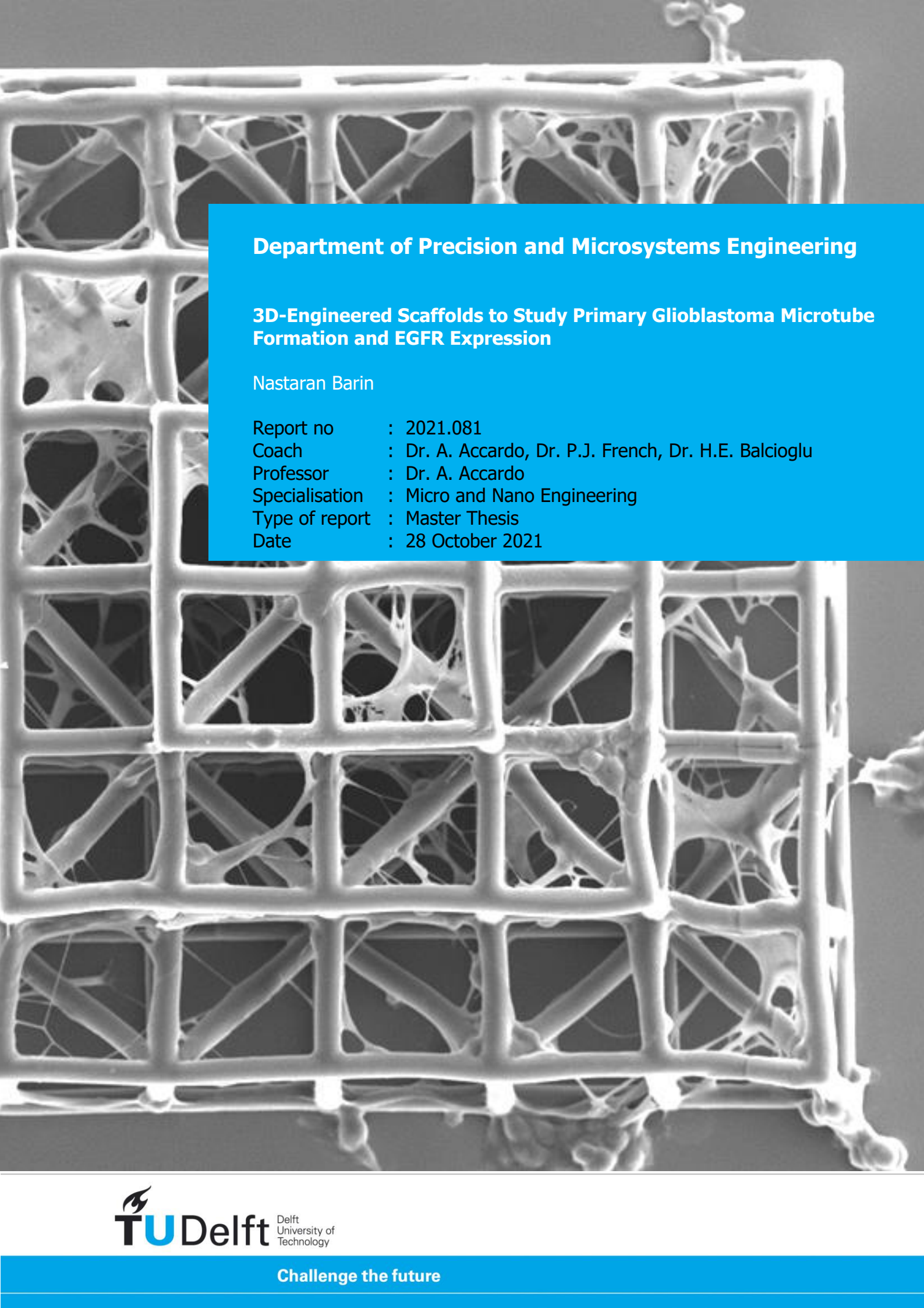




Department of Precision and Microsystems Engineering

3D-Engineered Scaffolds to Study Primary Glioblastoma Microtube Formation and EGFR Expression

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3D-Engineered Scaffolds to Study Primary Glioblastoma Microtube Formation and *EGFR* Expression

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PREFACE

Glioblastoma is the most fatal tumor of the central nervous system and so far, there are no effective treatments. The motivation for this research is to improve the current culturing platforms in order to adequately represent the natural environment of this tumor and, in the long-term, provide a tool to more accurately assess the effectiveness of different treatment methods. In this project, a 3D culturing model is proposed to better represent the glioblastoma environment.

This document is the final report of my Master's thesis project in the Department of Precision and Microsystems Engineering at Delft University of Technology (TU Delft). The nature of this work was multi-disciplinary and required knowledge of engineering, biology, as well as image and data analysis.

This was a collaborative project between TU Delft and Erasmus Medical Center (EMC). Dr. Angelo Accardo from TU Delft Department of Precision and Microsystems Engineering, Dr. Pim French from EMC Department of Neurology, and Dr. Hayri Emrah Balcioglu from EMC Department of Medical Oncology supervised this work.

The project started in October 2020 with a period of literature research. The literature survey lasted 4 months and helped in forming the research questions and design. The experimental part of the project started in February 2021. The fabrication and scanning electron microscopy parts of the experiments took place at TU Delft laboratories, while cell culturing and immuno-fluorescent microscopy tasks took place at EMC. The primary cells for this study were provided by the EMC Department of Neurosurgery.

The research was partially funded by a grant from the EMC Academic Centre of Expertise - Tumor Immunology and Immune Therapy and TU Delft Bioengineering Institute. The principal investigators were Drs. Accardo and French and I was one of the co-applicants of the grant proposal.

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ABSTRACT

Glioblastoma (GBM) is the most aggressive and malignant brain tumor with no effective treatment available so far. A major obstacle in GBM research is the lack of reliable *in vitro* models that can mimic the tumor cellular environment. The goal of this study was to develop a 3D-engineered scaffold as a new cell culture model capable of providing a more accurate representation of the tumor environment. 3D-engineered scaffolds, inspired by the geometry of brain blood vessels, were fabricated using two-photon polymerization. A newly developed biocompatible material with low auto-fluorescence was used for fabrication. To assess the ability of the 3D model in mimicking GBM's natural environment, cell colonization, cellular morphology, microtube formations, and epidermal growth factor receptor (*EGFR*) expressions of cells were derived from confocal microscopy images and compared with those in 2D control experiments. The *EGFR* expressions were further evaluated in the presence of two *EGFR* inhibitors. U-87 cell line and three primary GBM cell cultures successfully colonized the scaffolds. Morphological differences were observed between 2D and 3D models with 3D models more closely representing *in vivo* observations. Microtube-like protrusions were present in both 2D and 3D models but the 3D model better matched the morphology of microtubes *in vivo*. There were no significant differences in expressions and subcellular localizations of *EGFR* between 2D and 3D models. However, *EGFR* internalizations were not prevented in the presence of *EGFR* inhibitors. This previously-unreported observation may explain the *in vivo* resistance of GBM cells to inhibitors. The 3D-engineered scaffolds provided a new suitable model that better replicated the GBM's natural environment with advantages that were difficult to capture in 2D models.

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LIST OF ABBREVIATIONS

2D	2 dimensional
3D	3 dimensional
2PP	2 photon polymerization
ANOVA	One-way analysis of variance
ATP	Adenosine triphosphate
BBB	Brain-blood barrier
CNS	Central nervous system
Cx43	Connexin 43
DiLL	Dip-in laser lithography
DLW	Direct laser writing
DMEM	Dulbecco's Modified Eagle Medium
ECM	The extracellular matrix
EGF	Epidermal growth factor
<i>EGFR</i>	Epidermal growth factor receptor
EtOH	Ethanol
GAP-43	Growth associated protein-43
GBM	Glioblastoma
GA	Glutaraldehyde
GWL	General Writing Language
IDH	Isocitrate dehydrogenase
IPA	ISO-Propanol
ITO	Indium-tin oxide
MAPTMS	3-(trimethoxysilyl) propyl methacrylate
PBS	Phosphate buffered saline
PBS+	Phosphate Buffered Saline with bovine serum albumin
PBS-T	Phosphate Buffered Saline with Tween
PBS-triton	Phosphate-buffered saline with Triton
PFA	Paraformaldehyde
PEGDA	Poly (ethylene glycol) diacrylate
PGMEA	Propylene glycol methyl ether acetate

SEM	Scanning electron microscopy
STL	Standard Triangle Language
TKI	Tyrosine kinase inhibitors
Ttyh1	Tweety-homolog 1

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1 INTRODUCTION

Gliomas are brain tumors that stem from glial cells¹. Glioblastoma (GBM) is the most aggressive and life-threatening type of glioma²⁻⁴. GBM accounts for 56% of all gliomas and has the highest malignancy grade (grade IV)^{1,3}. The standard treatment for a GBM patient is surgery to remove the tumor as much as possible followed by chemotherapy and radiotherapy to kill and avoid the spread of the remaining tumor cells⁵. However, these methods, in all cases, fail to fully control this disease and less than 10% of the patients survive more than 5 years after diagnosis^{1,3}. Almost all patients experience relapse of the tumor⁴⁻⁶. Hence, there is a great need to find new effective therapeutic methods to combat this life-threatening condition. Unfortunately, GBM therapy outcomes in experimental models do not match clinical results. GBM tumors often respond better to therapeutic methods when they are tested *in vitro* compared to when they are used on patients⁴.

One contributor to this failure is the lack of cell culture methods that are able to faithfully replicate GBM behavior *in vitro*⁴. Although GBM cell culture methods have advanced greatly over the years, they still face many limitations^{2,4}. Cancer cell progression is affected by the cellular micro-environment⁷. GBM cells are highly sensitive to their environment and are capable of rapidly adapting themselves to their surroundings⁴. It can be hypothesized that the better replication of the GBM cellular environment will produce more consistent results with clinical trials.

With the technological advances in fabrication techniques, it is now possible to manufacture micro and nano scale 3D structures that more accurately resemble cellular micro-environments. New fabrication techniques, in particular the 2 photon polymerization (2PP) method, allow the production of novel 3D cell culture environments that were not feasible before⁸.

GBM heterogeneity, a well-known characteristic of the GBM cells, is another important contributing factor in failure to find an effective treatment. Standard cell lines, frequently used in GBM research, fail to capture the complexity of this tumor. The use of primary GBM cells and repeating experiments with a panel of such cells, may address this challenging aspect of GBM research. Other shortcomings of the current cell culture methods are the inability to form microtube-based networks and the failure to retain high-copy number epidermal growth factor receptor (*EGFR*) amplification^{6,9,10}. These are two important GBM features that promote tumor progression^{6,9,10}.

The motivation for this research was to address the current shortcomings by designing a 3D-engineered scaffold to provide a better model in mimicking the behavior of the GBM cells. After successful validation of this culturing method, the model can be used for further clinical research on GBM treatment methods such as radiotherapy, immunotherapy, and drug discovery.

2 REVIEW OF LITERATURE

2.1 GLIOBLASTOMA CELLS

GBM has been a challenging cell type to culture and study due to its variety of genotypes and phenotypes⁴. Some of the signature genetic changes for GBM are: *EGFR* amplification, trisomy of chromosome 7 combined with loss of one copy of chromosome 10, cyclin dependent kinase inhibitor 2A/B homozygous deletion, and phosphatase and tensin homolog mutation¹⁰⁻¹². In the 2016 World Health Organization classification of central nervous system (CNS) tumors, GBM cells are classified into two subtypes of isocitrate dehydrogenase (IDH)-wildtype and IDH-mutant¹³. IDH-wildtype tumors account for nearly 90% of all GBM cases. Patients with this tumor type are usually older adults and have dismal prognosis^{13,14}. IDH-mutant GBM tumors more common in younger patients compared to IDH-wildtype GBMs¹³. GBM cells can also be classified based on their gene expression profiles into classical, mesenchymal, and proneural^{4,10}. However, rigid classifications fail to account for tumor heterogeneity and plasticity. Samples from patients have shown that tumors can have more than one subtypes of GBM cells⁴. Tumor progression in different tumor subpopulations are not similar and these subpopulations may respond to treatments differently^{2,4}. Therefore, better models to capture the heterogeneity of these tumors are needed.

2.1.1 GBM Cellular Morphology

GBM cell morphologies vary between polygonal and spindle-like shapes¹⁵. These cells usually have increased nuclei sizes¹⁵. Research has shown that GBM cellular environment can affect its morphology^{16,17}. Koh et al (2018) studied morphology of GBM cells in 2 different 3D models (a patient tissue-derived decellularized ECM-based 3D tumor model and a collagen matrix model). They reported that GBM cells adapted their invasion modes to their environment and that the invasion mode correlated with cell morphology. Cells with mesenchymal mode of invasion had more spindle-like and elongated shapes and cells with amoeboid mode of invasion had rounder morphologies¹⁷.

Furthermore, Nafe et al (2005) showed a significant correlation between morphological features of GBM nuclei and the patient survival time¹⁸. For example, they reported that nuclei areas had negative correlation with the patient survival and that non-circular nuclei shapes were related to the GBM prognosis. Certain levels of nucleus non-circularity increased patient survival whereas other levels decreased it¹⁸. They claimed these correlations were more significant compared to the correlation between the patient age and the survival time¹⁸. Boruah et al (2013) studied the relationship between nucleus morphology and tumor grade in low grade and high grade gliomas. They used biopsies of 30 glioma patients in which 16 patients were diagnosed with GBM. They found that the larger nucleus area increased the probability of the glioma having a higher grade. On the other hand, a rounder nucleus decreased this probability but the effect was not as important as the nucleus area¹⁹. The exact mechanisms underlining the relationship between the nucleus morphology and tumor aggressiveness are not fully understood but one hypothesis is that nuclear size can affect chromatin organization and gene expression of the cells²⁰.

2.1.2 *EGFR* Expressions

EGFR is a member of the transmembrane proteins of the receptor tyrosine kinases superfamily^{21,22}. These receptors are responsible for initiating intracellular signaling and for regulating cell proliferation and survival²³. *EGFRs* are one of the most commonly altered receptors in GBM^{24–26}. Nearly 57% of all GBM tumors show *EGFR* alterations such as *EGFR* mutations, amplifications, and/or rearrangements^{22,23}. *EGFR* amplification, observed in nearly 40% of GBM tumors, increases the cell proliferation uncontrollably, which results in tumor progression^{21,23,25}. GBM cells demonstrate great heterogeneity at the cellular level in terms of *EGFR* expression²¹. *EGFR* amplifications correlate with tumor subtypes, tumor growth, and tumor resistance to therapy^{10,27}.

EGFR is a cell surface receptor for ligands such as the epidermal growth factor (EGF). So far, seven ligands, including EGF, have been identified for this receptor²³. Figure 1 demonstrates the extracellular, transmembrane and intracellular domains of *EGFR* and it shows the activation process of this receptor. *EGFR* in its inactive form exists as a monomer on the membrane of the cells with a closed configuration²⁴. Ligand-binding leads to configuration changes in *EGFR*'s external domains resulting in *EGFR* dimerization, tyrosine kinase activation, and phosphorylation of a range of downstream proteins^{23,24}. The dimerization occurs between two *EGFRs* monomers (homodimers) or with a different tyrosine kinase receptor (heterodimers)²³. This process commences the transduction of a cascade of signaling pathways, starting from the membrane and reaching the nucleus. These signals contribute to cell survival, migration, growth and invasion and are instrumental in the continuous proliferation of cancer cells. Strength and duration of these signals are controlled by *EGFR* internalization²³. *EGFR* internalization is then followed by *EGFR* recycling or degradation. The overexpression of *EGFR* in cancer cells can lead to its activation even in the absence of ligands, e.g. mediated by integrins or its activation caused by external stresses such as radiation²³. Recent studies investigated the possible new functions of *EGFR* and *EGFR* internalization²⁵.

EGFR amplifications are seen in various other types of cancers as well^{22,23}. Over the past two decades, targeting *EGFR* has been a focus area for developing treatment methods for GBM²¹.

After ligand-binding, changes in the intracellular domain of *EGFR* complex, including binding of adenosine triphosphate (ATP), lead to tyrosine kinase activation²⁴. Small molecule tyrosine kinase inhibitors (TKIs) stop the ATP binding by occupying the kinase binding pocket²⁴. TKIs are particularly responsive to certain mutations of *EGFR*. First generations of TKIs stop downstream *EGFR* signaling by outcompeting ATP for binding to the kinase activation pocket. Examples of these inhibitors are Erlotinib and Gefitinib²⁴. Tumors are initially responsive to these inhibitors but later develop certain mutations that cause resistance. To address this issue, second generation TKIs were developed. These inhibitors make changes to the ATP binding pocket hindering kinase activation²⁴. Examples of second generation TKIs are Dacomitinib and Neratinib²⁴. Unfortunately, these inhibitors target wild-type *EGFRs* that are present in normal tissues as well. This unintended targeting results in side effects such as rashes and diarrhea, which limits the dosing of these inhibitors. Third generation TKIs, such as Osimertinib, have been developed in response to the resistance mechanisms to the previous generations of TKIs. These inhibitors do not target *EGFR* in normal tissues and have better brain blood barrier (BBB) penetrations²⁴. However, after initial responses, *EGFR* mutations that were resistant to these inhibitors emerged. To

overcome this resistance, scientists are now working on the fourth generation of inhibitors. It should be mentioned that inhibitor categorizations are done slightly different in different journals^{24,28}.

Although these *EGFR* inhibitors have considerable therapeutic effects in *EGFR*-mutated lung cancer patients, they have not been particularly as effective in GBM patients^{21,27,29}. This is despite the fact that anti-*EGFR* drugs perform well on GBM cells in laboratory settings²¹. In literature, the commonly mentioned challenges of *EGFR* targeting are: emergence of new mutations, BBB penetration, *EGFR* heterogeneity, unintended targeting of *EGFR* in normal tissues, and escape mechanisms for signaling pathways²⁴.

To further study the potential of *EGFR*-targeting treatments in GBM, *in vitro* cell culture models that can retain *EGFR* amplification and monitor their reaction to *EGFR* inhibitors are needed²¹. The current cell culture systems *in vitro* are not able to maintain these amplifications and *EGFR* amplification disappears after certain number of *in vitro* passages (~1-5)^{21,30,31}. Passaging is the process whereby a fraction of multiplying cells is transferred to a new dish to provide more space and to facilitate their continued proliferation³². The mechanism behind the loss of *EGFR* amplification in *in vitro* cultures is not fully understood yet^{21,31}.

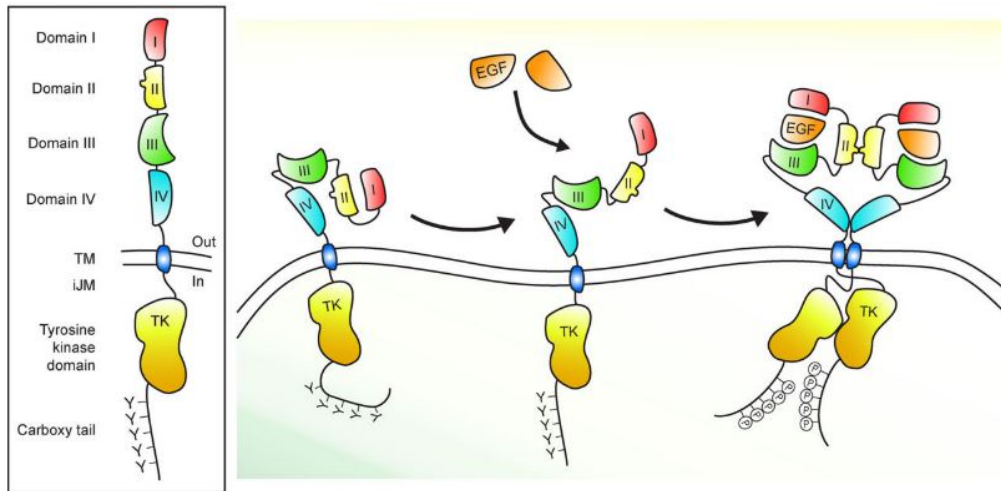


Figure 1: Process of EGF-induced activation of EGFR. Each EGFR consists of an extracellular, a transmembrane, and an intracellular domain²⁵

2.1.3 Microtubes

A recently discovered feature of the GBM cells is their ability to form nanotubes and microtubes^{6,33}. Microtubes are long and thin cellular protrusions that allow GBM cells to connect together, to form a cellular network, to maintain cellular homeostasis, and to colonize the brain⁹. Using these microtubes, cells are able to probe their environment and accelerate tumor growth. Microtubes are usually 1 to 2 μm wide, can be 10 to 500 μm long, and can live up to 200 days^{6,9,34}. Osswald et al (2016) demonstrated that aggressiveness and malignancy of glioma tumors increase with the increase in their microtube lengths⁹. On the other hand, nanotubes are very thin, open-ended cellular protrusions with diameter widths of less than 1 μm and length of up to 100 μm . They live up to 60 minutes^{6,35}.

The main difference between microtubes and nanotubes and other cellular protrusions is their ability to form functional cellular networks (Figure 2)^{6,9,35}. Both microtubes and nanotubes are capable of transferring mitochondria and propagate inter-cellular calcium waves⁹. They actively scan their environment and help tumor invasion. Osswald et al (2016) further claimed that nuclear material transfers are possible through microtubes⁹.

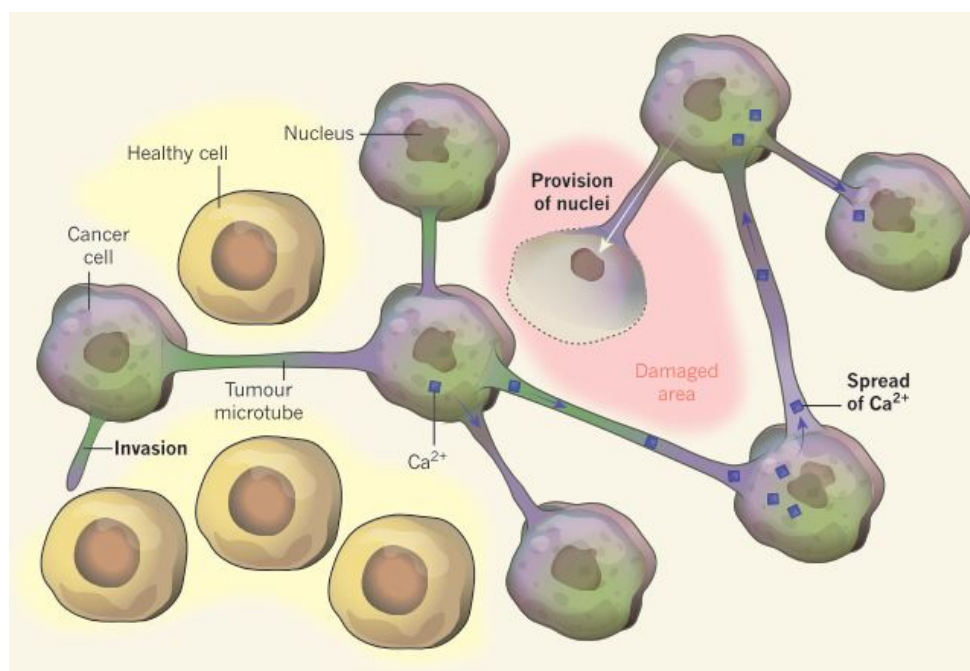


Figure 2: Microtube-based network of GBM cells³³

The ability of GBMs to form functionally resistant cellular networks makes them more resilient to therapeutic methods including radiotherapy and chemotherapy^{5,9}. In an experimental research on mice, Osswald et al (2017) showed that after surgical removal of GBM tumors, the remaining GBM cells extend microtube protrusions toward the lesion area and colonize it even more aggressively⁵. Figure 3 shows the infiltration of GBM cells after surgery.

Osswald et al (2019) claimed growth associated protein-43 (GAP-43) to be a biomarker for microtubes and stated that this protein has not been detected in molecular analyses of other protrusive structures such as filopodia, lamellipodia, or invadopodia⁶. Osswald et al (2017) further investigated the role of GAP-43 in microtube formation. By targeting GAP-43 and comparing the result of surgical removal of the tumor to a control experiment, they were able to show that GAP-43 deficient GBM tumors were not able to form as many microtubes and did not repopulate (Figure 4)⁵. They proposed that microtube connections are composed of connexin 43 (Cx43) gap junctions and tumors with higher number of microtubes and higher levels of Cx43 are more resistance to Temozolomide chemotherapy⁵. They further suggested targeting GAP-43 and inhibiting Cx43 gap junction formations as a method for lowering GBM resistance toward treatment methods.

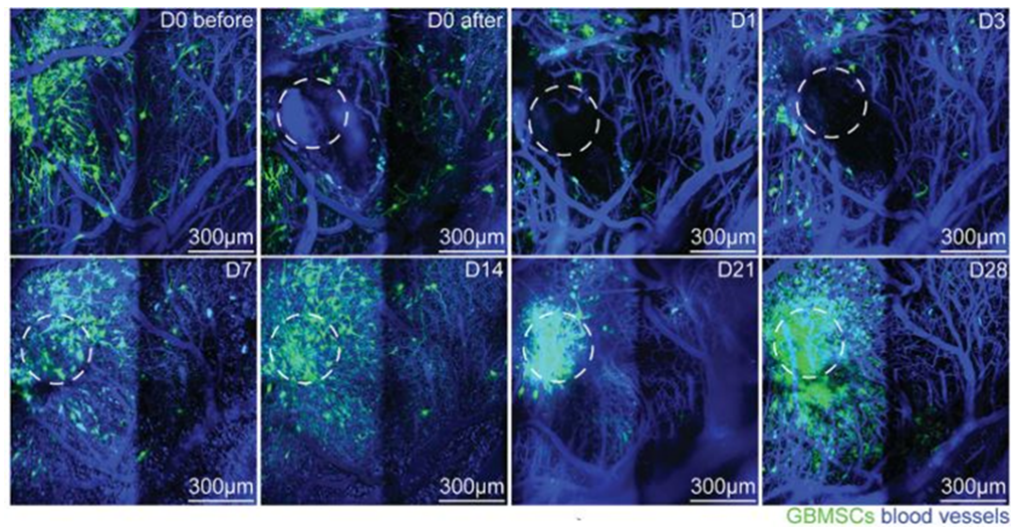


Figure 3: Tumor relapse following surgery after transplantation of patient-derived GBM stem-like cells (GBMSC) in the mouse brain⁵

A protein called tweety-homolog 1 (Ttyh1) has been detected in more invasive microtubes^{6,34}. Jung et al (2017) showed that Ttyh1 has a dominant role in microtube outgrowth and tumor invasiveness³⁴. It has been suggested that Ttyh1 down-regulation can be studied as a method for targeting microtubes for GBM therapy³⁴.

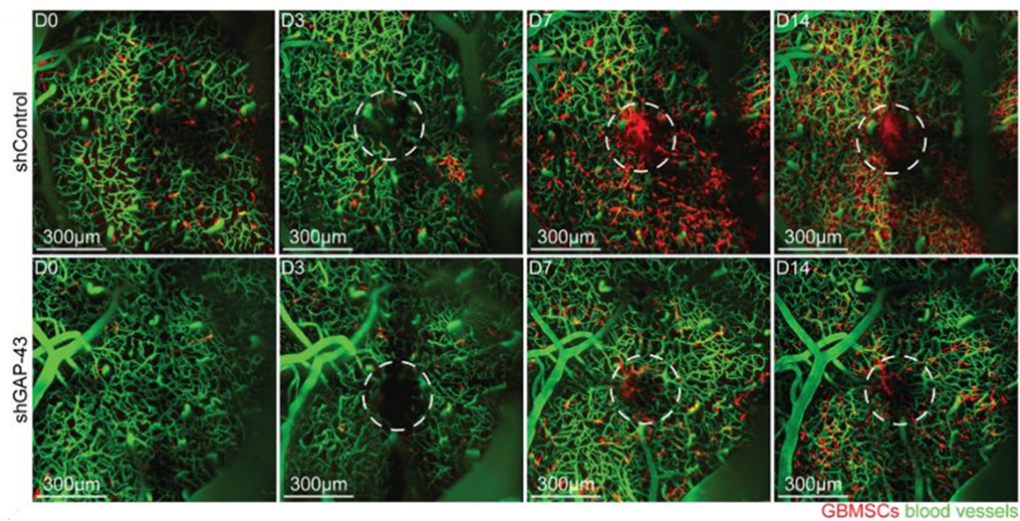


Figure 4: Top: control experiment, bottom: GAP-43 deficient glioma cells⁵

Recently the formation of nanotubes has been reported in 2D surfaces and in 3D tumor organoids using GBM primary cells (Figure 5)³⁵. Pinto et al (2021) reported formation of functional nanotubes that were capable of transferring mitochondria in both environments. Furthermore, they reported the formation of tube-like protrusions in micro scale only in the 3D organoids³⁵. The study did not report on the functionality of these protrusions.

Targeting microtubes can be a promising technique for advancing GBM treatment methods. To achieve this goal, GBM cell culturing models that are capable of capturing microtubule formation are needed.

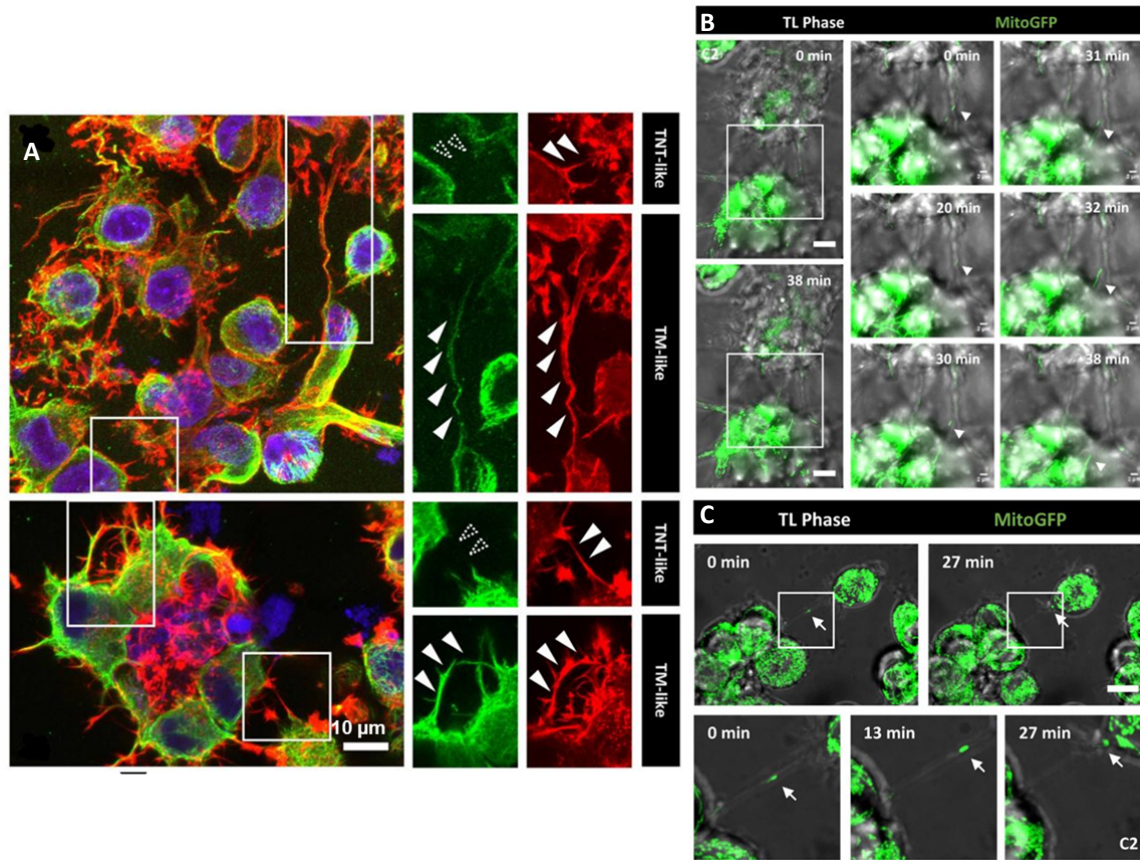


Figure 5: A) Formation of microtubes and nanotubes in 3D GBM tumor organoids. B) Mitochondria transfer in nanotubes in 3D GBM tumor organoids. C) Mitochondria transfer in nanotubes in the 2D GBM cell culture³⁵

2.1.4 GBM Cellular Micro-Environment

The extracellular matrix (ECM) is a 3D network of proteins that surrounds the cells and provides them with structural support^{7,36}. The ECM provides a fibrous 3D scaffold for the cell adhesion and has a central role in tissue development^{7,37}. The ECM also contains the growth factors that are necessary for cell growth⁷. Cells constantly interact with the ECM to maintain their cellular homeostasis³⁸. A computer simulation by Checa et al (2015) demonstrated that cellular behavior and organization are directly affected by the ECM-cell interactions and that mechanical properties of the ECM can influence tissue formation³⁸.

