like a liquid than a hydrogel at these concentrations.

The lowest concentration of fibrin made with thrombin diluted in F12K media was 2.5 mg/ml fibrinogen. This had a measured elasticity of 31 Pa (n=3; SEM= 15.1). The same formulation was used to prepare a 3mg/ml gel (120 Pa; n=3; SEM= 3.9). This could be directly
compared with the 3mg/ml gel which was prepared when thrombin was diluted in CaCl₂ instead of F12K, which resulted in a much higher modulus (601 Pa; n=3; SEM= 6.8).

A 4mg/ml fibrin hydrogel prepared with the addition of CaCl₂ had an average Young’s modulus of 1069 Pa (n= 4; SEM= 42.4) compared to the 4mg/ml F12K fibrin hydrogel which had an approximately four-fold lower average Young’s modulus of 231 Pa (n=5; SEM= 2.9). The 4mg/ml hydrogel prepared with CaCl₂ resulted in the closest measurement to the goal of 1kPa.

Since clearly the modulus was higher with a lower concentration of fibrinogen when CaCl₂ was used to dilute the thrombin, a 5mg/ml gel (1566 Pa; n=4; SEM= 141.8) and 6mg/ml (1892; n=3; SEM= 135) were also measured.

4.4.2 Laser interferometry analysis

The accurate transference of vibrations from the bioreactor through the gels was ensured by measuring each gel with laser interferometry. Firstly a calibration plate was measured, followed by the cell culture plates with the addition of hydrogels of different concentrations. The input voltage and frequency of the bioreactor was also altered to determine the effect of this on the different gels. The results of these measurements are presented in Sections 4.4.2.1-4.4.1.3.

4.4.2.1 Bioreactor calibration

When the bioreactor was tuned to vibrate at different frequencies, the amplitude increased alongside an increase in frequency. The range of displacement also increased as frequency increased. The 500Hz frequency induced an average displacement of 27.3nm (n=24; SD= 0.95) The average amplitude of the 1000Hz and 1500Hz frequency was very
similar, at 32nm (n=24; SD= 1.54) and 33nm (n=24; SD= 2.92) respectively. In contrast, the 2000Hz frequency had a much higher average amplitude at 48nm with a comparatively high standard deviation (n=24; SD= 12.85).

The range of displacement readings across the plate was also found to increase with the increasing frequency input. The table below details the range of displacement readings taken from each frequency.

<table>
<thead>
<tr>
<th>Frequency Applied, Hz</th>
<th>Average Displacement, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>30</td>
<td>35</td>
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<tr>
<td>35</td>
<td>40</td>
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<td>45</td>
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<td>50</td>
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<td>50</td>
<td>55</td>
</tr>
<tr>
<td>55</td>
<td>60</td>
</tr>
<tr>
<td>60</td>
<td>65</td>
</tr>
</tbody>
</table>

*Figure 4.16: Average displacement of calibration plate well surface under different frequencies*

Readings were taken from each well (n=24) and averaged. The vibration frequency was altered (shown on the x-axis), while input voltage remained the same at 12.1V. Points show mean displacement, error bars show SD.
Table 4.7: The range of recorded displacement across the entire plate with increasing frequency input

<table>
<thead>
<tr>
<th>FREQUENCY, Hz</th>
<th>500</th>
<th>1000</th>
<th>1500</th>
<th>2000</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Average displacement, nm</strong></td>
<td>27.3</td>
<td>32.4</td>
<td>33.6</td>
<td>47.9</td>
</tr>
<tr>
<td><strong>Lowest displacement reading, nm</strong></td>
<td>25.5</td>
<td>28.9</td>
<td>27.3</td>
<td>25.1</td>
</tr>
<tr>
<td><strong>Highest displacement reading, nm</strong></td>
<td>28.6</td>
<td>35.2</td>
<td>38</td>
<td>65.2</td>
</tr>
<tr>
<td><strong>Range of readings, nm</strong></td>
<td>3.1</td>
<td>6.3</td>
<td>10.7</td>
<td>40.1</td>
</tr>
</tbody>
</table>

This table clearly shows an increase in the range of displacement across the plate as frequency increased. At the 500Hz frequency, the range of displacement was 3.1nm (25.5 – 28.6 nm), while at 1000Hz the range of values increased to 6.3nm (28.9 – 35.2nm), increasing again at 1500 Hz to 10.7nm (27.3 – 38 nm). The largest range was seen when the plate was vibrated at a frequency of 2000 Hz (25.1 – 65.2 nm). This could be indicative of a resonance effect within the plastic material of the cell culture plate, in response to this specific frequency.
Amplitude was increased by increasing the input voltage. This corresponded to an increase in the average displacement of the wells. The standard deviation of the displacement for each of the amplitudes also increased as the voltage increased (shown on the graph).

The lowest input voltage was 8.7V, with an average displacement of 21.8nm (n=24; SD= 0.9) and this was increased to 12.1V, which resulted in an average displacement of 32.4nm (n=24; SD= 1.25). A voltage of 16.2V resulted in an average displacement of 42.2nm (n=25; SD= 1.29) and 18.3V had an average displacement of 51.1nm (n=24; SD= 1.55). Finally, the highest input voltage was 20.4V, which caused an average displacement of 62nm (n=24; SD= 1.61).
SD= 1.79). The SD remained low across each of the readings. However, the range of displacement readings across the plate was also found to increase with the increasing voltage input. The table below details the range of displacement readings taken from each voltage.

*Table 4.8: The range of recorded displacement across the entire plate with increasing voltage input*

<table>
<thead>
<tr>
<th>INPUT VOLTAGE, V</th>
<th>8.7</th>
<th>12.1</th>
<th>16.2</th>
<th>18.3</th>
<th>20.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average displacement, nm</td>
<td>21.8</td>
<td>32.4</td>
<td>42.4</td>
<td>51.1</td>
<td>62</td>
</tr>
<tr>
<td>Lowest displacement reading, nm</td>
<td>19.7</td>
<td>28.9</td>
<td>37.8</td>
<td>45.7</td>
<td>55</td>
</tr>
<tr>
<td>Highest displacement reading, nm</td>
<td>23.6</td>
<td>35.2</td>
<td>44.9</td>
<td>55.2</td>
<td>66.5</td>
</tr>
<tr>
<td>Range of readings, nm</td>
<td>3.9</td>
<td>6.3</td>
<td>7.1</td>
<td>9.5</td>
<td>11.5</td>
</tr>
</tbody>
</table>

This table shows an increase in the range of readings as the input voltage increased, although the range was much lower with the increase in voltage, than it was with the increase in frequency. At 8.7V the range of displacement readings was 3.9nm (19.7 – 23.6 nm), at 12.1V the range was 6.3nm (28.9 – 35.2 nm), increasing to 7.1nm (37.8 – 44.9nm) with 16.2V. An input voltage of 18.3V resulted in a range of 9.5nm (45.7 – 55.2 nm) and the highest range of displacement data was seen with a 20.4V input, with 11.5nm (55 – 66.5nm).
4.4.2.2 Hydrogel Interferometry

4.4.2.2.1 HT Agarose Hydrogel Interferometry

![Graph: Average Displacement of (HT) Agarose Gel Surface Under Different Frequencies]

*Figure 4.18: The average displacement of HT Agarose hydrogel under different frequencies*

Input voltage was 12.1V. Measurements were taken using hydrogels which were prepared with different concentrations of HT Agarose. Points represent the average displacement (n=5).

The average displacement of the hydrogels exposed to 500Hz, 1000Hz and 1500 Hz frequencies showed similar behaviour to each other, alongside the increasing hydrogel concentrations. As the HT Agarose concentration increased, the average displacement decreased, but ubiquitously, the 0.5% hydrogel correlated to an increase in average displacement across all of the different frequencies. At higher hydrogel concentrations, the average displacement of the hydrogel was very similar between each of the points, but especially the 500Hz, 1000Hz and 1500Hz frequencies. The 2000Hz frequency elicited a much higher average displacement from the hydrogels.
Figure 4.19: The average displacement of HT Agarose hydrogel under different input voltages

Frequency was 1kHz. Measurements were taken using hydrogels which were prepared with different concentrations of HT Agarose. Points represent the average displacement (n=5).

As the voltage increased, the average displacement also increased. Generally, the concentration of the hydrogel did not appear to have a significant impact on the average displacement, as the amplitude remained almost the same across each hydrogel. Although the 0.25% hydrogel was found to be the ideal stiffness of ~1kPa, this particular hydrogel concentration was not tested with interferometry.
4.4.2.2.2 LT Agarose Hydrogel Interferometry

![Graph showing average displacement of (LT) Agarose Gel Surface under different frequencies](image)

**Figure 4.20: The average displacement of (LT) Agarose hydrogel under different frequencies**

Input voltage was 12.1V. Measurements were taken using hydrogels which were prepared with different concentrations of LT Agarose. Points represent the average displacement (n=5).

The average displacement of the LT Agarose hydrogels under a variety of frequencies was measured. Each of the hydrogels tends to follow the same pattern of a slightly decreasing amplitude alongside the increase in hydrogel concentration, up to the 0.5% hydrogel where the amplitude increases. The average amplitude of the hydrogels are very similar for the 1 kHz and 1500 Hz frequencies, with a difference in average displacement of only around 1nm up to the 0.5% hydrogel.
Figure 4.21: The average displacement of (LT) Agarose hydrogel under different input voltages

Frequency was 1kHz. Measurements were taken using hydrogels which were prepared with different concentrations of LT Agarose. Points represent the average displacement (n=5).

The average displacement with changing input voltage follows the same pattern across each of the hydrogel concentrations. There is a small decrease in amplitude up to the 0.45% hydrogel, and a small increase with the 0.5% hydrogel, for each voltage.

Based on the rheology data, the 0.45% hydrogel was found to have the closest average stiffness to the aim of 1kPa, so it was highlighted across both graphs. The combined input of a 1kHz frequency and a 12.1V input correlated to an average amplitude of 30nm for the 0.45% hydrogel, which was the goal for average displacement of the hydrogel.
4.4.2.2.3 Alginate Hydrogel Interferometry

![Graph of Average Displacement of Alginate Gel Surface Under Different Frequencies](image)

**Figure 4.22: The average displacement of Alginate hydrogel under different frequencies**

Input voltage was 12.1V. Measurements were taken using hydrogels which were prepared with different concentrations of Alginate solution. Points represent the average displacement (n=5).

As the concentration of the hydrogel increased, the average displacement decreased, and this behaviour was common to each frequency. The average displacement for the hydrogels was higher than the calibration plate for each frequency.
Figure 4.23: The average displacement of Alginate hydrogel under different input voltages

Frequency was 1kHz. Measurements were taken using hydrogels which were prepared with different concentrations of Alginate solution. Points represent the average displacement (n=5).

The average displacement of the wells containing Alginate hydrogel increased linearly with the increase in voltage. Furthermore, as the alginate solution concentration of the alginate hydrogels increased, the average displacement decreased, and this pattern was seen across each of the voltages.

Across both graphs, the average displacement of the 0.5% Alginate hydrogel has been highlighted, since this correlates with the ~1 kPa hydrogel, confirmed using rheology. In general, the average displacement tends to decrease with increasing hydrogel concentration, with a small increase between the 0.25% - 0.5% hydrogel. The input voltage of 12.1V correlates to an average amplitude of 35nm for the 0.5% hydrogel at a frequency of 1kHz,
while the 500Hz frequency results in a lower average displacement of 29nm. This was slightly closer to the goal of a 30nm average displacement for the hydrogels.

4.4.2.2.4 Fibrin Hydrogel Interferometry

![Graph showing average displacement of fibrin gel surface under different frequencies](image)

*Figure 4.24: The average displacement of Fibrin hydrogel under different frequencies*

Input voltage was 12.1V. Measurements were taken using hydrogels which were prepared with different concentrations of Fibrinogen. Points represent the average displacement (n=5).

At the 500 Hz frequency, the average displacement does not appear to change much in relation to Fibrin hydrogel concentration. As the frequency increases, there is a greater variation in average displacement coinciding with an increase in hydrogel concentration.
Figure 4.25: The average displacement of Fibrin hydrogel under different input voltages

Frequency was 1kHz. Measurements were taken using hydrogels which were prepared with different concentrations of Fibrinogen. Points represent the average displacement (n=5).

There is a clear pattern followed by the average displacement of the hydrogels with increasing input voltage, although there does not appear a significant overall change in average displacement associated with increasing hydrogel concentration. The 4mg/ml hydrogel was highlighted across both graphs, since this is the concentration which was found to have the Young’s modulus of ~1kPa. For this hydrogel, the average displacement with an input of 12.1V and 1 kHz frequency was around 34nm.
4.4.3 Hydrogel Histology

Each of the hydrogels exhibits distinctive morphological features when observed under a microscope. Within the LT Agarose section, the presence of two discernible areas was apparent. Furthermore, the peripheral regions of some gel sections appear to have detached from the main Agarose structure, which could be attributed to melting during the wax processing phase.

The Alginate hydrogel, on the other hand, contains minute crystalline structures within its composition. These structures may be responsible for the observed striping patterns.
in the sections. These crystalline structures could correspond to calcium carbonate (CaCO3) crystals that have not completely dissolved within the hydrogel matrix.

The Fibrin hydrogel appears to have largely maintained its overall structure, although there are a couple of small holes which are visible in the material. It is impossible to say whether these holes existed in the hydrogel prior to wax embedding and sectioning process.

4.5 Discussion

4.5.1 Agarose Hydrogels

It has been established that hydrogels can be produced in a number of ways to adjust their suitability for different purposes. For example, increasing the cellular compatibility of hydrogels such as Agarose through the addition of collagen349, or modifying the drug delivery action of Fibrin gels by altering fibrinogen concentration405. Therefore the purpose of this study was to establish the most suitable protocol for the generation of hydrogels to support GBM stem cell culture, and vibration propagation.

Alginate and Agarose hydrogels, both derived from seaweed351, share the characteristic whereby cells are not able to attach directly to the gel structure. Thus ensuring that the cells would be encapsulated without the ability to bind to their immediate environment i.e. the hydrogel. Structurally however, the gels are very different. Alginate gel is formed by the ionic bonds between molecules within the gel, while Agarose forms a hydrogel through physical cross-linking and hydrogen bonds406.

HT and LT Agarose gels have a number of overlapping uses, including gel electrophoresis332 and tissue engineering scaffolds357,407. Their structural differences are related to modifications of HT Agarose, carried out through chemical processes, leading to
reduced hydrogen bonds between agarose strands. HT Agarose therefore has a much higher gel strength (referring to the force which must be applied to cause the hydrogel to fracture) than LT Agarose. According to the manufacturer of the Agarose used in this study, a 1% HT Agarose hydrogel has a six-fold higher gel strength (1200 g/cm²) compared to the same concentration of LT Agarose hydrogel (200 g/cm²). The gel strength is closely linked to the composition of the fibres within; a study by Tako et al. confirmed an increase in gel strength is associated with intermolecular ionic interactions. Therefore it is logical that the HT Agarose has as much higher gel strength that the LT Agarose, owing to the presence of a greater number of Hydrogen bonds present. This was also confirmed by Zhang et al., who produced several LT Agarose hydrogels through the oxyalkylation of HT Agarose, leading to reduced gel strength, melting and gelling temperature.

The thermotropic gelation process is the same for both of these types of hydrogel, in spite of their structural differences. However, since the Agarose gel strands form bonds as they cool, the difference between these two hydrogels becomes apparent. The LT Agarose has a melting point of 60 °C while the HT Agarose melts at 86.5-89.5 °C. Therefore as the solutions are heated at the same rate, the LT Agarose will melt sooner. Furthermore, LT Agarose has a gelling temperature of between 26-30°C while HT Agarose forms a gel at 36-40°C. Since HT Agarose may form a gel if cooled to 37°C, it could not have been maintained in solution at 37°C in preparation for the addition of GBM cells.

The aim of the rheology analysis was to ascertain the Young’s modulus of different hydrogel formulations, in order to obtain hydrogels of a specific elasticity: in this case, around 1kPa. This elasticity modulus was chosen due to its overlapping physiological relevance for both native brain tissue (200Pa – 1kPa) and the GBM tumour environment (1kPa –
26kPa). The HT Agarose hydrogel produced at a 0.25% concentration was very close to 1kPa, at 999 Pa while the nearest LT Agarose hydrogel in elasticity to the 1kPa goal was the 0.45% LT Agarose hydrogel.

### 4.5.2 Alginate Hydrogels

Alginate hydrogels were formed using two different methods in this study. Alginate gels are usually formed ionotropically with the addition of divalent cations like Ca$^{2+}$ or Cu$^{2+}$, although some thermotropic gelling ability has also been demonstrated$^{344}$. Commonly, the addition of CaCl$_2$ is employed to form Alginate beads for drug or cell encapsulation, since this results in almost instantaneous, uniform gel structures$^{378,411}$. However, while this approach is effective in the production of Alginate beads in particular, producing larger quantities of a uniform gel requires a different approach. When producing the Alginate gel inside the well of a cell culture plate, the addition of a CaCl$_2$-soaked piece of filter paper was effective at introducing the Ca$^{2+}$ ions into the Alginate solution, and therefore forming a gel. However, when the Alginate solution was of a relatively low concentration, and therefore less viscous, the filter paper sank into the gel as it formed and was impossible to remove without damaging the gel.

The addition of CaCO$_3$ and GDL is a very well-established method of Alginate production. It has been found to result in a slower rate of gel formation, since the Calcium ions are not readily available upon addition to the alginate solution, but instead are dissociated by the hydrogen atoms released by GDL$^{374,412}$. As expected, this method resulted in a slower gel formation overall, since it allows more time for CaCO$_3$ to distribute throughout the Alginate solution before gelation$^{374}$. 

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The Alginate hydrogel formed with the addition of CaCO$_3$/GDL powders followed a pattern of increasing elasticity alongside an increasing concentration of Alginate solution. A hydrogel prepared with a 0.5% alginate solution resulted in a stiffness of 1019 Pa, therefore this would be the ideal concentration of Alginate for use in studies with GBM cells.

4.5.3 Fibrin Hydrogels

Fibrin gel is very commonly utilised as a cell scaffold in tissue engineering owing to its inherent biocompatibility and highly tuneable mechanical properties. The role of Fibrin in the formation of blood clots and wound healing requires a balance of stiffness and elasticity. Fibrin is formed by the enzymatic cleavage of Fibrinogen by Thrombin, leading to the production of protofibrils which pack together to form fibres of Fibrin. There are many elements which can influence the mechanical properties of Fibrin gel, including the concentrations of both Fibrinogen and Thrombin, as well as the presence of calcium ions.

Increasing the concentration of Fibrinogen in the gel has been found to universally increase the stiffness (elasticity modulus) of the gel. This has been linked to an increased number of Fibrin fibres in the gel. Increased fibre thickness and branching has also been implicated in increasing elasticity, which has been linked to increasing Thrombin concentration, to a point, or including Calcium ions in the solution.

An elegant study carried out by Duong, Wu and Tawil, investigated the effect of different fibrinogen and thrombin concentrations in the Young’s Modulus of Fibrin gel formation. They found that increasing the Thrombin concentration increased the stiffness of the gel up to a point, while increasing fibrinogen concentration continued to increase the stiffness up to their highest tested concentration of 50 mg/ml.
The addition of Calcium ions in the formation of Fibrin hydrogels has been found to promote polymerisation, and increase stability of the hydrogel\textsuperscript{390,415}. The results of this study reflected this, since Fibrin hydrogel for which the Thrombin was diluted in CaCl\textsubscript{2} resulted in stiffer gels than the hydrogel prepared with Thrombin diluted in F12K. In fact, it was difficult to produce a hydrogel which was stiff enough to transfer onto the rheology plate in one piece without the addition of CaCl\textsubscript{2}. The hydrogels produced with just F12K were very soft, and behaved more like a liquid than a hydrogel. This was illustrated by the Young’s modulus which was obtained from hydrogels of the same Fibrinogen concentration, showing a significant difference in stiffness. For example, a 3mg/ml hydrogel produced with F12K had a Young’s modulus of 121 Pa compared to a 3mg/ml hydrogel produced with CaCl\textsubscript{2} which had a Young’s modulus of 601 Pa. The Fibrin gel produced with 4mg/ml Fibrinogen diluted in CaCl\textsubscript{2} had a Young’s modulus of 1069 Pa, which was the closest stiffness to the goal of 1kPa.

4.5.4 Further Considerations

It is clear from these results, and previous studies, that variations in the composition and type of material being vibrated, can impact the propagation of signals from the bioreactor through the hydrogel\textsuperscript{403}. Therefore the presence of cells within the gel may affect the vibrations, however this was not investigated in this study. Furthermore, the hydrogels may degrade over time with exposure to the physical stimulation\textsuperscript{416}, but this degradation was not investigated in this study.

There is evidence that there is a difference between the physical responses of each fel to the vibrational input, as can be seen in the interferometry data. The interferometry data in Figures 4.18, 4.20, 4.22 and 4.24 show that the same input frequency can result in a different displacement for each gel, which is likely due to a resonance response owing to the
different physical compositions of each gel type. This variation is also seen in the calibration plate (Figure 4.16), with increasingly large error bars alongside increasing frequency, suggesting that the plastic cultureware itself may add to this resonance response.

### 4.6 Conclusions

The HT Agarose generally had a more consistent stiffness according to readings taken with the rheometer, but the high gelling temperature of the material is largely incompatible with live cell culture, furthermore the experimental design required the Agarose to be maintained as a liquid at 37°C before cell seeding, which would not have been possible with the HT Agarose. Therefore the HT Agarose was disregarded as a material for further studies.

Contrastingly, due to its lower gelling temperature, the LT Agarose is more suitable for use in cell culture. Although, when the hydrogel was attached to the vibrational bioreactor and measurements were taken with the laser interferometer, the average displacement was exactly 30nm when the bioreactor was calibrated to 1kHz frequency at 12.1V input. This was the goal displacement for experimental purposes. When the hydrogel was embedded in wax, and sliced for histology, the material was easily sliced and visualised. The low gelling temperature (LT) Agarose was less consistent in rheology measurements, the 0.45% hydrogel had an average Young’s modulus of 664 Pa.

When preparing the Alginate hydrogel, the CaCO$_3$/GDL mixture had to be constantly agitated while the hydrogel formed, in order to encourage it to fully dissolve. Rheology measurements found a 0.5% hydrogel had a Young’s modulus of 1019 Pa, and this correlated to an average displacement of 37.98nm which was slightly higher than the goal displacement of 30nm. The histological slices showed grainy artefacts under the microscope, as well as some striations which may have been caused by incomplete dissolution of the CaCO$_3$.
A 4mg/ml Fibrin hydrogel made with CaCl$_2$ was found to have a Young’s modulus of 1069Pa, correlating to an average displacement of 34nm when the bioreactor was set to 12.1V input and 1 kHz frequency, which is only slightly higher than the goal displacement of 30nm. The histological slices taken from the hydrogel which was embedded in wax and sliced, showed some very small holes in the hydrogel which may have appeared during the hydrogel formation.

In summary, the Fibrin and Alginate hydrogels were more easily tuned to produce a hydrogel of specified stiffness. The (LT) Agarose hydrogel produced an average displacement of exactly 30nm, while the average displacement of the Fibrin and Alginate hydrogels was slightly higher. For the purposes of the study of neurospheres in 3D hydrogel culture, the goal was to establish the formation of hydrogels which were similar in elasticity, and could propagate signal vibrations to allow a known level of nanoscale vibrations to stimulate the cells. In this case, generating a gel which had a measured Young’s Modulus of 1kPa, to allow an average 30nm vertical cell displacement. According to the results of this study, these parameters were established to be best achieved using a 4mg/ml Fibrin gel (+CaCl$_2$), a 0.5% Alginate gel (+CaCO$_3$), and a 0.45% LT Agarose gel.
Chapter 5: MECHANICAL STIMULATION OF CANCER STEM CELLS IN A 3D MODEL

5.1 Chapter Overview

This chapter builds upon the cumulative knowledge gained in preceding chapters, applying it to the culture of Glioblastoma (GBM) stem cells in neurosphere formation and within hydrogels. These models offer a more physiologically relevant context for studying the impact of nanoscale vibrations on these cells.

In the hydrogel environment, cells were introduced either as single entities or as neurospheres, further diversifying the experimental approach. Additionally, the chapter explores methods for retrieving cells from the hydrogels, a critical aspect in developing and optimizing this 3D cell culture system. By integrating these elements, the research expands our understanding of the interplay between nanoscale mechanotransduction and GBM stem cells within more realistic physiological models.

As the key importance of the extracellular matrix on cell behaviour becomes clearer, there is a natural transition from observing cells in 2D culture, to their behaviour in a 3D model. Numerous studies have investigated the impact of culturing cells on surfaces with varying degrees of elasticity, revealing differences in cell motility, spreading, and protein expression\textsuperscript{202,417}. Therefore it is logical to consider methods of designing a more native environment for cell culture, \textit{in vitro}. 
5.2 Introduction

5.2.1 Isolating and culturing neural stem cells in neurospheres

The formation of neurospheres has been a staple of the neural stem cell isolation process since it was first described by Reynolds and Weiss in 1992\textsuperscript{113}. They demonstrated a simple and reproducible method of NSC culture, as well as the differentiation response of adult NSC upon growth factor withdrawal\textsuperscript{113,418}. The neurosphere formation assay (NFA) results in a large amount of cell death, leaving a tiny population of NSC which form neurospheres. One study found a decrease in cell number from 100 cells/cm\textsuperscript{2} to 2 cells/cm\textsuperscript{2}, across 7 independent culture preparations\textsuperscript{418}.

When cells cultured in a neurosphere undergo differentiation, for example by culturing in a differentiation medium, they may undergo asymmetric cell division (Figure 1.4) which maintains a population of stem cells while also producing differentiated cells\textsuperscript{419}. This type of cell division increases the population of cells overall, and the differentiated cells are likely to migrate away from the neurosphere\textsuperscript{275,420,421}.

5.2.2 GBM cells in 2D culture

Matrigel is a widely used substrate in 2D neural cell culture, including Glioblastoma (GBM) cell culture, primarily due to its ability to mimic certain aspects of the ECM, including laminin, collagen IV and heparin sulfate proteoglycans\textsuperscript{422}, enabling the support of cell growth and proliferation\textsuperscript{423}. Matrigel represents a valuable tool in GBM research, providing a substrate for 2D culture that partially replicates the complex ECM environment and supports the growth of GBM cells \textit{in vitro}\textsuperscript{417,424}. However, it poses challenges in terms of precisely characterizing its structure because there can be batch-to-batch variation, and the
manufacturer does not disclose its exact composition. Furthermore, since it is derived from Engelbreth–Holm–Swarm mouse sarcomas basement membranes, it is not a truly accurate representation of the GBM microenvironment.

5.2.2.1 How cells interact with their environment

It has been established that the physical environment in which a cell resides is as essential in cell survival and behaviour as the chemical environment. When cells are cultured in 3D environments, they are exposed to a variety of mechanical and biochemical signals from their interactions with elements of the 3D environment, as well as cell-cell signalling.

The ECM is made up of a complex web of interlinked proteins, containing signalling molecules like cytokines, growth factors and MMPs. These ECM is constantly remodelled by the cells within, as they produce and secrete the proteins which comprise it. The mechanical properties of the ECM are sensed by integrins, which connect the extracellular environment with the actin cytoskeleton.

Proteins which are found in the ECM include laminin, collagen, fibronectin and elastin. Additionally, a large family of proteins known as proteoglycans form part of the ECM, and can also be found on cell surfaces. They are involved in numerous signalling pathways, regulating proliferation, migration, differentiation and apoptosis. Furthermore, some abnormalities in their function can be observed as a prognostic marker in malignancies, including gliomas. Fibronectins interact with a large number of growth factors, cell surface receptors and other ECM molecules. These proteins exist in the ECM and on cell surfaces, enabling the interaction of cells via integrins, and through these interactions, the cell is able to detect mechanical cues.
5.2.3 GBM cells in 3D culture

As it has been established that the characteristics of cells can be altered by their microenvironment, it follows that altering hydrogel properties to mimic cellular environments could allow cells to be cultured in an environment which more closely resembles their native tissue. In glioma research, 3D models may be employed to investigate their susceptibility to anti-cancer drugs in particular. Interestingly, GBM cells cultured in 3D models have been found to show an increased resistance to treatment with chemotherapeutic drugs\textsuperscript{431,432}. This further highlights differences in behaviour between cells cultured in 2D and 3D environments. In this case, the resistance to chemotherapy could be due to the mechanical challenges introduced by 3D culture i.e. the need for chemotherapeutic agents to diffuse through the 3D scaffold, or into the centre of a neurosphere. However, there is also a difference in the characteristics of cells cultured in 2D and 3D in terms of their morphology and protein expression\textsuperscript{433}, which could also be impacting their response to chemotherapy, since most of the current methods for drug evaluation are based on 2D models of cell culture\textsuperscript{434,435}.

5.2.4 Examples of Hydrogels for 3D Culture

A common strategy of 3D culture involves incorporating multiple materials into the scaffold to mimic the complexities of the \textit{in vivo} environment. This may involve combining ECM proteins like Collagen or laminin with materials such as Hydroxypropyl methyl cellulose (HMC), Hyaluronic Acid (HA), Polyacrylamide (PA) or Poly(ethylene glycol) (PEG). Some examples of these are described in Table 5.1 below.
Table 5.1: Examples of common biomaterials for cell culture, relevant for brain studies

PEG: Poly- (ethylene glycol); HA: Hyaluronic Acid

<table>
<thead>
<tr>
<th>MATERIAL</th>
<th>CROSSLINKING</th>
<th>COMMON COMPOSITES</th>
<th>ELASTIC MODULUS:</th>
<th>REF</th>
</tr>
</thead>
<tbody>
<tr>
<td>MATRIGEL</td>
<td>Thermal</td>
<td>Alginate, Agarose</td>
<td>0.4kPa</td>
<td>195,33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>COLLAGEN</td>
<td>Thermal, Photocrosslinking</td>
<td>Matrigel, Fibrin, Agarose, HA, PA</td>
<td>0.9 – 3.6kPa</td>
<td>345,34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7,407</td>
</tr>
<tr>
<td>FIBRIN</td>
<td>Enzymatic</td>
<td>HA, Collagen, Laminin</td>
<td>0.058 – 4kPa</td>
<td>322,35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0,436</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>438</td>
</tr>
<tr>
<td>PEG</td>
<td>Photocrosslinking</td>
<td>HA, Laminin</td>
<td>1 – 26kPa</td>
<td>88,284</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>,309</td>
</tr>
<tr>
<td>ALGINATE</td>
<td>Ion mediated</td>
<td>Matrigel, PEG, Gelatin</td>
<td>&lt; 26 kPa</td>
<td>371,41</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1,439</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>441</td>
</tr>
<tr>
<td>HA</td>
<td>Photocrosslinking</td>
<td>Collagen, Gelatin, Fibrin, Laminin, Chitosan</td>
<td>0.011 – 3.5kPa</td>
<td>442,44</td>
</tr>
<tr>
<td>AGAROSE</td>
<td>Thermal</td>
<td>Matrigel, Agarose, HMC</td>
<td>0.005 - 1.3kPa</td>
<td>331,34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8,444</td>
</tr>
</tbody>
</table>
**5.2.4.1 Matrigel and Collagen**

One study investigated the migration of GBM CSCs through Matrigel and collagen type-I monomer matrices, concluding that the characteristics of migration were strongly influenced by the physical properties of the matrix\textsuperscript{445}. This included alterations in the stiffness, the presence of pseudo-vessels (synthetic polymer rods designed to mimic the topography introduced by blood vessels), the presence of Hyaluronic acid (HA), and the ratio of Matrigel to Collagen. This behaviour reflects the migration patterns of tumour cells \textit{in vivo}, which exhibit a strategy of optimal migration dependent on the physical and chemical obstacles faced by the cells, highlighting the effectiveness of constructing a biomimetic hydrogel for 3D tumour modelling\textsuperscript{445}.

**5.2.4.2 Fibrin gel**

Fibrin gel may be employed to provide similar support to cells as collagen matrices, since fibrinogen and collagen fibres can both be found in the ECM. Fibrin plays an essential role in wound healing in the body, and in 3D cell culture it has an advantage over collagen since its mechanical properties are highly tuneable\textsuperscript{437}. Fibres like collagen, fibronectin and fibrinogen\textsuperscript{446} exist in the ECM to provide structural support to cells, allowing cells \textit{in vivo} to interact with their extracellular environment.

**5.2.4.3 Collagen and Agarose**

For instance, in collagen-Agarose matrices, collagen fibres provide structural support for cell growth and motility, while Agarose forms a dense network surrounding these fibres. One particular study delved into the impact of varying Agarose concentration within this matrix on cell motility and invasion\textsuperscript{349}. The findings revealed that as Agarose concentration
increased, neurosphere invasion i.e. the migration of cells away from the neurosphere, was progressively reduced. This effect continued up to a concentration of 1% Agarose, at which point invasion was entirely inhibited. These results reveal the intricacies of 3D cell culture and highlight how the composition of the scaffold material can significantly influence cellular behaviours, offering valuable insights for tissue engineering and disease modelling applications.