The ECM composition includes collagen, fibronectin, vitronectin, and laminin³⁶. Compared to other tissues, the brain's ECM has relatively lower amounts of collagen and laminin and relatively higher levels of hyaluronic acid and heparin sulfate proteoglycans⁴. This composition reflects the relatively low Young's modulus (nearly 1 kPa) of the brain³⁹. Currently, in cell culture, the biochemical cues of the ECM are mimicked in a minimalistic way by using different substrate protein coatings. Coating materials are used to cover the surfaces that cells are in contact with. Another important feature of the brain ECM is that its composition and physical properties can vary across individuals and across tumors⁴. This adds to the difficulty of replicating the brain ECM *in vitro*. Furthermore, physical characteristics of the ECM, such as rigidity and porosity, which cannot be solely captured by varying the ECM composition, have proven to affect cellular behavior and tumor progression^{36,37,40}.

Another feature of the GBM cell microenvironment is the interaction between the vascular niche environment and the tumor cells. It is claimed that the GBM cells tend to move along the blood tracks^{3,33,41,42}.

2.2 MECHANOBIOLOGY

Mechanical characteristics of surroundings for the cells can influence their behavior^{7,8,38}. A computer simulation model by Werfel et al (2013) demonstrated that even with the lack of any gene mutation, physical alterations in the microenvironment of the cells can contribute to cancerous phenotypes and accelerate tumor progression⁴³. The four main features of a cell microenvironment are: stiffness, pore size, structure dimensionality, and surface topography.

The interaction of cells with their surrounding microenvironment is mainly divided into two mechanisms: mechanosensing and mechanotransduction. Mechanosensing is the process by means of which cells sense mechanical cues from their surroundings^{7,8}. These cues include force, stress, strain, material stiffness, adhesiveness, and surface topography⁴⁴. Mechanotransduction is translating these mechanical cues into biological responses^{7,8}. Figure 6 shows a schematic of these two processes.

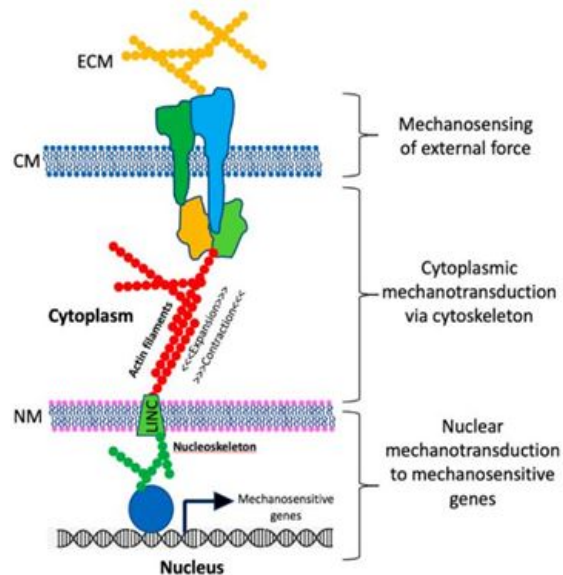


Figure 6: Cell-ECM interaction through mechanosensing and mechanotransduction⁴⁵

- **STIFFNESS**

Research has shown that variations in micro environmental stiffness can influence cell morphology, migration, and proliferation in 2D as well as in 3D models^{8,46}. The effects of ECM stiffness in cancer progression have been confirmed in many cancer types including GBM^{8,46-48}. Wang et al (2014) showed that the morphology, protrusion, invasion, and proliferation of GBM cells cultured in 3D PEG-based hydrogels changed with changes in the stiffness. With increased rigidity, the proliferation rate and the cellular network formation decreased and cells experienced rounder morphologies with shorter and thinner protrusions⁴⁸. Figure 7 demonstrates the differences in cellular morphology when cells are cultured in gels with Young's Moduli of 1 kPa and 26 kPa. Verstreet et al (2014) also studied the effects of material stiffness on U118 and U87R GBM cell lines cultured in gel-like 3D environments. Results

showed that cells proliferated more rapidly in softer substrates and had more stretched and elongated morphologies⁴⁷. Ghibaud et al (2008) showed that migration direction of fibroblastic cells can be determined and regulated with the control of substrate rigidity as fibroblast cells are more likely to move toward the more rigid end in a stiffness gradient⁴⁹. The migration of cells along gradients of stiffness is called durotaxis⁷. This migration is predominantly towards stiffer materials^{7,8}. Furthermore, research on patients has shown that elevated ECM stiffness directly correlates with the aggression of the GBM tumors, demonstrating the clinical relevance of 3D substrate stiffness⁵⁰.

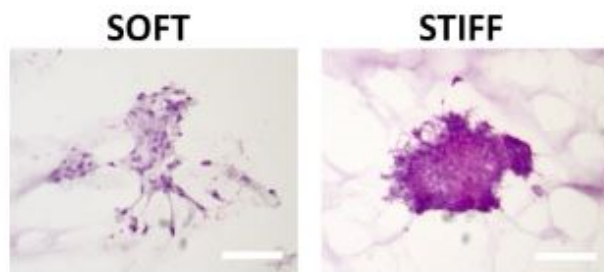


Figure 7: Glioma cells display different morphologies in gels with different rigidity, Left: 1kPa, Right: 26kPa⁴⁸

- **PORE SIZE**

Pore size is another feature of a 3D cell culturing environment that can affect the cell mobility, morphology, and invasion. Research has shown that glioma cells move differently when they are subjected to constraint-free spaces compared to spaces with small pores⁵⁰. Cells actively regulate their mode of migration depending on the optimal migration strategy depending on the substrate and the pore size. Experiments by Heffernan et al (2014) have shown that the pore size affects invasive distance of the glioma cells where larger pores increase invasiveness and small pores hinder it⁴⁷. Furthermore, cell migration is greatly hindered when the pore size is smaller than the cell nuclei⁷. In particular, for gliomas, Yang et al (2010) reported that pore sizes smaller than approximately 6 μ m hindered cell invasion in gels⁵¹.

- **STRUCTURE DIMENSIONALITY**

Some features related to the size of the cell environment, such as the fiber thickness, can affect how a single cell perceives dimensionality⁷. Cells that are in contact with thick fibers can sense their environment as a flat surface, thus experiencing mostly a 2D-like environment. On the other hand, cells in contact with multiple thin fibers will experience more of a 3D-like environment⁷. Cells that experience 2D environments have flatter shapes while the cell morphology in 3D environments depends on the cell type and the cellular environment⁷. For instance, cells when subjected to relatively less dense 3D environments tend to have more protrusions and to have star-like shapes. However, if the 3D environment is dense, as in dense gel substrates, they are more likely to have spherical shapes because they cannot form as many protrusions. Tolde et al (2012) showed that sarcoma cells demonstrated different adhesion and mobility when they were exposed to thin and thick (i.e. 0.3 μ m and 6 μ m) fibers⁵². Yang et al (2010) demonstrated that the invasion rate of glioma cells changed with the diameter of fibers that construct their cellular environments⁵¹. Hence, the perceived dimensionality by the cells can affect their movement mechanisms.

- **SURFACE MACRO AND NANO TOPOGRAPHY**

There is considerable evidence that surface topography or roughness can affect behavior of the cells, particularly their migration^{7,53–55}. Contact-guidance is the process of cells migrating along nano or micro ridges⁷. Beliveau et al (2016) demonstrated that aligned nano topography accelerated migration of GBM tumor cells but decreased cytoskeletal stiffness⁵⁶.

2.3 GLIOBLASTOMA CELL CULTURE MODELS

2.3.1 Primary versus Standard Cell Lines

GBM cell cultures can generally be classified into three models. In the order of increasing complexity, they are standard cell lines, primary cells, and xenografts. The first two can be *in vitro*, whereas the last one, xenografts, are *in vivo* models. Primary cell cultures are derived from living tissues and can be cultured in 2D monolayers or in 3D suspension. Standard cell lines are cells that are passaged (>30) over a longer period of time. Xenografts are cells that are implanted in living animal tissues⁵⁷. Xenografts offer several advantages over the *in vitro* models. First, the fate of cells can be followed over longer time periods. Second, they provide the opportunity to study the cellular behavior and treatment methods in conjunction with other cells and organs. Third, they provide realistic environments very similar to the human brain. On the other hand, they have a number of disadvantages. They are not suitable for high-throughput studies and they raise concerns regarding use of animals. Such considerations are a motivation for future *in vitro* research.

Compared to standard cell lines, both xenografts and primary cells more accurately represent the heterogeneity, proliferation and self-renewal abilities, and the gene profile of the parental tumor⁵⁷. However, GBM standard cell lines such as U-87 and U-252, are commonly used in experiments due to their low cost, ease of use, reproducibility, unlimited sources, relative homogeneity, and unlimited lifespan^{4,32,58}. Standard cell lines have been shown to display behaviors or gene patterns that are not similar to the behaviors of tumors *in vivo*³². With higher number of passages, the gene profile of the cell lines becomes less similar to that of tumors *in vivo*. This indicates the advantage of performing cell culturing and treatment discovery with primary cells. Primary cells generally can better represent the gene expression patterns, invasive nature, and the heterogeneity of the original tumor cells.

2.3.2 2D and 3D *In vitro* Cell Culture Models

Figure 8 shows an overview of recent GBM cell culture procedures.

The *in vitro* cell culture models can be classified based on their dimensionality: 2D, 2.5D, or 3D. Extensive research on the 2D monolayer cell culture models has been published and most drugs and treatment methods have been studied with 2D rigid surfaces⁴. Unfortunately, many of the treatment methods have failed in clinical settings demonstrating a mismatch between these models and *in vivo* environments⁴. With technological advances in micro and nano fabrication, 3D cell culturing has become more feasible and has gained considerable interest⁸. 2.5D models were created to bridge the gap between 2D and 3D models. An example of such a model is an array of micro-fabricated pillars on a flat substrate. Ghibaud et al (2008) used this setup to study the effects of surface rigidity on fibroblastic and epithelial cells⁴⁹.

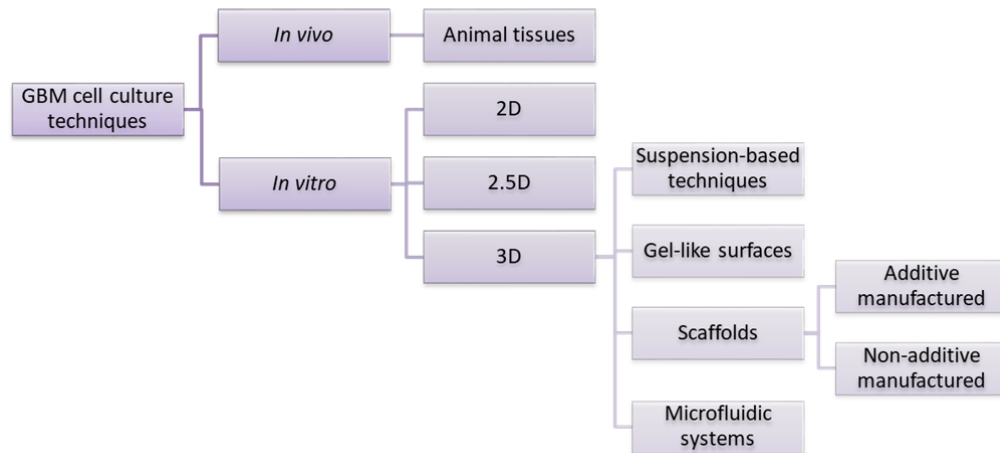


Figure 8: Schematic diagram of the available GBM cell culture models

In recent years, 3D cell culture models have improved considerably. One 3D model involves growing cells in suspension as spheres⁴. Different techniques can be applied to develop these spheres such as hanging-drop or agitation-based techniques³⁷. This model allows for quick results and generation of many replicas⁵⁹. Another 3D model involves growing cells in gel-like substrates. This model more accurately resembles the stiffness of the brain tissue. Heffernan et al (2014) used hydrogels to study the effects of stiffness on proliferation and invasion of U118 and U87R GBM cells⁴⁷.

Scaffold-based cell culturing is another 3D approach. Scaffolds can be additive-manufactured or non-additive-manufactured. Figure 9A depicts a porous non-additive-manufactured scaffold fabricated by a gas foaming technique to study carcinoma cells. Additive-manufactured scaffolds have precise geometries and usually offer more control over the physical features of the cellular micro-environment. Figure 9B shows such a scaffold fabricated by the 2PP method to study neuronal cell growth^{60,61}.

Another 3D platform for culturing cells is a microfluidic system. This model is capable of generating high numbers of capsulated samples from a small tumor⁴. Lee et al (2019) used a microfluidic system to encapsulate GBM cells in a fibrin-based solution⁶². The model response to chemical treatments was more similar to what was seen *in vivo* compared to what was previously achieved in 2D models⁶².

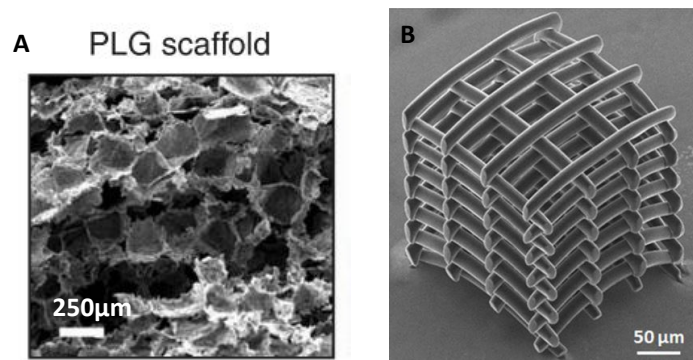


Figure 9: A) Porous 3D polymeric scaffold⁶⁰ B) 3D engineered hydrogel scaffold⁶¹

Nevertheless, research on cells in 3D-engineered environments is still in its early stages⁴⁶. It has been demonstrated that 3D cell culturing can better match the natural environment of the cells and produce results that are more consistent with the behavior of living organisms^{63,64}. Cells in their natural state are in contact with other cells and with the ECM in 3 dimensions⁴. Another difference between cells in 2D and 3D experiments is related to their respective morphologies. It has been reported that cells in 2D surfaces usually have a more flat and stretched morphology compared to those *in vivo* and in 3D experimental settings⁶³. This difference in morphology has also been reported in GBM (Figure 10)⁶⁴. Gomez-Romen et al (2015) also demonstrated that treatment responses to three different molecular targeted agents (temozolomide, bevacizumab, and erlotinib) in 3D GBM cell culture models better matched clinical results compared to 2D cell culture experiments⁶⁴. Furthermore, cells in 3D cell culture environments can be in various viable stages. For instance, it has been reported that in 3D spheroids, the outer cells are more proliferating while the inner cells are more in quiescent and hypoxic states. In contrast, cells in 2D surfaces are mostly proliferative⁶³. This difference can be due to the higher imbalance in distribution of the oxygen and nutrients in 3D cell cultures, as cells in the inner parts of the structures may have less access to oxygen and nutrients⁶³.

Another reported difference between the 2D and 3D cell cultures is the cell proliferation rate. While for some cell lines, lower proliferation rates in 3D have been reported⁶³, for other cell lines including gliomas, higher rates have been observed^{63,65}. Cells can also display different gene expression profiles in 2D and 3D experiments⁶³. While 3D cell cultures have the advantage of better mimicking the natural cell environment and producing more realistic results for drug discovery tests, they are more expensive, slower to produce, and more difficult to use in high-throughput settings^{4,46,63}.

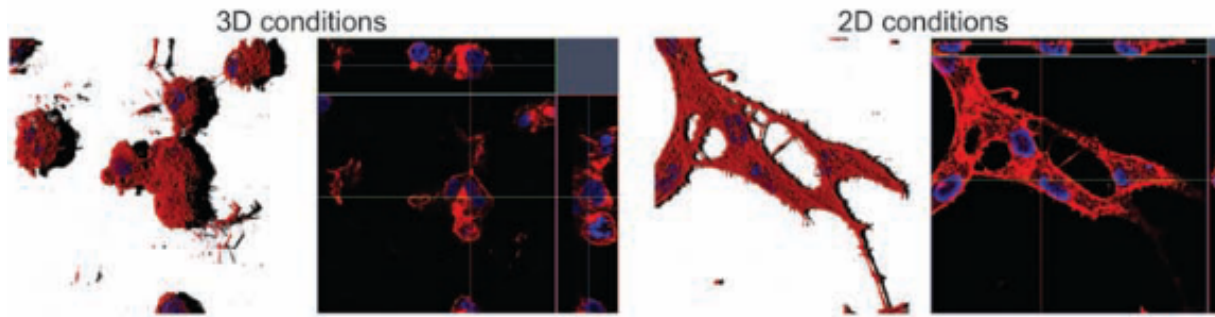


Figure 10: Glioblastoma G7 cells show different morphologies in 2D models vs. 3D porous non-additive manufactured scaffolds, red: actin filaments, blue: nuclei⁶⁴

2.4 2 PHOTON POLYMERIZATION (2PP) METHOD

2PP is a relatively recent light-assisted fabrication technique that offers a number of advantages in a variety of fields such as biology, optics, and micro-electro-mechanical systems. In direct-writing techniques, such as in the 2PP technique, the material is precisely transferred or modified in the desired voxels (volumetric grids in the 3D space) instead of depending on fabrication steps such as etching or using masks^{61,66}. The advantages of the 2PP technique include: high resolution (up to 100 nm), high reproducibility, precise and intricate control of structural features, and more freedom in design^{61,67–70}. However, this process is relatively slow and expensive. Also, because of its photolithographic nature,

the employed materials may contain cytotoxic byproducts^{68,70}. Another shortcoming is the limited photoresist options that resemble the kilopascal Young's modulus of ECM of the brain⁷⁰.

The 2PP technique is based on the two-photon absorption nonlinear phenomenon^{66,68}. This technique operates based on the interaction of a femtosecond-pulsed laser light with a polymeric photo-sensitive resin⁶⁷. In this process, two photons are simultaneously absorbed by the photo-sensitive resin⁸. In single photon absorption, upon illumination of the laser light to a photo-sensitive material, a photon will be absorbed if its energy level is higher or equal to the band gap between the excited electronic state and the ground state. In two-photon absorption, two photons are simultaneously absorbed provided that their collective energy level is higher than the band gap and that both photons are present at the same space at the same time⁷¹. This difference results in higher resolution and controllability for the 2PP method compared to other 3D light-assisted lithography techniques (Figure 11A).

In general, photosensitive resins that are used in 2PP consist of monomers, oligomers, solvents, and photo-initiators⁶⁸. Photo-initiators are the components added to the resin to make it cross-linkable and sensitive to light. Materials that become insoluble after laser exposure are termed negative photoresists⁸. In the two-photon absorption process, resin is only polymerized in the desired voxel and the rest of the resin is unaffected by the laser beam⁸. In this case, after laser exposure, the unpolymerized resin is rinsed away⁸. The writing occurs with the relative movement of the laser objective with respect to the stage that holds the resin⁸. This movement can be controlled with galvo mirrors and/or piezoelectric adjusters. Figure 11B shows a schematic of a 2PP device.

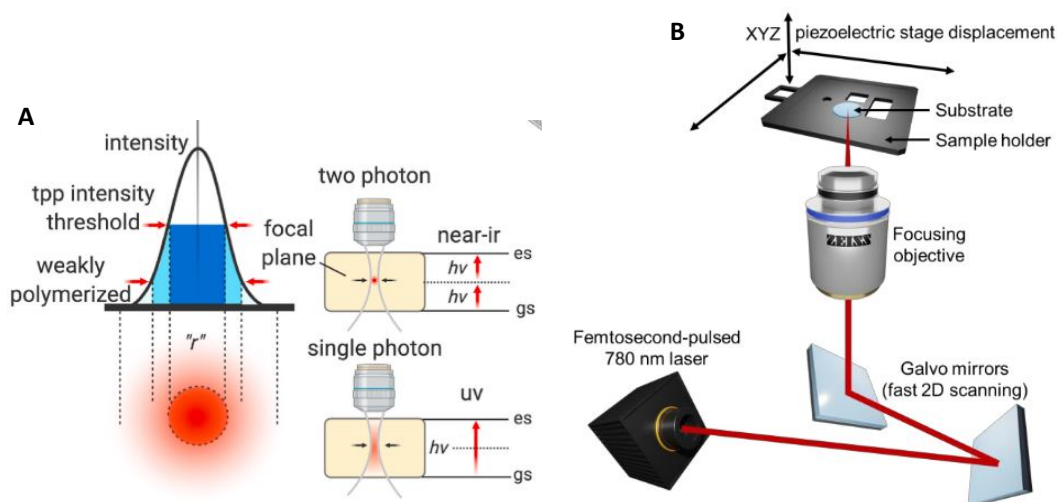


Figure 11: A) Light intensity distribution in the 2PP method⁶⁷ B) Schematic of the 2PP printer setup⁷²

Over the years, a wide range of materials for biological applications has become available for use in the 2PP technique⁵⁴. These materials can be categorized and utilized based on their different properties such as biocompatibility, rigidity, adhesiveness, fluorescent properties, bioactive properties, biodegradability, and porosity^{53,54}. They are either synthetically developed or are derived from nature⁵³. Synthetic materials are easy to use, they are reproducible, and they have tunable features. However, they are less similar to biological tissues⁵³. One such well-established and biocompatible synthetic material is poly (ethylene glycol) diacrylate (PEGDA)⁵³. On the other hand, naturally derived materials

are better in mimicking biological features and are more biocompatible. However, they have more batch-to-batch heterogeneity⁵³. Some examples of naturally-derived materials are collagen, hyaluronic acid, and chitosan^{53,67}.

2.4.1 Examples of 2PP in Cell Culture

The 2PP fabrication method offers the possibility of creating precise micro and nano scale structures with a variety of features for biological applications. Accardo et al (2018), using this technique, fabricated 3D PEGDA hydrogel woodpile scaffolds with micrometer feature sizes that were used for cell culturing and for studying neuronal cells *in vitro*⁶¹. Timashev et al (2016) also used the 2PP technique to culture patient-derived neural cells with a focus on demonstrating the role of porosity in cell culture and for studying biocompatibility of Zr-Si hybrid polymers⁷³. Worthington et al (2019) studied the effects of different nano-topographical patterns fabricated by the 2PP method on human-induced pluripotent stem cells⁵⁵. Tayalia et al (2008) used the 2PP fabrication method to precisely control the feature sizes of their woodpile structures and studied the effects of porosity of the cellular micro-environment on migration of fibrosarcoma cells. They showed that a decrease in pore size reduces average cell speed⁷⁴. Tricinci et al (2020) created a 3D model for the BBB and GBM tumors to study the effectiveness of drug delivery through the BBB *in vitro*⁷⁵. They were able to grow GBM cells on a 3D scaffold that was designed to foster the formation of a spherical tumor and to move it magnetically to the vicinity of the BBB⁷⁵.

Overall, the 2PP fabrication method presents the possibility of studying cells and tissues by creating precise 3D structures in the micro/nano range in a manner that has not been possible before. Physical features of the cellular micro-environment such as pore size, stiffness, surface topography and roughness, and feature sizes, can be controlled with the 2PP method. This technological advancement continues to offer new insights on cell-to-cell and cell-to-environment interactions.

3 RESEARCH DESIGN

3.1 PROBLEM STATEMENT

The review of literature demonstrates that despite extensive research, the GBM treatment options identified in *in vitro* studies do not translate well to the clinical settings⁴. One of the main reasons is that the current GBM cell culturing methods do not provide realistic models of the GBM behavior *in vivo*⁴. Therefore, more physiologic models for cell behaviors and their responses to drug therapies are needed.

Two of the main shortcomings of the current methods are, first, the inability of cells to maintain the amplified *EGFR* expressions and, second, the failure of cells to form microtubes^{5,27}. So far, microtube-like protrusions have been reported only once in *in vitro* 3D culture environments³⁵. However the observed protrusions were not expressed with the same frequency and morphology as those seen *in vivo*. In the context of *EGFR* targeting treatment methods, using *EGFR* inhibitors has been successful in clinical settings for other cancer types such as for lung cancer. However, this approach has failed to be effective for GBM tumors despite successful *in vitro* experiments. Culture models that can capture the natural *EGFR* expressions and their reaction to *EGFR* inhibitors are needed.

Moreover, *in vitro* GBM research has primarily used standard cell lines. To more accurately represent the gene profiles of the original tissue, it is important to establish a method that can culture both primary cell cultures as well as standard cell lines and to include multiple primary cultures in GBM research experiments.

Predominantly, *in vitro* cell culture research involves the use of 2D substrates, despite increasing evidence that cells behave differently in 3D environments^{7,47}. There is a need for novel 3D cell culturing methods that can overcome these limitations. The 2PP micro-fabrication technique can be used for this purpose for its high resolution, accuracy, and reproducibility⁵³.

In one experiment, a 3D scaffold fabricated with the 2PP technique was used for GBM culturing to assess the effectiveness of a BBB model⁷⁵. However, these experiments were carried out with a standard GBM cell line. Although, information on cell morphology, gene expression, *EGFR* expression persistence, or microtube formation was not provided, they nevertheless demonstrated that viable GBM cells can be cultured on such 3D engineered structures and are worthy of further research⁷⁵.

3.2 RESEARCH QUESTION

The goal of this project is to determine if a 3D-engineered scaffold fabricated by the 2PP technique can provide an accurate model system to study GBM cells.

The main research question is:

Can a 2PP 3D-engineered scaffold enable a primary GBM cell culture model that mimics *in-vivo* GBM morphology, maintenance of amplified *EGFR* expressions, and formation of tumor microtubes?

To answer this research question, the following sub-questions will be addressed:

- Can a standard GBM cell line colonize the 3D-engineered scaffolds?
- Can primary GBM cells colonize the 3D-engineered scaffolds?
- Are there any differences in the morphology of cells cultured on 3D scaffolds compared to those on 2D control models? If so, which one resembles the *in vivo* morphology more accurately?
- Can microtubes form in 3D or 2D structures?
- Are there any differences in microtube formation of the cells cultured on 3D scaffolds compared to those on 2D control models?
- Can *EGFR* expressions be maintained and imaged on these structures?
- Are there any differences in the *EGFR* expression of the cells cultured on 3D scaffolds compared to those on 2D control models?
- Are there any differences to the reaction of GBM cells to *EGFR* inhibitors in 3D or 2D models?

3.3 APPROACH

Figure 12 shows a flow chart of the workflow for this research.

A 3D-engineered scaffold and a flat plate for 2D control experiments were designed and fabricated with the 2PP technique. Next, the quality of these structures was evaluated with scanning electron microscopy (SEM). In case of any defects, new scaffolds and flat surfaces were manufactured in which geometrical features, printing parameters, and pre/post-treatments were modified. This process was repeated until acceptable results in terms of size accuracy, stability, and surface quality were achieved. Once the fabrication results were satisfactory, cell culturing was performed. Next, the cell colonization was assessed. The assessment firstly was done by regular brightfield microscopy and then with fixed confocal fluorescent cell imaging. In case of unsatisfactory cell colonization of the structures, the problem was addressed by either repeating the cell culturing, changing culturing protocols, or by changing the scaffold design and fabrication. Cell cultures started with a standard cell line i.e. U-87, and after confirming successful colonization of this cell line on the 3D scaffolds and the 2D plates, three primary GBM cultures were seeded on the samples. Next, the shape and network formation of the cells were studied by SEM. After confirming the cell colonization, cellular morphology, *EGFR* expression and microtube formation were investigated by fixed fluorescent cell imaging. Fixed cell imaging was repeated a number of times to confirm consistency. The fluorescence images/stacks were then analyzed and quantified with FIJI ImageJ software. The statistical analysis of the quantified data was performed via Microsoft Excel and SPSS software.

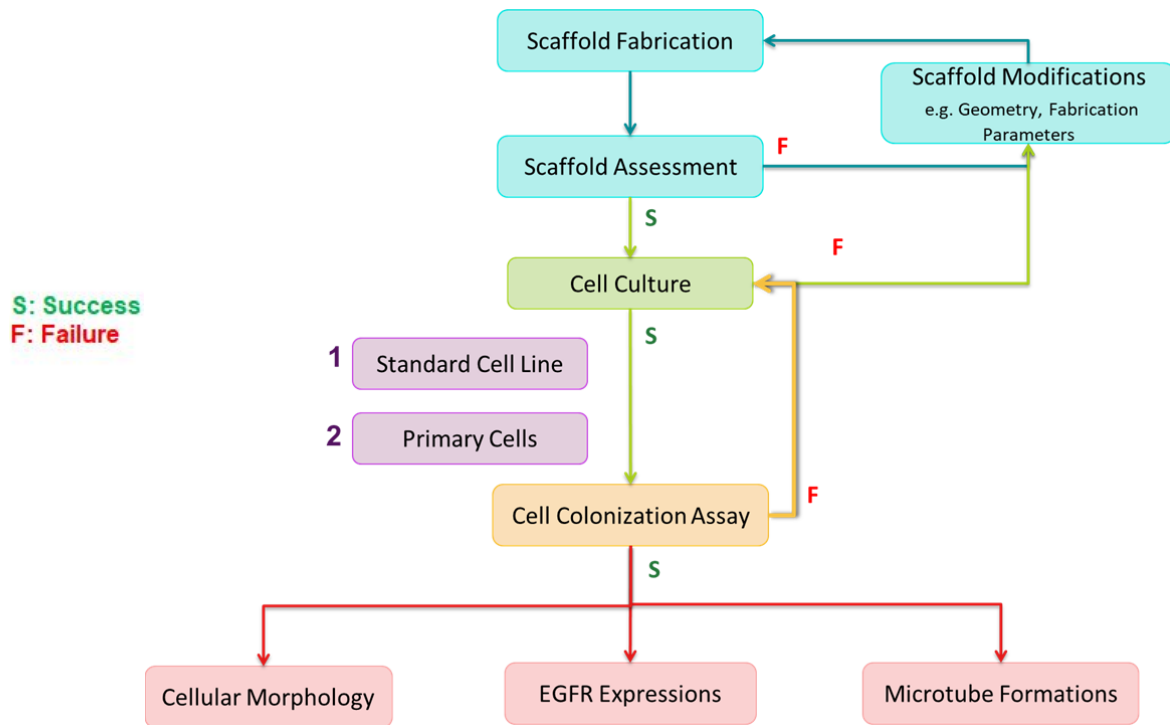


Figure 12: Flow chart of the research steps

4 METHODS

4.1 SCAFFOLD DESIGN CONSIDERATIONS

The first goal of the 3D scaffold design was to mimic the geometry of the natural GBM environment. The structure of the scaffolds was inspired by the characteristics of the blood vessels present in the GBM environment. Moreover, a porous structure was needed to allow cell migration into the structures and to allow for the flow of nutrients. The pore size had to be larger than the cell nuclei to allow for cell invasion and small enough to allow for cellular network formations⁵¹.

Another design criterion was the stability of the scaffolds. Overhanging structures were avoided to provide stability and ease of production. To avoid shadowing in the bottom and middle layers of the scaffolds during microscopy, highly dense structures were not considered. Surface patterning was not performed on the structures as its effect is beyond the scope of this project. The final scaffold design and modifications to the initial design will be discussed in the section 5.

4.2 SCAFFOLD FABRICATION

The 3D-engineered scaffolds and the 2D flat plates were fabricated by the 2PP method. For fabrication, the Nanoscribe Photonic Professional GT2 printer, which is available at TU Delft, was used.

4.2.1 Printer Set-Up

Nanoscribe printer allows for 3D and 2D direct laser writing in the macro and nano scale based on the 2PP technique. In the Nanoscribe printer set up, the photosensitive resin is placed on a substrate.

Two configurations (Figure 13) are used: conventional direct laser writing (DLW), also called oil immersion, and dip-in laser lithography (DiLL)⁷⁶.

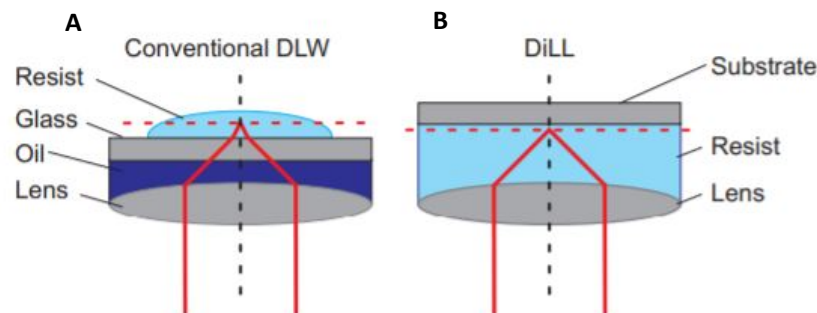


Figure 13 Schematic illustration of the writing configurations, A) conventional DLW configuration, B) DiLL configuration⁷⁷

In conventional DLW configuration, the laser objective is immersed in oil and the laser beam goes through first the immersion oil, next the substrate, and then reaches the photoresist resin. In the DiLL configuration, the laser objective is directly dipped in the photoresist resin. In this configuration, the writing starts at the interface between the substrate and the resin. The system automatically finds this interface based on the difference between the refractive indexes of the substrate and the resin. Therefore, when choosing the substrate and the resin, it is important to keep this difference large enough to be detectable by the printer.

4.2.2 Objective, Material, Substrate and Configuration Choices

Nanoscribe printer offers four objectives: 63x, 25x, 20x, and 10x. The resolution, the available writing field, and the writing speed vary with the objective. Because the scaffolds for this project are in the mesoscale, the 25x objective is the most suitable choice. Printing with 63x will increase the resolution but will also increase the printing time. Therefore, the 25x objective was chosen as the best compromise between the resolution and the printing time.

Three criteria were considered in choosing the suitable resin material. First, the material must be biocompatible to be suitable for cell studies. Second, the material should have minimal autofluorescence properties to minimize background noise during fluorescent microscopy. Third, the resin material used with the Nanoscribe machine should be able to create structures with features at least as small as 2 μ m. In addition, to save time and to stay within the scope of this project, only readily commercially available resins were considered.

IP-Visio, a newly developed resin by the Nanoscribe group for life sciences, meets all of the requirements⁷⁸. IP-Visio resin is classified as non-cytotoxic and its fluorescence is low making it ideal for fluorescent microscopy⁷⁹. IP-Visio in combination with indium-tin oxide (ITO) coated glass and the 25x objective with DiLL configuration creates a 3D micro-fabrication solution set for medium features⁷⁹. ITO-coated substrates are available in 25mm x 25mm x 0.7mm. According to the Nanoguide, this set can create structures with theoretical XY resolution of 0.6 μ m in writing fields of 285 μ m x 285 μ m (400 ϕ). Voxel aspect ratio of this set is 6, which means the voxel size in the Z direction could be 6 times larger than in the other two directions. These dimensional limitations are acceptable for the scaffolds.

IP-Visio is a negative tone resin. Because this resin is developed only recently, there are not many studies that report on its properties such as shrinkage, compatibility with alcohol, Young's modulus, and adhesion to substrates. Therefore, the effects of these properties could not be estimated prior to the fabrication.

4.2.3 Fabrication Process

The fabrication process consists of the following steps: Scaffold design, substrate preparation, printing, sample development, post-treatments, and quality control (Figure 14).

The scaffolds and the plates were designed and then modeled with SolidWorks. The geometrical features of the structures were defined at this step. Standard Triangle Language (STL) file was created and imported to the DeScribe software. DeScribe is a 3D printing job preparation software where the printing parameters are defined. The resulting General Writing Language (GWL) file was imported directly from DeScribe to the Nanowrite (Nanoscribe printing software).

To start the printing, the ITO-coated substrates, on which the structures were printed, went through a few preparation steps. The main purpose of these steps was to increase the adhesion of the structures to the substrates. First, the substrates were cleaned by acetone and ISO-Propanol (IPA). Next, the ITO-coated side of the substrates was exposed to oxygen plasma for further cleaning and it was exposed to a silanization solution. Silanization makes surfaces more hydrophobic and creates strong bonds between the IP resins and substrates⁸⁰. This increase in hydrophobicity may lower cell adhesion to the

glass. Therefore, the cells on glass part of the samples were not compared to cells on the printed scaffolds and plates. Silanization solution consisted of ethanol and 3-(trimethoxysilyl) propyl methacrylate (MAPTMS) as suggested by Nanoguide (Nanoscribe's user manual and supporting material). The sample exposure time and the concentration of the solution were optimized to be 1hr and 30ml of ethanol with 600µl of MAPTMS. The initial concentration of MAPTMS suggested by Nanoguide was 150µl in 30ml of ethanol (0.5%). However, this concentration failed to provide enough adhesion between the substrates and the structures. Liu et al (2018) reported on using 2% MAPTMS-ethanol concentration on ITO substrates⁸¹. This concentration provided much better adhesion for the structures and the substrates used in our research. After silanization, the samples were rinsed first with acetone and then with water. They were air-dried for approximately 5 minutes. Immediately after drying, the resin was placed on the substrates and samples were placed in the Nanoscribe printer for fabrication.

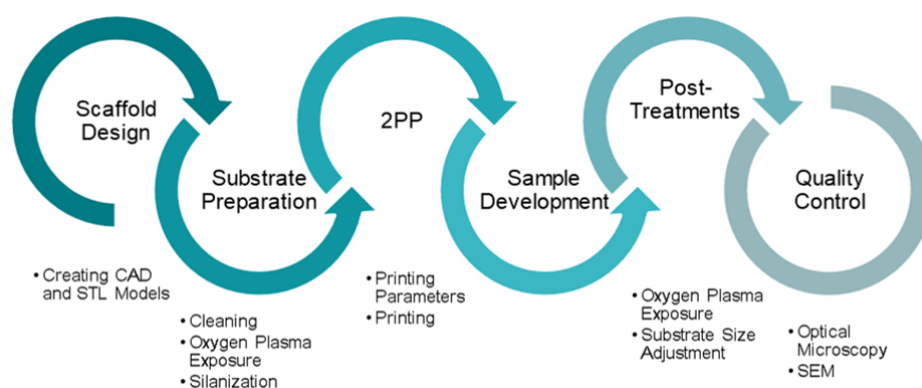


Figure 14: Workflow of the fabrication process

The optimization of the printing parameters is further discussed in section 5.1.3. After fabrication, to remove the un-polymerized resin, samples were placed in propylene glycol methyl ether acetate (PGMEA) for 25 minutes. Next, they were immediately placed in an IPA solution for 5 minutes. Later, samples were left in a fume hood to air-dry.

To fit the samples in 6-wells cell culture plates (needed for culturing and staining steps), the corners of the ITO-coated glass were cut off. This was done by creating diagonal scratches with a diamond pen cutter. To increase cell adhesion to the scaffolds and to the plates, the samples were placed again in the plasma cleaner and were exposed to oxygen plasma. This step makes the surface of the structures rougher. Appendix11.1 describes the fabrication protocol in more detail.

For quick analysis of the fabrication quality and to discard faulty samples, optical microscopy with 10x magnification was performed. Later, the fabrication quality was checked more extensively with SEM.

4.3 GLIOBLASTOMA CELL CULTURE

Cell cultures were performed at the EMC Neuro-Oncology Laboratory by Ms. Iris de Heer.

Cell cultures started with a commonly used standard human GBM cell line U-87 after which three primary GBM cell cultures, GS-830, GS-580, and GS-827, were tested. GS-830 is a primary GBM (grade IV) with wild type IDH. GS-580 and GS-827 are secondary GBMs with IDH mutations.

Prior to cell seeding, structures were coated with Cultrex 10X Spheroid Formation ECM (Trevigen, Catalog Number: 3500-096-01). Once cells reached 80% confluency in T75 flasks, they were collected in cell suspension of 10ml. Out of that volume 0.9ml was plated out in 6 wells plates. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)-based media in the presence of growth factors such as basic fibroblast growth factor and EGF. The cell culture protocols and instructions to apply the coating are available in Appendix 11.2.

4.4 IMAGING

The fabrication quality for the structures and the attachment of the cells to these structures were checked with SEM. Later, the cellular morphology and expression of proteins were tested by confocal fluorescent imaging. The cells were fixed 3 days after seeding on samples.

4.4.1 Scanning Electron Microscopy

A challenge with imaging non-conductive materials with SEM is that charge can build up in the samples. Consequently, the images will become distorted⁸². The traditional approach to fixing this issue is to coat the samples with a conductive layer⁸³. To avoid charge build-up in the samples, the samples were sputter-coated with a gold nano-layer. Sputter Coater JEOL JFC-1300 machine available in TU Delft was used for this purpose. All samples were coated once horizontally and two times in opposing 45° tilted configurations. Coating the samples in a tilted configuration ensured proper coating of the inner layers of the scaffolds. In the horizontal configuration, the samples were sputter-coated using a 20mA current for 20s adding approximately a 13nm thick gold layer. The duration was reduced to 10s for each tilted configuration adding approximately a 6.5nm thick gold layer.

For SEM imaging, Jeol JSM-6010LA scanning electron microscope available at TU Delft was used. Secondary electron images that demonstrated the topography of the surfaces were recorded. In-Touch Scope software was used to interact with the microscope and to tune the settings. For the recorded images, *accelerating voltage* ranged from 10kV to 20kV and the *spot size* (diameter of the electron beam) ranged from 50 nm down to 30 nm. Generally, smaller *spot sizes* and larger *acceleration voltages* resulted in higher resolution images. Tilted sample holders were used to image the structures from different angles.

For SEM imaging of cells seeded on these structures, the cells had to be fixed and dehydrated. After this process, the cells were ready for gold sputtering. The cell fixation protocol and the dehydration scheme are available in Appendix 11.3.1.

4.4.2 Fixed Confocal Imaging

To image the cell nuclei, DAPI staining was used. To visualize the cell cytoskeleton, anti-Beta-Tubulin staining (Rabbit Host, Abcam ab6046) in combination with a secondary antibody Alexa Fluor 488 (Life Technologies A11008, anti-rabbit) was applied. To image the GAP-43 proteins in microtubes, anti-GAP-43 staining (Rabbit host, Cell Signaling Company 8945) combined with Alexa Fluor 488 was used. *EGFR* proteins were stained with one of two anti-*EGFR* primary antibodies: DAKO with a mouse host (M3563) or Abcam with a rabbit host (ab76153). Secondary antibody Alexa Fluor 647 (Life Technologies A21240,

anti-mouse) was used with DAKO and Alexa Flour 488 was used with Abcam. The anti-EGFR made by Abcam provided stronger signals with less bleed-through and therefore was preferred. The detailed staining protocol is available in Appendix 11.3.2.

Two microscopes were used for the fixed cell imaging: water dipping microscope (Leica SP5 Intravital), and oil immersion upright microscope (Leica Stellaris 5 LIA).

The sample mounting configuration for a water dipping microscope is shown in Figure 15. Only an objective with 20x magnification (with numerical aperture of 1.00) was available for this set-up. Working distance of 1.95mm allowed for complete imaging of the scaffolds. 405, 488, and 633 nm laser light sources were used. For more detailed cell analysis and to obtain higher resolution images of EGFR proteins, this microscope was later replaced with Leica Stellaris 5 LIA.

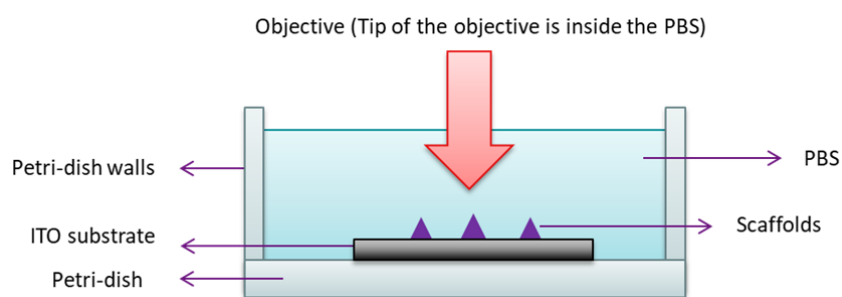


Figure 15: Set-up scheme for Leica SP5 Intravital microscope. The dimensions are not proportional

In the Leica Stellaris 5 LIA microscope, the tip of the objective was dipped in oil that was placed on a cover-glass on top of the scaffolds. In order to prevent damaging the scaffolds and to avoid bubble formations, two 120 μ m thick spacers (Imaging Spacers by Grace Bio-Labs, GBL654002) were used (Figure 16).

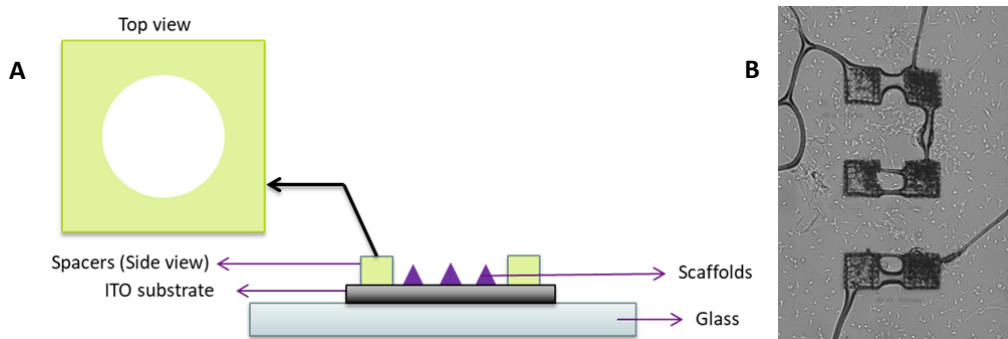


Figure 16: A) Schematics of the spacers B) Bubble formation without the use of spacers in Leica Stellaris 5 LIA microscope

In some cases, spacer delamination was observed. This was prevented by ~10 minutes of air-drying before placing the spacers. However, excessive drying time increased the background noise in the images. After completing the staining, a cover-glass was placed on the samples. To prevent movement of the cover-glass during microscopy, clear nail polish was applied between the spacers and the cover glass. Figure 17 demonstrates a schematic of this set-up.

With Leica Stellaris 5 LIA microscope, a 40x magnification was used. Four laser light sources were available with wavelengths of 405, 488, 561, and 633 nm. To avoid crosstalk between the channels, the

sequential detection mode was chosen. *Light intensity*, *gain*, and *offset* values were optimized during microscopy and were kept constant for each sample. It was attempted to keep these values constant between related experiments as much as possible. The microscope parameters for all images were 600Hz *speed*, *line average* of 3, and 1024x1024 *frame* (or 2048x2048 for some tests). For Z-stacks, 1 μ m step size was used.

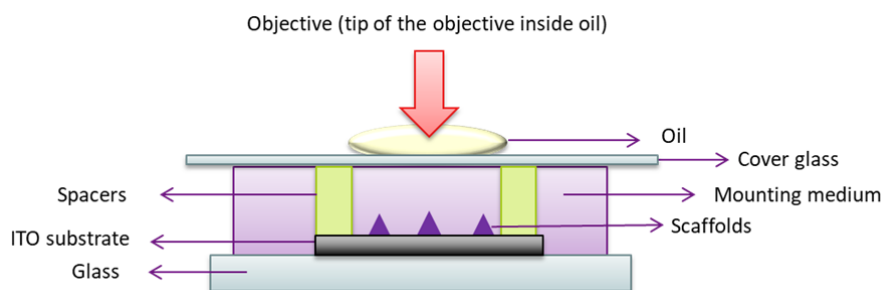


Figure 17: Set-up scheme for Leica Stellaris 5 LIA Microscope

4.5 IMAGE AND DATA ANALYSIS

Confocal images of all 4 cell cultures i.e. U-87, GS-580, GS-827, and GS-830, were recorded. All samples were seeded on the same day, stained on the same day, and imaged between consecutive days. The analysis was done based on creating the Z projection of stacks of image slices.

Images were analyzed using Fiji ImageJ software. Macro codes (programming language for ImageJ software) were developed to extract and quantify the features of interest from each image. A combination of ImageJ commands, built-in functions, and plug-ins were used in each macro code. The codes can be found in the Appendix.

The quantified data were analyzed with Excel and with the SPSS software. Bar graphs represent the mean values and the 95% confidence intervals (mean $\pm$ 2 x standard error). Statistical analysis consisted of one-way analysis of variance (ANOVA) using the SPSS software. Statistical significance was set at $p < 0.05$. In the bar graphs, statistically significant findings are marked by asterisks (*).

4.5.1 Cell colonization

To count the cells, Stardist plug-in (an automatic nuclei detection and segmentation method) from the ImageJ software was used. All settings in analyzing these images, including probability and overlap thresholds, were kept the same. This plug-in localizes nuclei with star-convex polygons resulting in minimized segmentation errors in comparison to earlier and simpler methods⁸⁴.

4.5.2 Nuclei Morphology

Cell nuclei were analyzed using a macro code in ImageJ software. The settings were the same for all images. For nuclei segmentation, Stardist plug-in was used. Before applying nuclei segmentation, all images were equally processed with *Subtract Background Noise* and *Enhance Contrast* commands. After the segmentation and identification of cell nuclei regions, original images (without any pre or post processes) were quantified for the nucleus area, the mean grey value, and the circularity. The code is shown in Appendix 11.4.

4.5.3 Cell Morphology

A macro code was written for cell detection and analysis using both tubulin and nuclei data. Several built-in cell segmentation plug-ins were tested to detect and individualize single cells. However, these plug-ins failed to provide reasonable estimates of individual cell boundaries. Thus, a combination of ImageJ commands and functions were used to build a suitable cell segmentation program.

After contrast enhancements on the nuclei channel, the nuclei and tubulin channels were merged. The *find maxima* function (with the output of *segmented particles*) was performed on the merged image. This step located an estimated boundary for the area that each individual cell occupies (Figure 18B). Next, a copy of the merged nuclei and tubulin image was used to create a binary mask that shows all cells in black and the rest of the surrounding area in white. With the *image calculator AND* function, the binary image and the segmented image were combined. The resulting image shows each cell in black and is separated from the neighboring cells with the established boundaries from the segmentation step (Figure 18C). Finally, the *analyze particle* function was performed on the combined image and the individual cell properties, such as area, circularity, and Feret's diameter, were displayed. Figure 18 shows the results of executing this code. The written macro code is in Appendix 11.5.

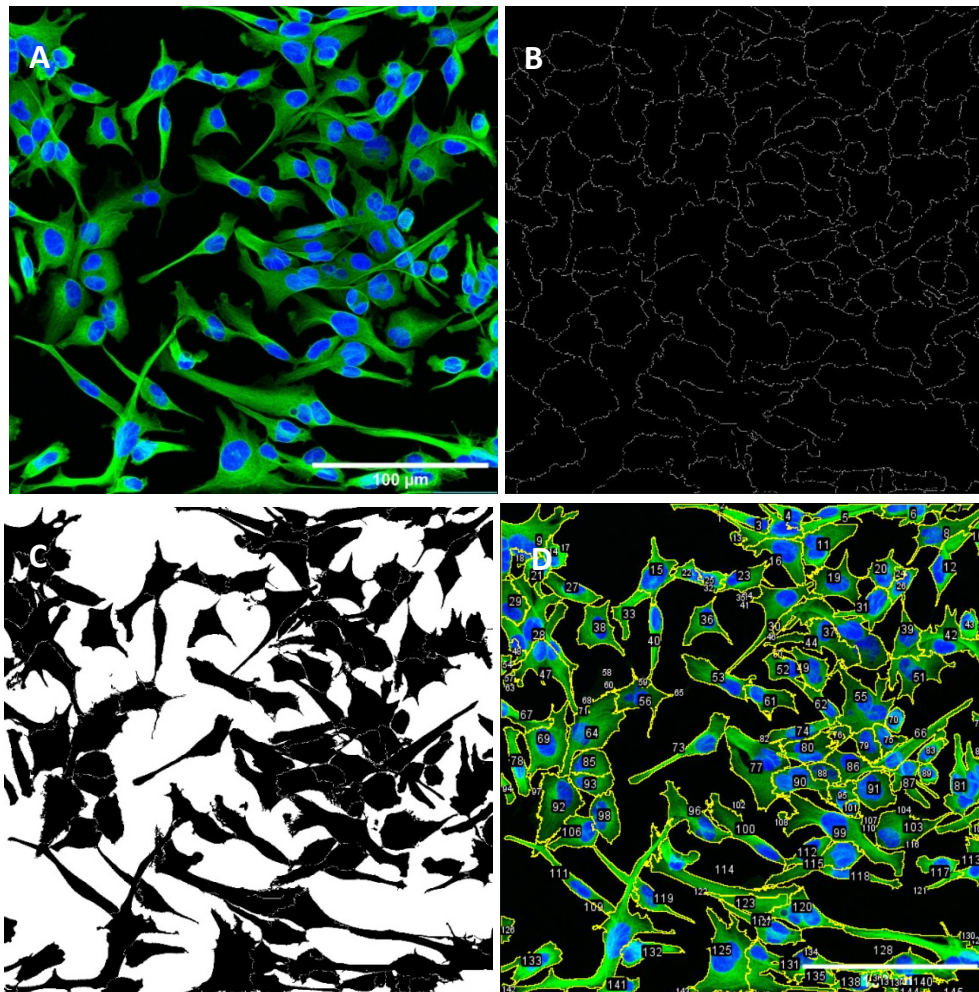


Figure 18: Steps of the cell segmentation code. A) An original image example B) Estimated boundary for individual cells C) Binary mask of the segmented cells D) Segmented cells

4.5.4 EGFR Spots

To study *EGFR* expression, a macro program in ImageJ was written. First the background noise was subtracted from the *EGFR* signal. In this step, most of the signal emitted from the scaffold material was removed from the images. Next, it was attempted to separate the *EGFR* regions that had a higher threshold from the rest of the image. For this, image was subjected to *Gaussian blur*, followed by *Set Threshold* command resulting in an image consisting of only the higher threshold areas of the *EGFR* signal (Figure 19B). Next, using the *Find Maxima* (with the output of *list*), the locations of the points with highest signal intensity were identified. These points were assumed to be the center of *EGFR* spots. Afterwards, a circle in the size of a single *EGFR* spot was drawn based on the achieved center locations (Figure 19C). A selection group was created and the circles were added to the selection group. Average *EGFR* spot size was estimated from the recorded images. *EGFR* spot counts as well as the quantified mean grey value of the individualized spots were determined using the *measure* command on the selected *EGFR* circles. Figure 19 displays the results of executing this code. The written macro code is in Appendix 11.6.

Later, the nuclei detection code was used to count the number of cells in order to normalize *EGFR* expression per cell. To distinguish the *EGFR* spots that are located near the nuclei, a binary mask of the nuclei was created where the nuclei were black and everything else was white. Next, the circles created to define *EGFR* area were superimposed on the binary image (Figure 19D). The mean grey values of the *EGFR* circles were recorded. If the value represented white, it meant that the *EGFR* spot was not near the nuclei. Any other value meant that the *EGFR* spot is close to the nuclei. Due to the noise variations between images, some settings were manually adapted as needed. In order to avoid bias and subjective manipulation of the images, all images were analyzed blindly.

4.5.1 Microtubes

Multiple ImageJ plug-ins, including ridge-detection, were tested to find a suitable method for quantifying microtube-like protrusions. Due to the considerable overlap of the cells and the inability of the plug-ins to differentiate tube-like protrusion from other tubulin signals, it was decided to perform this analysis manually. 380µm x 380µm images of the cells on 2D plates and 3D scaffolds were analyzed blindly. All tube-like protrusions were marked in ImageJ in all samples and were quantified for length and frequency. The images were analyzed in 2 dimensions. Therefore the underestimation of the length of the protrusions in the 3D scaffolds is expected.

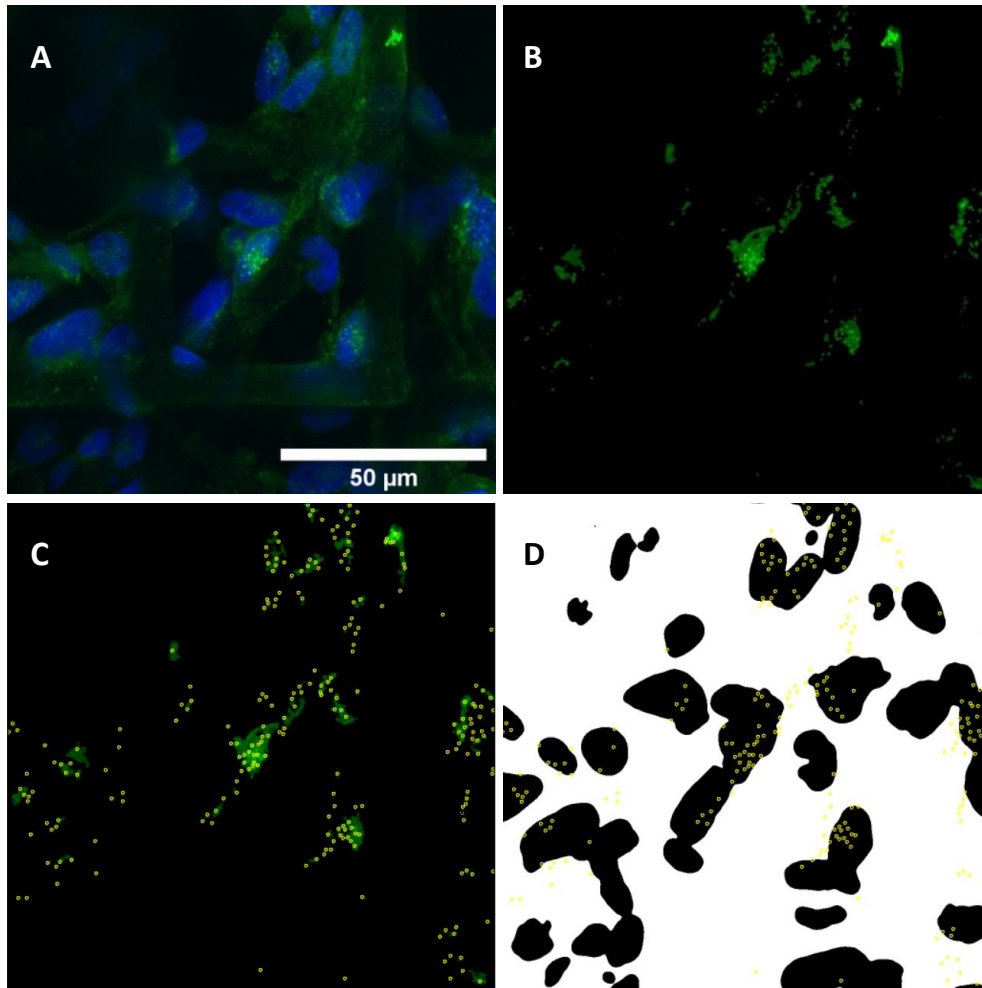


Figure 19: Steps of the EGFR quantification code Blue: nuclei Green: EGFR. A) Example of a of an image B) Higher threshold areas of the EGFR signal C) Circles drawn around the EGFR signals D) Yellow circles show the EGFR spots and black is the cell nuclei

5 EXPERIMENTAL RESULTS

5.1 SCAFFOLD DESIGN AND FABRICATION

5.1.1 Overview of the Scaffold Design

To achieve the design requirements, the geometry shown in Figure 20A was selected for the 3D scaffolds. This structure resembles the brain blood vessels obtained by confocal microscopy *in vivo*, as shown in Figure 20B⁵.

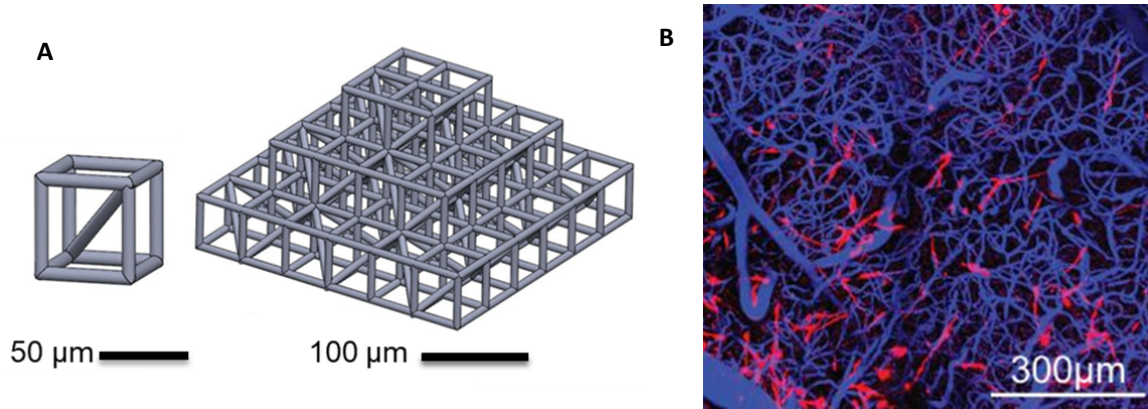


Figure 20: A) The geometry of 3D-engineered scaffolds B) In vivo image of GBM cells, Blue: blood vessels Red: GBM cells⁵

The base element of the scaffolds consisted of rods connected as cubic unit cells with additional interconnecting diagonal rods. Cylindrical rods were chosen as the building blocks of the unit cells to resemble the circular cross-section of the blood vessels. Diagonal rods were added to provide angles in the structure that resemble more closely those observed in blood vessels. Each scaffold consisted of multiple unit cells arranged to form a large pyramid. The pyramid arrangement was chosen to decrease density in the Z direction.

Three copies of the scaffold as well as 4 copies of 2D flat plates were printed on each ITO-coated substrate creating one sample set.

5.1.2 Geometrical Parameters

Two main complications were encountered during the printing process. First was the blockage of scaffold pores. This blockage was more prominent in the inner units and was occurring from top to bottom (Figure 21A). To address this issue, geometrical dimensions and printing parameters were changed. The first geometrical adjustment was to round the corners of the scaffolds (Figure 21B). Live camera images during the printing process showed that the printer was intentionally blocking certain pores. According to the Nanoguide, the DeScribe software attempts to repair surfaces with assumed defects. The software considers surfaces with gaps or sharp edges as defective surfaces⁸⁵. The rounded corners fixed this automatic surface modification issue.

Initially rod size of 5 μm and pore size of 30 μm x 30 μm were selected for the scaffolds. The pore size was increased to 50 μm x 50 μm and the rod diameter to 10 μm to reduce blockage of the pores. The

subsequent cell culture experiments showed that these new dimensions better suited for the GBM cell dimensions. The third geometrical adjustment was to change the cross-section of the top rods of the unit cells from circular to oval. As can be seen in Figure 21C, this change made the rods narrower in the Z direction. This modification compensated for the larger voxel size in the Z direction compared to those in the X and Y directions. These geometrical adjustments in combination with optimizing printing parameters (discussed in section 5.1.3) resolved the problem of pore blockage.