### 5.2.5 Aims & Hypotheses of this Chapter

**Aims:**

1. To optimise an experimental design to produce viable neurospheres for hydrogel culture
2. To establish the effect of culturing GBM CSC neurospheres in different hydrogels and different media
3. To establish the effect of culturing GBM CSC neurospheres in different hydrogels and different media under the influence of nanoscale vibrations

**Hypotheses:**

1. The neurosphere size will affect its survivability and characteristics
2. Neurospheres cultured in Fibrin hydrogel will form cell-matrix attachments, while neurospheres in Agarose and Alginate will not
3. The neurospheres cultured in Fibrin hydrogel will be more responsive to nanoscale vibrations

### 5.2.6 Summary of Methodology

Flowchart shows the goals for this chapter. The methods are expanded on in section 5.3.
5.3 Methods

5.3.1 Media preparation for different experimental conditions

5.3.1.1 Full Expansion Media

M-cells were used for the following experiments. Stem cell culture media (henceforth referred to as Full Expansion Media) was prepared using Advanced DMEM-F12 (Gibco), supplemented with 0.5% L-glutamine (Gibco), 1% 200mM Amphotericin B (Gibco), 1% B27 (Gibco), 0.5% N2 (Gibco), 1% Penicillin-Streptomycin (Sigma-Aldrich) and 0.5% Heparin (Sigma-Aldrich) with the addition of the following growth factors: Recombinant Human FGF-basic (Peprotech, Thermo Fisher Scientific, Cramlington, UK) and Recombinant Murine EGF (Peprotech) at 10ng/ml each. When culturing cells in flasks for expansion, 50% of the full expansion media was changed every 2-3 days, and cells were passaged at confluence, approximately 5-7 days. Full Expansion Media was stored in the fridge (4°C) for up to two weeks, or in the freezer (-20°C) for up to 4 weeks. This was used in experiments in cell expansion, and in experiments described as ‘Control +GF’ or ‘NMT +GF’.

5.3.1.2 Full Expansion Media without growth factors

Stem cell culture media without growth factors was prepared using Advanced DMEM-F12 (Gibco), supplemented with 0.5% L-glutamine (Gibco), 1% 200mM Amphotericin B (Gibco), 1% B27 (Gibco), 0.5% N2 (Gibco), 1% Penicillin-Streptomycin (Sigma-Aldrich) and 0.5% Heparin (Sigma-Aldrich). Media was stored in the fridge (4°C) for up to two weeks, or in the freezer (-20°C) for up to 4 weeks. This was prepared in a sterile environment, and used in experiments described as ‘Control –GF’ or ‘NMT –GF’.
5.3.1.3 DMEM/F-12 +BMP4

Advanced DMEM-F12 (Gibco) was prepared with the addition of BMP4 (Gibco) to produce a final concentration of 20ng/ml, 1% 200mM Amphotericin B (Gibco) and 1% Penicillin-Streptomycin (Sigma-Aldrich). Media was stored in the fridge (4°C) for up to two weeks, or in the freezer (-20°C) for up to 4 weeks. This was prepared in a sterile environment, and used in experiments described as ‘Control +BMP4’ or ‘NMT +BMP4’.

5.3.1.4 DMEM/F-12 +FBS

Advanced DMEM-F12 (Gibco) was prepared with the addition of 10% Foetal Bovine Serum (FBS; Labtech, Heathfield, UK), 1% 200mM Amphotericin B (Gibco) and 1% Penicillin-Streptomycin (Sigma-Aldrich). Media was stored in the fridge (4°C) for up to two weeks, or in the freezer (-20°C) for up to 4 weeks. This was prepared in a sterile environment, and used in experiments described as ‘Control +FBS’ or ‘NMT +FBS’.

5.3.1.5 Experimental Conditions

Table 5.2: Media preparation for different experimental conditions

<table>
<thead>
<tr>
<th>CONDITION</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>NMT</td>
</tr>
<tr>
<td></td>
<td>‘Control’ plate is not receiving any vibrational stimulation.</td>
</tr>
<tr>
<td></td>
<td>‘NMT’ cells are receiving nanoscale vibrational stimulation.</td>
</tr>
<tr>
<td>CONT +GF</td>
<td>NMT +GF</td>
</tr>
<tr>
<td></td>
<td>Full expansion media (See Section 2.1): Contains growth factors EGF and FGF</td>
</tr>
<tr>
<td>CONT -GF</td>
<td>NMT -GF</td>
</tr>
<tr>
<td>CONT + BMP4</td>
<td>NMT +BMP4</td>
</tr>
<tr>
<td>CONT+ FBS</td>
<td>NMT +FBS</td>
</tr>
</tbody>
</table>

5.3.2 Seeding Gels with Single Cells

The viability of cells in hydrogels was explored by initially seeding single cells into the three different hydrogel types. Cells were prepared and counted as described in Sections 2.1 - 2.3. After counting, approximately 20,000 cells were mixed into the Fibrin gel, Alginate gel and Agarose gel each as they formed. The gels were prepared as described in Sections 4.3.4.2, 4.3.3.3 and 4.3.2. Once the gels had formed, 500 µl of full expansion media (As described in Section 2.1) was added to each well. The cell-seeded gels were transferred to an incubator and maintained at 37°C, 5% CO₂ [v/v]. Cells were cultured 7 days. 50% of the media was removed and replaced every 2 days.

5.3.3 Single Cell Retrieval from Gels

5.3.3.1 Agarose (Low Temp): Melt gel

To retrieve the single cells from Agarose, the media was first carefully aspirated from the wells, and the gel was washed twice with 200µl PBS. The low temp gel has a melting temperature of around 60°C, therefore it was transferred to an oven set to this temperature,
and left until the gel melted, up to 60 minutes. The melted gel was then quickly pipetted into a 1.5ml Eppendorf, and centrifuged at 330 xg for 1 minute to encourage the cells to sink to the bottom of the Eppendorf. However, individual cell removal was a challenge - this is expanded on in Section 5.4.1.2.1.

5.3.3.2 Alginate: Dissolve gel in Sodium Citrate

To retrieve cells from Alginate, media was first carefully aspirated from the wells, and the gel was washed twice with PBS. 500μl of a 10mM solution of sodium citrate buffer was applied (Prepared as described in Appendix 3), and the hydrogels were returned to the incubator to allow the sodium citrate to break down the alginate gel. After an hour, the liquid was collected into a 1.5ml Eppendorf, and this was centrifuged at 330 xg for 1 minute. The supernatant was discarded, leaving ~10μl of liquid and a pellet in the bottom of the Eppendorf. 100μl of PBS was carefully added, and then gently removed. This pellet was then resuspended in 100μl DMEM/F12 and gently pipetted to resuspend the cells.

5.3.3.3 Fibrin: Digestion with Trypsin-EDTA

To retrieve cells from Fibrin, media was first carefully aspirated from the wells, and the gel was washed twice with PBS. 500μl of 0.05% Trypsin-EDTA was applied, and the gels were returned to the incubator for between 30-60 minutes, to allow the Trypsin to break down the Fibrin. After this time, the contents of the well were pipetted up to 5 times and then collected into a 1.5ml Eppendorf. This was centrifuged at 330 xg for 1 minute. The supernatant was discarded, leaving ~10μl of liquid in the bottom of the Eppendorf. 100μl of PBS was carefully added, and then gently removed. This pellet was then resuspended in 100μl DMEM/F12 and gently pipetted to resuspend the cells.
5.3.4 Neurosphere Formation

According to the cell culture protocol in Section 2.1, a T75 Flask of M-cells was cultured to confluence. The media was aspirated and the flask was washed twice in 500μl PBS. 3ml Accutase was added to the T75 flask, and this was incubated to detach the cells. Once the cells detached, they were collected in 7ml fresh DMEM-F12 and transferred to a 15 ml falcon tube. The cells were centrifuged at 330 xg for 3 mins, and the supernatant was discarded. The cells were resuspended in 2ml full expansion media and counted using a haemocytometer. After counting, a specific number of cells was transferred to the 96-well, U-bottomed cell-repellent plates to form neurospheres, ranging from 15,000 to 60,000 cells. 200μl of full expansion media (details in Section 2.1) was added to each well and 100μl of this media was changed every day. Neurospheres were allowed to form for 4 days, with measurements of their size taken daily.
5.3.4.1 Measuring Neurosphere Size

A Microscope Micrometer was set under a Leica DMI1 light microscope (Leica), with photographs taken using the built-in Leica MC170 HD camera (Leica). The photographs were taken with the magnification at each setting i.e. 5x, 10x, 20x and 40x. Using ImageJ software the micrometer image, at a magnification correlating to the magnification used for the target cells being measured, was opened and using the line tool, a line was drawn between two 100um points on the micrometer. The measurement for this line was taken, and this was used to set the scale for the micrometer image, using the known size of the micrometer spaces. The exact size of the image in pixels was also recorded, to ensure the compared pictures were of the same scale. This process is demonstrated in Figure 5.1 below.

![Image of ImageJ software and a neurosphere measurement](image)

*Figure 5.1: ImageJ was used to measure the neurosphere*

Measurements were taken in triplicate by so that the mean size could be calculated for each neurosphere.

The file of samples to be measured was opened with ImageJ, and first the image scale was set to the same as the micrometer. Three diameter measurements were taken by drawing a line from one side of the neurosphere across the middle, and to the other side. The length of
these lines was in μm, using the scale previously set. This data was exported to an Excel spreadsheet, and the length of the diameter was averaged (n=3).

5.3.4.2 Initial Studies of Neurospheres in Hydrogels

Initial experiments to examine the culture of neurospheres in the hydrogels were carried out using the neurospheres which were produced as described in Section 5.3.4. After four days of neurosphere formation, the media was carefully removed and the neurospheres were washed gently with DPBS and resuspended in 20μl of full expansion media (Full details in Section 2.1.). This was collected into a 200μl pipette, with the tip cut off to avoid damaging the neurosphere. A diagram of the neurosphere encapsulation process is below (Figure 5.2).

A 3mg/ml fibrinogen solution was prepared (as described in Section 4.3.4) and 250μl of fibrinogen was pipetted into four wells of a 12-well plate. 250μl of thrombin mix was added to this, and this was incubated at 37°C to allow the gel to form. One or two of the neurospheres of each cell seeding density were placed on top of this first gel layer, depending on the number of neurospheres formed. A second layer of gel was prepared as described above, and layered on top of the neurosphere. This was incubated again to allow the gel to form, before 500μl of full expansion media was added on top.

In four other wells, a 0.5% alginate gel was added, and in the remaining four wells, a 0.45% LT Agarose gel was added, encasing the neurospheres within the gel.

These gels which contained the neurospheres were transferred to an incubator for 7 days, with photographs taken of the neurospheres on day 1, day 3 and day 7.
Methods

**Figure 5.2: Transferring neurospheres into hydrogels**

This method ensured the cells would not attach to the bottom of the well, and was suspended within the gels. Gel is approximately 12mm thick, with neurospheres in the middle at approximately 6mm.

5.3.5 Live/Dead Staining

Preparation was required to be carried out in the dark (Room lights switched off, carried out in tissue culture hood with light switched off), due to the photosensitive nature of the materials. Cells remained in the incubator while the working solutions were prepared. Sufficient working solution was prepared to fully cover each sample, accounting for 250µl/well (24-well plate) or 500µl/well (12-well plate). Solution was prepared with 7 µl of Calcein AM [50 µg/ml] (Invitrogen) per 1 ml of DMEM, and 20 µl of Propidium Iodide [1 mg/ml] (Invitrogen) per 1 ml of DMEM.
Samples were retrieved from the incubator, and the media was aspirated. The samples were then washed three times in 500μl PBS, before adding the Live/Dead working solution to the sample. This was incubated at 37°C for 10 mins for monolayer samples, and 60 minutes for 3D hydrogel samples. Again, this was carried out in the dark. After the allotted time, the Live/Dead solution was aspirated, and the sample was washed twice in PBS. The samples were then imaged using a confocal microscope (Leica TCS SP8) to capture a Z-stack.

### 5.3.5.1 Live/Dead Quantification

To quantify cells from a Z-stack image, the image was first opened in ImageJ, importing all of the stacked images. The Z-stacked images were merged into a single 8-bit image using Image > Stacks > Z-project, setting the projection type at Max intensity.

To quantify, the channels were split into red and green using Image > Colour > Split Channels. Background ‘noise’ was removed by adjusting the brightness and contrast using Image > Adjust > Brightness/Contrast.

The threshold was then set to highlight the cells of each channel, using Image > Adjust > Threshold. This produced a black and white image for each channel. In order to ensure individual cells were counted in densely packed areas, the cells were separated using the function: Process > Binary > Watershed.

Finally, the data was collected using Analyse > Analyse Particles for each channel, resulting in the data below, indicating the count, area and size of live and dead cells.
Methods

Figure 5.3: Quantification of Live/Dead cells in Neurosphere

The number of live/dead cells were quantified using ImageJ. While this gives a whole number, it was used as an approximation since it is sometimes difficult for the software to highlight the individual cells.

5.3.6 3D Nanoscale Mechanotransduction Experiment

5.3.6.1 Preparing Materials

Neurospheres were cultured for at least four days. After this time, the media was carefully aspirated from each well before rinsing with sterile PBS. The PBS was removed carefully to avoid disturbing the neurospheres, and 20μl of the appropriate cell culture media, corresponding to the experimental condition for each neurosphere was added to each well, in preparation for the neurosphere to be added to the cell culture plate with prepared gels.

Two 24-well cell culture plates were then prepared by labelling them appropriately in order to identify the contents of each well. A magnet was attached to the bottom of one cell culture plate, which was to be used in NMT conditions, while the other plate was used as a
control and therefore did not require a magnet. The gels were prepared as described in Sections 5.3.6.2-5.3.6.4 with the neurospheres prepared as described above.

5.3.6.2 LT Agarose Gel

LT Agarose gel was prepared by dissolving LT Agarose in ddH$_2$O to the appropriate dilution [as described in Section 4.3.2] and this was melted by heating and agitating the mixture. The gel was maintained at 37°C before use in the experiment.

500μl of melted LT Agarose gel was added to the well, and left at room temperature to allow the gel to form. When transferring the neurospheres into gels, the contents of each well, prepared as described in Section 5.3.6.1, were collected into a 200μl pipette, with the tip cut off to avoid damaging the neurosphere. The neurospheres (1-3 per well) were carefully added on top of this gel, before pipetting another 500μl of LT Agarose gel (maintained in liquid state at 37°C) on top to sandwich the neurosphere inside the gel layers. This was again left to form a gel at room temperature, at which point 500μl of cell culture media was added on top, corresponding to the experimental conditions. This process is presented as a diagram in Figure 5.4 below. The experiment length was 7 days, with 50% of the media changed every 2-3 days.
5.3.6.3 Alginate Gel

Alginate gel was prepared by dissolving alginic acid sodium salt in ddH$_2$O to form a solution of the appropriate dilution, while GDL and CaCO$_3$ were weighed [as described in Section 4.3.3.3]. The alginic acid was maintained at 37°C before use in the experiment.

Figure 5.4: Neurospheres sandwiched between gel layers for 3D experiments

The image represents a single well view. 500μl of each gel was first added to each well, and neurosphere(s) were then placed on top of this gel once it formed. Another layer of gel was added on top so that the neurosphere was sandwiched between two layers of the gel to make a total volume of 1 ml. Once the final gel formed, 500μl of cell culture media was added on top before transferring the cell culture plate to the incubator, or bioreactor inside the incubator. Image created using Biorender.com.
The alginic acid was mixed with the GDL/CaCO$_3$ powders and continuously agitated to ensure an even distribution in the mixture before 500 μl was added to each well. This was transferred to an incubator to allow the alginate gel to form. When transferring the neurospheres into gels, the contents of each well, prepared as described in Section 5.3.6.1, were collected into a 200μl pipette, with the tip cut off to avoid damaging the neurosphere. The neurospheres (1-3 per well) were carefully added on top of this gel, before pipetting another 500 μl of fresh alginic acid mixed with GDL/CaCO$_3$ on top to sandwich the neurosphere inside the gel layers. This was again transferred to the incubator to allow the gel to form, before adding 500 μl of cell culture media on top, corresponding to the experimental conditions. This process is presented as a diagram in Figure 5.4. The experiment length was 7 days, with 50% of the media changed every 2-3 days.

5.3.6.4 Fibrin Gel

Fibrin gel was prepared [as described in Section 4.3.4] by preparing fibrinogen and Thrombin mixes at the required concentrations. These mixtures were maintained at 37°C before use in the experiment.

250 μl of Fibrinogen was first added to the well, followed by 250 μl of Thrombin mix. This was gently swirled to allow the solutions to mix, before being transferred to the incubator to allow the gel to form. When transferring the neurospheres into gels, the contents of each well, prepared as described in Section 5.3.6.1, were collected into a 200μl pipette, with the tip cut off to avoid damaging the neurosphere. The neurospheres (1-3 per well) were carefully added on top of this gel, before pipetting another 250 μl of fibrinogen on top, and quickly adding 250 μl of Thrombin mix to sandwich the neurosphere inside the gel layers. This was again transferred to the incubator to allow the gel to form, before adding 500μl of cell culture media.
media on top, corresponding to the experimental conditions. This process is presented as a diagram in Figure 5.4. The experiment length was 7 days, with 50% of the media changed every 2-3 days.