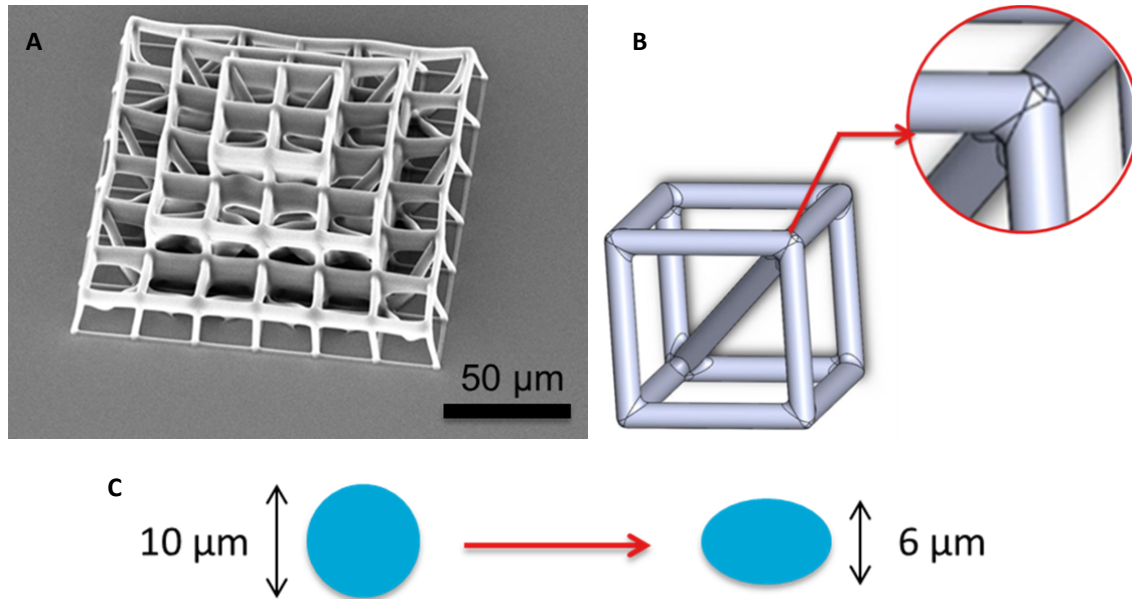


Figure 21: A) SEM image of the 3D-engineered scaffold with vertical blocked pores. B) Corners of the unit cells were rounded with the use of the fillet function in Solidworks C) Rods were made narrower in the Z direction

The second complication was occasional detachment of the scaffolds and the plates after exposure to cell culture media. To address this and to increase stability of the 3D structures, the cross-sections of the rods connected to the substrate were changed. Figure 22 shows five cross-sections that were tested. The cross-sections 3, 4, and 5 resulted in steadier structures and increased attachment to the substrate. Cross-section 3 was chosen as there were minimal differences among these three cross-sections. This adjustment also fixed the occasional issue of fragile bottom rods in the scaffolds (Figure 22B).

To address the detachment of the 2D plates, a number of different geometries were tested. The detachment in the plates almost always started from the edges. The geometry shown in Figure 23 reduced the detachment issue. The rationale behind this choice was that the opposing delamination of corners will cancel each other and would provide more adhesion sites around the corners. Furthermore, increasing the thickness of the 2D plates increased the incidents of delamination. Thus, the thickness was kept at its lowest acceptable value i.e. 10μm. For 2D structures, plates of 500μm x 500μm x 10μm were chosen. These modifications alone did not completely fix the detachment problem. Further adjustments to the printing parameters and to the silanization solution were required to solve this issue. Table 1 shows a summary of the final scaffold dimensions.

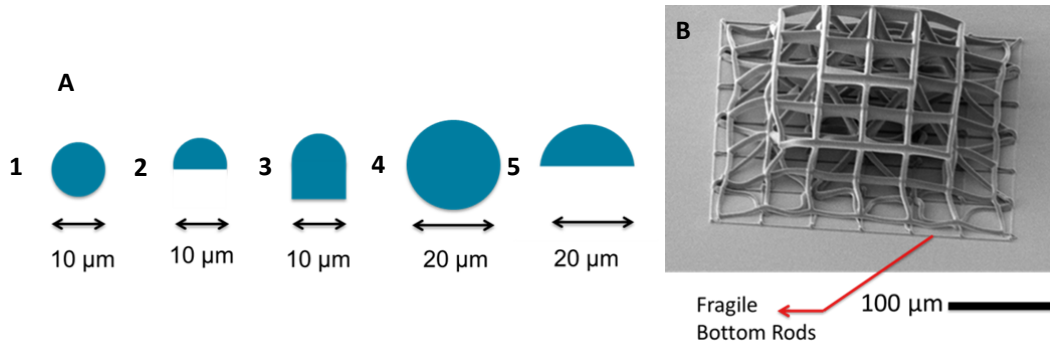


Figure 22: A) Five cross-sections tested for the bottom rods of the scaffolds B) Fragile bottom rods of the scaffolds

Table 1: Summary of the 3D-engineered scaffold dimensions, values are in μm

Scaffold Dimensions (μm^3)	Unit Size (μm^2)	Pore Size (μm^2)	Rod Diameter	Diameter of the Adjusted Rods	Fillet Diameter	Bottom Rods' Geometry
370x370x190	60x60	50x50	10 μm	6 μm	1 μm	

The large Young's Modulus of the IP-Visio material (estimated as 1.5 GPa⁸⁶) caused the stiffness of the structures not to be similar to that of the brain tissue. However, the Young's modulus was still closer to that of the brain (estimated as 0.1 to 1 kPa⁴⁸) when compared to the 2D cell culture plates where cells were in contact with glass (approximate Young's Modulus of 70 GPa, changes depending on the glass type) or other rigid surfaces. The effects of the stiffness on GBM cells were not investigated in this research.

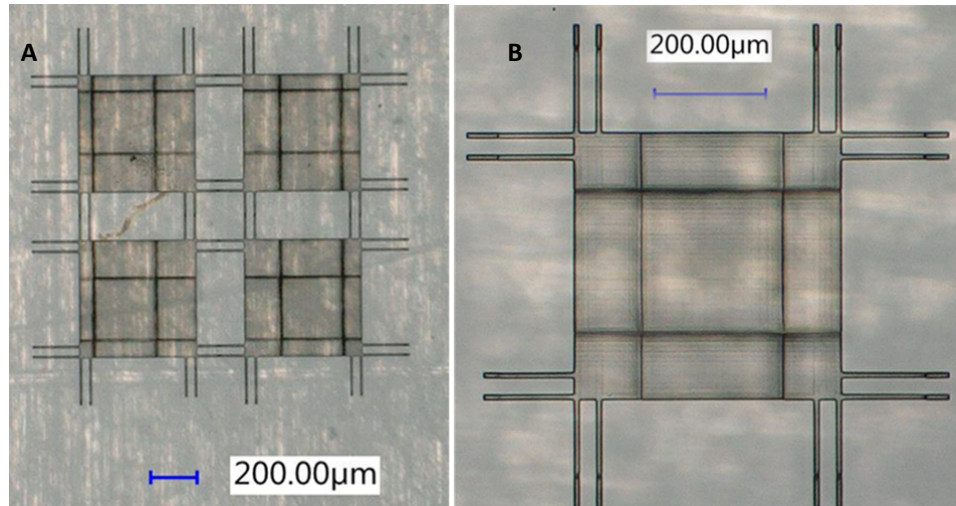


Figure 23: A) Four 2D plates on one substrate B) Geometry of a single 2D plate

5.1.3 Printing Parameters

Among the printing parameters that needed optimization were the *scan speed*, the *laser power*, the *slicing and hatching*. The *scan speed* refers to the velocity of laser movements during printing and the *laser power* refers to the percentage of the overall power of the laser (0% to 100%). The maximum available power is 50mW. *Hatching* is the spacing distance between voxels in the X or Y directions and *slicing* is the spacing distance between voxels in the Z direction (provided that Z is the direction of the

laser beam)⁷⁰. Figure 24 represents how these parameters are defined. Decreasing slicing and hatching values will increase the shape accuracy and steadiness of the structures but will noticeably increase the printing time.

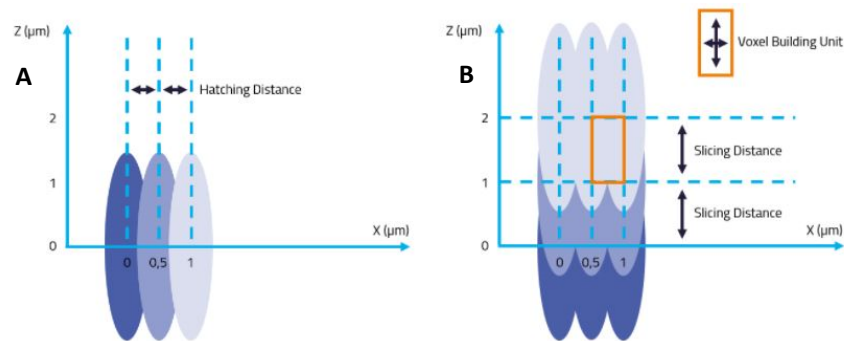


Figure 24: Printing distance of voxels in the 2PP method A) Hatching B) Slicing⁸⁷

To optimize the *scan speed* and the *laser power*, dose tests were conducted. In order to reduce the printing time for the 3D structures, the dose test was initially performed on a reduced scale scaffold consisting of only 3 unit cells. The *scan speed* and the *laser power* values were tested using the suggested values by Nanoguide (*laser power*: 100%, *scan speed*: 50Kμm/s) as a reference point. These parameters were increased or decreased from the reference point in multiple steps.

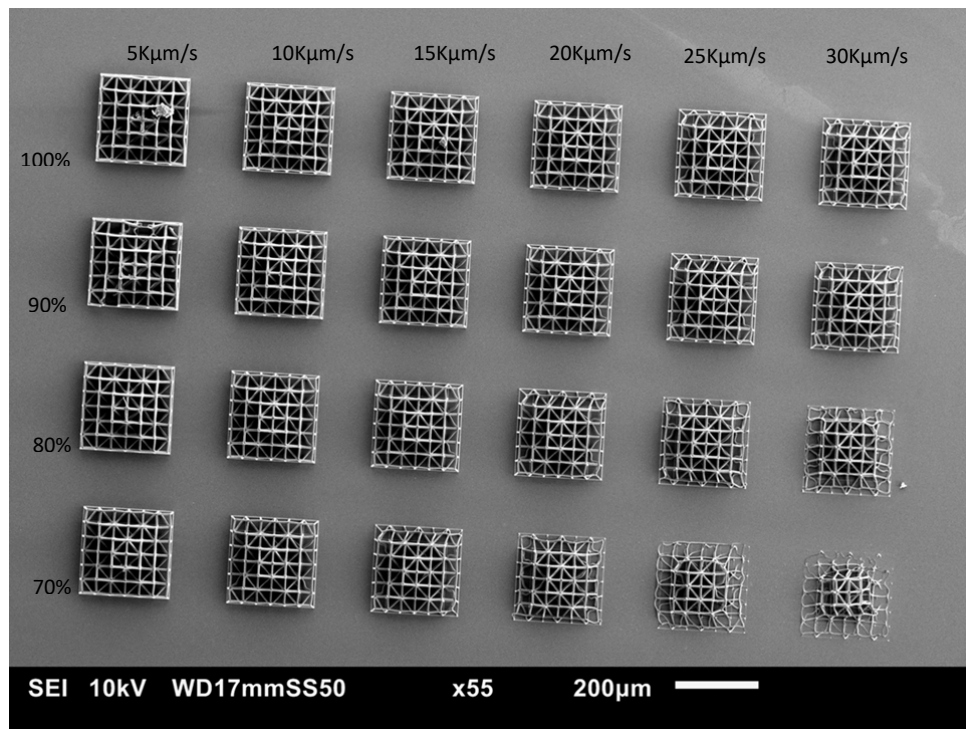


Figure 25: SEM image of a dose test to find the optimal laser power and scan speed combination. Laser power is descending from 100% to 70% from top to bottom. Scan speed is ascending from 5Kμm/s to 30Kμm/s from left to right.

Generally, higher *scan speeds* and lower *laser powers* resulted in more fragile but more intricate structures. In contrast, lower *scan speeds* and higher *laser powers* resulted in sturdier but bulkier structures while at the same time increasing chances of bubble formation. The optimization was then performed for the whole scaffold with parameter settings close to the optimal values. This process was

repeated several times until the suitable *laser power* and *scan speed* were determined. Changes in the geometry and other printing parameters as well as changes due to the maintenance of the Nanoscribe printer led to more repetitions. Figure 25 shows an SEM image of a dose test. Table 2 shows the iterations needed to find the optimal *scan speed* and *laser power*.

For the final scaffolds, the 10K $\mu\text{m/s}$ scan speed and 60% laser power provided the optimal combination of precision and stability. The dose test was repeated for the 2D plates. The 50K $\mu\text{m/s}$ scan speed and 80% laser power provided the optimal plate quality. The process for the plates was quicker as the final values were found after one dose test.

Table 2: List of tests conducted to optimize the printing parameters

Test number	Structure	Scan speed (SS) ($\mu\text{m/s}$)	Laser Power (LP) (%)	Notes	Optimal values
1	Three units of the scaffold	10K to 90K	20 to 100	-	SS:30K & LP:100
2	Three units of the scaffold	10K to 55K	70 to 100	Smaller steps	SS: 30K & LP:80
3	Entire scaffold	20K to 40K	80 to 100	Three units did not exactly represent the whole scaffold, lower SS was needed	SS: 20K & LP:80
4	Entire scaffold	5 to 30	70 to 100	Fragile bottom rods and blocked pores were observed	SS:5K & LP:80 SS:10K & LP:90 SS: 15K & LP:100
5	Entire scaffold + adjusted bottom rods	5 to 15	80 to 100	Blocked pores were observed	SS:5K & LP:80
6	Entire scaffold + new rod diameter + new pore size + new slicing value	5 to 20	40 to 100	Lowest LP meant least blocked pores, First test after Nanoscribe maintenance	SS:10K & LP:60
7	Entire scaffold + adjusted oval shaped rods	5 to 15	50 to 70	Adequate results were achieved	SS:10K & LP:60

Initially, the prints started with the values of slicing and hatching recommended by Nanoguide (*hatching*: 0.5 μm and *slicing*: 1 μm). To further address the blocked pore issue with increasing the accuracy in the Z direction, the *slicing* value for the scaffolds was reduced to 0.4 μm . The *hatching* value for the scaffolds and both *slicing* and *hatching* values for the plates remained unchanged from the recommended values.

Both 3D scaffolds and 2D plates exceeded the addressable printing range of galvo mirrors. Therefore, the structures needed to be sectioned into smaller pieces (block splitting) and these pieces needed to be bonded together during the printing process (stitching). Block splitting should be avoided at locations where it can lead to creation of overhanging rods. The block splitting locations can be adjusted in DeScribe with changing the block offset values. The block splitting was optimized with the help of DeScribe visualization option. In order to increase adhesion between blocks, instead of a straight line, a shear angle interface was selected. The optimal value was found by printing scaffolds and plates with different shear angles starting from 15° to 45°. Larger shear angles increased adhesion between blocks but decreased the block size. This decrease increased the number of blocks and the printing time.

Increased number of blocks required more blocks to be stitched and resulted in creation of more overhanging rods in the 3D scaffolds. The optimal shear stitching angles for 2D plates and 3D scaffolds were found to be 15° and 45° respectively. In order to avoid disjointed blocks due to un-polymerized resin, overlaps between the stitched blocks were set. *Layer overlap* (Z direction) and *block overlap* (X and Y directions) settings were determined in Describe. After optimization tests, the *layer overlap* and *block overlap* values for the 2D plates and the 3D scaffolds were found to be 2 and 3, 2 and 1 µm, respectively. Figure 26A and Figure 26B demonstrate the adequacy of stitched rods and the effect of shear angles on the plates respectively.

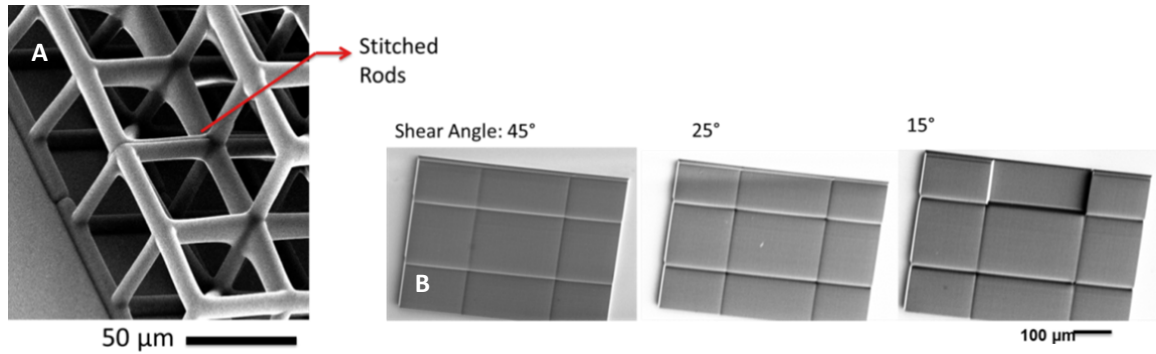


Figure 26: A) Adequately stitched rods of the scaffolds B) Effect of shear angle values on the stitching of the 2D plates

Summaries of the printing parameters for the 3D scaffolds and 2D plates are presented in Table 3 and Table 4 respectively. Other parameters such as *InterfacePosition*, *GalvoAcceleration*, *PiezoSettlingTime*, *StageVelocity* were also tuned and they are presented in

Table 5. These parameters did not require substantial alteration from the initial recommended values.

Each sample set consists of three scaffolds and four plates and it took 95 minutes to print. To be specific, each scaffold took approximately 25 minutes and each plate took nearly 5 minutes to be fabricated. The final scaffolds and 2D plates are shown in Figure 27. The resulting structures are steady, reproducible, and have the needed dimensional accuracy.

Table 3: Summary of printing parameters for the 3D engineered scaffolds

Slicing	Slicing Simplification Tolerance	Hatching	Splitting	Bounding Box	Block Size	Block Offset	Shear Angle	Overlap
0.4µm (Fixed Mode)	0.05µm	0.5µm	Rectangular Mode	X: -185:185 Y: -185:185 Z: 0:189	X: 230 Y: 230 Z: 190	X: 124.5 Y: 187.5 Z: 0	15°	XY:2 Z:1

Table 4: Summary of printing parameters for the 2D plates

Slicing	Slicing Simplification Tolerance	Hatching	Splitting	Bounding Box	Block Size	Block Offset	Shear Angle	Overlap
0.8µm (Fixed Mode)	0.05µm	0.5µm	Rectangular Mode	X: -800:800 Y: -800:800 Z: 0:10	X: 269 Y: 269 Z: 11	X: 165.5 Y: 172.5 Z: 0	45°	XY:2 Z:3

Table 5: Other printing parameters for both 2D plates and 3D scaffolds

Piezo Settling Time	Galvo Acceleration Time	Stage Velocity	Power Scaling	Laser Power	Scan Speed ($\mu\text{m/s}$)	Interface Position
10ms	10ms	200 $\mu\text{m/s}$	1.0	Scaffolds: 60% Plates: 100%	Scaffolds: 10K Plates: 50K	1 μm

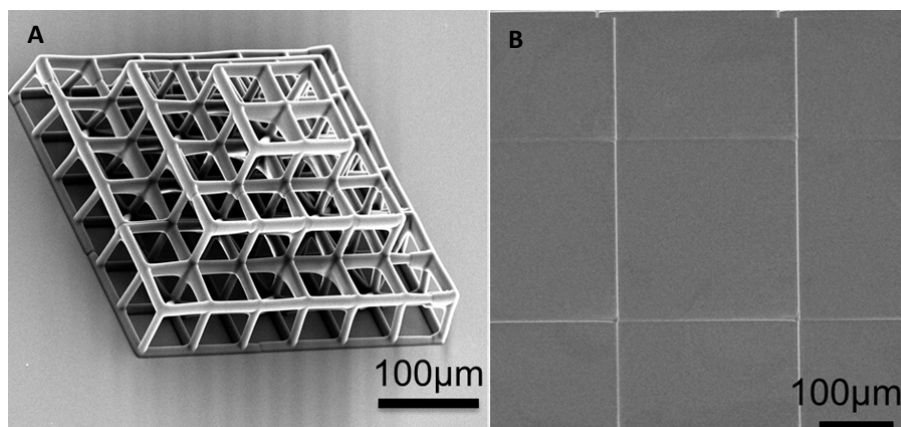


Figure 27: A) SEM image of the final 3D-engineered scaffold B) SEM Image of the final 2D plate

5.2 SCAFFOLD COLONIZATION

Initially, the standard cell line U-87 was cultured on the 3D scaffolds and the 2D flat plates. Figure 28 shows the confocal images of U-87 cells that successfully reached and colonized all layers of the scaffolds and formed a cellular network. Tubulin protrusions were observed with some lengths exceeding 50 μm .

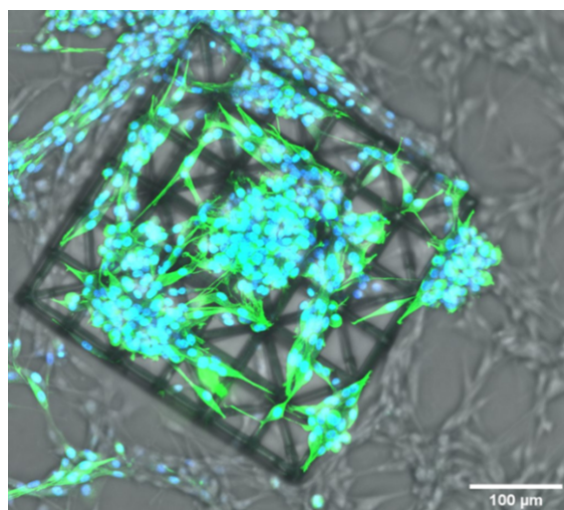


Figure 28: U-87 cell colonization of the scaffolds. Cyan: nuclei, Green: tubulin. Images are taken with a water dipping microscope with a 20x objective

SEM images of the samples shows that the U-87 cells colonized the scaffolds and formed tube like protrusions between the pores and along the scaffold rods (Figure 29). Also, Figure 29B shows that U-87 cells grew on the 2D plates and on the glass area surrounding the 3D scaffolds.

After successful culture of the U-87 cell line, the three primary cells (GS-580, GS-827, and GS-830) were cultured on the structures. All three primary cell cultures also successfully colonized the scaffolds. Cells formed long protrusions and clearly invaded the inner regions of the scaffolds. Figure 30 shows the SEM images of the GS-830 and GS-580 primary cells 3 days after seeding.

Figure 31 depicts the confocal fluorescent images of the GS-830 primary cells cultured on the scaffold and fixed 3 days after seeding. The same procedure was repeated for the other two primary cells (GS-580 and GS-827). Additionally, the experiment was repeated for all three primary cell cultures to check for consistency. Figure 32 demonstrates the images for all four cell cultures.

Figure 33A shows the number of cells cultured on 2D flat plates and 3D scaffolds for the four cell cultures on equal areas ($380\mu\text{m} \times 380\mu\text{m}$). The number of cells on the 3D scaffolds exceeds the number of cells on the 2D plates except for the GS-827 cell culture.

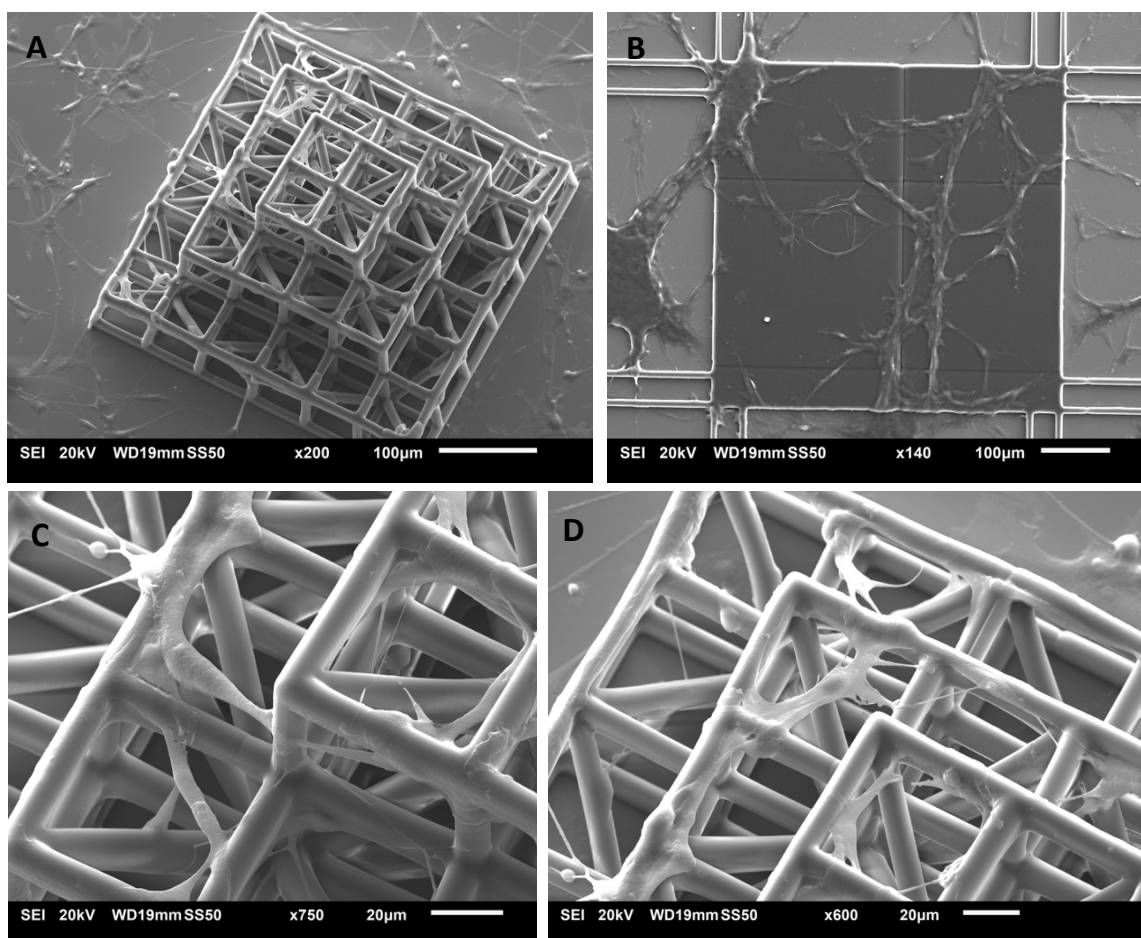


Figure 29: SEM images of U-87 cells cultured on the scaffolds and plates. A) Overview of 3D scaffolds B) Overview of the 2D flat plate. C, D) Zoomed in images of the 3D scaffold

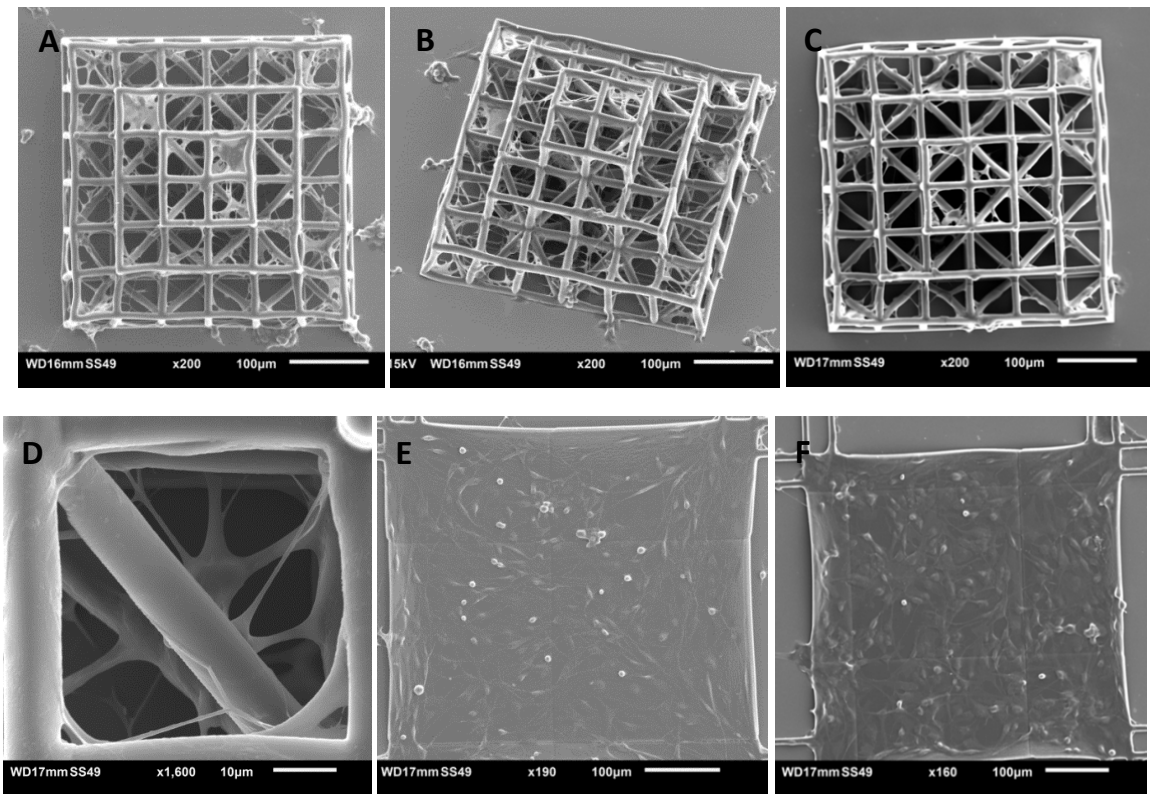


Figure 30: SEM images of two primary GBM cells. A) Top view of GS-830 cells on the 3D scaffolds B) Tilted view of GS-830 cells on 3D scaffolds C) Top view of GS-580 cells on the 3D scaffolds D) Zoomed in Image of the GS-830 cells on the 3D scaffolds showing that the cells have reached the inner units E) GS-830 cells on the 2D plates F) GS-580 cells on the 2D plates