5.3.7 Neurosphere Retrieval from Gels

5.3.7.1 LT Agarose: Melt gel

To retrieve the neurosphere from LT Agarose, media was first carefully aspirated from the wells, and the gel was washed twice with 200μl PBS. The low temp gel has a melting temperature of around 60°C, therefore it was transferred to an oven set to this temperature, and left for up to 60 minutes. The neurosphere was pipetted up using a P200 pipette with the tip cut off. The retrieved Neurosphere was then rinsed with 500μl PBS, and resuspended in PBS before immediate use for analysis.

5.3.7.2 Alginate: Dissolve gel in Sodium Citrate

To retrieve cells from Alginate, media was first carefully aspirated from the wells, and the gel was washed twice with PBS. 500μl of a 10mM solution of sodium citrate was applied (Prepared as described in Appendix III), and the gels were returned to the incubator to allow the sodium citrate to break down the alginate gel. This took up to an hour, and was carefully monitored. The neurosphere was retrieved as soon as it was able to be removed from the gel. The retrieved Neurosphere was then washed carefully with 500μl PBS, and resuspended in PBS before immediate use for analysis.

5.3.7.3 Fibrin: Digestion with Trypsin-EDTA

To retrieve cells from Fibrin, media was first carefully aspirated from the wells, and the gel was washed twice with PBS. 500μl of 0.05% Trypsin-EDTA was applied, and the gels
were returned to the incubator for up to two hours, to allow the Trypsin to break down the Fibrin. This was carefully monitored to ensure the Neurosphere was released from the gel, but not broken down into single cells. The neurospheres were then collected using a P200 pipette with the tip chopped off, and rinsed in 500μl PBS and resuspended in PBS before immediate use for analysis.

![Image of Fibrin gel with Trypsin/EDTA added on top]

**Figure 5.5: Fibrin gel with Trypsin/EDTA added on top**

There are small wisps, highlighted by the BLACK arrows, at the surface of the gel which indicates that the gel is being broken down.

### 5.3.8 Neurospheres Trypsinised for Immunocytochemistry

Glass coverslips were placed into the wells of a 12-well cell culture plate, and a dot of Matrigel [prepared as described in Section 2.1] was added to each coverslip, this was incubated for at least 30 minutes before rinsing twice with PBS. Meanwhile, the neurospheres
which had previously been retrieved from the gels were rinsed in PBS. 500 μl of Accutase was added to each neurosphere to separate it into individual cells, this was incubated at 37°C until the cells began to detach from the neurosphere. The cells were gently pipetted to encourage a single-cell suspension, then the cells were suspended in expansion media corresponding to their experimental condition, and pipetted onto the prepared coverslip. The cells were left to adhere for several hours, before immunocytochemistry was carried out.

5.3.9 Neurospheres Collected for Western Blotting

RIPA Buffer + Protease Inhibitor (RIPA Buffer/PI) was prepared in advance for each neurosphere, at a ratio of 1000:1. The neurospheres which were retrieved from the gels were rinsed in 500μl PBS and then collected in 200μl RIPA Buffer/PI and transferred to an Eppendorf tube. This could then be frozen at -80°C until required. Before use in Western Blotting, the cells were defrosted and the cell membranes were sheared by pipetting the sample using a small needle.

5.3.9.1 Western Blotting

Neurospheres which were retrieved from Fibrin gel were used for protein analysis with Western Blotting. The full protocol for western blotting is described in Section 2.3. Briefly, BCA quantification was carried out to establish the quantity of protein within the sample, and this information was used to prepare samples for electrophoresis. The proteins were separated by molecular weight via gel electrophoresis, and transferred to a membrane for staining (Figure 5.6).
First, the retrieved membrane was stained with Revert Total Protein Stain in order to confirm protein transfer, and this was later used for normalisation. The membrane was then stained for specific protein antibodies.
Methods

Unfortunately in every attempt, the antibody staining was unsuccessful with no bands visible for the targets: SOX2, NESTIN or GFAP. This could be due to an insufficient transfer of proteins: the total protein stain in Figure 5.7 did not have the same protein bands visible across all the channels, and the signal was generally low. Furthermore, the quantity of protein in the initial sample was relatively low, as measured using the BCA assay. This could be due to the fact that only one or two neurospheres were retrieved from the gel for western blot analysis. While the cell number was not counted in the neurosphere, the total number of cells present in the neurosphere may not have been as high as when cells are cultured in 2D culture, where the cells are usually confluent across the entire well. Furthermore, the neurospheres may have been collected in too-large a volume of RIPA buffer, which would result in a diluted protein concentration.

Figure 5.7: Total protein stained membrane

Proteins in the sample are separated by molecular weight, and Revert Total Protein Stain was used to highlight all of the protein bands for detection and quantification.

Unfortunately in every attempt, the antibody staining was unsuccessful with no bands visible for the targets: SOX2, NESTIN or GFAP. This could be due to an insufficient transfer of proteins: the total protein stain in Figure 5.7 did not have the same protein bands visible across all the channels, and the signal was generally low. Furthermore, the quantity of protein in the initial sample was relatively low, as measured using the BCA assay. This could be due to the fact that only one or two neurospheres were retrieved from the gel for western blot analysis. While the cell number was not counted in the neurosphere, the total number of cells present in the neurosphere may not have been as high as when cells are cultured in 2D culture, where the cells are usually confluent across the entire well. Furthermore, the neurospheres may have been collected in too-large a volume of RIPA buffer, which would result in a diluted protein concentration.
Another problem could be with the gel digestion process, where neurospheres embedded in gels are left in Fibrin+ EDTA for up to two hours. The total amount of time depended on how quickly the neurosphere was released from the gel. Trypsin is an enzyme which digests proteins, and cells which are left in Trypsin for extended periods of time are liable to damage caused by trypsin stripping surface proteins. Due to limited resources, it was not possible to carry out more optimisation steps.

5.3.10 Histology for Neurospheres

Neurospheres were retrieved from the hydrogel as described in Sections 5.3.7. The ddH₂O was first aspirated from the well, and 500μl 4% PFA was added. The neurosphere in PFA was left at room temperature for 15 minutes before the PFA was aspirated. The neurosphere was then washed twice in 500μl ddH₂O.

Before encasing the neurosphere in the Agarose gel, it was very briefly (<30 seconds) stained with 1% water-based Eosin to aid visualisation of the neurosphere for histology (Figure 5.8). This was washed in 500μl ddH₂O. A 2% Agarose gel by dissolving 2g of Agarose in 100ml distilled water, which was heated until dissolved. This was allowed to cool slightly until it began to thicken, at which point a droplet was pipetted onto a slide. The Neurosphere was then quickly added on top, followed by a second droplet of Agarose to trap the Neurosphere within. This was left to gel at room temperature, before the gel was trimmed into a cube using a blade (Figure 5.9). These encapsulated neurospheres were transferred to cassettes and placed in a jar containing 70% methanol, and stored at 4°C for wax processing. Due to the fluorescent properties of Eosin, these neurospheres were used for histology, but not immunohistochemistry.
Figure 5.8: Neurospheres extracted from gels and suspended in Agarose for histology

Neurospheres have been stained with Eosin (RED arrow), so they are visible with a slightly pink colour in the cell culture well. Here, they have been resuspended in a 1% Agarose gel.

Figure 5.9: Neurosphere was cut out of the Agarose gel into a small cube shape

In the photograph, the neurosphere (RED arrow) is sat on the tip of a spatula, ready to transfer into the cassette for histology. This cassette was then placed in 70% ETOH and stored at 4°C.

5.3.10.1 Wax Embedding Neurospheres Retrieved from Gels

After wax embedding, the blocks were sliced in 10μm thick slices, and mounted on a Poly-d-Lysine coated slide, and left to dry completely.
5.3.10.2 Wax Embedding Neurospheres Left in Gels

Some neurospheres were left in the gels in which they were cultured, and these were then embedded in wax directly. Media was aspirated from the wells containing the hydrogels, and the gel was washed 3 times with 500μl PBS. 500μl 4% PFA was added to the gel, and this was left for 15 minutes at room temperature. This was aspirated, and the gel was washed twice in ddH2O.

The gels containing neurospheres were transferred to cassettes (Figure 5.10) and placed in a jar containing 70% methanol, and stored at 4°C for wax processing. After wax embedding, the blocks were sliced in 10μm thick slices, and mounted on a Poly-d-Lysine coated slide, and left to dry completely.

![Figure 5.10: Neurospheres embedded in Fibrin gel transferred to cassette for histology.](image)

Fibrin gel (LEFT) extracted from the cell culture well was deposited into the cassettes (RIGHT) for wax embedding, without the requirement to extract the neurosphere, or transfer it into Agarose gel.

5.3.10.3 Dewaxing Histology Slides

To prepare slides for staining, it was essential to remove the wax from the samples first. The dry slides were first washed in Xylene for 20 minutes, then transferred to fresh
Methods

Xylene for another 15 minutes. The sample was then rehydrated in stages, with 5 minute washes in this order: 100% (Absolute) Alcohol, 100% (Absolute) Alcohol, 95% Alcohol, 70% Alcohol, 50% Alcohol. The slides were then washed in cool running tap water and then used for either H&E staining or IHC.

5.3.10.4 Antigen Retrieval

For Immunohistochemistry, antigen retrieval was carried out following the dewaxing step. The slides were placed into a rack, and transferred to a box filled with 10mM Sodium citrate buffer (pH 6.0) and left at RT for 10 minutes. This was microwaved on high power (700W) for 20 minutes in 5 minute intervals to check the liquid volume- the evaporated water from the citrate buffer was replaced between stages. These slides were cooled on ice for at least 20 minutes before beginning IHC.

5.3.10.5 Immunohistochemistry

Immunohistochemistry was carried out on the prepared slides after antigen retrieval. The cooled slides were rinsed in ddH₂O and then permeabilised in PBS-Tx (PBS + 0.1% Triton-X) for 10 minutes, before being placed in blocking solution (PBS + 0.05% Tween + 3% BSA) for 1 hour at RT. After an hour, the blocking solution was removed and the primary antibody was added, diluted in the same blocking solution in the following concentrations:

GFAP (Invitrogen; Rabbit Polyclonal) diluted 1:500
SOX2 (Invitrogen; Rabbit Polyclonal) diluted 1:100

This was covered to reduce evaporation, and incubated overnight at 4°C. After the incubation period, the primary antibody solution was removed. The slide was washed in PBS-Tx for 10 minutes, repeated three times. The following steps were carried out in the dark, due
to the photosensitive nature of the secondary antibodies. The secondary antibody was added to the slide, diluted 1:500 in the same blocking solution, and covered to reduce evaporation before being left to incubate for an hour at RT.

After the incubation period, the secondary antibody (Invitrogen; Mouse anti-rabbit) was removed and the slide was washed in PBS-Tx for 10 minutes, repeated three times. DAPI (Invitrogen) was diluted 1:3000 in PBS and added to the slide, this was left to stain for 1 minute before being rinsed in PBS. The slide was dipped into ddH$_2$O and dried slightly before mounting a coverslip using a few drops of MOWIOL mounting medium (Mowiol® 4-88, Sigma-Aldrich). The slide was left to dry overnight in the dark, before imaging with a confocal microscope (Leica TCS SP8).

5.3.10.6 Haematoxylin & Eosin Staining

The structure of the cells within gels was analysed using H&E staining. Following the dewaxing step, the slides were dipped into filtered Haematoxylin for 4 minutes, then washed in running water. The slides were dipped in Acid Alcohol, and washed again in running water, then dipped in STWS for 3 minutes and washed again in running water for 3 minutes. The staining was checked at this stage to ensure the nuclei were visible.

The retrieved slide was then dipped into filtered, 1% water-based Eosin for 1 min and washed in running water. Finally, the slide is dipped into Potassium Alum for 2 minutes, before the final wash in running water. Staining was checked at this stage, before the sample was dehydrated in stages by transferring the slides through 5 minute washes in this order: 50% Alcohol, 70% Alcohol, 90% Alcohol, 95% Alcohol, 100% (Absolute) Alcohol, 100% (Absolute) Alcohol. The slide was then dipped into Xylene for another 5 minutes, before
mounting a coverslip using DPX mountant (Sigma). The slide was left to dry overnight in the dark, before imaging with an upright light microscope (Leica).

5.3.11 Image Analysis

Outlines of H&E stained neurospheres were prepared using ImageJ software. First, the image of the appropriate scale bar from the micrometre was loaded, and the scale of this photograph was recorded. The distance between two points of the micrometre was measured, to set the pixels scale.

Images of the neurosphere slices were loaded using ImageJ, and set to the same scale as the micrometre image. They were then converted to 32-bit images (Type > 32-bit). The contrast was increased to remove the Fibrin gel from the background (Image > Adjust > Brightness/ Contrast) before the shape outlines were highlighted (Process > Find Edges). The threshold was then set manually to ensure only cells were highlighted (Image > Adjust > Threshold) to give the final outline of the shapes. This was measured using the ‘Analyse particles’ function to give the area of the neurosphere, including migrated cells.

An outline was then drawn around the neurosphere, excluding cells which had migrated away, and this was measured using the ‘Analyse particles’ function to calculate the total area of the neurosphere.

5.4 Results

5.4.1 Optimisation

Single cells were seeded into the hydrogels in order to establish the viability of this method for nanoscale mechanotransduction experiments. This section details their
embedding within the gels, followed by their retrieval after 7 days in culture in full expansion media. The results are shown in Sections 5.4.1.1 – 5.4.1.2.

5.4.1.1 Seeding Gels with Single Cells

![Figure 5.11: Light micrograph of single cell suspension seeded in hydrogels](image)

Single cells seeded into LT Agarose (left), Alginate (Middle) and Fibrin gel (Right). The cells were mixed with the gels as they were forming to ensure a suspension throughout the gels, and to minimise cell adhesion to the bottom of the well. Cells encapsulated in LT Agarose and Alginate did not put out neurites in the gel, instead remaining in a spherical shape. In the Fibrin gel, the cells appeared to grow more normally, branching out with neurites to form connections and proliferate. Magnification 5x. Scale bar represents 200μm.

A comparative overview of the light micrographs taken of single cells seeded into each type of hydrogel (Figure 5.11) clearly appear quite different, despite the same microscope settings used to photograph each gel. The shape of some cells can be identified in the LT Agarose and Alginate gels, while the Fibrin gel contains dark patches which make it difficult to identify individual cells. These micrographs are investigated individually the following sections.
5.4.1.1.1 LT Agarose Gel

Cells were cultured for 7 days in full expansion media. The RED arrows point to cells within the gel. Magnification 5x. Scale bar represents 200μm. n=1

The distribution of cells within LT Agarose gel is shown in Figure 5.12, using an upright light microscope set to 5x magnification. Some cells appear in focus while others are slightly blurry, due to the nature of the microscope which presents a 2D image of a 3D shape. This confirms that there is a distribution of cells across multiple layers of the gel i.e. they are not adhering to the bottom of the well.

The shape of the cells, as shown by the RED arrows, is relatively uniform with cells displaying a spherical shape, and lacking the neurites which are observed in adherent cell culture.
5.4.1.1.2 Alginate Gel

![Image of Alginate Gel]

*Figure 5.13: Light micrograph of single cells seeded into Alginate gel*

Cells were cultured for 7 days in full expansion media. The red arrows point to cells within the gel. The yellow arrow points to a crystalline structure within the gel. Magnification 5x. Scale bar represents 200μm. n=1

The distribution of cells within Alginate gel is shown here in *Figure 5.13* using a light microscope. Some cells appear in focus while others are slightly blurry. This confirms that there is a distribution of cells across multiple layers of the gel.

The shape of the cells, as shown by the RED arrows, is relatively uniform with cells displaying a spherical shape, and lacking the neurites which are observed in adherent cell culture. The WHITE arrow points to a small crystal, which is likely to be a crystal of CaCO₃ which was not fully dissolved during formation of the alginate gel.
5.4.1.1.3 Fibrin Gel

*Figure 5.14: Light micrograph of single cells seeded into Fibrin gel*

Cells were cultured for 7 days in full expansion media. The RED arrows point to elongated cells and YELLOW arrows point to rounded cells within the gel. There are some dark areas of fibrin gel highlighted with circles, which are likely to be denser areas of gel formation. Magnification 5x. Scale bar represents 200μm. n=1

The cells grown in Fibrin gel are shown here in *Figure 5.14* using a light microscope. The fact that some cells are clearly defined while others are out of focus is indicative of the distributed of cells within layers of the gel. It can therefore be concluded that the cells are not simply adhering to the bottom of the well.

The black circles indicate darker areas within the gel, likely areas of more dense fibres formed during the formation of the gel. These areas make it difficult to identify the entire length of some individual cells. The shape of the cells in the Fibrin gel is more typical of the


2D structure seen in adherent cells, whereby the cell body extends long processes, shown by the RED arrows in this image. The YELLOW arrows indicate cells which have remained in a spherical shape.

5.4.1.2 Retrieving Single Cells from Gels

5.4.1.2.1 LT Agarose Gel

As described in Section 5.3.7.1, the LT Agarose hydrogel was melted to retrieve the cells. However, while melting the gel did allow the cells to move within the gel, it was impossible to extract the individual cells from the liquefied LT Agarose gel (Data not shown). Furthermore, the temperature of 60°C which was required to melt the gel, would likely have resulted in cell death.
5.4.1.2.2 Alginate Gel

Figure 5.15: Retrieving single cells from Alginate gel

After 7 days of growth in full expansion media the cells were retrieved using Sodium citrate. The RED arrows point to cells within the gel. The WHITE arrows point to debris and small crystals of undissolved CaCO₃. Magnification 5x. Scale bar represents 200µm.
While there are some single cells visible in Figure 5.15, most of the cells appear to have remained in small clumps. These have been identified by their rounded shape, as opposed to the crystals of CaCO$_3$ which are a rectangular shape. The RED arrows show clumps which could be cells, based on their rounded shape and size. The YELLOW arrows show debris, which could be from cells or the gel, and undissolved crystals of CaCO$_3$. 
5.4.1.2.3 Fibrin Gel

Figure 5.16: Retrieving single cells from Fibrin gel

After 7 days of growth in full expansion media. The gel was dissolved in Trypsin/EDTA to retrieve the cells. The RED arrows point to cells retrieved from the gel. The WHITE arrows point to debris, which could be from the Fibrin gel or cells. Magnification 5x. Scale bar represents 200μm.
The RED arrows show single cells retrieved from the gel in Figure 5.16. There appear to be a few clumps of cells, but the majority are single cells. There is a large amount of debris in the background, indicated by the WHITE arrows, which may be cell debris or remnants of the Fibrin gel.

5.4.1.3 Initial Neurosphere Culture

Initial formation of neurospheres in full expansion media, cultured in a cell-repellent, U-bottomed, 96-well plate was carried out to optimise seeding density and ascertain the typical neurosphere size. Neurospheres effectively represent a tumour microenvironment allowing cell-cell and cell-matrix interaction which is more comparable to the in vivo environment than a monolayer\textsuperscript{448}. Photographs of the neurospheres were taken daily, and the neurospheres were measured across the four days. Results are shown in Table 5.3 and Figure 5.17.
Table 5.3: Seeding densities of neurospheres

Light micrographs were taken of the neurosphere formation up to four days. Magnification was 10x. Scale bar represents 100μm. N=3 per seeding density

**Initial Seeding Density of Neurospheres**

<table>
<thead>
<tr>
<th>Day</th>
<th>15,000</th>
<th>30,000</th>
<th>45,000</th>
<th>60,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
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<tr>
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<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td>Day 3</td>
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<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 5.17: The average diameter of neurospheres several days after cell seeding

A higher initial cell seeding density correlated with a larger diameter, which decreased daily in the case of each of the initial cell seeding densities. Error bars on graph show SD (n=3).

Over 4 days from initial cell seeding on day 0 (no measurement), there was a clear decrease in neurosphere size. This is apparent from both measurements (Figure 5.17), and
from the photographs light micrographs, which include a scale bar (Table 5.3). A higher number of initial cells seeded resulted in overall larger neurospheres, but regardless of the initial size, the average neurosphere diameter continued to decrease each day of measurement. This is due to the cells incorporating into the body of the neurosphere as they cannot adhere to the cell-repellent plate while the U-bottom shape encourages them to form a sphere.

From day 1 to day 4, the neurosphere made from the smallest number of cells seeded (15,000 cells), decreased in diameter from 296μm (n=3; SD=2.2) to 227μm (n=3; SD= 8.8) which was an average decrease of 68μm. The 20,000 cell neurosphere diameter decreased from 438μm (n=3; SD=4.7) to 304μm (n=3; SD=4.5) which was a decrease of 129μm. The largest decrease in size was seen in the 45,000 cell neurosphere, which decreased from 563μm (n=3; SD=5.1) to 348μm (n=3; SD=14). The largest overall neurosphere was seeded with the most cells: 60,000 cells. This neurosphere decreased in diameter from 604μm (n=3; SD=12.2) on day 1, to 397μm (n=3; SD= 7.3) on day 4.

In the pictures of the neurospheres taken on day 1, it is clear that the edges of the neurospheres have not fully incorporated all of the surrounding cells, since a thin veil of cells are visible surrounding the neurosphere. By day four, almost all of the cells appear to have incorporated into the neurospheres. Although there appears to be some shedding around the neurospheres, particularly in the 45,000 cell neurosphere, and the 60,000 cell neurosphere—which could be debris from dead cells.

5.4.1.4 Initial Neurospheres in Hydrogels Study

Neurospheres from the previous section were transferred into Fibrin gel and Alginate gel and cultured for 7 days in full expansion media. As three neurospheres were produced for each
seeding density, there were initially enough neurospheres to embed them into one hydrogel of each type; however, several neurospheres were lost (broke apart, stuck in pipette etc.) during the transfer into gels. Therefore only Alginate and Fibrin gels were used in this section.

**Table 5.4: Neurospheres suspended in Alginate hydrogels**

Light micrographs were taken with an upright light microscope. Scale bars not included since the distance of the neurosphere from the lens was not known.

<table>
<thead>
<tr>
<th>ALGINATE</th>
<th>15,000</th>
<th>30,000</th>
<th>45,000</th>
<th>60,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAY 1</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>DAY 3</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
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<tr>
<td>DAY 7</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>

The true size of the neurospheres could not be measured in this case, since the neurospheres may be sat at different depths within the gel, and therefore they are not a known distance from the camera lens. Neurospheres in the Alginate gel (Table 5.4) maintain a rounded shape, not protruding into the gel. This is due to the lack of adhesion proteins in Alginate gel, meaning there is no extracellular protein to which the cells can anchor themselves.
Results

**Table 5.5: Neurospheres suspended in Fibrin hydrogels**

Light micrographs were taken with an upright light microscope. Scale bars not included since the distance of the neurosphere from the lens was not known.

<table>
<thead>
<tr>
<th>FIBRIN</th>
<th>15,000</th>
<th>30,000</th>
<th>45,000</th>
<th>60,000</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DAY 1</strong></td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td><strong>DAY 3</strong></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
<tr>
<td><strong>DAY 7</strong></td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
</tbody>
</table>

In the Fibrin gel (Table 5.5), the neurospheres made with the lowest cell seeding density (15,000 and 30,000 cells) appear to put out more extensive protrusions than the larger neurospheres (45,000 and 60,000 cells). As would be expected, each of the neurospheres in the Fibrin gel put protrusions out into the gel, and this increases as the days go on. However, the 60,000 cell neurosphere appears to demonstrate single cell migration, as opposed to neurites from the neurosphere itself, compared to the lower density neurospheres.

The shape of the neurospheres clearly shows a difference in morphology between the gel types, as the neurospheres grown in Alginate gel (Table 5.4) maintain a very rounded
shape, while the Fibrin gel allows the neurospheres to put out neurites (Table 5.5). At day one, the neurospheres in each gel show a very similar morphology in terms of size and shape, but by day three there are some noticeable differences between each of the gels. Specifically, it is clear that the Fibrin gel allowed some spreading of cells from the neurosphere out into the gel.

5.4.1.5 Live/Dead Staining of Neurospheres in Different Hydrogels

This section shows the live/dead staining carried out on neurospheres embedded in the three types of gel. Since the aim was to visualise the neurosphere in situ with live/dead staining, the entire gel in which the neurosphere resided was stained. This meant that the confocal microscope was used to locate and photograph the neurosphere, which was impossible if the neurosphere was deeply embedded in the gel, due to the limitation of the light penetration. Because of this, live/dead stained neurospheres were retrieved for Fibrin and Agarose cultured in full expansion media, but only the neurosphere cultured in DMEM+FBS could be stained for the Alginate gel.

The results of the live/dead staining are shown in Figure 5.18, including a graph which represents the living and dead cells within the image as a proportion of the total cell number.
Figure 5.18: Live/Dead staining of neurospheres

Neurospheres has been stained with Propidium Iodide to indicate dead cells (Red) and Calcein to indicate live cells (Green). Neurospheres cultured in A: Agarose and C: Fibrin in full expansion media. B: Alginate in DMEM + FBS. The area of the live and dead cells was calculated and is represented as a proportion of all of the stained cells in the neurosphere. n=1 for each gel.

LT Agarose (A): The spherical shape of the neurosphere is clear in this image, as is the round morphology of the cells within. The live cells (green) are much larger than the dead cells (red), and the area of live cells (75.1%) is three times greater than the area of the dead cells (24.9%). There are only a small number of cells stained in this particular neurosphere.
Alginate (B): The neurosphere is a very rounded shape, with a small number of cells which have detached and migrated, or been shed into the gel. A large number of cells were stained by the Live/Dead solution, revealing small holes between the cells within the neurosphere. In this neurosphere, there was a greater area of dead cells (65%) than live cells (35%). All of the cells exhibit a rounded morphology, and the live and dead cells appear to be a similar size to each other.

Fibrin (C): The centre of the neurosphere appears to have a higher number of dead cells than the outer edge, with more live cells towards the edge of the neurosphere. There are proportionally a lot more live (82%) cells than dead (18%) indicating that there is a much higher rate of cell survival than cell death.

The neurosphere cultured in Fibrin gels appears to have elongated neurites of live cells protruding into the gel. This is reflective of the micrograph images of neurospheres cultured in Fibrin gels (Table 5.5). Conversely, the dead cells exhibit a rounded morphology.

5.4.1.6 Live/Dead Staining of Fibrin Neurospheres

Based on the literature, it is expected that the neurospheres cultured in Agarose and Alginate gels would not extend neurites out into the surrounding gel, since there are no proteins available in these hydrogels to which the cells can attach. This was also confirmed experimentally in Figure 5.18. However, the neurospheres cultured in Fibrin gel can attach to the Fibrin fibres surrounding them. Therefore, in order to visualise the cell shape when cells were cultured in Fibrin gel in the different experimental conditions, the neurospheres were stained with Calcein and Propidium Iodide i.e. Live/Dead staining. The results are shown below in Figure 5.19.
‘Control’ neurospheres in Fibrin gel were cultured in full expansion media which contained growth factors (A), media which did not contain any growth factors (B), media which contained growth factors and 10ng/ml BMP4 (C), and media which contained growth factors and 10% FBS (D) – the green channel represents live cells which have been stained with Calcein. Red cells are dead, and were stained with Propidium Iodide. N=1 per condition.

The neurosphere cultured in +GF media (A) had a necrotic centre indicated by a high red signal, surrounded by a ring of live cells. The cells cultured in −GF media (B) did not appear to have any areas of necrosis, the neurosphere cultured in media +FBS (D) contained a small number of single dead cells, and the neurosphere cultured in media +BMP4 (C) had a relatively large necrotic area visible in this picture.
While the precise shapes of the neurospheres are difficult to elucidate, the lack of cells in the centre of the neurosphere grown in media –GF suggests that the shape of the neurosphere may be donut-shaped, or else the dye may have struggled to penetrate this area of the neurosphere.

Cells cultured in full expansion media with growth factors (+GF), and with media + FBS, showed the cells on the outer edges of the neurosphere appear to be elongated, and growing outwards into the Fibrin gel. The addition of BMP4 in the media did not appear to cause this elongation of the cells, nor did the withdrawal of growth factors.

5.4.2 Nanoscale Mechanotransduction Experiments

The following section details the response of the GBM stem cells to being cultured in the three established hydrogel types: LT Agarose, Alginate and Fibrin gels, with the four media conditions: full expansion media plus growth factors, full media without growth factors, DMEM + BMP4 and DMEM + FBS. The neurospheres in these conditions were then treated either as control (unstimulated), or vibrated (NMT). The neurospheres were stained with H&E staining for morphological analysis (Section 5.4.2.1) and immunofluorescence staining for protein analysis (Section 5.4.2.2). Data from previous studies, Live/Dead data and behaviour of the cells in different gels, and morphological data (presented below), alongside the pressure of limited resources, culminated in the decision to focus on Fibrin gels for future studies.

5.4.2.1 Haematoxylin & Eosin Staining

Below are the images of histological slices of neurospheres which have been stained with Haematoxylin (Purple) which stains the nucleus and some organelles such as ribosomes
and the rough endoplasmic reticulum, while Eosin (Pink) stains proteins of the cytoplasm. Initial experiments featured one neurosphere per condition per gel. Due to the limited number of samples however, neurospheres could not be stained for each condition for Agarose hydrogels due to loss of some samples during the processing phase, which will limit the conclusions which can be drawn. These include Control +GF & Control +FBS; NMT -GF & NMT +FBS for the LT Agarose gels. The LT Agarose and Alginate results are presented in Sections 5.4.2.1.1 and 5.4.2.1.2.

5.4.2.1.1 LT Agarose Gel

5.4.2.1.1.1 Control Conditions

![Figure 5.20: Neurospheres cultured in LT Agarose gel under control conditions without growth factors (CONT -GF), maintained a rounded shape with small gaps between some of the cells. The cells maintained a rounded shape of different sizes within the neurosphere (RED arrows). There are some lighter areas stained by the Eosin (Pink) which surround some of the cells without visible nuclei (Purple).](image)

Figure 5.20: CONT -GF neurosphere cultured in LT Agarose gel.

Magnification 40x. Scale bar represents 100µm.
Figure 5.21: Neurospheres cultured in LT Agarose gel under control conditions with the addition of BMP4 (CONT +BMP4) maintained a generally rounded shape (RED ARROW), with small gaps visible between some cell clumps. There are also some smaller cells which have detached from the main neurosphere (BLACK ARROW) which are typically only nuclei.

Figure 5.21: CONT +BMP4 neurosphere cultured in LT Agarose gel

Magnification 40x. Scale bar represents 100µm.
5.4.2.1.1.2 Nanoscale Mechanotransduction Conditions

**Figure 5.22:** Neurospheres cultured in LT Agarose gel under the NMT condition with the addition of full expansion media (NMT +GF), maintained a very rounded shape in the gel (RED ARROW). There are also small gaps between some clusters of cells. There are some smaller cells which have detached from the main neurosphere, which are mostly stained purple, indicating the cell nucleus.

*Figure 5.22: NMT +GF neurosphere cultured in LT Agarose gel.*

Magnification 40x. Scale bar represents 100μm.

**Figure 5.23:** Neurospheres cultured in LT Agarose gel under the NMT condition with the addition of BMP4 (NMT +BMP4) appear to shed a large number of cells (BLACK ARROW), with large gaps between cell clusters of the main neurosphere. The cells display atypical morphology with a variety of sizes of cell, and different morphologies.

*Figure 5.23: NMT +BMP4 neurosphere cultured in LT Agarose gel.*

Magnification: b=40x; a= 20x. Scale bar represents 100μm.
5.4.2.1.2 Alginate Gel

5.4.2.1.2.1 Control Conditions

**Figure 5.24:** Neurospheres cultured in Alginate gel under control conditions with the addition of full expansion media (CONT +GF) exhibit a very rounded shape, especially on the edge of the neurosphere (RED ARROW). The cells are generally a similar size throughout the neurosphere.

![CONT +GF Neurosphere](image)

*Figure 5.24: CONT +GF neurosphere cultured in Alginate gel.*

*Magnification 40x. Scale bar represents 100μm*

**Figure 5.25:** Neurospheres cultured in Alginate gel under control conditions with the addition of full expansion media without growth factors (CONT -GF) also maintain a rounded shape in the gel. The cells appear to be very round in shape (RED ARROW), and are generally a similar size throughout the neurosphere. There are gaps visible between clusters of cells. A protrusion which may be a neurite is highlighted by the GREEN ARROW.

![CONT -GF Neurosphere](image)

*Figure 5.25: CONT-GF neurosphere cultured in Alginate gel.*

*Green arrow represent neurites. Magnification 40x. Scale bar represents 100μm*
Figure 5.26: CONT +BMP4 neurosphere cultured in Alginate gel.

The green arrows highlight protrusions from the cells, possibly neurites. Magnification 40x. Scale bar represents 100µm.

Figure 5.26: Neurospheres cultured in Alginate gel under control conditions with the addition of media containing BMP4 (CONT +BMP4) maintain a rounded shape in the gel. A couple of cells appear to have detached from the main neurosphere, and these cells display a rounded shape, without any clear protrusions or neurites (RED ARROW). Towards the centre of the neurosphere, some small protrusions between the cells are visible, as indicated by the GREEN arrows. There appear to be fewer large gaps between the cells of the neurosphere in this condition than in the Control +GF (Fig. 5.24) or Control –GF (Fig. 5.25), although some small gaps are visible.

Figure 5.27: CONT +FBS neurosphere cultured in Alginate gel.

Green arrows highlight some protrusions between cells, possibly neurites. Magnification 40x. Scale bar represents 100µm.
rounded shape in the gel. There are some large gaps visible between groups of cells in the neurosphere. While the majority of the cells have a very round morphology, towards the centre of the neurosphere there are small protrusions visible between the cells, as indicated by the GREEN arrows.