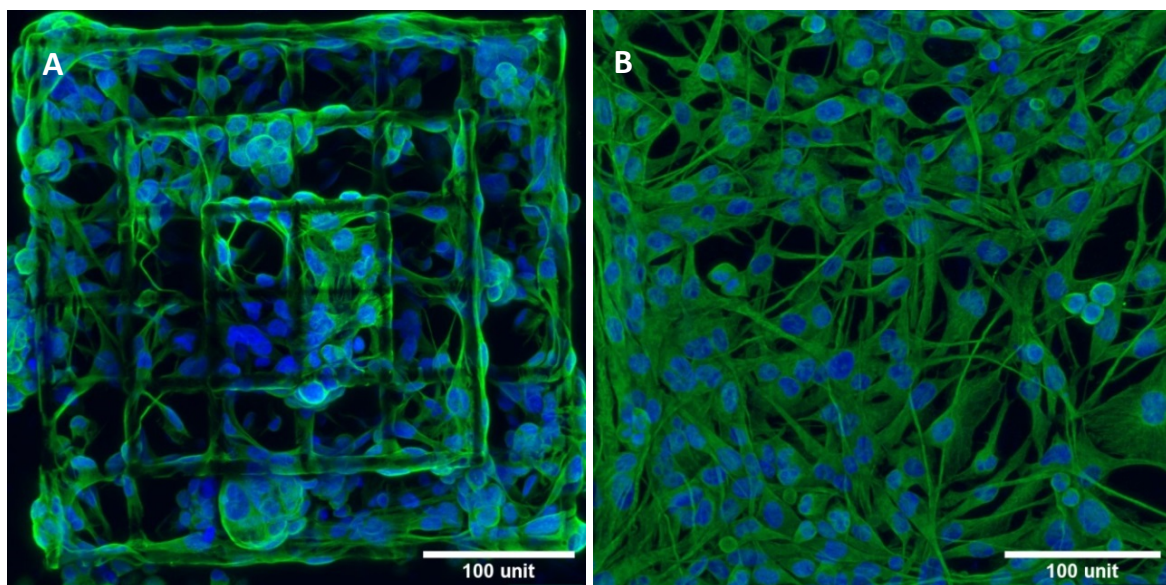
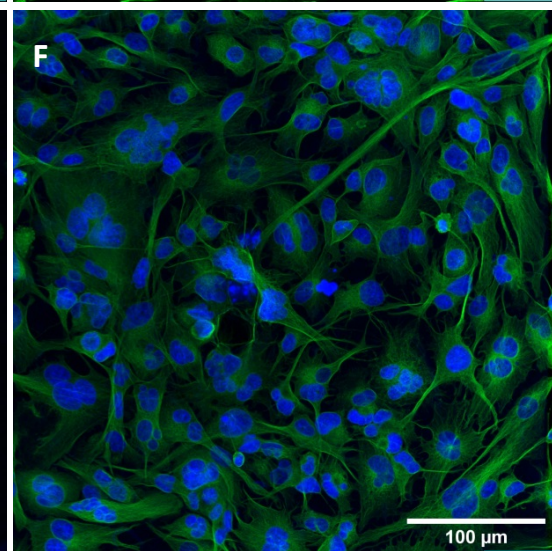
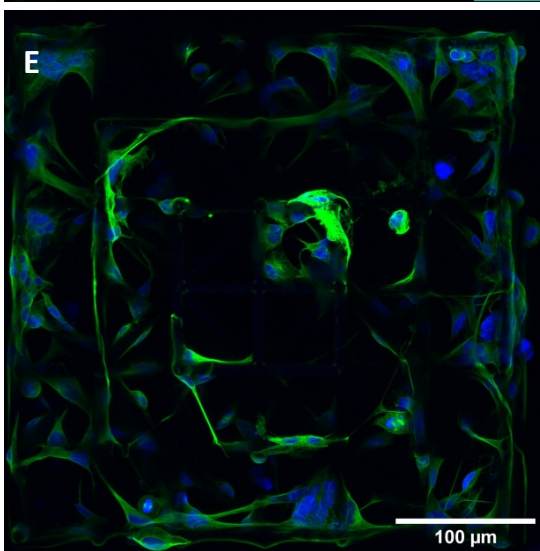
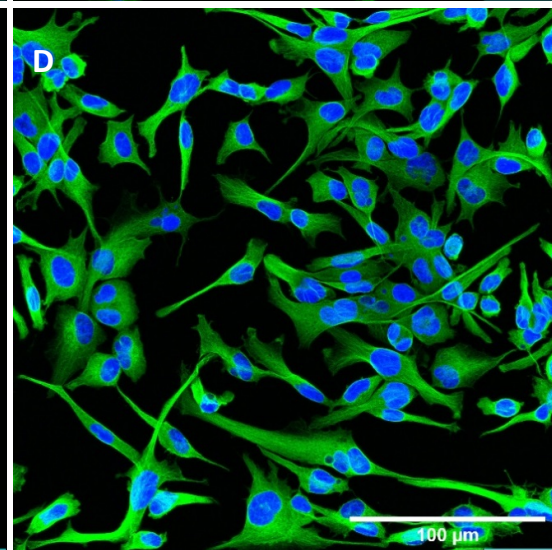
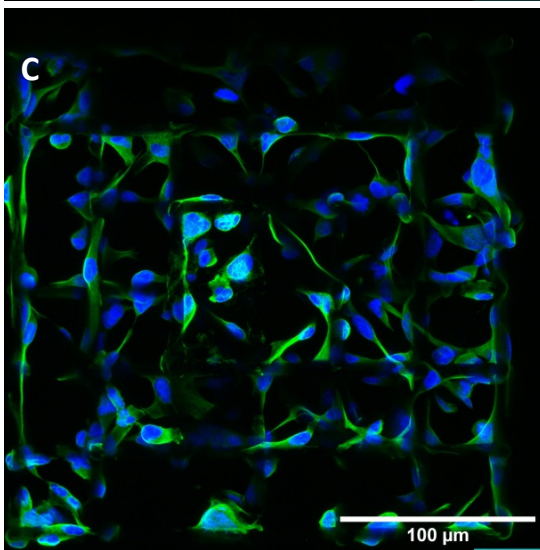
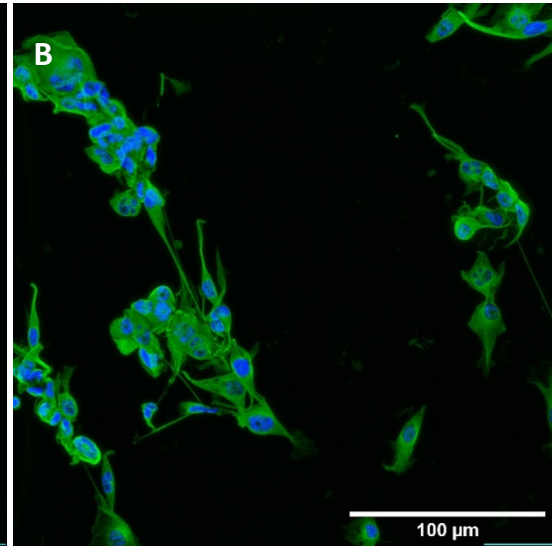
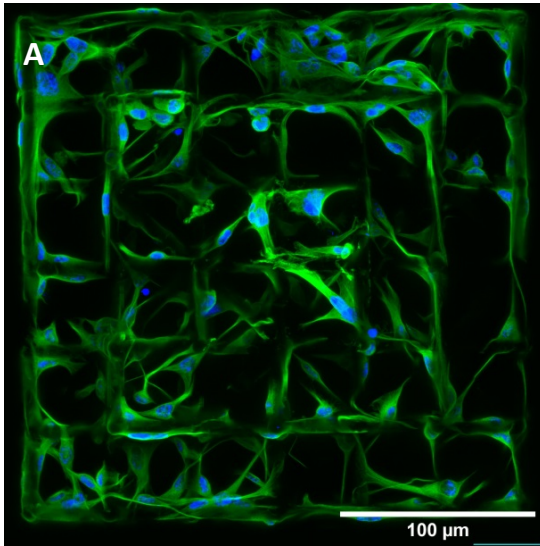


Figure 31: Confocal images of GS-830 primary GBM cells. Blue: nuclei Green: tubulin. A) Cells cultured on the 3D scaffolds. B) Cells cultured on the 2D plates



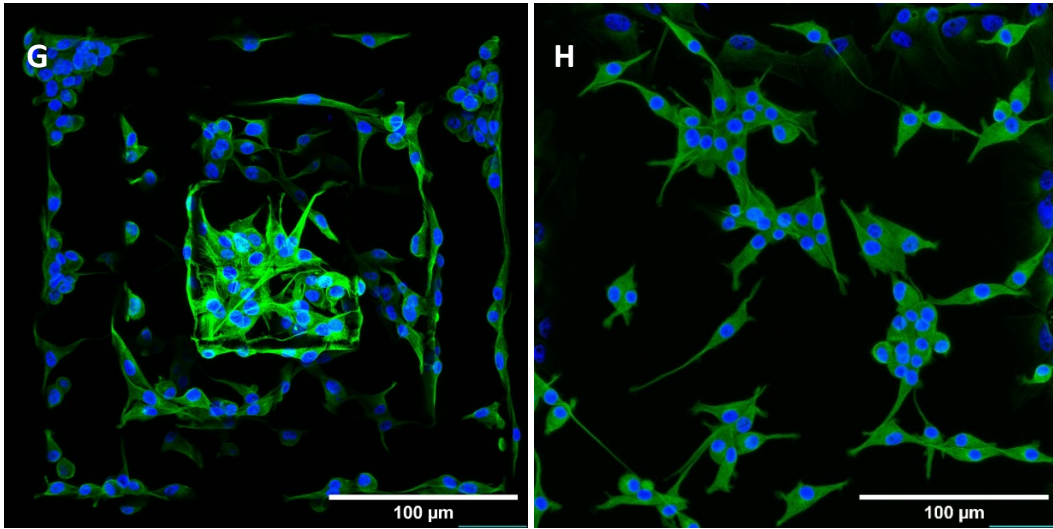


Figure 32: Confocal images of GBM cell. Blue: nuclei Green: tubulin. A) GS-830 cells on a 3D scaffold B) GS-830 cells on a 2D flat plate C) GS-580 cells on a 3D scaffold. D) GS-580 cells on a 2D flat plate E) GS-827 cells on a 3D scaffold F) GS-827 cells on a 2D flat plate G) U-87 cells on a 3D scaffold H) U-87 cells on a 2D flat plate

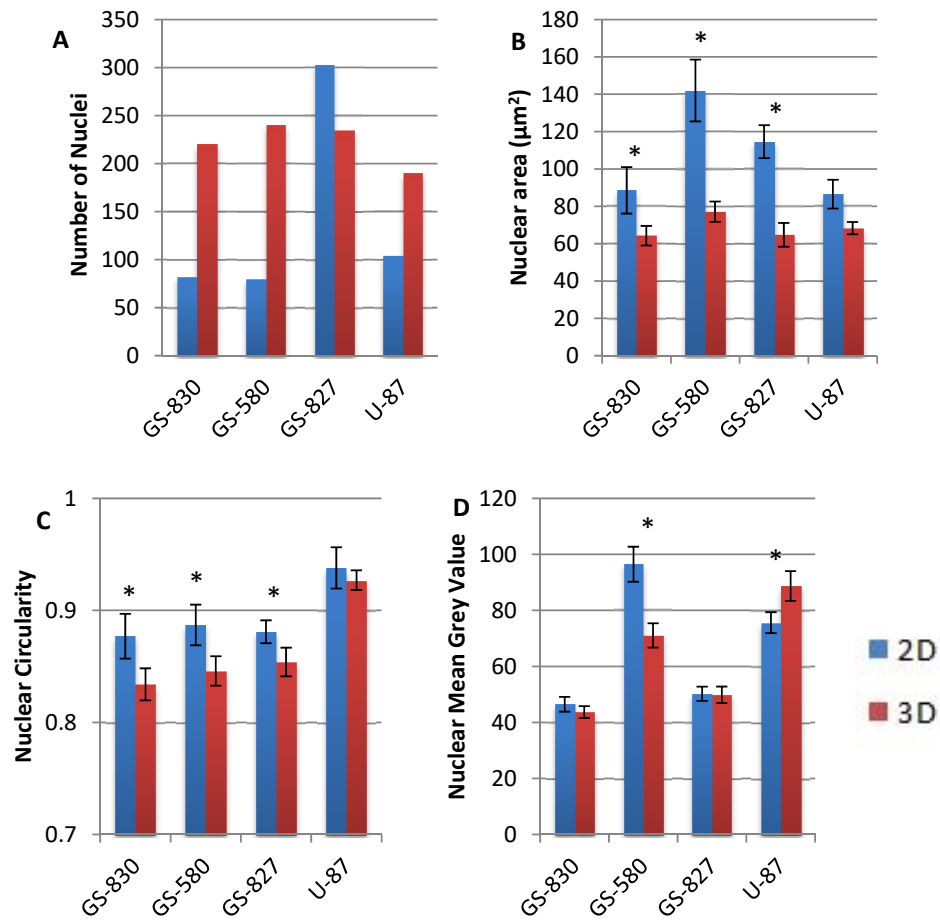


Figure 33: Means and 95% confidence intervals for A) Number of nuclei B) Nuclear area C) Nuclear circularity D) Nuclear mean grey value.

5.3 GBM MORPHOLOGY

Cellular morphology was studied using confocal images of the GBM cells (representative images in Figure 32).

5.3.1 Nuclear Morphology

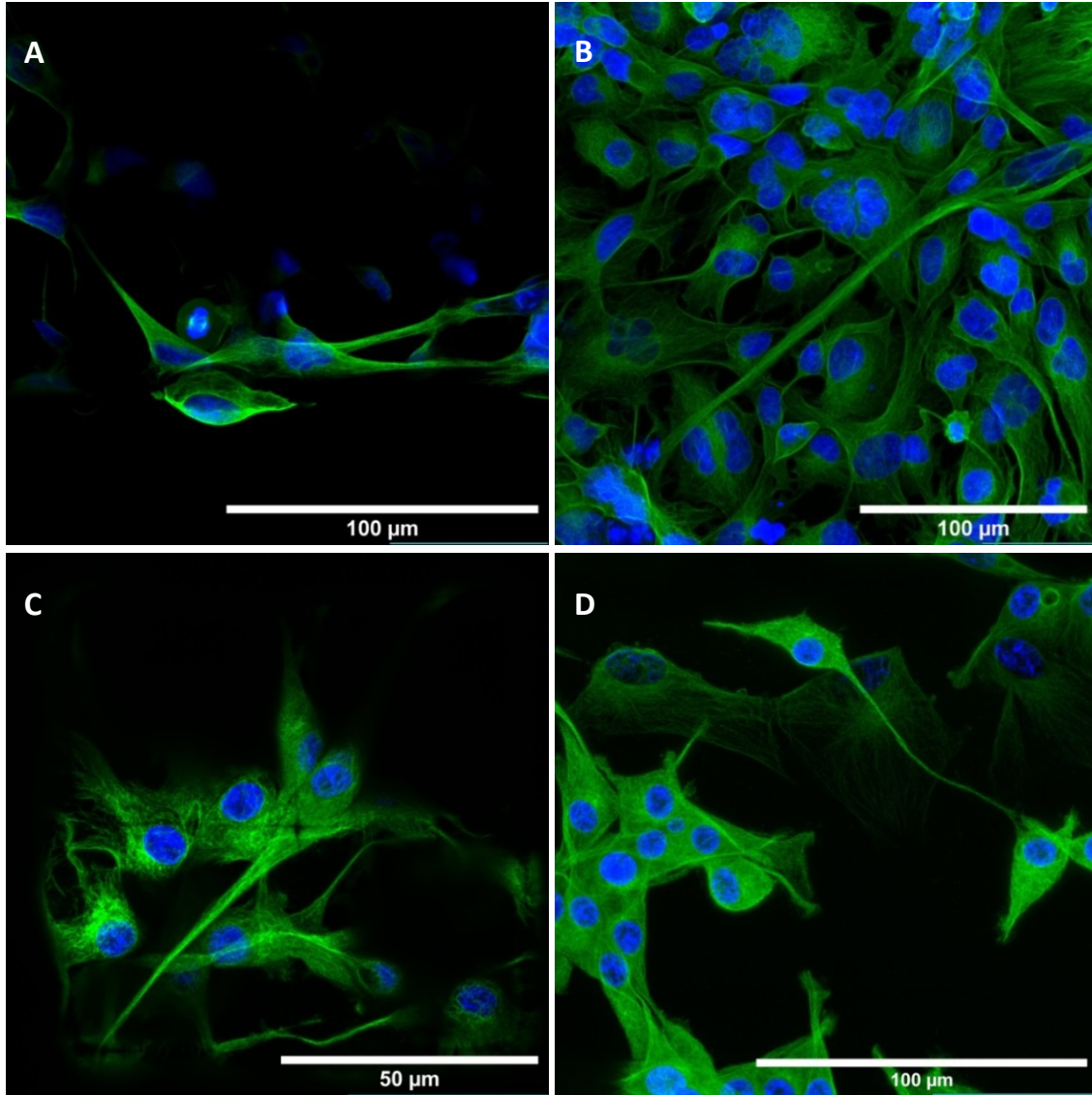


Figure 34: Zoomed-in confocal images of GBM cell. Blue: nuclei Green: tubulin. A) GS-827 cells on a 3D scaffold B) GS-827 cells on a 2D flat plate C) U-87 cells on a 3D scaffold D) U-87 cells on a 2D flat plate

Figure 33B shows the nuclear area for all four cell cultures on 2D flat plates and 3D scaffolds. As can be seen, the cells in 2D plates had larger nuclear area projected in the X-Y directions compared to those in the 3D scaffolds. The differences between the mean values in 2D plates and 3D scaffolds for primary cells were statistically significant. The same difference for the standard cell line U87 was not significant ($p = 0.097$). The three primary cell cultures also had significant nuclear area variability compared to that of the standard U-87 cell line (variability was assessed by F-test in Appendix 11.7).

Figure 33C reports on the circularity of GBM nuclei for all four cell cultures on 2D plates and 3D scaffolds. The variable “circularity” in ImageJ is defined by Equation 1. A circularity number closer to 1 means the nucleus has a rounder and more circular shape whereas a number closer to 0 means the nucleus is more elongated or irregular. As can be seen in Figure 33C, the nuclei of cells on 2D substrates were on average more circular than cells on 3D and this difference was significant in primary cultures. The nuclei of the standard cell line U-87 were more circular compared to the ones of the primary cell cultures both in plates and in scaffolds. However, the p-value for the mean circularity difference between 2D plates and 3D scaffolds was not significant for the standard cell line. Furthermore, the circularity variability in the 3D scaffolds was significantly lower in the standard cell line compared to the primary cells. However, the variability between the standard cell line and primary cells was not significantly different in the 2D plates (variability was assessed by F-test in Appendix 11.8).

$$Circularity = 4\pi\left(\frac{Area}{Perimeter^2}\right)$$

Equation 1

Figure 34 shows more magnified images of GS-827 and U-87 cells. From these images, it appears that nuclear shape is not as circular for the primary cells compared to the U-87 cell line.

Figure 33D depicts the mean grey value (the average intensity of fluorescent emissions) of the nuclei for the four cell lines on 2D plates and 3D scaffolds. Consistent differences in nucleus intensity signals between 2D and 3D cultures were not observed.

5.3.2 Cell Morphology

The cell area was defined as the combination of nuclei and tubulin areas. Figure 35A shows the single cell area projected in X-Y directions for all four cell cultures on 2D plates and 3D scaffolds. All cell areas on 2D plates exceeded cell areas on the 3D scaffolds. This difference was significant in all cultures except in GS-830 and was most prominent in the GS-827 cell line. For the cell culture GS-580, ANOVA showed that the difference between 2D and 3D structures was approaching significance (p-value=0.06).

Figure 35B shows the circularity of single cells for all cell cultures. All four cell cultures in plates and scaffolds had an average circularity of less than 0.4, which means the cell shapes were far from a perfect circle. Although the circularity difference in plates and scaffolds was significant in all cell cultures, we did not find consistently different cell circularity for 2D plates and 3D scaffolds.

Figure 35C displays the average Feret’s diameter for single cells for all cell cultures. Feret’s diameter represents the longest distance between two points in the specified cell boundary. In other words, this parameter shows the length of each cell. Figure 35C shows that cells in all cell cultures were more elongated in 2D plates compared to 3D scaffolds. This difference was most prominent in the GS-827 cells. The longest recorded length for a single cell in 2D plates was 245 µm (GS-827) and in 3D scaffolds was 180µm (GS-830). These values in 3D scaffolds were definitely underestimated due to suboptimal cell segmentation and because the measured diameters were based on Z projections.

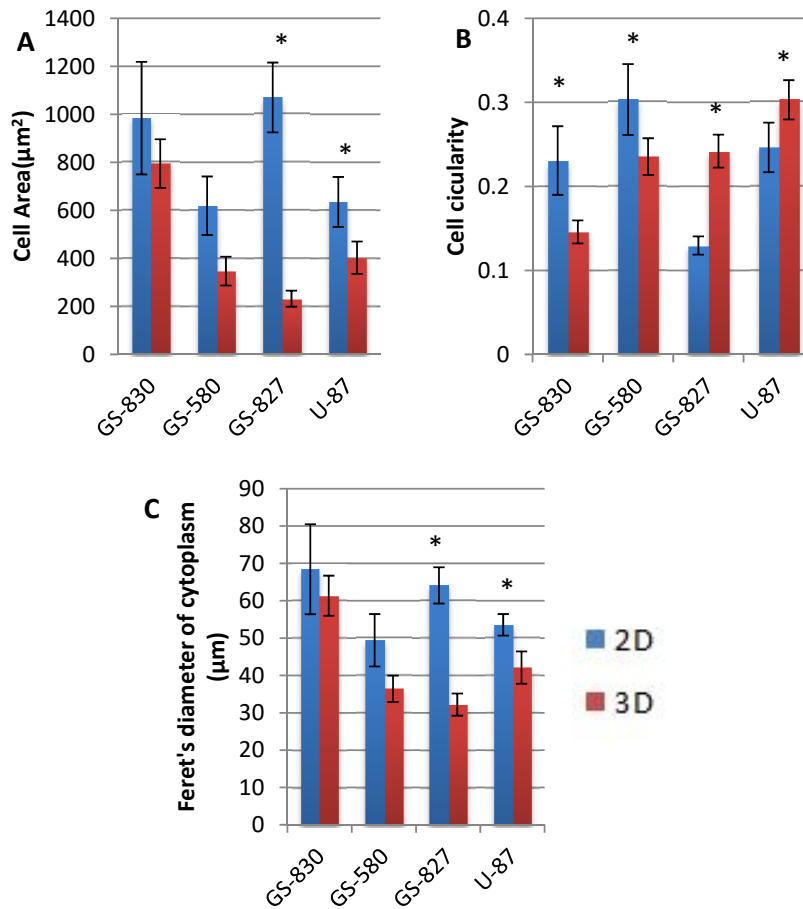


Figure 35: Means and 95% confidence intervals for A) Average single cell area B) Circularity of cells C) Feret's diameter of single cells

5.4 EGFR EXPRESSION

During the first experiment, the *EGFR* signal was observed in the form of distributed spots that were mostly concentrated near the nuclei (Figure 36). This experiment was repeated for consistency and the same effect was observed. It was hypothesized that the *EGFR* spots seen during the initial experiments were internalized *EGFR* proteins. Two experiments were designed to verify that the observed signals were indeed *EGFR* spots and the staining was specific to this protein.

In the first experiment, two different anti-*EGFR* antibodies were used (anti-*EGFR* by DAKO M3563 and anti-*EGFR* by Abcam ab76153). The aim of this test was to determine if signals of the two anti-*EGFR* antibodies would co-localize. Figure 37 shows an example image of this experiment. In this figure, green and red are the two *EGFR* antibodies. This and similar images were analyzed to check if the detected spots were highlighted similarly with both antibodies. Both antibodies showed *EGFR* as spot-like entities. In addition, similar to the previous experiment, the density of these spots was higher near the nuclei. Overall, both antibodies demonstrated similar *EGFR* patterns.

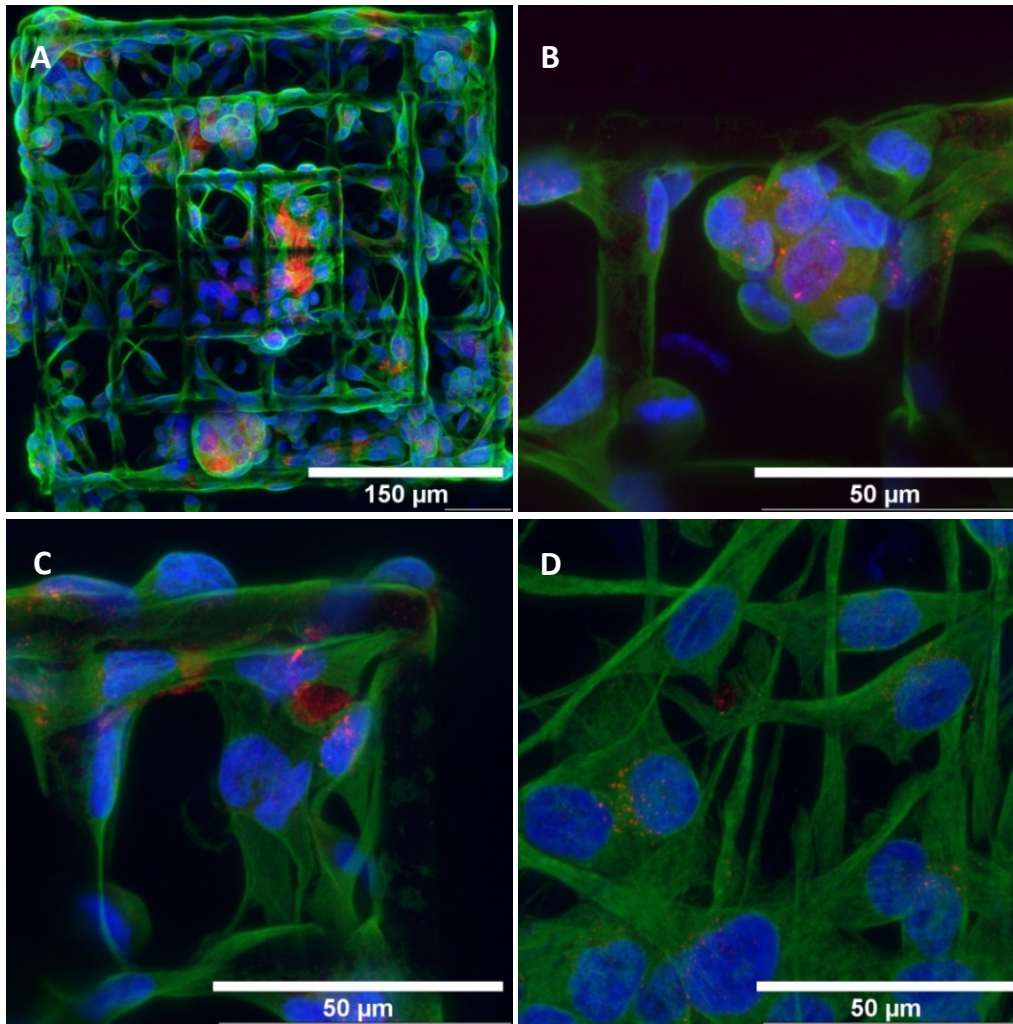


Figure 36: GS-830 cells cultured on 2D plates and 3D scaffolds. Blue: nuclei, Green: tubulin, Red: EGFR. A) Top view of the 3D scaffold B, C) zoomed-in images of the 3D scaffold D) zoomed in image of the 2D plate

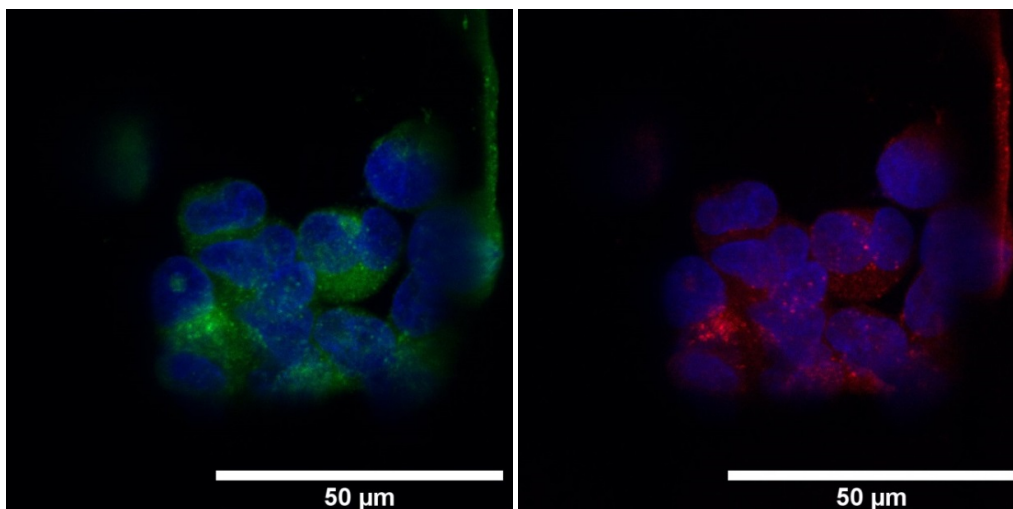


Figure 37: GS-830 cells stained with two different EGFR antibodies and cultured on the 3D scaffolds. Blue: nuclei, Green: with M3563 anti-EGFR, Red: with ab76153 anti-EGFR

In the second experiment, EGF was removed from the culture media and was compared to a controlled experiment in which EGF was not removed. Without EGF, only membrane staining was visible and no *EGFR* spots surrounding the nucleus were visible (Figure 38A and Figure 38B). These two experiments confirmed that the observed signal was specific to the *EGFR* protein.

To further study *EGFR* and analyze *EGFR* expressions in 2D plates and in 3D scaffolds, the experiments were repeated with *EGFR* inhibitors, Erlotinib and Dacomitinib, in the presence of EGF. In this experiment, GS-830 cells were cultured on the plates and the scaffolds under four conditions: i) with EGF in the culture media (control experiment), ii) removing EGF from the culture media, iii) adding Erlotinib for 2 hours to the culture media, and iv) adding Dacomitinib for 2 hours to the culture media. Figure 38 shows some sample images of the experiment.

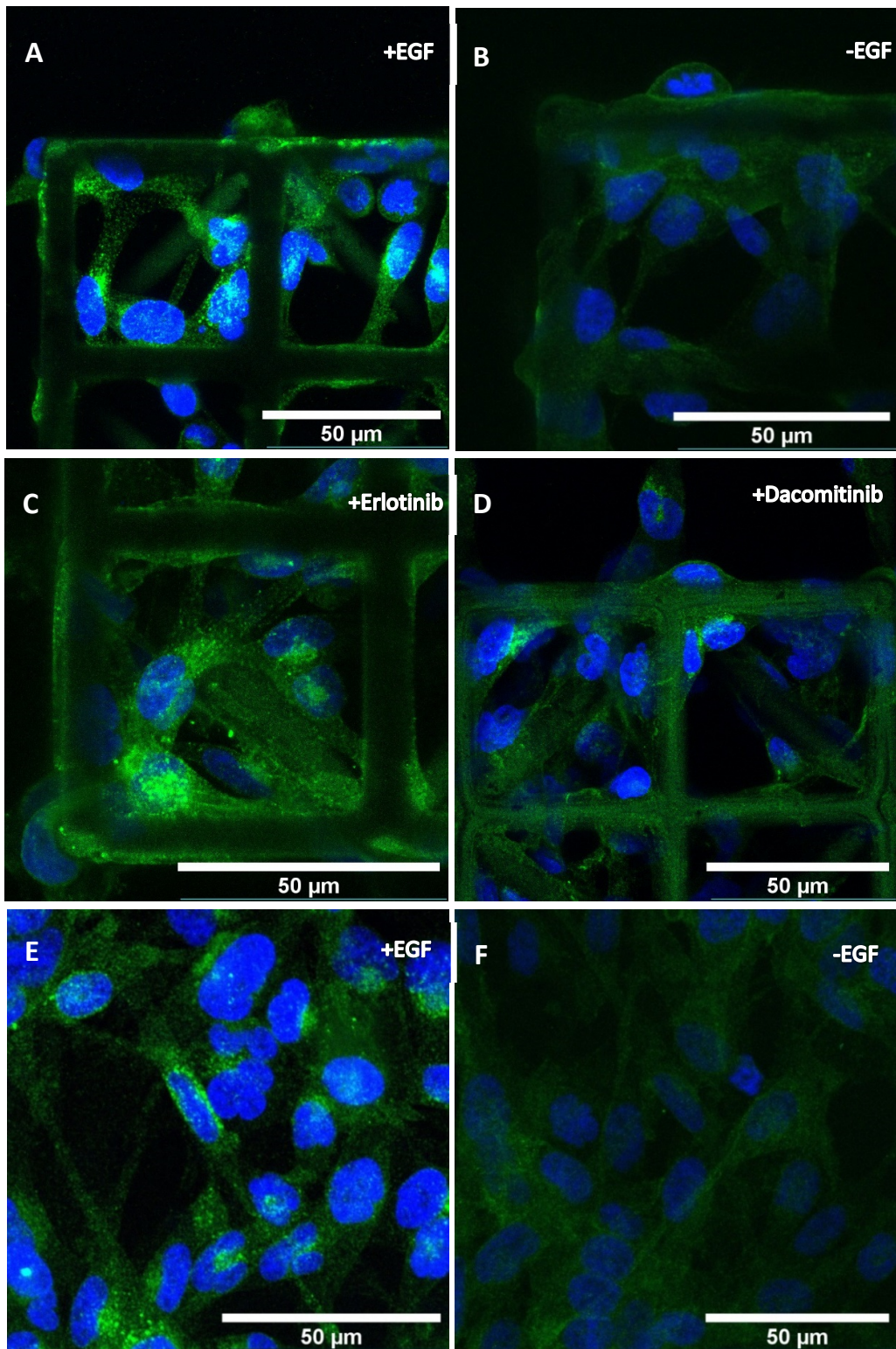
In general in cell cultures without EGF, the *EGFR* signal was more concentrated on the cell membranes both in the plates and in the scaffolds. With the addition of the inhibitors, there were fewer internalized *EGFR* spots but the spots did not completely disappear. It was further observed that *EGFR* internalized spots were more concentrated near the cell nuclei. All experiments were repeated with the GS-580 cell culture (Appendix 11.9.2) and also repeated with increasing the exposure time to the inhibitors (Appendix 11.9.3). The results were similar to the previous findings.

Figure 39A depicts the frequency of detected *EGFR* spots for GS-830 cell culture under the four conditions. Because the number of cells was not constant between the four conditions, the frequency of *EGFR* spots was normalized (the number of *EGFR* spots divided by the number of nuclei).

As discussed later in section 6.5.4, meaningful statistical comparisons were not possible and the following statements are general observations based on the mean number of spots in the images. It can be observed that the number of *EGFR* spots with EGF exceeded that without EGF. This effect did not change in 2D or 3D structures. The number of spots without EGF in both cases was close to zero. The number of spots was higher with both inhibitors when compared to the experiments without EGF. When the Erlotinib inhibitor was used, the number of spots was reduced only slightly. The reduction with the Dacomitinib inhibitor was more pronounced.

Figure 39B shows the normalized frequency of *EGFR* spots detected near the cell nuclei for GS-830. Similar to the previous analysis, the number of spots near the nuclei was higher in the experiment with EGF compared to the experiment without EGF. This number was reduced when the inhibitors were added to the media but still exceeded that of the experiment without EGF. However, the general trend in all conditions was similar in plates and in scaffolds.