5.4.2.1.2.2 Nanoscale Mechanotransduction Conditions

Figure 5.28: Neurospheres cultured in Alginate gel under NMT conditions with the addition of full expansion media (NMT+GF) exhibit a very rounded shape, especially on the edge of the neurosphere, without any clear neurites, highlighted by the RED ARROW. The cells are generally a similar size throughout the neurosphere.
Results

Figure 5.29: Neurospheres cultured in Alginate gel under NMT conditions with the addition of full expansion media without growth factors (NMT -GF) maintain a rounded shape in the gel. The cells appear to be very round in shape (RED ARROW), and are generally a similar size throughout the neurosphere. There are small holes visible between some clusters of cells, although the neurosphere generally maintained a dense shape.

Figure 5.29: NMT -GF neurosphere cultured in Alginate gel.
Magnification x40. Magnification 40x. Scale bar represents 100μm

Figure 5.30: Neurospheres cultured in Alginate gel under NMT conditions with the addition of media containing BMP4 (NMT +BMP4) maintain a rounded shape in the gel. The cells display a rounded shape, without any clear protrusions or neurites (RED ARROW). There are more gaps between the cells of the neurosphere in this condition than in the NMT +GF or NMT –GF.
Figure 5.31: Neurospheres cultured in Alginate gel under NMT conditions with the addition of media containing FBS (NMT +FBS) maintain a rounded shape in the gel, although there are some large gaps visible between groups of cells in the neurosphere. The cells generally have a very round morphology (RED ARROW), although there are some areas (GREEN ARROW) which could be neurites from the cells, or wrinkles in the gel which have retained the dye.

![Figure 5.31: NMT +FBS neurosphere cultured in Alginate gel.](image)

Green arrows highlight some protrusions, which could be neurites. Magnification x40. Magnification 40x. Scale bar represents 100μm

5.4.2.1.3 Fibrin Gel

In the following section, the number of neurospheres cultured in fibrin gel is detailed in each figure legend in the H&E staining section (Sections 5.4.2.1.3.1 and 5.4.2.1.3.2).

5.4.2.1.3.1 Control Conditions
Figure 5.32: Control +GF Neurospheres in Fibrin, stained with Haematoxylin and Eosin

A-D = neurosphere 1   |   E-F = neurosphere 2

Magnification 10X (A+B); 40X (D+F); 5X (E).

Zoomed in section (C) shows less-rounded cells, some extending neurites into the gel, highlighted by the GREEN arrows. (B) Has been made binary in ImageJ to highlight the cells and eliminate the background. (N=2)
Results

Neurospheres cultured in Fibrin gel under control conditions with the addition of full expansion media (Figure 5.32) appear to maintain a generally rounded shape, with a few cells extending out into the surrounding gel. Some cells exhibiting an elongated shape towards the edge of the neurosphere, highlighted by a GREEN arrow and magnified (C). The cells inside the neurosphere appear to maintain a more rounded shape overall.

Figure 5.33: Control -GF Neurospheres in Fibrin, stained with Haematoxylin and Eosin

A-D= neurosphere 1/2
E = neurosphere 3
Magnification 10X (A); 40X (D); 5X (E).

Zoomed in section (C) shows less-rounded cells, some extending neurites into the gel. The YELLOW arrows highlight ‘layers’ of cells in (D). (B) Has been made binary in ImageJ to highlight the cells and eliminate the background. (N=3)
Neurospheres cultured in Fibrin gel under control conditions with the addition of expansion media without growth factors (Figure 5.33) appear to extend or migrate out into the surrounding gel, with some cells exhibiting an elongated shape towards the edge of the neurosphere, examples in this area have been magnified. Some of the cells inside the neurosphere appear to maintain a more rounded shape, while some elongated ‘layers’ of cells are also visible, highlighted with the YELLOW arrows.
Figure 5.34: Control +BMP4 Neurospheres in Fibrin, stained with Haematoxylin and Eosin

A-C = Neurosphere 1 | D= Neurosphere 2 | E= Neurosphere 3

Magnification 10X (A, D, E); 40X (C, F)

(B) Has been made binary in ImageJ to highlight the cells and eliminate the background. (N=3)
Neurospheres cultured in Fibrin gel under control conditions with the addition of media +BMP4 (Figure 5.34) appear to remain in the neurosphere, with some single cells migrating out into the Fibrin gel. Again, many cells within the neurosphere exhibit a rounded shape, while some appear elongated and forming ‘stripes’ within the neurosphere, particularly towards the edge of the neurosphere- highlighted with a BLACK ARROW.
Figure 5.35: Control +FBS Neurospheres in Fibrin, stained with Haematoxylin and Eosin

A-C = Neurosphere 1  
D= Neurosphere 2  
E-F= Neurosphere 3  

Magnification 10X (A,D); 40X (C,E); 5X (F).

Some cells in (C) AND (E) are extending neurites into the gel, highlighted by the GREEN arrows.

(B) Has been made binary in ImageJ to highlight the cells and eliminate the background. (N=3)
Neurospheres cultured in Fibrin gel under control conditions with the addition of media +FBS (Figure 5.35) appear to extend out into the surrounding gel, resulting in a more star-shaped neurosphere, as opposed to the round shape that was initially embedded in the gel. As was the case with cells in the other control conditions, the cells inside the neurosphere appear to maintain a rounded shape, with some cells elongating to form ‘stripes’ within the neurosphere. The cells which are reaching out into the gel present as elongated shapes, highlighted by the GREEN arrow.
5.4.2.1.3.2 Nanoscale Mechatransduction Conditions

**Figure 5.36: NMT +GF Neurospheres in Fibrin, stained with Haematoxylin and Eosin**

A-C = Neurosphere 1  
D-F = Neurosphere 2  

Magnification 10X (A,D); 40X (C,E,F)  
(B) Has been made binary in ImageJ to highlight the cells and eliminate the background. (N=2)
Neurospheres cultured in Fibrin gel under NMT conditions with the addition of full expansion media (Figure 5.36) appear to extend out into the surrounding gel, although the morphology still appears to be quite round. The cells inside the neurosphere appear to maintain a more rounded shape overall.

*Figure 5.37: NMT –GF Neurospheres in Fibrin, stained with Haematoxylin and Eosin*

A-C= Neurosphere 1
Magnification 10X (A); 40X (C)
The GREEN arrows highlight protrusions within the neurosphere which may be neurites. (B) Has been made binary in ImageJ to highlight the cells and eliminate the background. (N=1)

Neurospheres cultured in Fibrin gel under NMT conditions with the addition of expansion media without growth factors (Figure 5.37) do not appear to extend out into the surrounding gel. However, a small number of cells appear to have migrated from the neurosphere into the gel. Most of the cells inside the neurosphere appear to maintain a more
rounded shape, while a small number of elongated cells are also visible, highlighted with the GREEN arrows.

Figure 5.38: NMT +BMP4 Neurospheres in Fibrin, stained with Haematoxylin and Eosin
A-D= Neurosphere 1
E= Neurosphere 2
Magnification 10X (A, E); 40X (C, D)
The WHITE arrows point out some cells which have migrated from the neurosphere. (B) Has been made binary in ImageJ to highlight the cells and eliminate the background. (N=2)

Neurospheres cultured in Fibrin gel under NMT conditions with the addition of media +BMP4 (Figure 5.38) do not appear to extend out into the surrounding gel. The neurosphere
Results

maintained a very compact, rounded shape, and the cells on the edge of the neurosphere do not appear to be extending neurites out into the gel. Some cells have been shed or have migrated from the neurosphere, some of which are these are highlighted by the WHITE arrows in micrograph B.
Figure 5.39: NMT +FBS Neurospheres in Fibrin, stained with Haematoxylin and Eosin

A-D= Neurosphere 1
E-F= Neurosphere 2
Magnification 10X (A,E); 40X (D,F)
(B) Has been made binary in ImageJ to highlight the cells and eliminate the background. (C) is a zoomed in on a section of (A) to highlight cell morphology (N=2)

Neurospheres cultured in Fibrin gel under NMT conditions with the addition of media +FBS (Figure 5.39). The lower left micrograph shows a neurosphere slice where the Fibrin gel
surrounds the neurosphere, whereas the neurosphere in the top left micrograph appears to have detached from the surrounding gel. In the lower left micrograph, cells from the neurosphere appear to be extending out into the gel.

### 5.4.2.1.4 Comparison of Neurosphere Size in Fibrin Gel

**Figure 5.40: A comparison of the size of neurospheres in Fibrin gels**

The size of the neurospheres are presented here in a bar chart, with the entire neurosphere, including migrated cells (BLUE) and neurosphere, excluding migrated cells (ORANGE). Error bars represent SEM. N numbers in Table 5.6.

The average neurosphere size under different conditions is shown in Figure 5.40, which indicates the effect of each condition on cell spreading and proliferation rate. However, as shown in the table below (Table 5.6), there was not always an n=3. This is due to neurosphere loss during the steps from neurosphere formation, to histology, leading to a smaller final number of neurospheres. Again, due to resource limitations, it was not possible to perform more experimental repeats in these cases.
Table 5.6: Comparison of average neurosphere sizes for each condition

The NMT-treated neurospheres are consistently smaller than the Control neurospheres. Given as % of the neurosphere in Control condition. Data from entire neurosphere, including migrated cells.

<table>
<thead>
<tr>
<th>CONTROL VS NMT</th>
<th>NMT NS % DIFFERENCE</th>
<th>CONTROL N=</th>
<th>NMT N=</th>
</tr>
</thead>
<tbody>
<tr>
<td>+GF</td>
<td>-52%</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>-GF</td>
<td>-41%</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>+BMP4</td>
<td>-61%</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>+FBS</td>
<td>-41%</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

The largest neurosphere in Fibrin gel was cultured in media containing FBS under control conditions (CONT +FBS) which was 41% larger than the area of the same neurosphere cultured with the application of vibrations (NMT +FBS). For each condition, the average area of NMT treated neurospheres was smaller than the control. The NMT neurosphere with growth factors (NMT +GF) was just less than half the size of the CONT +GF neurosphere. The NMT −GF neurosphere was 41% of the size of the CONT −GF neurosphere. And finally, the NMT +BMP4 neurosphere was 61% of the size of the CONT +BMP4 neurosphere.

5.4.2.2 Immunofluorescence of neurospheres cultured in Fibrin gels

Data from previous studies, Live/Dead data and behaviour of the cells in different gels, and morphological data, alongside the pressure of limited resources, culminated in the decision to focus on Fibrin gels for future studies. Therefore, immunofluorescence was carried out on neurospheres cultured in Fibrin gels. Furthermore, optimisation was not possible for many samples due to the low number of samples obtained. This was due to difficulty in visualising the neurospheres owing to the opacity of the Fibrin gel, which meant that some of the samples could not be stained. Therefore, some data points were not able to be retrieved.
for immunofluorescence data, which will limit the conclusions which can be drawn. The results are presented below in Sections 5.4.2.2.1 – 5.4.2.2.4
5.4.2.2.1 Control – GF

Figure 5.41: Neurosphere in Fibrin gel: CONTROL – GF, SOX2

Neurosphere cultured in Fibrin gel under control conditions, in expansion media without growth factors (CONT – GF). Magnification x20 (Bottom right magnification x40). Stained for SOX2 (RED) and counterstained with DAPI (BLUE). YELLOW arrows indicate nuclei without any SOX2 staining. Limitation: No Control + GF sample, which limits the findings.

The neurosphere in Figure 5.41 was cultured under control conditions in expansion media without growth factors (CONT – GF) contain a small number of cells (85 cell nuclei) relative to the other neurospheres, in this slice. The BLUE signal (DAPI) highlights the nuclei,
while the RED signal shows the SOX2 stain. There is a very low red signal which surrounds some nuclei, but SOX2 is not present on all of the cells. Nuclei which are stained with DAPI without any SOX2 surrounding them are indicated with a YELLOW arrow. The cells are very spread out, with only some small clusters of cells visible. Some cells individually exhibit a round morphology, while others demonstrate a more typical neural shape with the extension of neurites, some of which are highlighted in the white magnified box. Control +GF neurospheres could not be retrieved for staining, and therefore this limits the conclusions which are drawn. Future work would aim to address this by obtaining a Control +GF sample, and using a larger sample size.
5.4.2.2.2 NMT -GF

*Figure 5.42: Neurosphere in Fibrin gel: NMT –GF, SOX2*

Neurosphere cultured in Fibrin gel under NMT conditions, in expansion media without growth factors (NMT –GF). Magnification x40. Stained for SOX2 (RED) and counterstained with DAPI (BLUE). Limitation: No NMT +GF Sample. This will limit the findings.
This slice of neurosphere contains a large number of cells, and generally remained in a rounded shape, with many cells spreading out to the sides of the main sphere. Again, the upper edge of the neurosphere in the micrograph above contains cells which have neurites and an elongated shape, typical of adherent neural cells. Since the cells within the main neurosphere are very tightly packed together, the morphology of individual cells is not always clear. However, the separate SOX2 stained image (Upper left image) indicates that the shape of the cells within the neurosphere is highly spherical, and not typical of the elongated morphology of neural cells in culture. Control -GF neurospheres could not be retrieved for staining, and therefore this limits the conclusions which are drawn. Future work would aim to address this by obtaining a Control -GF sample, and using a larger sample size.
The neurosphere in figure 5.43 were cultured under NMT conditions in expansion media without growth factors (NMT –GF) contain many more cells, and maintained a round shape, similar to the original shape of the neurosphere that was seeded in the gel. The shape

Figure 5.43: Neurosphere in Fibrin gel: NMT –GF, GFAP

Neurosphere cultured in Fibrin gel under NMT conditions, in expansion media without growth factors (NMT –GF). Magnification x40. Stained for GFAP (GREEN) and counterstained with DAPI (BLUE). Limitation: No NMT +GF Sample. This will limit the findings.
of most individual cells is difficult to discern since the cells are in tight clusters. However, the upper left section of the neurosphere, highlighted with the ‘zoomed in’ box, has spread out into the gel, and the shape of the cells in this section is typical of the neural cell lineage, with an elongated shape and neurites.

Control -GF neurospheres could not be retrieved for staining, and therefore this limits the conclusions which are drawn. Future work would aim to address this by obtaining a Control -GF sample, and using a larger sample size.
5.4.2.2.3 Control +BMP4

*Figure 5.44: Neurosphere in Fibrin gel: CONTROL +BMP4, GFAP*

Neurosphere cultured in Fibrin gel under NMT conditions, in media +BMP4 (CONT +BMP4). Magnification x40. Stained for GFAP (GREEN) and counterstained with DAPI (BLUE). Limitation: No NMT +BMP4 sample. This limits the findings.
This neurosphere in figure 5.44 was cultured under control conditions with BMP4 (CONT +BMP4), and the centre of the neurosphere has remained quite tightly packed, but layers of cells are visible which have detached or migrated from the central neurosphere shape. This has typically occurred in large clusters, with a small number of individual cells visible which are not attached to the rest of the neurosphere. There is significant GFAP staining, which appears to surround nearly every cell, although a few of the individual cells express a low signal. The cells which are on the edges of the neurosphere, highlighted by the white ‘zoomed in’ box, vary in their morphology, with most cells presenting a rounded shape, while some are elongated or appear to be sprouting neurites. Control +BMP4 neurospheres could not be retrieved for staining, and therefore this limits the conclusions which are drawn. Future work would aim to address this by obtaining a Control +BMP4 sample, and using a larger sample size.
5.4.2.2.4 NMT + FBS

Figure 5.45: Neurosphere in Fibrin gel: NMT + FBS, SOX2

Neurosphere cultured in Fibrin gel under NMT conditions, in media + FBS (NMT + FBS). Magnification x20. Stained for SOX2 (RED) and counterstained with DAPI (BLUE). The YELLOW arrow highlights small protrusions which could be neurites, while the GREEN arrow highlights stained nuclei in the absence of SOX2. Limitation: No Control + FBS Sample. This limits the findings.
The SOX2 signalling cells in this neurosphere (Figure 5.46) are typically spherical in shape, with a couple of neurites visible on only a small number of cells, which are highlighted with a YELLOW arrow. There are several cells which have a visibly stained nucleus, but there is no SOX2 signal around the nucleus, these are highlighted with a GREEN arrow. There are a couple of points of SOX2 signal without a stained nucleus attached, which could be debris.

Control +FBS neurospheres could not be retrieved for staining, and therefore this limits the conclusions which are drawn. Future work would aim to address this by obtaining a Control +FBS sample, and using a larger sample size.
Neurosphere cultured in Fibrin gel under NMT conditions, in media +BMP4 (CONT +BMP4). Magnification x20 (Bottom Right Magnification x40). Stained for GFAP (GREEN) and counterstained with DAPI (BLUE). Limitation: No control +FBS Sample. This limits the findings.
The neurosphere in figure 5.45 was cultured under NMT conditions, in media containing FBS (NMT +FBS) and it maintained a generally rounded shape, with some gaps in the cells especially towards the edge of the neurosphere. The shape of the cells on the edge of the neurosphere is a mix of spherical and elongated, with some neurites visible. These are highlighted in the magnified boxes underneath the left neurosphere micrograph. The GFAP staining within the centre of the neurosphere highlights the elongated shape of the cells within. There are a small number of individual cells outside of the main neurosphere, although generally the cells appear to have remained attached to the neurosphere.