Figure 40 shows the mean grey value of the *EGFR* spots for the GS-830 cells under the four mentioned conditions. In all conditions except for the experiment without EGF, the signal value in *EGFR* spots near the nuclei was significantly higher compared to the rest of *EGFR* spots. This is most prominent for the Erlotinib condition in the 3D scaffolds. Highest intensities were observed for the experiments with EGF and with Erlotinib, whereas the Dacomitinib experiment had the lowest signal.



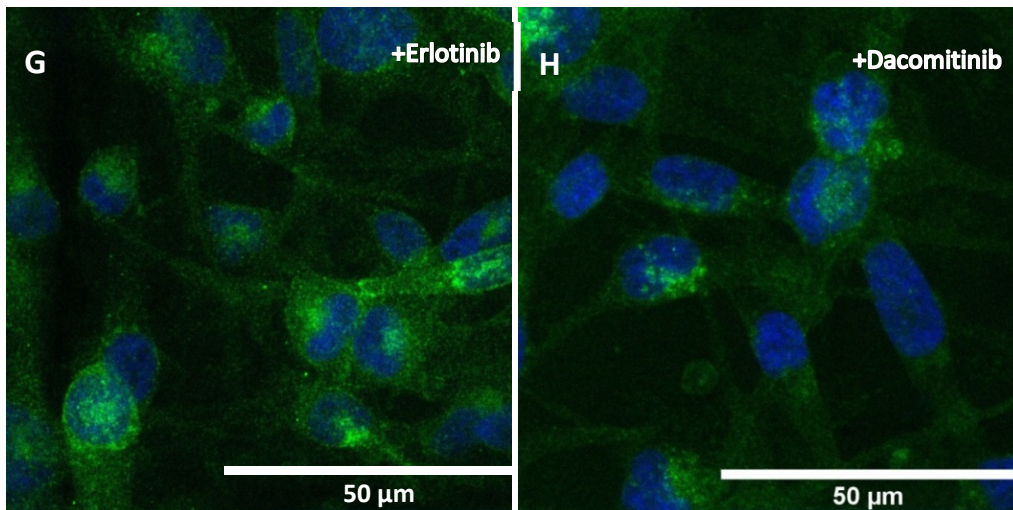


Figure 38: Confocal images of Gs-830 cells. Exposure to inhibitors was EGFR 2 hours. Blue: nuclei Green: EGFR A) Zoomed-in image of the scaffold with EGF B) Zoomed-in image of the scaffold without EGF C) Zoomed-in image of the scaffold with Erlotinib D) Zoomed-in image of the scaffold with Dacomitinib E) Zoomed-in image of the 2D plate with EGF F) Zoomed-in image of the 2D plate without EGF G) Zoomed-in image of the 2D plate with Erlotinib H) Zoomed-in image of the 2D plate with Dacomitinib

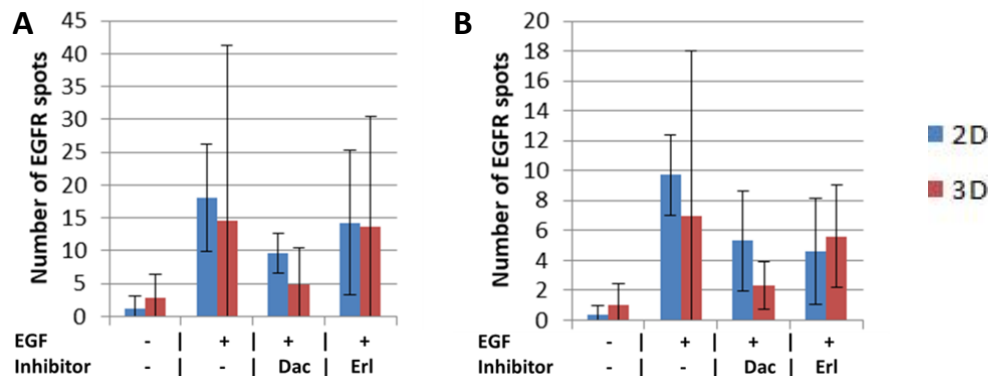


Figure 39: Means and 95% confidence intervals for A) Average number of the EGFR spots detected per cell B) Average number of the EGFR spots detected near the cell nuclei, *Dac*: Dacomitinib, *Erl*: Erlotinib

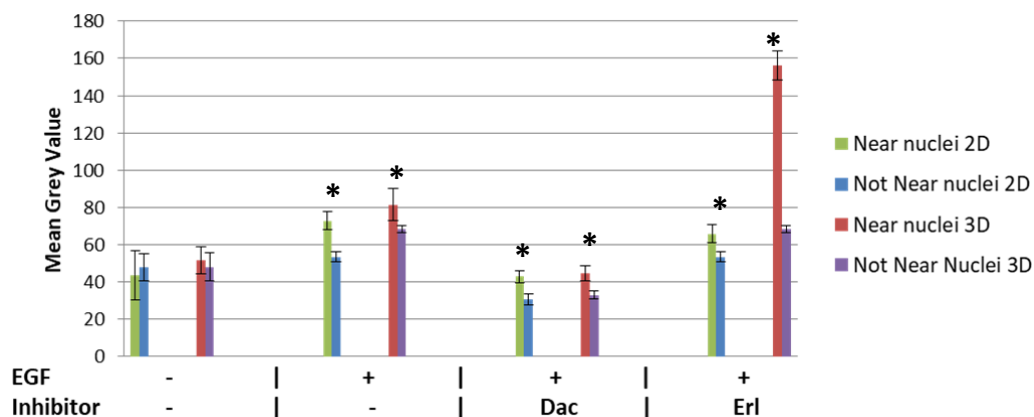


Figure 40: Means and 95% confidence intervals for the mean grey value of EGFR spots for the GS-830 cell culture, *Dac*: Dacomitinib, *Erl*: Erlotinib

5.5 MICROTUBE FORMATION

SEM and confocal images of the GBM cells were used to observe microtube formation *in vitro*. SEM images show tube-like protrusions that connected cells to each other and to their environment. Figure 41 shows SEM images of the GS-830 cell culture on the 3D scaffolds and 2D plates. Protrusions longer than 50 μm were observed. For example, Figure 41B shows a tube-like protrusion over 70 μm long. Nanotubes, tube-like protrusions in the nano scale (<1 μm wide), were also detected (Figure 41C). Figure 41D and E show that occasionally a single cell may have multiple tube-like protrusions. The tube-like protrusions were also observed in 2D but they were harder to detect and monitor (Figure 41F).

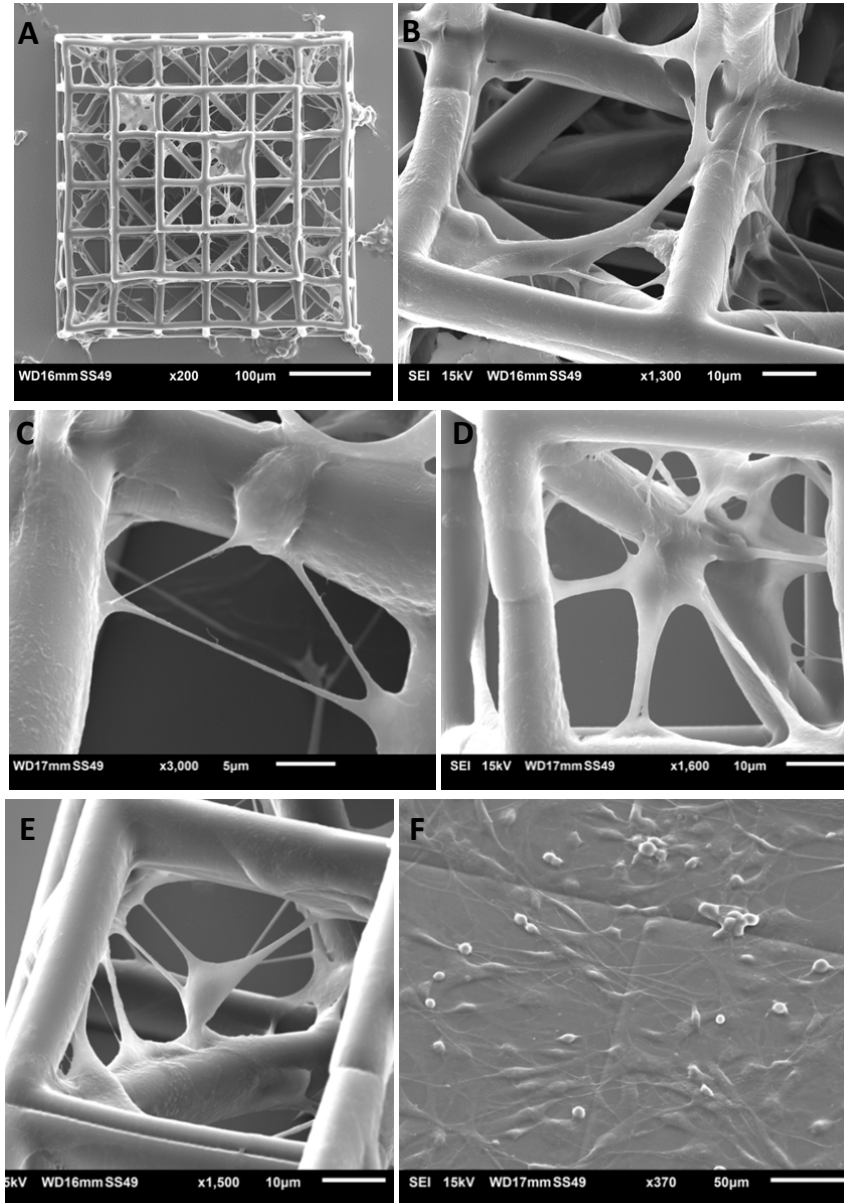


Figure 41: SEM images of GS-830 cells on 2D plates and 3D scaffolds. A) An overview of the 3D scaffold showing several tube-like protrusions B) Zoomed-in image of cells cultured on the 3D scaffold showing protrusions in the macro range C) Zoomed-in image of cells cultured on the 3D scaffold showing protrusions in the nano range D, E) Zoomed-in image of cells cultured on the 3D scaffold showing single cells with multiple protrusions F) Cells cultured on 2D flat plate

Figure 34 shows the confocal images (stained for tubulin and nuclei) of GS-827 and U-87 cells. These images demonstrate that the long tube-like protrusions were present both in the scaffolds and in the plates. Moreover, these protrusions were observed both in primary cultures and in the U-87 standard cell-line. Based on definitions provided by Osswald et al (2019), these protrusions in both 2D and 3D structures match the geometrical characteristics of microtubes or nanotubes⁶.

To determine if these protrusions are indeed microtubes, the cells were stained with the GAP-43 antibody (Figure 42). This protein was detected in the GS-830 cells and seemed like it surrounded the membranes all throughout the cells. Some increased signal was observed at the end-cones of the protrusions. However, GAP-43 did not seem to be exclusive to the microtube-like protrusions.

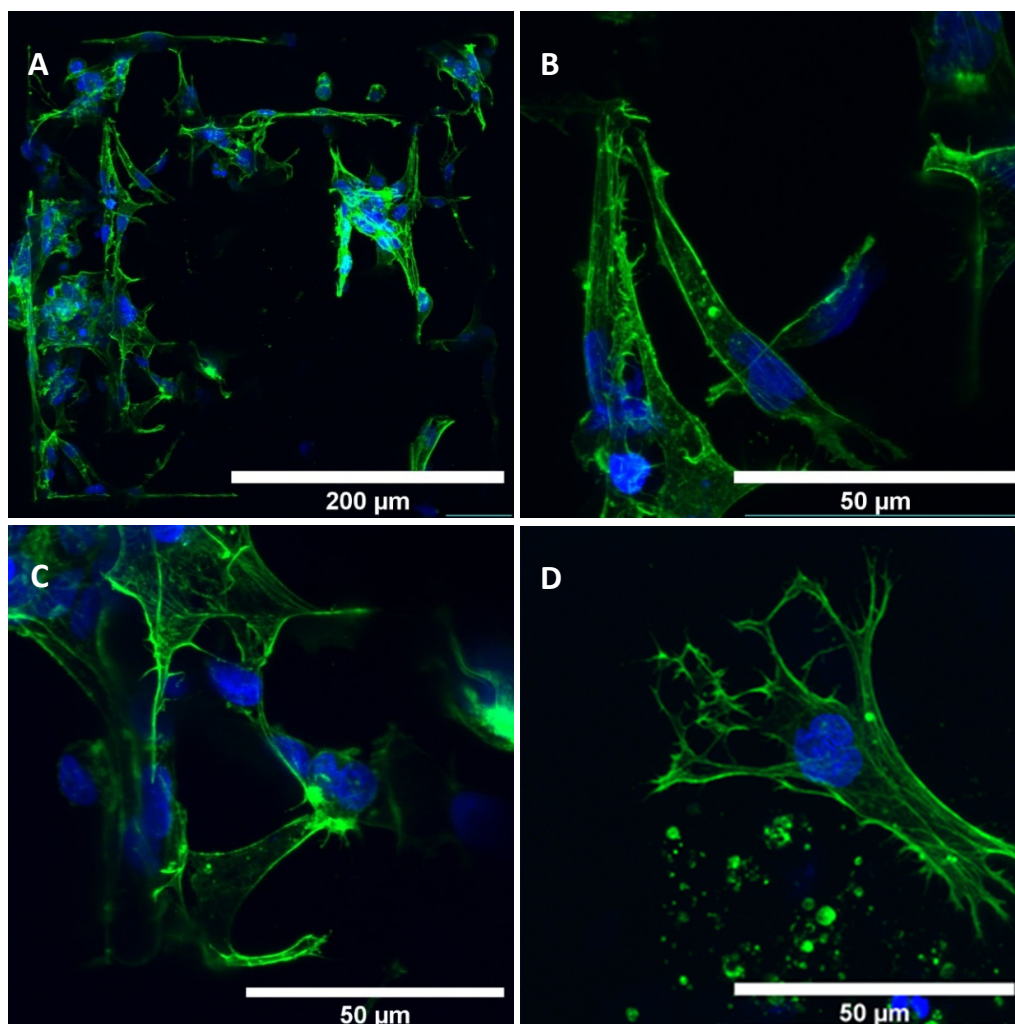


Figure 42: Fluorescent images of GS-830 cells Blue: nuclei, Green: GAP-43. A) Overview of the entire scaffold B, C) Zoomed-in image of the scaffolds D) Zoomed-in image of the 2D plate

Because GAP-43 did not show specificity for microtubes, confocal images (stained for tubulin and nuclei) of all four cell cultures were used to quantify the tube-like protrusions in 2D plates and 3D scaffolds. Figure 43A reports the average length of the detected protrusions. For all cell cultures, the lengths of the tube-like protrusions in 2D plates and 3D scaffolds were similar. However this length in scaffolds may be underestimated. Except for GS-827, the remaining cultures all had average protrusion lengths of nearly 40µm in both 2D and 3D structures.

Figure 43B shows the longest protrusions detected in each of the four cell cultures. GS-827 had the longest protrusion among all cultures. Protrusions in this cell culture reached lengths as long as 249 μm in 2D plates and 179 μm in 3D scaffolds. The remaining cell lines had maximum protrusion lengths of nearly 100 μm . Figure 43C shows the average protrusion width in all four cell cultures. Excluding GS-830, protrusion width in all cultures was larger in 2D plates than in 3D scaffolds.

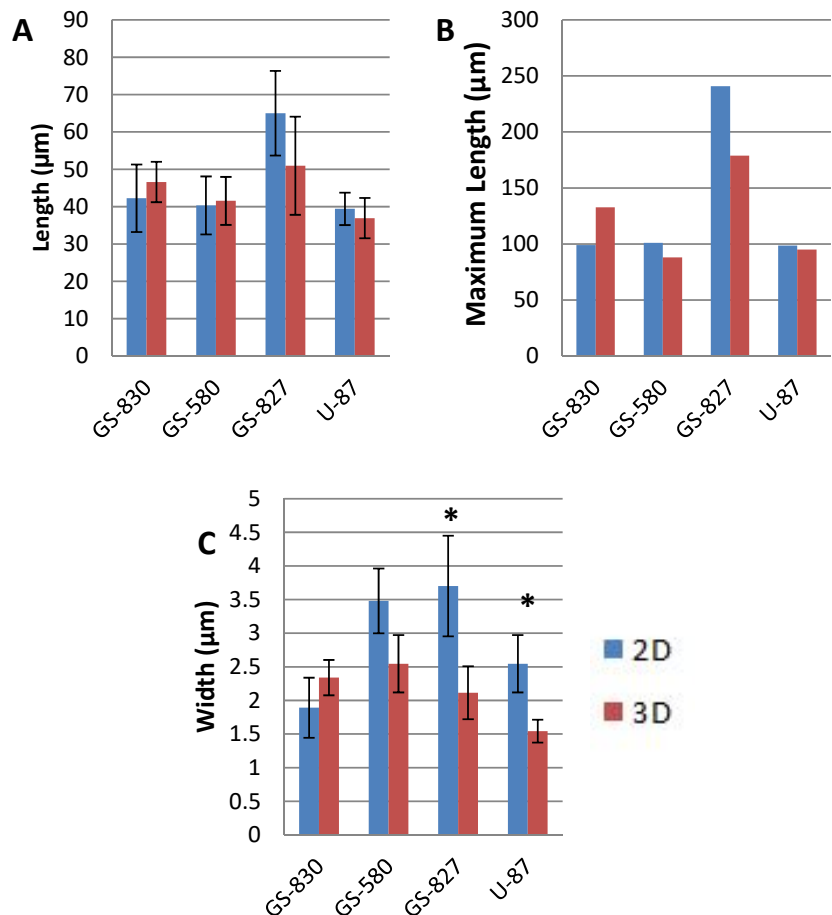


Figure 43: Means and 95% confidence intervals for A) Average protrusion length B) Maximum detected length of the tube-like cellular protrusions C) Average protrusion width

6 DISCUSSION

6.1 3D-ENGINEERED SCAFFOLDS AS A GBM CULTURE MODEL

The designed 3D-engineered scaffolds provide cell culture platforms that are reproducible, low auto-fluorescent, non-cytotoxic, steady, and compatible with cell culture protocols. Higher number of cell colonization was observed for most 3D scaffolds compared to the 2D surfaces showing that the cell colonization was not hindered in the scaffolds. Because of the higher volume of the scaffolds, this observation was not surprising.

In some experiments, GBM primary cells appeared round, isolated, and unable to colonize the 2D plates. However, the cells were colonizing the scaffolds in higher numbers and were forming more protrusions. Confocal images in Appendix 11.10 show these observations but they were not pursued in this research and are subjects for future studies.

The 3D-engineered scaffolds, fabricated by 2PP, have been used with other cell types such as neuronal cells⁶¹. There is only one report of such structure being used for GBM cells by Tricinci et al (2020). Their scaffolds were fabricated with a different material (IP-L), had a spherical geometry, and were tested only with a standard cell line⁷⁵. In our research, for the first time, in addition to a standard cell line, three primary cell cultures successfully colonized the scaffolds. Tricinci et al (2020) reported the successful cell colonization but the appropriateness of their model was unclear because they did not provide details about cell characteristics such as morphology and gene expressions. In our research, we first compared the functionality of our 3D structures with the more established 2D models before using them for treatment assessment. One difference between our model and the model used by Tricinci et al (2020) was that they modified the IP-L material to be magnetic, thus allowing the structures to be movable with an external magnet. Their goal was to easily move the spherical scaffolds closer to their BBB model so that they could study the effects of different drugs. Such a goal was not part of our research requirements and was not pursued at this time.

We used a structure with a fixed cubic unit cell as the base element to control geometrical features, such as rod and pore sizes, and keep them constant within each scaffold. This choice reduced the number of model parameters to a manageable level and will allow us to study the effects of these parameters on the behavior of GBM cells in the future. Moreover, the size of the entire scaffolds could be easily increased or decreased by adding or removing cubic unit cells. Lastly, the pyramid geometry of our design allowed for better imaging of different scaffold layers.

One drawback of these scaffolds is that they do not have the similar stiffness to the brain tissue as the Young's Modulus of IP-Visio is considerably larger than that of the brain. Thus in this regard, the scaffolds do not fully mimic the natural GBM environment and cannot outperform gel-based culture models.

6.2 CELL MORPHOLOGY

In general, cells in the 2D plates had flatter morphologies compared to those in the 3D scaffolds. This finding is consistent with previous research⁶³. The morphological differences, especially those related to

the nuclei, can be important in replicating the behavior of GBM cells *in vivo*. For example, Nafe et al (2005) showed the survival time for GBM patients has a relationship with the nuclei circularity and area¹⁸. Culture platforms that can more accurately capture the cell morphology, such as the proposed 3D scaffolds, can lead to more realistic cell culture environments *in vitro*.

Analyzing and comparing cellular morphology showed that cells had different nuclear area and nuclear circularity in 2D and in 3D structures. Boruah et al (2013) report that the average nucleus area of high grade gliomas is $55.22 \pm 13.48 \mu\text{m}^2$ ¹⁹. In their study, 16 biopsies of 16 GBM patients were used and analyzed on 5 μm thick CNS tissues. As seen in Figure 33B, the measured nuclear area in our 3D scaffolds was closer to what was measured by Boruah et al (2013) representing the actual GBM nuclear area.

In this document, the nuclear area is the 2D representation of the Z projection of 3D images. Therefore, the larger nuclear area observed in 2D plates could either represent larger nuclear volume or a nucleus that has a flatter shape. The latter explanation seems to be more likely as it is more consistent with previous research. Nevertheless, the data show the cellular nuclei in 2D and in 3D structures generally have different shapes. The same argument is also applicable to the cell area.

6.3 EGFR EXPRESSIONS

Overall, the use of 3D scaffolds did not drastically change the expression of *EGFR* in any of the experimental conditions when compared to the 2D plates. However, the 3D scaffolds offered the possibility of studying *EGFR* expressions in single cells connected in a network of cells without the errors caused by the cell overlap.

In the initial experiments, in the presence of EGF, the *EGFR* signal was unexpectedly high near the nuclei and was in the form of spot-like entities. This was surprising because *EGFR* is a transmembrane protein and was expected to be observed as a signal mainly on the membrane. One possible reason for these observations is that the *EGFR* spots are activated and internalized so that they can be degraded, recycled, or possibly to serve another unknown or lesser-known function. It was further observed that *EGFR* internalized spots were more concentrated near the cell nuclei and had a higher mean grey value in most experiments. This may be related to a feature of *EGFR* that needs further studying.

The reaction to inhibitors in this project is noteworthy and deserves more research. As mentioned in section 2.1.2, *EGFR* inhibitors fail to function as an effective treatment in clinical settings despite successful results *in vitro*. This seems to be the first case of reported *in vitro* failure of *EGFR* inhibitors to stop *EGFR* internalization for GBM cells. More research on this topic may lead to the discovery of root cause of unsuccessful clinical trials. Some of the frequently named reasons for unsuccessful *EGFR* inhibitor treatments in clinical settings are: secondary mutations, poor BBB penetration, and dose limitations²⁴. In this research, the inhibitors failed to stop *EGFR* internalization even in the absence of these factors, which may point to a more fundamental reason for unsuccessful clinical results.

In a study on Hela cells, Scharaw et al (2016) described a reaction that was similar to our observations with GBM cells when EGF was added to the cell culture media⁸⁸. They reported the depletion of *EGFR* on membranes and also the formation of *EGFR* spot-like entities in the inner cellular layers. In their experiment, increasing the EGF concentration (from 1ng/ml to 200ng/ml) showed higher *EGFR*

expressions near the nuclei (Figure 44). For comparison, *EGFR* concentration was 20ng/ml in our research. Our findings are consistent with those of Scharaw et al (2016)⁸⁸.

In another study, researchers reported the detection of *EGFR* near the nuclei for breast cancer cells⁸⁹. In that study, higher expression of *EGFR* near the nuclei correlated with the tumor size and with poor patient survival. *EGFR* was detected near the nuclei for %40 of the samples. Similarly in another study, *EGFR* was detected near the nuclei in ovarian cancer cells⁹⁰. In that research, nearly 28.3% of the samples had *EGFR* near the nuclei. High levels of *EGFR* near the nuclei correlated inversely with the overall survival. These results show the potential importance of *EGFR* localization near the nuclei in cancer studies. Since only a limited percentage of tumor cells have been reported to have *EGFR* spots near the nuclei, it is important to include a large number of primary GBM cultures in such studies.

Recently, researchers have also shown more interest in studying the unidentified functions of *EGFR* for different tumors²⁵. More research regarding the role of localized *EGFR* near the nuclei is needed particularly for GBM. Such studies may broaden our understanding of GBM resistance to *EGFR*-targeting inhibitors. The established setup with the 3D-engineered scaffolds can be used to further study the *EGFR* mechanisms and reactions to *EGFR* inhibitors in GBM.

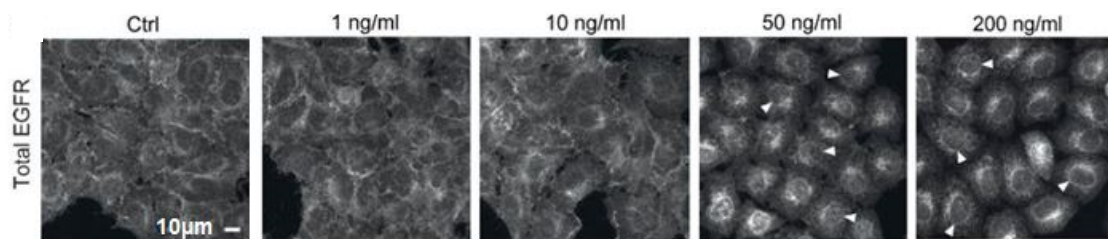


Figure 44: *EGFR* expressions in response to different *EGF* concentration. HeLa cells exposed to 1, 10, 50, or 200 ng/ml *EGF* in media for 18 hours⁸⁸

6.4 MICROTUBE FORMATIONS

Protrusive structures with geometrical features of nanotubes and microtubes were observed on both the 3D scaffolds and 2D plates. The recorded length of the microtube-like protrusions was not significantly different in 2D plates versus 3D scaffolds. However the width of these protrusions was larger on 2D compared to 3D structures. This can point to the more flat morphology of cells in 2D settings. The average protrusion width in the 3D scaffolds was closer to the *in vivo* value of 1.7µm reported by Osswald et al (2019)⁶.

In this study, GAP-43 (biomarkers for microtubes) did not seem to exclusively localize the tube-like protrusions. Instead, it was detected throughout the cell membranes and was more prominent at the end cone of protrusions (Figure 42). This appears to be consistent with how GAP-43 is distributed through GBM cells in the images published by Osswald et al (2015)²⁶. Figure 45A shows an example of the expression of GAP-43 in microtubes captured by Osswald et al (2015) *in vivo*. In their study it is hard to distinguish microtubes from other cell cytoskeletal features as their images are not as magnified. The lack of high resolution *in vivo* images in Osswald et al (2015) study prevents adequate comparison of their findings with ours. In a recent research, Pinto et al (2021) also used GAP-43 to identify microtubes in 3D GBM tumor organoids³⁵. Figure 45B shows GAP-43 expression in the detected microtube-like

protrusion in their study where the GAP-43 seems to be more exclusive to the tube-like protrusion. In our experiments however, there was not a distinct difference between GAP-43 expressions among cells (compare Figure 45B to Figure 42A). Our observation may challenge the claim by Osswald et al (2019) that GAP-43 is a biomarker for micro and nanotubes in all GBM cultures⁶. Other possibilities are that the observed protrusions in our experiments were not true microtubes or nanotubes, or the staining and imaging were faulty, or that microtubes were expressed too frequently in our structures.

Comparing our results (Figure 45D) to the microtubes formed *in vivo* (Figure 45C), to 2D *in vitro* models (Figure 45E), and to the other 3D *in vitro* model (Figure 45B), the protrusive structures detected on our 3D-engineered scaffolds better match the morphology of microtubes *in vivo*. Moreover, the microtube-like protrusions seem to be more abundant in our 3D-engineered scaffolds compared to the GBM organoids (compare Figure 45B to Figure 45D).

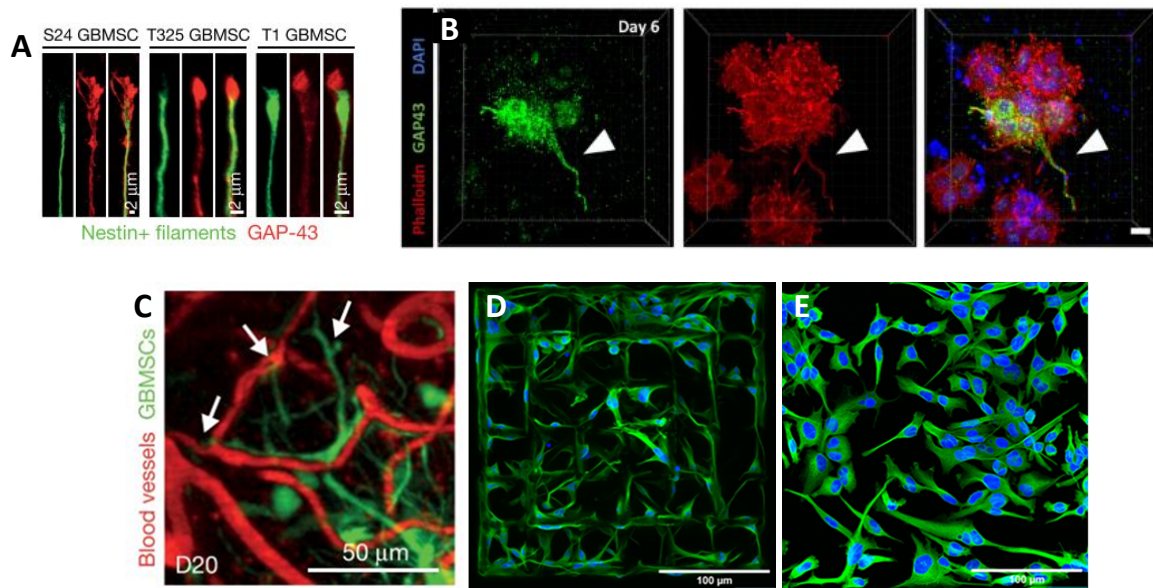


Figure 45: A) GAP-43 localization at the tip of the microtubes in GBM cells²⁶ B) Microtube-like protrusion expressing GAP-43 in GBM organoids³⁵ C) Microtubes formed *in vivo* in mouse brain²⁶ D) Microtube-like protrusions observed in 3D-engineered scaffolds E) Protrusions observed in 2D plates

Due to the insufficient literature in this topic and the lack of exact definition for these protrusions, identification of microtubes and nanotubes is still challenging. The results of our research are promising but need further validation. To categorize these protrusions as microtubes and nanotubes, their functionality can be tested. This is discussed in more detail in Section 8.

6.5 LIMITATIONS

6.5.1 Data Limitations

In this project, experiments were repeated to demonstrate consistency and repeatability of the results. However, repeated measure statistical analysis was not possible because all cell cultures were not always performed at the same time and variables could not always be strictly controlled between different experiments. Doing that required additional time that exceeded this project's timeline.

The experiments were performed with only three primary cultures. To better capture the heterogeneity of GBM, especially regarding microtubule formation and *EGFR* expression, data from higher numbers of primary cultures are needed.

6.5.2 Model Limitations

The 3D-engineered scaffolds focused on mimicking the geometry of the natural environment of the GBM. The model was not accurate in mimicking the composition of the tumor environment. For instance, mono-culturing of GBM cells is an over-simplification that fails to account for the influence of non-tumor cells such as neurons, endothelial cells, and immune cells⁴. Furthermore, effects of different geometrical features, such as rod diameter, pore size, were not separately investigated. In addition, composition and geometrical features were kept constant throughout the structures, which do not fully represent the heterogeneity of the tumor environment.

Morphology, microtubule formation, and *EGFR* expression evaluations are not the only ways to assess this model. More methods to evaluate whether the scaffolds better represent the tumor environment, such as gene expression and response to different treatments assessments, exist and should be further investigated.

Moreover, in this study, the 3D engineered scaffolds were only compared to 2D surfaces, and not to the other 3D models. Lastly, both 2D and 3D models were fabricated with IP-Visio and therefore the effects of the material on cells were not separately studied.

6.5.3 Imaging

The top layers of the scaffold blocked the signal from the inner units in confocal imaging. This caused features such as the cell count and protrusion length in 3D to be underestimated. Increasing the laser intensity to gain more insight resulted in the bleaching of the outer units.