Control +FBS neurospheres could not be retrieved for staining, and therefore this limits the conclusions which are drawn. Future work would aim to address this by obtaining a Control +FBS sample, and using a larger sample size.

5.5 Discussion

5.5.1 Summary

In the following discussion, a comparison of the structures seen in neurospheres cultured in different hydrogels will be compared, based on the data collected in this chapter. The results will be compared to what is known in the literature, in order to establish where these results fit into existing work.

5.5.2 Single cells vs Neurospheres

Single cells seeded in gels were ultimately disregarded in favour of neurospheres as a model for this study, for several reasons. First and foremost, neurospheres are regarded as the most comparable model of tumours\cite{449,450}, and therefore the cells were exposed to a more accurate replication of their \textit{in vivo} environment. Furthermore, studies of single cell retrieval
from the hydrogels highlighted the difficulty off efficiently removing the cells from the hydrogels, and separating cells from debris therein.

### 5.5.3 Optimal neurosphere size

When producing neurospheres of various sizes, it was observed that their size consistently decreased from day 1 to day 4, regardless of the initial seeding density. Notably, a higher initial cell seeding density resulted in larger neurospheres. This size reduction can be attributed to the physical interactions and attachments formed among the constituent cells within the neurospheres, which is visually evident in the microscope images presented in Table 5.3. These images reveal that the early neurospheres, especially the smaller ones, have 'loose' cells around their periphery. However, for the 60,000 cell neurosphere on day 1, this observation is less conclusive. By day 3, all neurospheres adopt spherical shapes, but the edges of these structures do not exhibit tight binding to the rounded shape until day 4. This suggests that cells gradually migrate into the spherical shape, leading to a decrease in size primarily on a single plane.

The size of a neurosphere may correlate with the survival and proliferation capabilities of the constituent cells. It is frequently observed in studies involving neurospheres that live cells tend to concentrate towards the periphery of the neurosphere\(^{451}\). This observation is consistent with the Live/Dead staining results presented here, which suggest increased apoptosis and the presence of a necrotic core within the neurosphere. This phenomenon could be attributed to the limited diffusion of necessary nutrients into the central region of the neurosphere, a phenomenon reported in some studies\(^{451}\). It's worth noting that one study found optimal cell proliferation in neurospheres with a diameter under 100μm, with a decrease in proliferation as the diameter exceeded this threshold\(^{452}\).
When seeding neurospheres of varying sizes into Fibrin gels, distinct differences were observed in the behaviour of the constituent cells, depending on neurosphere size. After 7 days of culture in Fibrin gel, smaller neurospheres displayed prominent neurite extension into the hydrogel, with elongated extensions reaching into the Fibrin. In contrast, as neurosphere size increased, there were fewer elongated cells, and a higher proportion of cells appeared to detach from the neurosphere in a rounder shape. This observation might be attributed to increased cell death, potentially leading to debris shedding from the neurosphere, suggesting that smaller neurospheres may be more likely to contain viable cells. This is supported by existing literature, which records a decrease in proliferation rate correlating with an increase in neurosphere diameter, linked with metabolism and cell viability in these conditions\textsuperscript{452}. There are multiple assays for detecting cell death; for example, the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide)) assay, Trypan Blue assay or measurement of ATP\textsuperscript{453,454}.

Moreover, when passaging cells, an average of just under 1 million cells was contained in a T75 flask. In setting up experiments, some neurospheres were occasionally lost due to damage or pipetting errors. Consequently, determining the number of cells to include in a neurosphere was a delicate balance between achieving the optimal neurosphere size and the available cell count. Seeding neurospheres with 20,000 cells enabled the production of approximately 40-50 neurospheres from each flask, ensuring a robust number of experimental models. This seeding density aligns with the number of cells used in the earlier 2D monolayer experiments, ensuring consistency between the studies.
5.5.4 Fibrin Gel vs LT Agarose and Alginate Gels

As described in Section 5.2, different hydrogels may be employed for different uses. Hydrogels which do not contain proteins which promote cell adhesion are often combined with another material which will facilitate cell adhesion. In the present investigation, the primary objective was to assess the significance of cell-matrix interactions on cell viability, proliferation, and differentiation. Consequently, no supplementary materials were combined with the LT Agarose or Alginate hydrogels. The nanoscale vibrations applied to the hydrogels are expected to be faithfully propagated through the gel medium, owing to the predominantly incompressible nature of the gel, which is primarily composed of water. As a result, the gel is expected to efficiently transmit and convey the applied force to the cells within.

Fibrin has two pairs of RGD sites (See Figure 1.9) which represent integrin binding sites. These play a critical role in the formation of focal adhesions, thus allowing cells to adhere to the surrounding hydrogel, and receive physical signals from their environment, as described in Chapter 1. Since LT Agarose and Alginate hydrogels do not contain proteins required for cell adhesion, the cells and neurospheres grown in these environments exhibit a very spherical shape. This difference in morphology was starkly apparent in Tables 5.4 and 5.5. These images depict cells cultured within Fibrin and Alginate hydrogels, revealing a distinctly spherical morphology for the Alginate neurospheres. In contrast, the Fibrin neurospheres clearly exhibited neurites extending into the surrounding gel as early as day 3, thereby highlighting a considerable disparity in their morphological characteristics.

However, in some cases there were visible neurites within the neurospheres cultured in Alginate gels. These were visible in the control conditions with BMP4 and FBS, and the NMT condition with FBS. This could be indicative of cells producing ECM to which neighbouring
cells could attach. In these cases, the neurites could suggest that since the cells are forming adhesions, they could be susceptible to mechanotransduction from nanoscale vibrations.

The structure of neurospheres have been reported to contain layers of cells surrounding the inner core, with gap-junctions existing to connect the adjacent cells. In this study, these cell layers are visible in the H&E stained models in Fibrin gels, but not in the gels cultured in Agarose or Alginate. This could be linked to the large amounts of cell death which were seen in the live/dead staining model of Alginate and Agarose neurospheres.

Gap-junctions are formed by hemichannels in neighbouring cells, consisting of proteins called connexins (Cx) or pannexins (PanX). They allow passive diffusion of ions and molecules between the cells to aid communication and coordinate activities, particularly regulation of cell propagation or death via the Cx43 and Cx46 hemichannels in astrocytes, as well as regulating neurite outgrowth in neurons. Interestingly, mechanical stimulation using patch-clamping techniques has been found to open some hemichannels, including Cx43 and PanX1 channels, which allow the influx of Adenosine Triphosphate (ATP) and thus aids cell proliferation. Expression of these PanX1 proteins have been confirmed in neural stem cells, as well as GFAP-expressing cells.

These gap-junctions have been found to exist not only between cells of the same type, but also between undifferentiated and differentiated cells. Therefore, differentiated astrocytes may remain attached to the neurosphere for some time. This is further compounded by research which confirms that a heterogeneous population of cells can exist within neurospheres. Though in the in vivo environment, differentiated progeny will migrate away from the stem cell niche to reach their final destinations, often using the vascular system.
5.5.5 Neurospheres cultured in LT Agarose and Alginate

The neurospheres cultured in Agarose gel in full expansion media appear to form a dense spherical shape, although there are some gaps visible between clusters of cells, as seen in Figure 5.21. This suggests a propensity for small cell clusters to preferentially aggregate rather than maintaining a singular large spheroidal configuration. This may aid survivability of the cells\(^4\); a theory which is recapitulated by the Live/dead staining of the Agarose neurosphere (Figure 5.18), in which large clumps of live cells are surrounded by smaller, dead cells. This is the case for each of the neurospheres cultured in Agarose gel.

However, the Agarose hydrogel neurosphere cultured in NMT +BMP4 display a notably distinct morphology, with their spherical shape almost entirely disrupted, as evidenced in Figure 5.23. This figure shows a large amount of small particles of debris surrounding the larger cells which remain attached together. This is supported by studies which have found that differentiated cells which are unable to migrate from the neurosphere may not survive\(^2\). This could explain the morphology of the cells in the NMT +BMP4 neurosphere in particular. In which case, debris surrounding the neurosphere could be indicative of differentiated cells. In this case, the cells were not analysed further, but protein analysis (such as western blot or immunofluorescence) or genetic analysis (such as PCR) could be used to confirm cell differentiation.

Neurospheres which were cultured in Alginate gel also exhibited similar behaviour, with gaps visible between clusters of cells in each example, although there did not tend to be any debris of dead cells surrounding the neurospheres. Interestingly, there appear to be some neurites extended between cells within the neurosphere. This was particularly noticeable in the Control +BMP4 neurosphere (Figure 5.26), and the control +FBS neurosphere (Figure
5.27), and the NMT +FBS neurosphere (Figure 5.30). This could indicate that the cells are forming connections between cells inside the neurosphere. In neurosphere formation studies, similar extensions have been identified as expressing Nestin through immunostaining. Nestin expression in these cells was not confirmed in this study.

### 5.5.6 Culturing neurospheres in Fibrin gel in different media

Neurospheres were cultured in Fibrin gel for 7 days in the four types of cell culture media: full expansion media with growth factors or without growth factors, and DMEM with BMP4 or FBS. These neurospheres were stained with a Live/Dead protocol to investigate the survivability of cells under these conditions.

The neurosphere cultured in media containing BMP4 contained a mixed population of live and dead cells, with a large number of live cells and a generally localised area of cell death. The overall staining signal in this neurosphere was quite low compared to the other conditions, but the morphology of the live cells appeared to be heterogeneous. This indicates some differentiation has taken place, which may have been induced by the BMP4 signalling.

Interestingly, the cells grown in expansion media, which contains growth factors, had a necrotic core in the neurosphere. This observation is also seen in the literature: since the cells of the neurosphere were likely to be proliferating due to the influence of growth factors, this could suggest that the number of cells within the neurosphere was increasing. This may have increased the size or density of the neurosphere, thus decreasing the ease of diffusion for necessary nutrients like glucose, resulting in some cell death in the centre. The live cells cultured in full expansion media with growth factors also show an elongated morphology, with cells stretching from the neurosphere, out into the gel. As has been established from
previous literature, Fibrin gel contains the integrin binding sites required to allow cells to attach to it, therefore facilitating cell migration in the gel, which is demonstrated in this study. In these images, the cells cultured in full expansion media with growth factors and DMEM with FBS, clearly exhibit cell migration. The appearance of some GFAP-positive cells which have detached from the cells could indicate migration of these differentiated astrocytes.

Interestingly, the cells cultured in FBS appear much more elongated than the cells in the full expansion media. This phenomenon has been documented in previous studies, which have found that serum-induced differentiation of GBM CSCs is associated with an increased invasive potential. This indicates that the cells on the edge of the FBS neurosphere are not only more invasive than GBM CSCs, but they may have also differentiated into astrocytes.

Surprisingly, the neurospheres cultured in full expansion media without growth factors did not contain any dead cells at all. The gross morphology of the neurosphere was also quite different to the other neurospheres, appearing as a ‘donut’ shape. This could be due to the actual shape of the neurosphere, or it could be that the centre was particularly dense and did not allow the penetration of the Propidium Iodide. However, based on H&E and immunofluorescence images, it seems likely that this is the shape of the neurosphere; this could be due to a hole or tear in the Fibrin gel. This gap would then be filled with the cell culture media, directly next to the cells, so the cells would receive nutrients easily. Additionally, the lack of growth factors reduces the ability of the cells within the neurosphere to proliferate (aside from any aberrantly active growth factor signalling mechanisms within cells on account of the fact that these are tumour cells) and therefore the same population of cells may be maintained in the culture.
**5.5.7 Neurospheres in Fibrin: Control +GF and NMT +GF Conditions**

It is accepted that the optimal conditions required to maintain GBM stem cell characteristics include serum-free media, and the addition of the growth factors, EGF and FGF-2. Interestingly, the inhibition of growth factors including FGF-2 has been found to reduce cell adhesion, neurite extension and cell migration distance in spheroids produced from neural progenitor cells\textsuperscript{468,469}. The neurospheres cultured in full expansion media with growth factors therefore may have been expected to be the largest and most invasive. EGF has also been demonstrated to induce rapid actin depolymerisation, and concurrently myosin polymerisation, creating ruffles in the cell membrane which aid in cell migration\textsuperscript{261}.

Full expansion media containing growth factors ensured that cells were cultured under conditions which were ideal for the continuing proliferation and propagation of stem cells. Unfortunately, it was not possible to retrieve samples of these for immunostaining, but the H&E stained neurospheres allowed a comparison of neurosphere shape, cell morphology and cell number.

In both Control +GF and NMT +GF cases, the neurosphere remained very round within the Fibrin gel, but with some migration of cells from the edges out into the gel. The neurosphere in Control +GF gel had a greater instance of migrated cells (n=60 cells) compared to the NMT +GF cells (n=30 cells). These were defined as cells which were not visibly attached to the main neurosphere. This is noteworthy that cell migration is associated with differentiated cells in the neurosphere environment, and could therefore suggest that the control cells underwent more differentiation than the NMT cells. Neurospheres cultured in full media +GF were also comparatively large, in relation to the neurospheres cultured in the other experimental conditions. This could be expected since EGF and FGF-2 are linked to cell
Discussion

proliferation, and the maintenance of stem cell characteristics\textsuperscript{470,471}, therefore the cells cultured in this condition were cultured in typical neural stem cell maintenance media. Interestingly, the Control and NMT neurospheres were a similar size, demonstrated as 89\% and 81\%, respectively, of the largest neurosphere (CONT +FBS). The Control neurosphere is the larger of the two, however, which is a pattern repeated across each condition.

When GBM CSC adhere to a surface, their morphology changes from the spherical shape of a cell in suspension, to a more typical neural cell shape, with the extension of neurites. This is notable in the Live/Dead stained neurosphere, which shows a mix of round and elongated live cells which are migrating out into the Fibrin hydrogel. The centre of the neurosphere cultured in +GF conditions contains a large population of dead cells, which could be due to a lack of diffusion of the required nutrients into the centre of the neurosphere as it grows in size\textsuperscript{451}.

Control +GF and NMT +GF neurospheres did not show an increased amount of cell migration relative to the neurospheres cultured in media which did not contain any added growth factors. In fact, the NMT +GF neurosphere surrounded by a much smaller number of migrated cells compared to the Control +GF. This could indicate that the NMT condition is affecting some cell migration pathways. However, the investigation should be repeated to confirm this finding, since it is based on a single neurosphere in this case.

In summary, the Control neurosphere in Fibrin hydrogel was slightly larger, with a greater number of migrated cells, compared to the NMT treated cells.
5.5.8 Neurospheres in Fibrin: Control –GF and NMT- GF

As has been established, growth factors play an important role in stem cell maintenance and cell proliferation. Therefore the cells of a neurosphere cultured in media without the addition of growth factors may not be expected to undergo proliferation (although aberrant pathways may be active in cancer cells\textsuperscript{252,293,295}) and this could explain the comparatively smaller size of the neurosphere cultured in media without growth factors, in relation to the neurospheres cultured in media containing growth factors. Furthermore, Control neurosphere was larger than then NMT treated neurosphere,

Both NMT -GF and Control -GF neurospheres express SOX2, which suggests that some cells remain undifferentiated. The SOX2 staining on the NMT –GF neurosphere is reduced, particularly towards the edges of the neurosphere, compared to the Control –GF neurosphere. There are a large number of cells in the NMT-GF neurosphere which have a visibly stained nucleus, but little to no SOX2 signal surrounding it. In contrast, nearly every cell in the Control –GF neurosphere stained for SOX2, with only a couple of visibly stained nuclei with little to no SOX2 detected. Due to the shape of the neurosphere in the immunofluorescence stained Control –GF condition, it appears that this maybe a slice taken from the edge of the neurosphere. This is apparent since there are a much smaller number of cells than in the NMT –GF neurosphere slice, and the shape of the cells is less spherical that those seen in the centre of the neurospheres.

As would be expected, the cells towards the edge of the neurospheres which are embedded in gels tend to show the greatest response to differentiation stimuli: The cells on the edge of the NMT -GF neurosphere have stained less for SOX2 and more for GFAP, and some of the cells exhibit a classic astrocytic shape, with multiple neurites extending from the
cell body. The fact that the edges of the neurospheres which are growing into the fibrin gel appear to exhibit reduced SOX2 expression, as well as increased GFAP expression, is suggestive of astrocytic differentiation in these areas. This could be anticipated, since cells would be able to adhere to the fibrinogen using FAK, thus producing a focal adhesion which can induce differentiation. In the NMT neurosphere, the shape of the neurosphere is very clear and so the ‘edge’ is clearly defined. In the case of the control neurosphere, there are few cells visible which could suggest that the neurosphere disintegrated, or that this slice is in fact the edge of the neurosphere. It may therefore be difficult to compare the effect of the NMT condition compared to control. However, the NMT neurosphere does appear to have a higher number of stained nuclei which do not also contain SOX2, which could suggest that there are more differentiated cells in this NMT condition without growth factors.

In differentiation media, neurospheres may contain a heterogeneous population of cells. It is therefore possible for some cells to express stem cell markers like SOX2 or Nestin, whilst also expressing astrocytic markers such as GFAP. This could explain why the neurospheres which were stained for GFAP and SOX2 can express both markers.

**5.5.9 Neurospheres in Fibrin: Control +BMP4 and NMT +BMP4**

Interestingly, the neurospheres cultured in media containing BMP4 were a comparable size to the neurospheres cultured in growth factor-free media. The relationship between cell proliferation, growth factors and BMP4 is a complicated one. It has been established that growth factors play an important role in stem cell maintenance and cell proliferation, while BMP4 has also been linked to apoptosis and proliferation suppression\textsuperscript{472}. This could explain the smaller neurospheres, and the large number of dead cells which were detected with the Calcein/Propidium Iodide staining.
BMP4 has also been found to increase the sensitivity of cells to mechanical cues\textsuperscript{289}, which could explain the fact that some cells have migrated quite far from the main neurosphere, since this behaviour is typical of a differentiated cell.

Immunohistochemistry of the Control +BMP4 neurosphere indicates the presence of cells which express GFAP, suggesting these cells have differentiated into astrocytes. This is true for cells throughout the neurosphere, with GFAP staining in the centre and edge. Some of the cells towards the edge of the neurosphere display clear neurite growths, indicating they have adhered to the Fibrin scaffold.

The morphology of the neurospheres and individual cells therein was very similar between the Control and NMT cells. Both the Control and NMT neurospheres remained in a tight spherical shape, with clear layers of cells visible in the fluorescent micrograph as well as the H&E stained images. In the Control +BMP4 H&E stained image, a hole formed in the Fibrin gel which appears to have pulled some cells away from the main neurosphere shape, and it was therefore impossible to determine the extent to which those particular cells migrated on their own. However, the other side of the neurosphere remained embedded in the Fibrin gel, and there are some cells which migrated out of the neurosphere. Some of these migrated cells exhibit an elongated shape, suggesting that they have adhered to proteins within the hydrogel. Generally, these cells which have migrated from the neurosphere are single cells, and therefore they would not be linked to other cells by gap-junctions. However, they may form focal adhesion complexes with the proteins in the Fibrin scaffold.

It is interesting that the ‘rounded’ cells also express GFAP, suggesting that they have differentiated without forming adhesions. This could be due to the BMP4 in the media, which is can induce differentiation of the cells, as described in Section 3.2.3.3. The GFAP signal was
detected throughout the neurosphere, suggesting that the location of the cell did not affect its differentiation potential.

5.5.10 Neurospheres in Fibrin: Control +FBS and NMT +FBS

The size of the neurospheres cultured in media containing FBS is very large, and is comparable to the size of the neurosphere cultured in full expansion media with growth factors. This is interesting given that this media does not contain growth factors, and therefore the cells would not be expected to proliferate as successfully as those in media containing growth factors. In fact, the average size of the Control +FBS neurosphere was found to be twice the area of the NMT +FBS neurosphere. Therefore, there is the possibility that the neurosphere consists of larger cells, since astrocytes are typically larger than stem cells, although it could also be the case that the cells are differentiating and migrating out of the NMT neurosphere, resulting in a smaller sphere. The enhanced expression of Matrix Metalloproteinase-9 (MM9) in serum-treated cells is linked to increased cell invasion, which could indicate an increased amount of cell migration compared to neurospheres cultured in serum-free media\textsuperscript{276,473}.

There is a striking difference in the morphology of cells cultured in Control +FBS and NMT +FBS conditions. The H&E stained neurospheres for both conditions, as well as the immunofluorescently labelled neurosphere for NMT+ FBS, show a pattern of more rounded cell shapes in the NMT condition, and more elongated cell shapes in the Control condition. The Control +FBS neurosphere contains layers of cells similar to the rounded neurospheres, but the edges have established protrusions out into the gel, with many elongated individual cells. However, whilst the NMT +FBS neurosphere has also spread out cells from the main neurosphere, they appear to be much rounder in shape. This is in contrast to the literature
which reports elongation of the cells, which typically extend neurites into the hydrogel on the edge of the neurosphere. This compounds the idea that the NMT condition may affect the ability of cells to adhere to the Fibrin gels. Interestingly, several of the cells within the layers inside the neurosphere appear to have formed neurites, suggesting that these cells are forming adhesions. This may be occurring between the cells and fibrin proteins intertwined with the neurosphere, or with other matrix proteins secreted by the cells themselves. However, within the neurospheres, the majority of cells typically maintain a spherical shape.

The neurosphere which was exposed to NMT conditions, and cultured in media containing 10% FBS, was stained for GFAP and SOX2. Although confirming the difference between the control and NMT-treated cells in these conditions would require further analysis at the protein and genetic level, some conclusions can be drawn based on morphological data. The SOX2 staining of the NMT neurosphere appears to be a much lower signal than the GFAP, with several stained nuclei visible which are not encompassed by SOX2, and some expressing only a very small amount, which suggests that these are differentiated cells. Furthermore, the GFAP expression is very strong with a high level of GFAP staining visible around nearly every cell in the neurosphere. Overall, both the control and NMT FBS neurospheres appears to have resulted in a large amount of differentiation, which was to be expected given the differentiation-inducing effect of FBS on GBM CSCs.

### 5.6 Conclusions

In summary, this chapter has shed light on key insights regarding the behaviour of neurospheres and cancer stem cells in various culture conditions. As discussed in Chapter 3, cancer cells often exhibit aberrantly active signalling pathways, allowing them to produce their own growth factors or activate growth factor receptors even in the absence of
exogenous growth factors. This property has been crucial in enabling neurospheres cultured in media without added growth factors to proliferate effectively.

Neurospheres, primarily used to isolate a population of stem cells in vitro, may retain some differentiated cells temporarily. It's worth noting that differentiated cells that fail to migrate away from the neurosphere typically undergo apoptosis. Consequently, neurospheres cultured in Agarose and Alginate media may contain a subpopulation of apoptotic astrocytes. However, precisely distinguishing cells that have differentiated and died from those that perished due to nutrient deprivation would require further analysis.

The Live/Dead staining of the Agarose neurosphere cultured in full media with growth factors revealed a significant number of dead cells alongside clusters of live stem cells, likely resulting from nutrient depletion. Similarly, the Alginate neurosphere cultured in media with FBS also exhibited a substantial population of necrotic cells, some of which might have undergone astrocytic differentiation due to the presence of FBS. This is supported by studies which have concluded that the size of the neurosphere can be correlated with cell survival, in that larger neurospheres result in higher levels of apoptosis.

Notably, in Fibrin gels, the addition of FBS induced pronounced cell spreading and migration, which may suggest a propensity for differentiation and the subsequent migration of astrocytes away from the neurosphere. This observation aligns with previous research demonstrating that cells induced to differentiate with serum exhibit increased invasive behaviour. Furthermore, BMP4 has been associated with heightened sensitivity to mechanical cues, potentially explaining the extensive migration of cells from the neurosphere into the Fibrin gel.
Conclusions

One of the most remarkable findings in this chapter is the distinct morphology of cells in Alginate gel which were exposed to nanoscale vibrations. The cells of these neurospheres exhibited a notably rounder shape compared to their control counterparts, which extended neurites between the cells of the neurosphere.

In Fibrin gels, the NMT neurospheres themselves were also smaller than the control for each of the media conditions, suggesting there may have been reduced proliferation of the cells in response to the nanovibrations. This is particularly interesting, since it has been established that the FAK pathway can promote both proliferation and differentiation. In this case, a reduced rate of proliferation may correspond to increased differentiation of the stem cells. This was also in direct contradiction of the findings in Chapter 3, where the NMT condition led to increased proliferation.

It is difficult to elucidate further information from morphological analysis alone. A protein or genetic analysis would provide further insight into the mechanisms underlying the cell behaviour, and to confirm the effects of the nanovibrational stimulation in a 3D model.
Chapter 6: FINAL CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Summary of Key Findings

Below is a summary of the methodology which was applied in this study. This table encompasses the effects of the chemical and physical environment in which the cells were cultured, as well as the physical stimulation which was applied.

<table>
<thead>
<tr>
<th>MEDIA CONDITIONS</th>
<th>+ Growth Factors</th>
<th>- Growth Factors</th>
<th>+ BMP4</th>
<th>+FBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actively Prevents Differentiation</td>
<td>Actively Promotes Differentiation</td>
<td>Actively Promotes Differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Passive Differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**2D Study**

**FOR EACH MEDIA CONDITION, CELLS WERE:**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NMT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not vibrated</td>
<td>Vibrated</td>
</tr>
</tbody>
</table>

**3D Study**

**THOSE CONDITIONS WERE APPLIED TO CELLS CULTURED IN:**

<table>
<thead>
<tr>
<th>LT Agarose</th>
<th>Alginate</th>
<th>Fibrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Did not allow cells to attach to gel</td>
<td>Did not allow cells to attach to gel</td>
<td>Allowed cells to attach to gel</td>
</tr>
</tbody>
</table>

Nanoscale vibrations were found to have some effect on cells in nearly every condition. Beginning with cells cultured in a 2D monolayer, when the cells were cultured in a 2D environment in media which did not contain growth factors, both the control and NMT populations expressed a mix of stem cell and astrocytic markers. But perhaps most notably, the increase in GFAP was significant when the growth factor-free cells were exposed to nanoscale vibrations. The effect of chemical mediators of differentiation, BMP4 and FBS, was similarly impacted by the nanoscale vibrations, with a notable increase in differentiation with the addition of BMP4 in the media in the 2D culture.
It is interesting to note that across all media conditions, the neurospheres cultured in Fibrin gel which received nanoscale vibrational simulation were smaller in size than the control neurospheres, which may be as a result of differentiation of the cells, encouraged by the vibrational stimulation, which normally results in decreased proliferation. This discrepancy between the results of 2D and 3D stimulation could indicate that the cells do respond differently to mechanical cues when cultured in a 2D or 3D environment, suggesting that GBM stem cells in a 2D culture may proliferate and differentiate in response to vibrations, while cells in 3D culture tend only to differentiate. It must be noted that the findings are based on a small sample size, and therefore the strength of the conclusions may be limited by this.

Surprisingly, the 2D cell culture population cultured with FBS did not have a marked increase in GFAP expression according the qPCR data, with a smaller increase in expression than simply removing growth factors. This was an unexpected finding, since FBS appeared to have a much more significant impact on cell behaviour when the cells were cultured in 3D hydrogels. In this case, the neurospheres cultured with FBS in Fibrin gels strongly expressed GFAP, and only a very small amount of the stem cell marker, SOX2. This indicates that FBS as a chemical mediator has a stronger effect on 3D cultured cells than a monolayer.

Overall, treatment of the cells with nanoscale vibrations resulted in a general decrease in stem cell characteristics, and an increase in astrocytic differentiation. Interestingly, the treatment of cells with vibrations appeared to universally affect the morphology and size of neurospheres in 3D culture, indicating that this treatment has an effect on signalling pathways associated with these mechanisms. There is a clear difference between the size of the neurospheres cultured in control and NMT conditions, whereby the control condition results in a larger neurosphere overall. This may be due to increased migration from the NMT
neurosphere, and reduced proliferation in response to the NMT stimulation\(^{474}\). The idea that this is due to differentiation is supported by many studies, which have found that differentiated cancer stem cells will migrate away from a neurosphere\(^{275}\). In a clinical setting, this may be linked to increased susceptibility to chemotherapy, highlighting the significance of culturing the cancer stem cells in a more biomimetic, neurosphere environment, as opposed to the more typical monolayer approach to \textit{in vitro} cell culture.

In order to quantify the differences in protein expression which were detected using immunofluorescence of the neurospheres, a larger sample size would be required. The stained cells responded in a way which is in line with the published literature on the topic\(^{419,476–478}\), with GFAP expression indicative of astrocytic differentiation, and SOX2 expression indicative of a stem cell population. In both 2D and 3D culture, these markers were expressed appropriately when cultured in media designed to encourage or suppress differentiation, and this was also reflected in the 3D culture conditions. Differences between levels of expression between control and NMT treated neurospheres are not obvious, however.

Morphologically, there are clear differences between the cells of the neurosphere cultured in Fibrin gel compared to those cultured in Alginate or Agarose. These findings are supported by the literature, which highlights the structural differences between the gels which either encourage the formation of neurites, as in the case of Fibrin gel, or does not allow neurite formation, in the case of Agarose and Alginate. The purpose of utilising these vastly structurally different materials, was to investigate the response of neurospheres which were, or were not, able to physically attach to their environment. As was expected, the neurospheres cultured in fibrin gel did attach to their environment, and were generally
Limitations and Future Work

elongated on the edge of the neurosphere, while the cells on the edge of the neurospheres cultured in Agarose and Alginate gels remained rounded in shape. It is interesting to note, however, that the cells of the neurospheres in the Fibrin gels exhibited more neurite growth in the control group than the NMT group. This could indicate that the NMT condition affects the cell’s ability to bind with its environment, and produce neurites.

Overall, there is an interesting contradiction between the behaviour of the monolayer of cells in comparison to the 3D cultured cells, in which the physical stimulation encouraged proliferation of cells in 2D culture, and the differentiation of cells in 3D culture. This may have significant implications for the treatment of cancer stem cells in vitro, suggesting that the physical environment can greatly impact the expected behaviour of cells. This may be particularly important for in vivo studies, since the situation of these cells is much more closely related to the neurosphere environment in vitro.

6.2 Limitations and Future Work

A deeper investigation into the mechanisms behind the observations recorded in this thesis could include genetic analysis of the response to nanoscale mechanotransduction in a 3D model. This study was primarily focused on morphological assessment, and some protein analysis. Furthermore, some of the experimental conditions within this study were not able to be analysed due to difficulties in collecting samples, or through loss during the experiment. This limits the conclusions which can be drawn from these results. Therefore, further repeats of these studies to would give a more comprehensive overview of the results, and allow for more robust conclusions to be drawn.

While preliminary studies were carried out to optimise Agarose and Alginate gels in order to examine the impact of culturing cells in an environment in which they could not form cell-
matrix attachments, these were not taken further due to the need to streamline the investigation of cell behaviour. These should be revisited to explore the effect of these conditions on cells within a neurosphere.

Future research aimed at comprehending the fundamental mechanisms governing NSC responses to physical stimulation should seek to identify specific genetic or signalling pathways. This includes a detailed examination of how nanoscale vibrations impact cell-matrix interactions, particularly the adhesion of NSCs to relevant matrix proteins.

Additionally, further studies could be carried out to determine how varying scales of nanoscale vibration affect these cells. Previous research has indicated that different frequencies and corresponding physical displacements may influence various cell types in distinct ways\textsuperscript{184,479}.

The implications of this study in a clinical setting are yet to be explored, and therefore while the importance of more \textit{in vitro} studies is highlighted above, the heterogenous nature of brain tissue may hinder attempts to faithfully mimic the in vivo environment. As was described in the introduction, the presence of cancer stem cells has been linked to the recurrence of tumours and the ineffectuality of chemotherapeutic drugs. Therefore, the ability to induce the differentiation of cancer stem cells using nanoscale mechanotransduction could result in increased effectiveness of chemotherapy drugs, and more impactful cancer treatments overall.
Chapter 7: BIBLIOGRAPHY


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# Appendix I

**SDS PAGE RUNNING BUFFER (10X)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>30.3g</td>
<td>0.25M</td>
</tr>
<tr>
<td>Glycine</td>
<td>44.4g</td>
<td>2M</td>
</tr>
<tr>
<td>SDS</td>
<td>10g</td>
<td>0.035M</td>
</tr>
</tbody>
</table>

800 mL of distilled water, to which add all the above components

Add distilled water until the volume is 1 L
Appendix II

**TB Buffer Recipe**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EDTA</strong></td>
<td>0.37g</td>
<td>0.001M</td>
</tr>
<tr>
<td><strong>Potassium Chloride</strong></td>
<td>13.64g</td>
<td>0.2M</td>
</tr>
<tr>
<td><strong>Sodium Chloride</strong></td>
<td>2.74g</td>
<td>0.047M</td>
</tr>
<tr>
<td><strong>Phenylmethylsulfonyl fluoride (PMSF)</strong></td>
<td>0.17g</td>
<td>0.001M</td>
</tr>
<tr>
<td><strong>Tris- hydrochloride</strong></td>
<td>1.21g</td>
<td>0.01M</td>
</tr>
</tbody>
</table>

800 mL of distilled water, to which add all the above components.

Adjust pH to 6.8

Add distilled water until volume is 1L

Sterilise by filtration
Appendix III

Citrate Buffer Recipe

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium citrate dihydrate</td>
<td>25.7g</td>
<td>0.0874 M</td>
</tr>
<tr>
<td>Citric acid</td>
<td>2.431g</td>
<td>0.0126 M</td>
</tr>
</tbody>
</table>

800mL distilled water, to which add all the above components.

Adjust to required pH using HCl or NaOH

Add distilled water until volume is 1L