After 3 days of culture, it was difficult to identify the number of individual cells on the 2D structures because of the high cell density. Unfortunately, fixing the cells at an earlier time before reaching high confluency was not an option as this greatly reduced cell colonization before the cellular network could be fully formed.

Occasionally the intensity of the fluorescent signal from a fixed region changed slightly from one image to another with no change in microscope settings. This could have affected the mean grey value measurements for the nuclei (section 5.3.1) and for the *EGFR* spots (section 5.4). Fixing this issue was beyond the scope of this project. The result might have been slightly different if the images were taken with a microscope with less signal variations.

SEM allowed for imaging the inner parts of the scaffolds and provided high resolution images of cell features such as nanotubes in 3D scaffolds. However, SEM did not provide detailed images of cells cultured on 2D surfaces for studying their morphologies or protrusions. Nanotube-like protrusions were not detectable in 2D plates due to the merging of cell boundaries with the plate surfaces in SEM images.

6.5.4 Image and Data Analysis

For image analysis, 2D image analysis programs with Z projections of 3D image stacks were used. The reason for this decision was that 3D image analysis programs did not accurately estimate the cell parameters. Meanwhile, the 2D analysis method resulted in misestimating the size of cellular features by increasing the cell overlap, which falsely merged cells into a unit. Moreover, using 2D projections could have affected some cell feature measurements that extend to different Z layers. Furthermore, the overlapping of cells made it more challenging to detect if the tube-like protrusions were open-ended or if they connected multiple cells.

Better 2D and 3D analysis programs are needed for *EGFR* expression and microtubule detection quantifications that involve less manual work. Such programs will lower the random human errors and the systematic subjective errors.

In this analysis, the number of cells was quantified by counting the number of nuclei assuming that each cell had only one nucleus. Although on average this assumption is mostly true, for primary cells it may not always be the case. Even though more cells appeared to be multinuclear in the primary cells, this could not be reliably quantified due to the high seeding density and the lack of membrane staining.

We quantified the *EGFR* expression differences for different experiments in Figure 39 using the normalized frequency of the *EGFR* spots. However, this variable does not accurately capture the observed image differences thus preventing statistically meaningful comparison of the results. For example, the 95% confidence intervals in section 5.4 were large because 1) *EGFR* expressions were highly variable among different scaffold units, 2) *EGFR* expressions were highly variable among individual cells, 3) the number of images was small due to time limitations, and 4) the underlying variables were assumed to have normal distributions, which is unlikely to be the case. A better quantification of what was observed in Figure 38 and more appropriate statistical tests are needed but were not carried out due to time limitations.

7 CONCLUSIONS AND RECOMMENDATIONS

In this research, for the first time, primary GBM cells were successfully cultured on 3D-engineered scaffolds. The fabricated scaffolds are reproducible, steady, biocompatible, and have the needed dimensional accuracy. Furthermore, these scaffolds are compatible with common laboratory substances such as alcohol and cell culture media. The low-auto-fluorescence nature of the material makes fluorescent microscopy possible. The use of ITO-coated glass as the substrate beneath the scaffolds does not prevent the light transfer. This makes the set up appropriate for both upright and inverted microscopy. A set-up for confocal fluorescent microscopy and SEM was developed that allowed high-resolution imaging of the cells.

The U-87 standard cell line as well as all three primary cell cultures successfully colonized the scaffolds. The use of primary cells allowed studies of *EGFR* expression, showed more variety in cellular morphological features, and demonstrated cell features closer to those observed in actual GBM tumors.

As for the research question, morphological features of the cells were more similar to the actual tumors in 3D-engineered scaffolds compared to the 2D models. This shows that the 3D-engineered scaffolds better replicate the actual GBM cellular environment in terms of geometry. Morphological features such as the nuclei area and the protrusion width were shown to be closer to the *in vivo* experiments on the scaffolds compared to those on the 2D plates.

The question of whether the microtube formation can be captured on the scaffolds or on the 2D plates is still inconclusive. Based on the described dimensions of the microtubes and nanotubes, similar protrusions have been observed both in the 2D and in the 3D models. The morphology of the tube-like protrusions observed in 3D scaffolds better mimics what is described *in vivo* as microtubes compared to those formed in 2D plates or even in other 3D culture models such as tumor spheroids. However, we were unable to confirm presence GAP-43 exclusively within these protrusions, which was previously reported as a biomarker for microtubes⁶. The next step is to investigate the functionality and connectivity of these protrusions. This topic is further discussed in section 8. Currently, microtubes seem to be an emerging concept that is still not fully defined. Thus, identifying these protrusions as microtubes or nanotubes seems premature at this time.

In this study, *EGFR* expressions were not significantly different for the 2D plates and the 3D scaffolds. This finding needs further research by repeating the experiments at different points in time or by tracking individual *EGFR* spots. Another recommendation is to use primary cells directly from the operating theatre to see if *EGFR* amplification can be retained. Regardless, *EGFR* expressions as spot-like entities residing near the cell nuclei require more investigation. Furthermore, the reaction of the GBM cells to the *EGFR* inhibitors *in vitro* was unexpected and has not been reported previously. To date, most *in vitro* *EGFR* inhibitor tests have been successful and the unsuccessful results have been limited to *in vivo* experiments. In this study, the inhibitors failed to stop *EGFR* internalization even in the absence of factors such as cell mutations, drug penetration issues, and dose limitations. This observation may indicate presence of un-recognized *EGFR* functions or un-identified mechanisms specific for GBMs that can explain how cells cope with inhibitors.

One recommendation is to reduce the variability by repeating the experiments and producing additional images. That will increase the power of statistical analysis. Future studies should include more repetitions of the experiments. It is also recommended to carry out the experiments with more primary cell cultures. Lastly a better quantification method for *EGFR* expression evaluation is recommended. One possible solution is to count the cells that have high amounts of internalized *EGFR* spots near the nuclei and use them for comparison.

Table 6 lists the advantages and disadvantages of using 3D-engineered scaffolds versus using traditional 2D models. The first advantage of the scaffolds is that they better replicate the actual environment of the GBM cells in terms of geometry. Cell-to-cell connections or cell-to-ECM connections are not limited to 2D networks when using 3D-engineered scaffolds. This is the basis for closer resemblance of the morphological features of GBM in 3D scaffolds to *in vivo* conditions. Furthermore, scaffolds offer the opportunity to study individual cells while they are in a connected cellular network. In 2D models, by the time cells form a cellular network, the formation and overlap of cell clusters make studying individual cells difficult. Lastly, the 3D-engineered scaffolds offer a more advanced platform for cell culturing, which can be modified and improved to better replicate the actual environment of the GBM cells. For instance, co-culturing of endothelial cells or T-cells could increase the similarity to *in vivo* conditions. This topic is further discussed in section 8.

One disadvantage of 3D-engineered scaffolds is that their fabrication is more time-consuming and more expensive. The limited access to the fabrication material and the 2PP printers makes the scaffolds less accessible. Currently, it is not possible to manufacture 3D-engineered structures in high quantities. Lastly, compared to the 2D models, the 3D engineered scaffolds require more complex imaging setups. Another limitation in using these scaffolds is the inaccessibility to the inner layers.

Overall, the 3D-engineered scaffolds provide a new model with advantages that are difficult to capture in commercially available 2D models. 2D models fail to accurately represent the GBM tumor behavior. The 3D-engineered scaffolds have the potential of better replicating the GBM's natural environment and better predicting GBM's reaction to different treatments. More research is needed to identify additional benefits and drawbacks of 3D-engineered scaffolds and to define if they can better represent the behavior of GBM tumors. The 3D-engineered scaffolds can be particularly useful in experiments where high-resolution imaging, morphological analysis, studying cellular connections, and understanding network formations are important. This research introduces the 3D-engineered scaffolds as the building block of what can become a more accurate and complex 3D cell culture model for GBM tumors.

Table 6: List of advantage and disadvantages of using 3D-engineered scaffolds versus 2D traditional flat plates

Advantages	Disadvantages
Closer resemblance to actual environment of the cells (in terms of geometry)	More time-consuming and expensive
The opportunity to study cells individually while in a cellular network	No high-throughput fabrication capability at this time
Opportunity for model growth	Requires a more complex set-up for imaging

8 FUTURE WORK

Future work should include further study of the microtubule formations on the scaffolds. The functionality of the protrusions can be investigated to identify the microtubules. As stated earlier, microtubules and nanotubes are claimed to be cellular protrusions contributing to a functional cellular network. One function of these protrusions is their ability to transfer mitochondria between cells⁹. This function can be examined by using live cell imaging and marking of mitochondria with a GFP stain³⁵. Another function is the propagation of inter-cellular calcium waves²⁶. Their assessment is also possible with live cell fluorescent imaging and by using small molecule calcium indicators such as Rhod-2AM²⁶. Cx43 gap junctions are claimed to connect microtubules²⁶. Cx43 can also be quantified by immunofluorescence microscopy. However, similar to GAP-43, there is not enough research to clarify if Cx43s are specific to microtubule connections. Nonetheless, they can be useful in assessing cellular connectivity.

One of the noteworthy observations in this research was the formation of *EGFR* spots in the inner cellular areas and their reactions to *EGFR* inhibitors. Continuing these experiments may add to our understanding of unidentified *EGFR* functions and mechanisms that make GBMs resistant to *EGFR*-targeting treatment methods. To observe whether the internalized *EGFR* spots are indeed activated and phosphorylated, GBM cells can be stained with anti-phospho-*EGFR* stains and imaged with confocal fluorescent microscopy. Furthermore, live imaging of the *EGFR* spots and tracking their transfer to the nuclear area may add to our understanding of their functions. For this, eGFP-tagged *EGFR* constructs need to be (stably) transfected into the primary cell cultures. The experiments can be repeated at different points in time and with more advanced *EGFR* inhibitors. In addition, the effects of other *EGFR* targeted therapies, such as monoclonal humanized antibodies that target the extracellular domain of *EGFR*, can also be studied²⁵. It would be interesting to investigate whether targeting the extra-cellular domain can stop the *EGFR* internalization. As stated before, targeting lung cancer tumors with the *EGFR* inhibitors has been successful both in clinical and experimental settings. Therefore, repeating these experiments with lung cancer cells and comparing the results to the results of GBM cells, may offer more insight on the differences between these two types of cancer and clarify why *EGFR* inhibitors fail to be effective in GBM treatments.

To further mimic the *in vivo* culture environment of the GBM cells, the culture model based on the 3D-engineered scaffolds can be further developed. The 3D-engineered culture models can be improved by co-culturing of GBM cells with other non-tumor cells, for example, the endothelial cells that are adjacent to the GBM cells in their natural environment. In addition, co-culturing may include immune cells, such as T-cells, that are predicted to have a role in GBM prognosis. Moreover, other features of the natural cellular environment, such as the BBB, can be replicated by the 2PP fabrication method⁷⁵. Also, the effects of different geometrical features on the behavior and morphology of GBM cell should be considered. These features include the pore size, the surface nano-topography, and the rod size. One of the limitations of the current scaffolds is their relatively high stiffness. Developing materials for 2PP with lower Young's modulus or structures in which cells perceive a lower stiffness can be further investigated. One example of lowering the local stiffness is to add surface nano patterning⁴⁹.

Lastly, the current commonly available 3D image analysis programs fail to offer adequately accurate analysis of 3D images. In this research, it was observed that making Z projection of images and analyzing the results in 2D settings still offered better estimation of the properties of the cells. There is a need for more advanced 3D image analysis programs to be combined with the 3D cell culture models. Two candidates for such an analysis is Imaris Microscopy Image Analysis Software.

9 SELF-REFLECTION

This project opened my eyes to two of my main research approach flaws. First, I tend to jump to conclusions based on a few observations and make strong claims without having enough statistical data to back up my claims. Second, because of my lack of knowledge in biological topics, I use terminologies for describing observations that in biology have specific meanings different from what I aim to describe. The guidance of my supervisors has made me more aware of these issues and I have tried to avoid them in this report. However, I am aware that there is room for further improvements.

For me, the most challenging aspects that still require further improvements were the image and data analysis. My lack of knowledge in image analysis and biostatistics complicated the interpretation of results and led to unproven claims. With the help of my supervisory team, I have gradually become aware of these issues. I am now highly motivated to take part in advanced image analysis and statistics courses to build a solid foundation for future studies.

Another topic that could have been addressed more effectively was the topic of microtubule formations. As stated before, the microtubule formation is an emerging concept supported by few research papers. I may have put too much emphasis on the claims of those papers. This taught me to have a more critical outlook on the available literature and also on my own assumptions.

Managing the experiments and the timeline of the project became of high importance after literature survey. I gradually learned that experiments tend to take more time than initially assumed. After obtaining suitable experimental setups, most of my time was spent on imaging and repeating experiments. I later realized that I should have better planned the experiment from the start of the project. In the beginning, keeping track of all the images and the quantified data was challenging. Gradually I developed better skills to organize the images and the quantified data.

Time limitations caused me to narrow the initial scope of my project. For example, initially it was decided to study the effects of rod size on cells as a sub-question. Thus, three scaffolds with different rod diameters were planned to be built. Later due to time limitations it was decided to focus only on comparing 3D scaffolds to 2D plates without separately studying the effect of specific geometrical features. I realize that if I had narrowed my topic further, I could have provided more specific conclusions. Preliminary results also directed the experiment planning. For instance, observing the *EGFR* spot formation was noteworthy and led to including experiments with *EGFR* inhibitors.

Furthermore, some experiments were carried out but were not included in this report because they were unsuccessful. One example is that we tried to perform live imaging of cell colonization. This experiment failed because the cells could not be kept alive with the microscope that was being used. Some test parameters such the cytotoxicity of the glues (used to keep the substrates in place), were also considered as a cause of this failure. Since our findings pointed to the microscope as the main reason for the failure, it was decided that fixing this issue was not a priority. Another example is that to find the reason behind the blockage of scaffold pores during fabrication, variables such as development time were investigated. It was found out that the blockage was not due to un-polymerized resin not being washed away and thus the development time did not solve the issue. Last example was using a phospho-*EGFR* antibody to detect phosphorylated *EGFR* spots. The resulting images showed that either

the staining had been performed poorly or the antibody had expired. Nevertheless, it was decided further investigation of this topic was not a priority. Even though these experiments were not used in this report, they still broaden my understanding of the topics and the findings may be useful in future works. For instance, working with the Opera inverted microscope showed us that the ITO-coated substrates let the light pass through and are suitable for inverted microscopy.

During the starting weeks of the project, I was worried by my lack of knowledge and the amount of new information particularly on biological topics. I am happy that with the help of my supervisors I was able to gradually acquire enough knowledge to work on the project and gain an understanding of the direction of the research plan. Working with a multi-disciplinary team on topic spanning biology and high-tech engineering has always been an interest of mine. This has been an important milestone for me that showed me with scientific attitude and a curious mindset it is possible to learn new disciplines and work with experts from different fields in such multidisciplinary research.

This project has been a valuable journey for me where I have been able to use my academic knowledge to address a real-life problem. I hope that this research will lead to better diagnosis and treatment of GBM patients. I am deeply indebted to my supervisors for the trust they put on me and for their continued support through all stages of the project. It has been a privilege to be a part of this dynamic research team and to have the opportunity to participate in state-of-the-art projects.

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11 APPENDIX

11.1 FABRICATION PROTOCOL

Scaffold Fabrication Protocol

- It is highly advised to preform sample preparation, pre-treatment, printing, and sample development back to back.

Sample Preparation:

1. The resin should be placed on the ITO-coated side of the substrate. Find ITO-coated side by using an ohm-meter. The ITO-coated side will read the resistance of 100Ω to 400Ω. The side that is not coated will have a very large resistance, thus will read OL. From this point onward always keep the ITO-coated sides facing upward. The sample preparation steps and pre-treatment should be applied to this side.
2. Clean the substrate with a fine tissue and rinse the substrate with acetone followed by IPA.
3. Either air-dry the substrate or blow-dry it with the nitrogen or air gun, so that moisture is removed from the substrate surface.

Pre-treatments:

1. Further clean the substrate by exposing the ITO-coated side to oxygen plasma. This step will prepare the surface of the samples for silanization.
Use the plasma cleaner by Diener Femtois available at TU Delft, PME Labs, MNE lab, main room (F-0-310) .
 - a. Put the samples (ITO-coated side facing up) on the tray inside the plasma cleaner.
 - b. Follow the Diener Femto Plasma Cleaner Quick Guide for instructions.
 - c. Expose the samples to oxygen plasma (represented as **Gas 1**) for the duration of **10 minutes** and with maximum power (the maximum power available is 80% of 100W).
 - d. Set the gauge of *Gas 1* to 5-6 sccm.
2. Immediately after oxygen plasma exposure, the samples should be put in a silanization solution. Therefore, start silanization solution preparation, 5 minutes before the exposure time runs out.
 - a. Mix 600μl of 3-(trimethoxysilyl)propyl methacrylate (MAPTMS) with 30 mL ethanol in a glass petri-dish⁸¹.
 - b. Put the substrates in the petri-dish (ITO-coated facing up). The solution should completely cover the surface of the substrates.
 - A 10 cm petri-dish covers 9 substrates and the 30mL solution is enough to cover the surface of all substrates.
 - c. Cover the petri-dish with a lid and let the substrates merge in the silanization solution for 1 to 2 hours.
 - d. Take the samples out of the petri-dish and rinse them first with action and after with distilled water.
 - Provided that the silanization steps are performed correctly, the substrate should have become hydrophobic.
 - e. Let the substrates air-dry for 5 to 10 minutes until no moisture is visible on their surfaces.

Printing:

1. Place the substrates on the sample holders (ITO-coated side facing up).
2. Ensure the substrates are fixed on their place with using tape.
3. Put a drop of IP-Visio resin in the middle of the substrates.
 - a. Try to avoid creating bubbles in the substrate. If bubbles are formed, remove them with using pipets or sharp tweezers.
 - b. Don't let the resin be exposed to UV-light.
4. Put the 25x objective inside the Nanoscribe machine.
 - a. Before placing the objective, make sure a felt ring is put on the objective.
 - b. Make sure the immersion adjustment ring of the 25x objective is set at the correct position : *Glyc ///*
 - c. Make sure the objective is at its lowest position.
5. Initiate the Nanowrite software.
6. Place the sample holder inside the Nanoscribe machine.
 - a. Make sure the objective is at its lowest position by clicking the "exchange holder".
 - b. The ITO-coated side should face down (face the objective)
 - c. Select the used sample holder type and the desired sample position.
7. Follow Nanoguide instructions for Printing:
<https://support.nanoscribe.com/hc/en-gb/articles/360001342793>
 - a. Printing each 3D-engineered scaffold takes nearly 25 minutes and each 2D plate takes nearly 5 minutes.
8. After printing is finished, press exchange holder to unload the substrates.
9. Follow Nanoguide instructions to clean the objective.

Sample development:

1. After fabrication put all samples (ITO-coated side facing up) immediately in PGMEA for duration of 25 minutes.
 - a. Samples could be developed horizontally or vertically.
 - b. Put Maximum of 5 substrates in a 10 cm petri-dish at once.
 - c. If needed renew the PGMEA solution.
2. After 25 minutes of PGMEA exposure, place the substrates (ITO-coated substrates) in IPA for 5 Minutes.
3. Leave the substrates in the fume hood to air-dry.

Post-treatments:

1. To roughen the surface of the structures, expose them to oxygen plasma.
 - a. Put the samples (ITO-coated side facing up) on the tray inside the plasma cleaner.
 - b. Follow the Diener Femto Plasma Cleaner Quick Guide for instructions.
 - c. Expose the samples to oxygen plasma (represented as **Gas 1**) for the duration of **30 seconds** and with maximum power (the maximum power available is 80% of 100W).
 - d. Set the gauge of *Gas 1* to 5-6 sccm.
2. To be able to fit the substrates inside 6-wells plates cut the corners of the substrates with a diamond pen glass cutter.
 - a. For this step, two tweezers are needed.
 - b. Hold the substrates with one tweezer and draw a diagonal scratch on each corner of the substrate (a scratch with nearly 5mm length)

- c. Hold the substrate with one tweezer and break the corner with the second tweezer at the location of the scratch.
 - d. Discard the broken glass in a petri-dish specified for broken glass in the MNE lab.
- 3. To quickly check the quality of the fabrication and separate faulty samples use Keyence optical microscope.
- 4. To avoid sample movement during transportation, place tape on bottom of a petri-dish and glue the substrates (from the glass side) to the petri-dish.

11.2 CELL CULTURE PROTOCOL

11.2.1 Standard Cell Lines

Protocol copied from the cell culture protocol developed and provided by EMC Neuro-Oncology laboratory.

- DMEM++ = DMEM medium + 1% Pen/Strep + 10% FBS
- ECM coating = Cultrex® 10X Spheroid Formation ECM → Trevigen, Catalog Number: 3500-096-01

Sterilizing + coating of scaffolds:

1. Put scaffolds in well
2. Wash 2 mins with 70% EtOH
3. Wash 2x with PBS
4. Add ECM Coating 1:100 (diluted in DMEM++), incubate 30+ mins @ 37 degrees stove
5. Add cells (2 ml cell suspension in 5cm petri dish (19.63 cm²) or 0.9 ml cell suspension in 6 wells plate (9.6 cm²))

Culturing:

1. Remove medium
2. Wash cells with PBS
3. Add 1,5 ml Trypsin, incubate +-5 mins @ 37 degrees stove
4. Add 8,5 ml DMEM++ and make a cell suspension (10 ml total)
5. Plate out cells on scaffolds

Some notes:

- This is the protocol for non-primary cells. The medium and Trypsin are dependent on the cell type being cultured and can be changed.
- ECM is needed for the primary cells, but can be excluded when culturing the cell lines. In our experiments all cultures were performed in the presence of the ECM coating.

11.2.2 Primary Cells

This section consists of four protocols: ECM coating, storage, medium, and passaging.

11.2.2.1 ECM Coating

Protocol copied from “Protocol 2.1 - Primary Culture - ECM coating (NECH)” developed by EMC Neurosurgery laboratory.

PROTOCOL 01 – Coating culture flasks with ECM (NS cultures only)

ECM (Extracellular matrix)

Cultrex – Trevigem

#product

Only performed by Tessa, Cassandra and Trisha. If stocks are almost empty, ask one of them to prepare new stocks.

ECM stock preparation:

1. Thaw 5 ml vial of cultrex overnight in ice at 4°C
2. Dilute 1:10 in plane & cold DMEM-F12 (5 ml cultrex + 45 ml DMEM-F12)
3. Aliquot 10 ml in 15 ml bluecap tubes and 0,5 ml in Eppendorf tubes
4. Store at -20°C

Coating flasks:

1. Wash flask with appropriate amount of PBS (2-5 ml) to remove electrostatic tension.
2. Remove PBS.
3. Dilute the ECM with NS medium (remember: stock is already 1:10).
4. Add 1:100 diluted ECM in the appropriate amount (see table below).
5. Write initials and coating sign on the upper right corner of the flask.
6. Incubate at 37°C for 30 minutes.
7. Coated flasks can be stored up to 7 days at 37°C, and up to three weeks if stored at 4°C.

		96-well	48-well	24-well	12-well	6-well	T25 flask	T75 flask	T175 flask
general info	cm2	0,34	1	1,9	3,9	9,6	25	75	175
	working volume	100 - 150 µl	500 µl	1 ml	2 ml	3 ml	3,5 ml	10 - 15 ml	20 ml
coating	PBS rinse	NA	0,5 ml	0,5 ml	1 ml	1 ml	2 ml	5 ml	10 ml
	ECM	40 µl	40 µl	50 µl	50 µl	100 µl	300 µl	500 µl	1 ml

11.2.2.2 Storage

Protocol copied from “Protocol 2.2 - Primary Culture - Starting cultures from storage (NECH)” developed by EMC Neurosurgery laboratory.

PROTOCOL 02 – Starting new cultures from -80°C or N2 storage

Preferably start in the beginning of the week so you can follow the growth process during the week.

1. Take vials from -80°C freezer or N2.
 - a. Don't forget to cross out the sample in the sample overviews!
2. Place at RT until thawed (or place in 37°C water bath for 1 minute).
 - a. If thawed you should immediately proceed, since DMSO is toxic for the cells
3. Transfer mixture of cells/DMSO to a 15ml bluecap tube.
4. Slowly (dropwise) add 5 ml of the appropriate medium (to reduce osmolysis).
5. Rotate tube thrice and spin down at 1500 rpm for 3 min.
6. Remove supernatant.

7. Add 3,5 ml of medium and loosen cell pellet by carefully pipetting up and down till it is a homogeneous suspension.
8. Transfer to T25 culture flask.
 - a. For NS cultures this should be an ECM coated flask (see protocol _01).
 - b. Copy the passage number from the vial to the T25 flask
 - c. If the cell pellet is large enough and it is a very stable and fast growing cell culture you may start in a T75, but then the passage number is +1
9. Place flask containing cells in incubator.
10. Sign the culture labjournal with the following information.
 - a. Date + initials
 - b. Write down "started from -80°C / N₂
 - c. Name culture + passage number
 - d. Date it was frozen + initial previous user !! – **don't forget this!**

Example:

- EMC 61 NS WB p14 (20-2-15, JK)
 - EGT 18 NS pen. WB p17 (14-5-14, TP)
 - EMC 125 NS WB p17 (15-4-15, AP)
 - EGT 33 NS WB p13 (4-8-15, TP)

} Started from N₂

Check the confluency the next day

11.2.2.3 Medium

Protocol copied from "Protocol 2.3 - Primary Culture - Medium (NECH)" developed by EMC Neurosurgery laboratory.

PROTOCOL 03 – Medium

Neurosphere medium (NS-Medium)

A full NS medium is stable for one month. Prepare a 50 ml tube containing the supplements below if you don't have a lot of cultures (you can prepare a few tubes in once). In this case, add 5 ml Pen/Strep to a 500 ml DMEM-F12 bottle (this is stable for a very long time) and use this solution to make the whole medium by adding the remaining supplements.

Prepare a 500 ml bottle if you have a lot of cultures and will be able to finish the whole bottle within a month.

DMEM-F12	500 ml	50 ml
Pen/Strep (1%)	5 ml	(0.5 ml)
B27 (2%)	10 ml	1.0 ml
bFGF (20 ng/ml)	2.0 ml	0.2 ml
EGF (20 ng/ml)	2.0 ml	0.2 ml
Heparine (5 µg/ml)	500 µl	50 µl

Stock solution growth factors

- **bFGF: Basic Fibroblast Growth Factor**, Human, recombinant (invitrogen Gibco, #PHG0263)
 - 1 mg is dissolved in 200 ml sterile PBS 0.1% BSA (5 µg/ml)
 - Aliquots of 1 ml are stored at -20°C in F2, Ee2277a.
- **EGF: Epidermal Growth Factor**, Human, recombinant (invitrogen Gibco, #PHG0313)
 - 1 mg is dissolved in 200 ml sterile PBS 0.1% BSA (5 µg/ml)
 - Aliquots of 1 ml are stored at -20°C in F2, Ee2277a.
- **Sterile 0,1% BSA/PBS**
 - BSA, bovine serum albumin, (company product), stored at 4°C R1
 - Let BSA first become RT before opening!
 - BSA is non-sterile
 - Make stocks of 1% BSA in PBS (1000 mg in 100 ml = 1%)
 - Filter with 0,22 µm filter
 - Store aliquotes of 10 ml at -20°C in F1
 - When dissolving new bFGF/EGF, first dilute BSA/PBS 1:10 to get 0,1%
- **Heparin** (Alfa Aesar, #A16198).
 - Dissolve a small portion in sterile milliQ to a concentration of 5 mg/ml.
 - Aliquots of 500 µl are stored at -20°C in the cell culture lab
- **B27 supplement without vitamin A 50x** (Gibco, #12587-010).

Serum medium

DMEM	500 ml
Pen/strep(1%)	5 ml
FBS	50 ml

11.2.2.4 Passaging

Protocol copied from “Protocol 2.4 - Primary Culture - Passaging Cultures (NECH)” developed by EMC Neurosurgery laboratory.

PROTOCOL 04 - Passaging cultures

S = serum supplemented medium (DMEM, 10% FBS)

NS = Neurosphere medium, Serum free (DMEM-F12, B27, bFGF, EGF, Heparin)

For NS cultures: Coat new flasks with ECM (see protocol 01) in advance.

Before removing the medium, make a picture using the fluorescence microscope at passage 0 (or 1) and at the final passage 5.

1. Remove medium
2. *S-only: wash cells once with PBS + remove PBS*
3. Loosen cells from surface
 - S-culture > trypsin (for volume see volume overview below)
 - NS-culture > accutase (for volume see volume overview below)
4. Incubate at 37°C
 - S-culture > incubate 3 - 5 min (max 15) with trypsin

- NS-culture > incubate 5 – 10 min (max 30) with accutase
5. Add a volume to dilute
- S-culture > add 3× volume of serum medium to inactivate the trypsin
 - NS-culture > add PBS (choose between method A or B)
 - A. Example: you will pass the cells 1:3. In a T75 2 ml of accutase is already in the flasks. Flush the flask with 7 ml of PBS and transfer the total 9 ml to a 15 ml bluecap tube. Transfer from this 9 ml 3 ml to a new tube. So now you have one 15 ml bluecap tube containing 3 ml (1/3 of the cells for passing to another T75) and one 15 ml bluecap tube containing 6 ml (2/3 of the cells for freezing at -80°C). *So to split before centrifuging, make sure the final volume is a fold of the amount you want to pass.*

OR:

- B. Add PBS to each flask to get a final volume of 10ml and transfer to a 15 ml tube. Spin down the tube at 1500 rpm for 3 min. Resuspend pellet in appropriate amount of medium. To leave **1 ml** for freezing, use the table below for appropriate amount of NS:

Split	Total medium (ml) for resuspending	Transfer to new tube (ml)
1:2	2	1
1:3	1,5	0,5
1:4	1,33	0,33
1:5	1,25	0,25
1:6	1,2	0,2
1:8	1,14	0,14
1:10	1,1	~0,1

If following this protocol, skip the next steps and go directly to step 10

6. Transfer to a 15ml bluecap tube.
7. Spin down at 1500 RPM for 3 min.
8. Remove supernatant.
9. Resuspend pellet in new medium. *For the pellet that will be frozen in -80°C see 'PROTOCOL 05 – Freezing cells'.*
10. Add to new (coated) flask.
11. Place at 37°C.

Volume overview:

Flask	Amount of accutase/trypsin
T25	1 ml
T75	2 ml
T175	4 ml

11.3 IMAGING PROTOCOL

11.3.1 Sample Preparation Protocol for Scanning Electron Microscopy

- If cells are cultured on the structures, cells should be fixed and dehydrated. If cells are not cultured on the scaffolds skip steps “fixation” and “dehydration”.

Materials:

Phosphate buffered saline (PBS):

- Use 1x PBS solution concentration
- 1L 10x PBS in 10L demi water

2% Paraformaldehyde (2% PFA):

- PFA diluted in PBS (dilution 2:100).

4% Glutaraldehyde (4% GA):

- Glutaraldehyde diluted in Phosphate buffer pH 7.4 (dilution 4:100)

Ethanol (EtOH) 50%, 70%, and 96%:

- All diluted in demi water.

Fixation:

Remove DMEM.

Add PBS and leave for 5 minutes. Then remove PBS. Repeat this step once more.

Add 2% PFA and leave for 30 minutes. Then remove 2% PFA.

Add PFA 4% and leave for 2 hours. Then remove 4% GA.

Dehydration:

Add distilled water and leave for 5 minutes. Then remove distilled water. Repeat this step once more.

Add EtOH 50% and leave for 15 minutes. Then remove EtOH 50%.

Add EtOH 70% and leave for 20 minutes. Then remove EtOH 70%.

Add EtOH 96% and leave for 20 minutes. Then remove EtOH 96%.

Gold sputtering:

Place the sample on a SEM horizontal sample holder. The sample should be fixed at its place using carbon tapes.

Put the sample in the Sputter Coater JEOL JFC-1300 machine.

Use 20 mA current for 20s to sputter coat the samples with gold. This will result in an approximately 13 nm thick gold coating layer.

Take the sample out and place it on a 45° tilted SEM sample holder. Put the samples in the sputter coater machine and use 20 mA current for 10s. This will add a 6.5 nm thick coating layer. Repeat this step with putting the sample in an opposing 45° tilted configuration.

11.3.2 Immunostaining Protocol for Confocal Imaging

- This protocol was developed with the help of EMC Neuro-Oncology Laboratory.
- Immunostaining was performed with samples placed in 6-well plates.

Materials:

Phosphate buffered saline (PBS):

- Use 1x PBS solution concentration
- 1L 10x PBS in 10L demi water

Phosphate-buffered saline with Triton (PBS-triton):

- 1mL 10% triton in 99mL 1x PBS

Phosphate Buffered Saline with Tween (PBS-T):

- 0.25 ml Tween 20 in 500 ml 1x PBS

Phosphate Buffered Saline with bovine serum albumin (PBS+)

- 1gr bovine serum albumin (BSA) in 100 mL 1x PBS

Paraformaldehyde 4% (PFA 4%):

- PFA 4% diluted in PBS.

List of Primary and Secondary Antibodies:

Table-A 1 and Table-A 2 show the lists of primary and secondary antibodies used in the experiments.

Table-A 1: List of primary antibodies

Antigen	Source	Company	Catalog Number	Dilution in PBS+	Paired with #
Tubulin	Rabbit	Abcam	Ab6046	1:200	1
GAP-43	Rabbit	Cell Signaling Technologies	8945	1:200	1
EGFR	Mouse	DAKO	M3563	1:500	2
EGFR	Rabbit	Abcam	Ab76153	1:200	3

Table-A 2: List of secondary antibodies

#	Antigen	Source	Anti	Company	Catalog Number	Dilution in PBS+
1	Alexa fluor 488	Goat	Rabbit	Life Technologies	A11008	1:500
2	Alexa fluor 568	Donkey	Mouse	Life Technologies	A10037	1:500
3	Alexa fluor 647	Goat	Rabbit	Life Technologies	A21240	1:500

Fixation:

Remove Medium.

Add 100µl PFA 4% and leave for 15 minutes at room temperature. Then, remove PFA 4%.

Add PBS for at least 5 minutes. At this stage the samples could be left in the fridge for further use.

Permeablize:

Remove PBS and add PBS again for 5 minutes. Then, remove PBS.

Add PBS-triton and leave for 15 minutes in room temperature. Then remove PBS-triton.

Add PBS-T and leave for 5 minutes. Then remove PBS-T. Repeat this step once more.

Add PBS and leave for 5 minutes. Then remove PBS. Repeat this step once more.

Block:

Add PBS+ and leave for 1 hour minutes in room temperature. Then remove PBS+.

Add PBS-T and leave for 5 minutes. Then remove PBS-T. Repeat this step once more.

Spacer placement:

Let the samples air dry for 10 minutes. You can additionally dry the area around the structures with using a Q-tip and a tissue. Wrap the tissue around the tip of the Q-tip and pat it where the spacers will be placed. Not using a tissue may cause the Q-tip treads to entangle with the structures.

After samples are dried put two spacers on top of each other to ensure a height larger than the height of the scaffolds. Handling of spacers should be done with sharp tweezers. Spacers are sticky on both sides. Remove the cover on both sides and immediately place them on the samples. Give the spacers time to adhere to the substrates and make sure they are not already delaminating. If delamination happens replace the spacers.

Primary antibody:

Primary antibodies are diluted in PBS+. Refer to List of Primary and Secondary Antibodies section to see concentrations.

Per sample 80µl to 100µl solution of primary and body and PBS+ is needed. Add the solution in the middle of the spacers and make sure no leaking is observed. Otherwise the spacers should be changed or the samples should be regularly checked to have enough solution.

Put the samples in a humidity chamber for one hour. Then, remove the primary antibody solution.

Secondary antibody:

Add PBS-T and leave for 5 minutes. Then remove PBS-T. Repeat this step once more.

Primary antibodies are diluted in PBS+. Refer to List of Primary and Secondary Antibodies section to see concentrations.

Per sample 80µl to 100µl of solution is needed. Add the solution in the middle of the spacers and make sure no leaking is observed. Otherwise samples should be regularly checked to have enough solution.

Staining the nuclei:

Add PBS-T and leave for 5 minutes. Then remove PBS-T. Repeat this step once more.

Add PBS and leave for 5 minutes. Then remove PBS.

Remove the samples from the 6 well-plates and place the samples on a glass microscope slide. Then put the slides in a wider petri-dish

Add Mounting medium (which contains DAPI) to the middle of the scaffolds. Add nearly 80µl per sample. In this project Vectashield Mounting Medium with DAPI from Vector Laboratories was used.

Put clear nail polish around the spacers to increase adhesion between the cover-glass and the spacers. Then put a cover-glass on the samples directly from top. Keep the cover-glass horizontal, when placing it on the samples. Tilting of the cover-glass creates bubbles.

Wrap the petri-dishes in foil and let the samples dry for at least 24 hours in the fridge before microscopy.

11.4 NUCLEI MORPHOLOGY ANALYSIS MACRO CODE

```
1 roiManager("Show All");
2 roiManager("Deselect");
3 roiManager("Delete");
4
5 run("Clear Results");
6 rename("sample");
7
8 run("Z Project...", "projection=[Max Intensity]");
9 run("Split Channels");
10 selectWindow("C2-MAX_sample");
11 close();
12 selectWindow("C3-MAX_sample");
13 close();
14 selectWindow("C1-MAX_sample");
15 rename("nuclei");
16 run("Duplicate...", " ");
17 selectImage("nuclei-1");
18
19 run("Subtract Background...", "rolling=60 disable");
20 run("Enhance Contrast...", "saturated=0.3");
21
22 run("Command From Macro",
    "command=[de.csbdresden.stardist.StarDist2D], args=['input':'nuclei', 'modelChoice':
    'Versatile (fluorescent nuclei)', 'normalizeInput':'true', 'percentileBottom':
    '1.0', 'percentileTop':'100.0', 'probThresh':'0.65', 'nmsThresh':'0.70000000000000
    001', 'outputType':'Both', 'nTiles':'1', 'excludeBoundary':'2', 'roiPosition':'Au
    tomatic', 'verbose':'false', 'showCsbdeepProgress':'false', 'showProbAndDist':'fa
    lse'], process=[false]");
23
24 run("Set Measurements...",
    "area mean standard min shape median redirect=None decimal=3");
25
26 selectWindow("nuclei");
27 roiManager("Show All");
28 roiManager("Measure");
29
30 selectWindow("nuclei-1");
31 close();
32 selectWindow("Label Image");
33 close();
34
35 run("Summarize");
36 String.copyResults();
```

11.5 CELL MORPHOLOGY ANALYSIS MACRO CODE

```
1 roiManager("Show All");
2 roiManager("Deselect");
3 roiManager("Delete");
4
5 run("Clear Results");
6 rename("sample");
7
8 run("Z Project...", "projection=[Max Intensity]");
9 run("Split Channels");
10 selectWindow("C2-MAX_sample");
11 close();
12 selectWindow("C3-MAX_sample");
13 rename("tubulin");
14 selectWindow("C1-MAX_sample");
15 rename("nuclei");
16 run("Enhance Contrast...", "saturated=0.3");
17
18 run("Merge Channels...", "c2=tubulin c3=nuclei create");
19 rename("Composite");
20 run("RGB Color");
21 rename("Composite-0");
22
23 selectWindow("Composite");
24 close();
25
26 selectWindow("Composite-0");
27 run("Duplicate...", " ");
28 rename("Composite-1");
29
30 selectImage("Composite-1");
31 run("Find Maxima...", "prominence=45 output=[Segmented Particles]");
32 rename("Composite-2 Segmented");
33
34 selectWindow("Composite-1");
35 run("8-bit");
36 run("Smooth");
37 setThreshold(14, 255);
38 run("Convert to Mask");
39
40 imageCalculator("AND create", "Composite-2 Segmented", "Composite-1");
41 rename("and image");
42
43 run("Set Measurements...", "area shape feret's redirect=None decimal=3");
44
45 selectImage("and image");
46 run("Analyze Particles...", "size=30-Infinity show=Masks display add in_situ");
47
48
49 run("Clear Results");
50
51 selectWindow("Composite-0");
52 roiManager("Show All");
53 roiManager("Measure");
54 run("Summarize");
55 String.copyResults();
56
57 selectWindow("Composite-1");
58 close();
59 selectWindow("Composite-2 Segmented");
60 close();
```

11.6 EGFR ANALYSIS MACRO CODE

Three codes are written that should be run consecutively.

Code 1:

```
1 // Choose the original tiff image
2 roiManager("Show All");
3 roiManager("Deselect");
4 roiManager("Delete");
5
6 run("Clear Results");
7 rename("sample");
8
9 run("Z Project...", "projection=[Max Intensity]");
10 run("Split Channels");
11 selectWindow("C1-MAX_sample");
12 close();
13 selectWindow("C2-MAX_sample");
14 close();
15 selectWindow("C4-MAX_sample");
16 close();
17 selectWindow("C5-MAX_sample");
18 close();
19 selectWindow("C3-MAX_sample");
20 rename("egfr");
21 run("Duplicate...", " ");
22 selectImage("egfr-1");
23 rename("egfr-mask");
24
25 selectImage("egfr-mask");
26 run("Subtract Background...", "rolling=80 sliding");
27 run("Gaussian Blur...", "sigma=2");
28 //run("Threshold...");
29 setThreshold(35, 255);
30 run("Create Selection");
31 roiManager("Add");
32 run("Make Inverse");
33 roiManager("Add");
34 selectWindow("egfr");
35 roiManager("Select", 0);
36 run("Set Measurements...",
    "area mean standard integrated median redirect=None decimal=3");
37 roiManager("Measure");
38 roiManager("Select", 1);
39 run("Clear", "slice");
40
41 roiManager("Show None");
42
43 selectWindow("egfr-mask");
44 close();
45 String.copyResults();
```

Code 2:

```
1 // Choose the window called EGFR
2
3 run("Set Measurements...", "mean standard min median redirect=None decimal=3");
4 run("Clear Results");
5 roiManager("Show All");
6 roiManager("Deselect");
7 roiManager("Delete");
8
9 run("Find Maxima...", "prominence=50 output=List");
10
11 roiManager("reset");
12 for (i=0; i<nResults; i++){
13     x=getResult("X", i);
14     y=getResult("Y", i);
15     print(x,y);
16     makeOval(x-3.5, y-3.5, 7, 7);
17     roiManager("add");
18 }
19 }
20 run("Clear Results");
21 roiManager("Measure");
22
23 String.copyResults();
```

Code 3:

```
1 // Choose the original photo (now called sample)
2
3 selectWindow("sample");
4
5 run("Z Project...", "projection=[Max Intensity]");
6 run("Split Channels");
7 selectWindow("C2-MAX_sample");
8 close();
9 selectWindow("C3-MAX_sample");
10 close();
11 selectWindow("C4-MAX_sample");
12 close();
13 selectWindow("C5-MAX_sample");
14 close();
15 selectWindow("C1-MAX_sample");
16 run("Gaussian Blur...", "sigma=5");
17 //run("Threshold...");
18 setAutoThreshold("Huang dark");
19 //setThreshold(37, 255);
20 run("Convert to Mask");
21 //run("Close");
22 roiManager("Show All");
23 run("Clear Results");
24 roiManager("Measure");
25 String.copyResults();
26
27 selectWindow("view0");
28 close();
29 selectWindow("egfr");
30 close();
31
```

11.7 NUCLEAR AREA F-TEST

	Group1(Standard cell line)				Group2(Primary cells)				F value	95% region of acceptance	P-value
	Cell line	N	Mean	Std. Deviation	Cell line	N	Mean	Std. Deviation			
2D	U87	100	86.483	38.706	GS-580	76	141.98	72.194	0.287	0.656 : 1.543	0.000*
					GS-827	299	114.61	76.413	0.257	0.714 : 1.363	0.000*
					GS-830	79	88.578	55.434	0.488	0.659 : 1.534	0.001*
3D	U87	186	68.309	22.465	GS-580	236	77.117	41.740	0.290	0.759 : 1.311	0.000*
					GS-827	231	64.738	48.631	0.213	0.758 : 1.313	0.000*
					GS-830	216	64.277	38.610	0.338	0.756 : 1.319	0.000*

Calculated from: <https://www.statskingdom.com/220VarF2.html>

P<0.05 shows statistical significant between standard deviations of Group1 and Group2 (marked by *).

11.8 NUCLEAR CIRCULARITY F-TEST

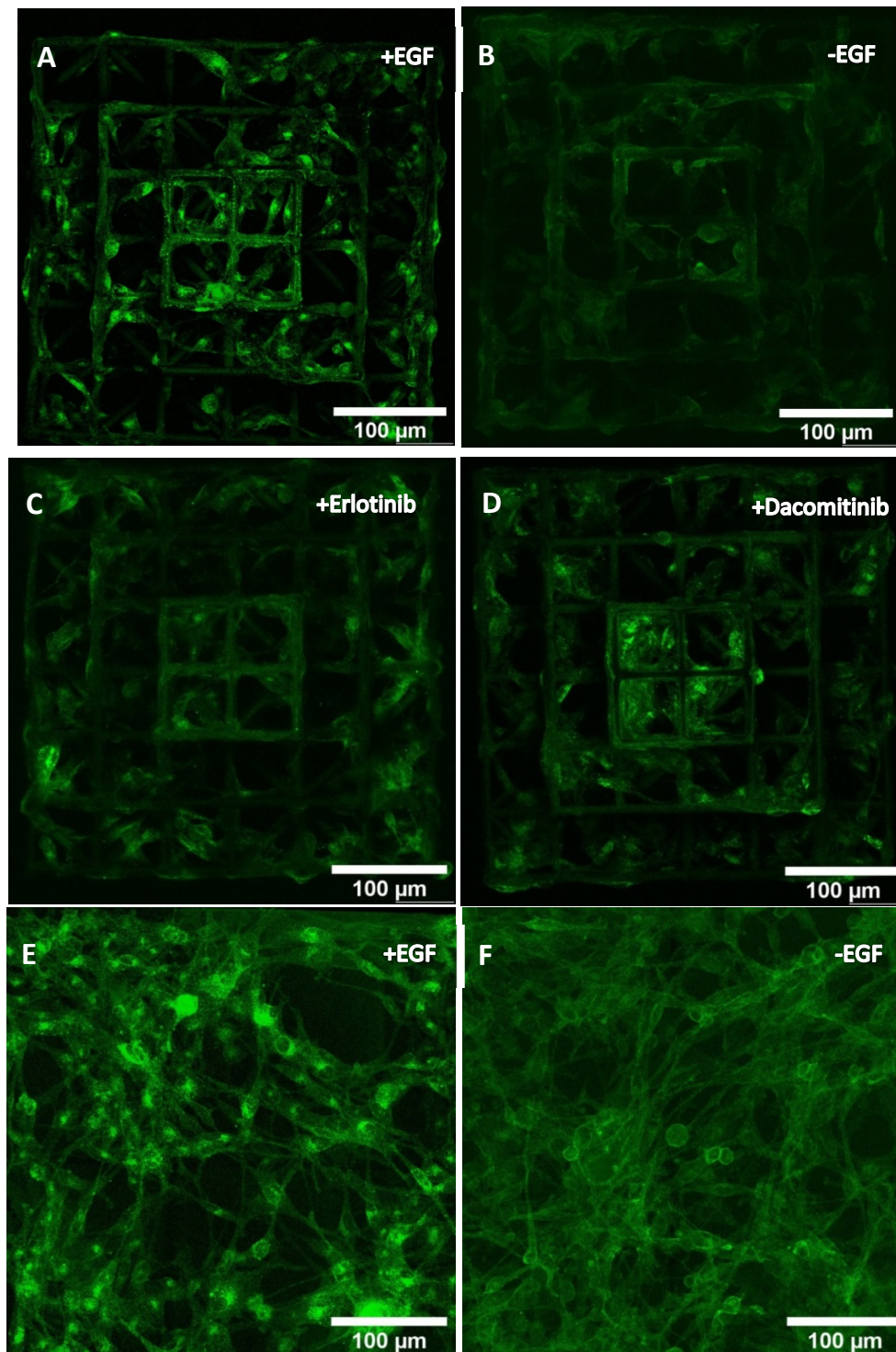
	Group1 (Standard cell line)				Group2 (Primary cells)				F value	95% region of acceptance	P-value
	Cell line	N	Mean	Std. Deviation	Cell line	N	Mean	Std. Deviation			
2D	U87	100	.9381	.09243	GS-580	76	.8865	.0789	1.374	0.656 : 1.543	0.150
					GS-827	299	.8813	.0879	1.105	0.714 : 1.363	0.523
					GS-830	79	.8767	.0895	1.067	0.659 : 1.534	0.770
3D	U87	186	.9270	.06014	GS-580	236	.8465	.1011	0.354	0.759 : 1.311	0.000*
					GS-827	231	.8535	.0973	0.382	0.758 : 1.313	0.000*
					GS-830	216	.8344	.1052	0.327	0.756 : 1.319	0.000*

Calculated from: <https://www.statskingdom.com/220VarF2.html>

P<0.05 shows statistical significant between standard deviations of Group1 and Group2 (marked by *).

11.9 *EGFR* IMAGES

11.9.1 GS-830, 2 Hours Exposure to Inhibitors



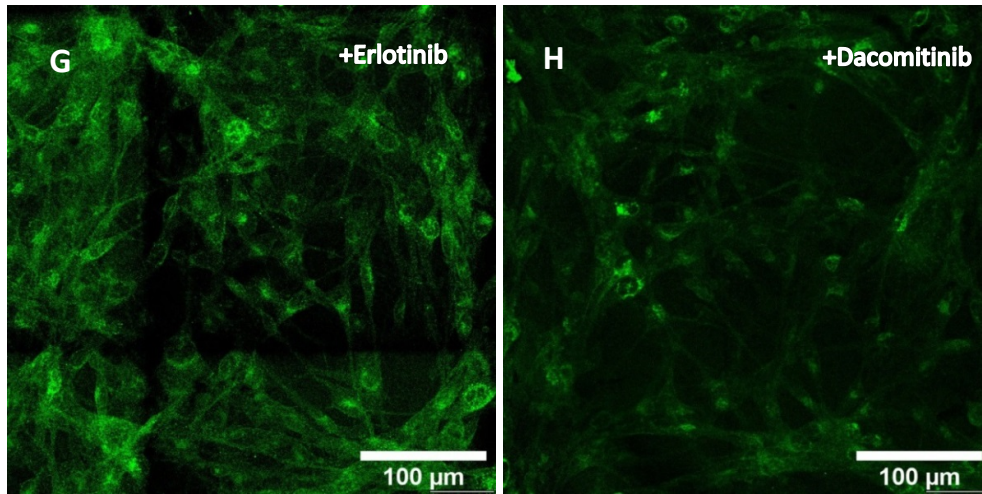
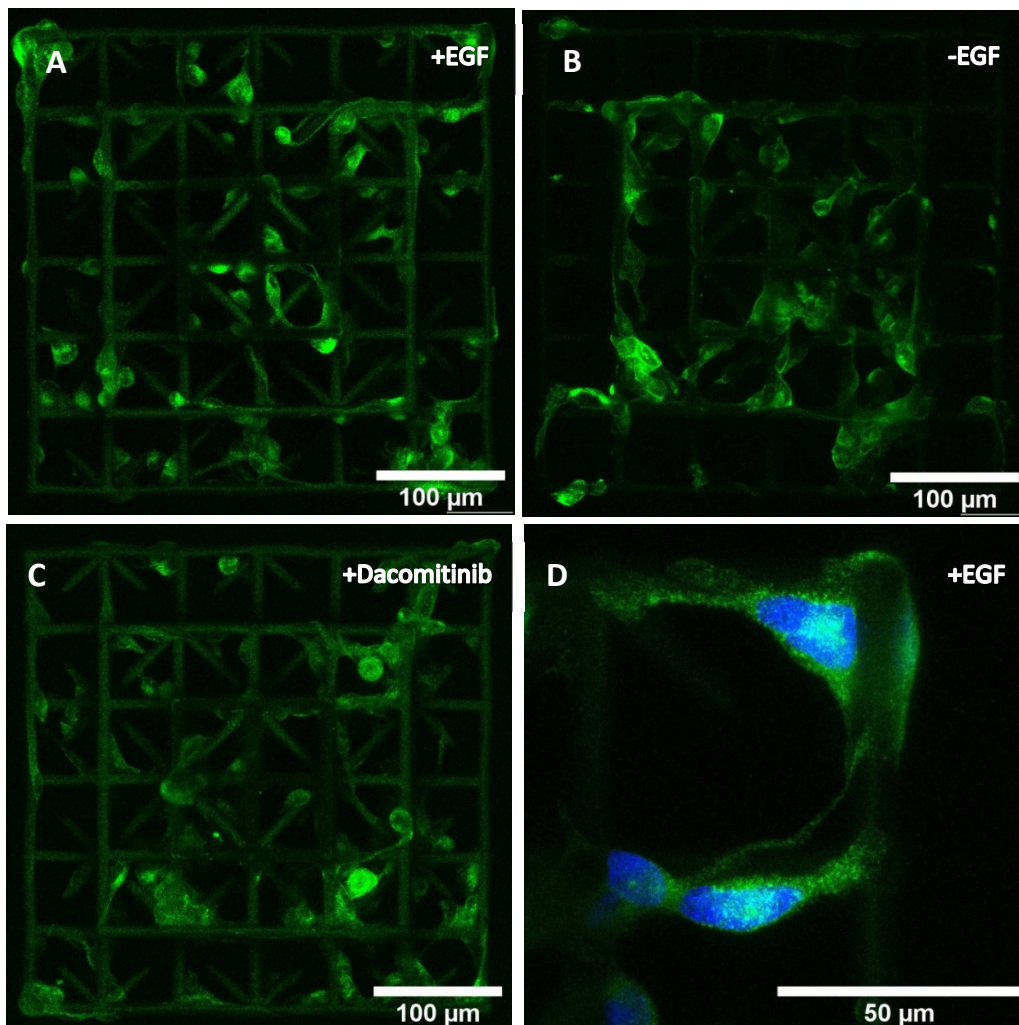


Figure A- 1: Confocal images of Gs-830 cells. Exposure to inhibitors was 2 hours. Blue: nuclei Green: EGFR A) Image of the entire scaffold with EGF B) Image of the entire scaffold without EGF C) Image of the entire scaffold with Erlotinib D) Image of the entire scaffold with Dacomitinib E) Image of the flat plate with EGF F) Image of the flat plate without EGF G) Image of the flat plate with Erlotinib H) Image of the flat plate with Dacomitinib

11.9.2 GS-580, 2 Hours Exposure to Inhibitors



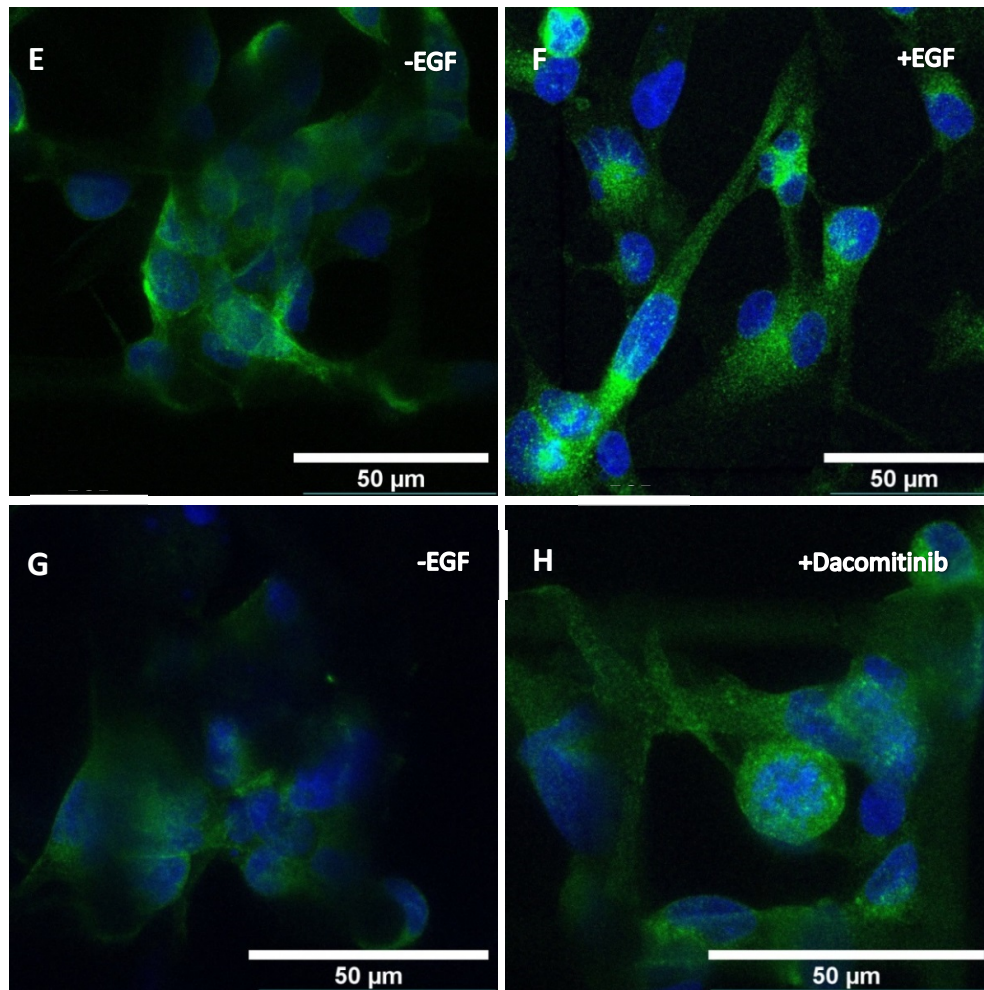
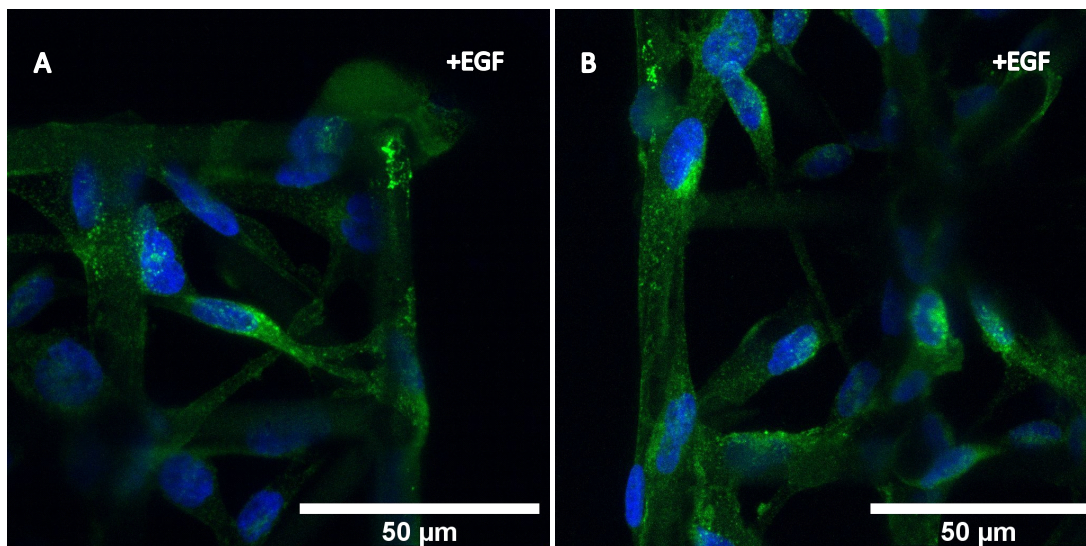


Figure A- 2: Confocal images of Gs-580 cells. Exposure to inhibitors was 2 hours. Blue: nuclei Green: EGFR A) Image of the entire scaffold with EGF B) Image of the entire scaffold without EGF C) Image of the entire scaffold with Dacomitinib D) Zoomed-in image of the scaffold with EGF E) Zoomed-in image of the scaffold without EGF F) Zoomed-in image of the scaffold with EGF G) Zoomed-in image of the scaffold without EGF H) Zoomed-in image of the scaffold with Dacomitinib

11.9.3 GS-830, Overnight Exposure to Inhibitors



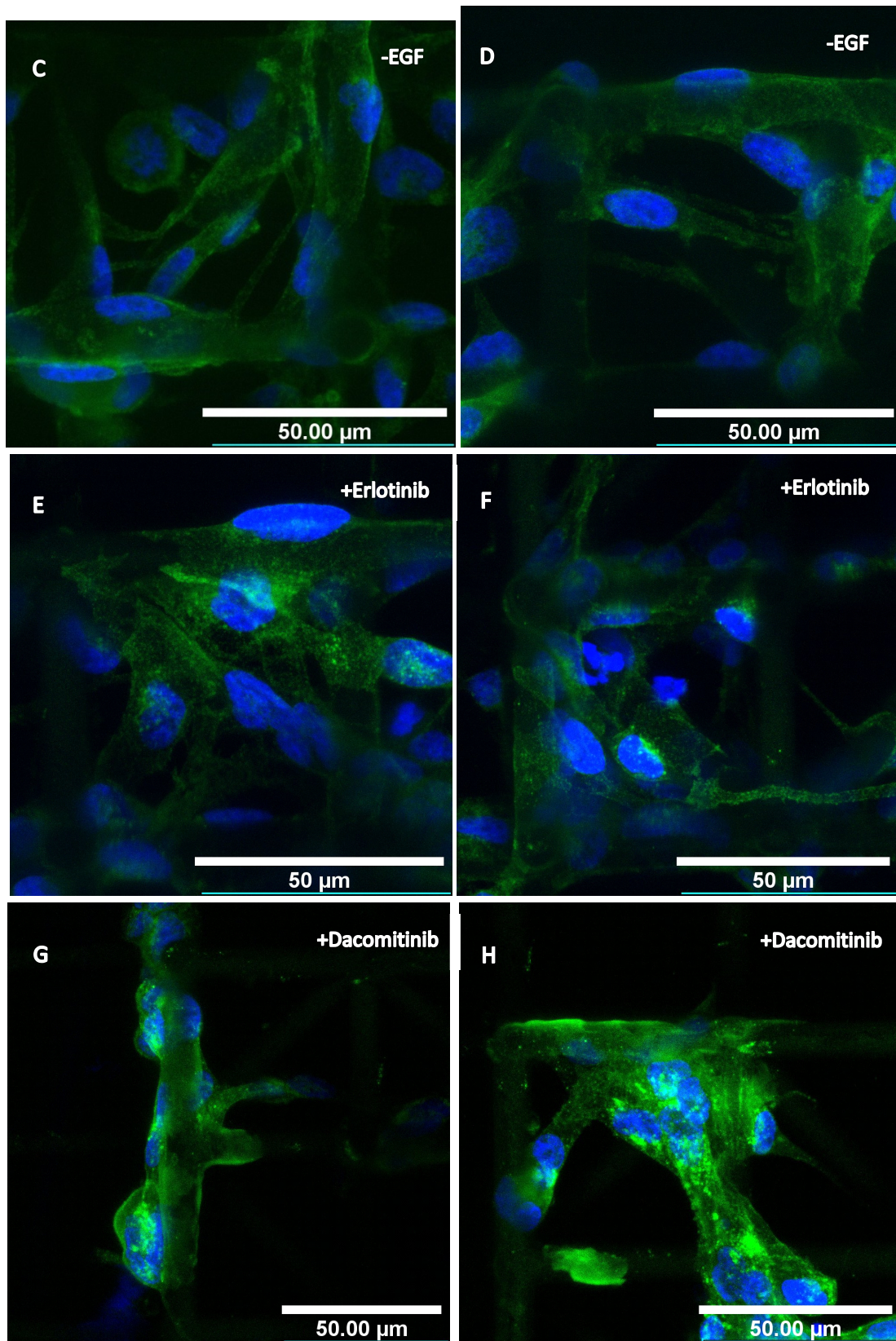


Figure A- 3: Confocal images of Gs-830 cells. Exposure to inhibitors was overnight. Blue: nuclei Green: EGFR A,B) Zoomed-in image of the scaffold with EGF C,D) Zoomed-in image of the scaffold without EGF E,F) Zoomed-in image of the scaffold with Erlotinib G,H) Zoomed-in image of the scaffold with Dacomitinib

11.10 CELL COLONIZATION WAS LOWER IN 2D

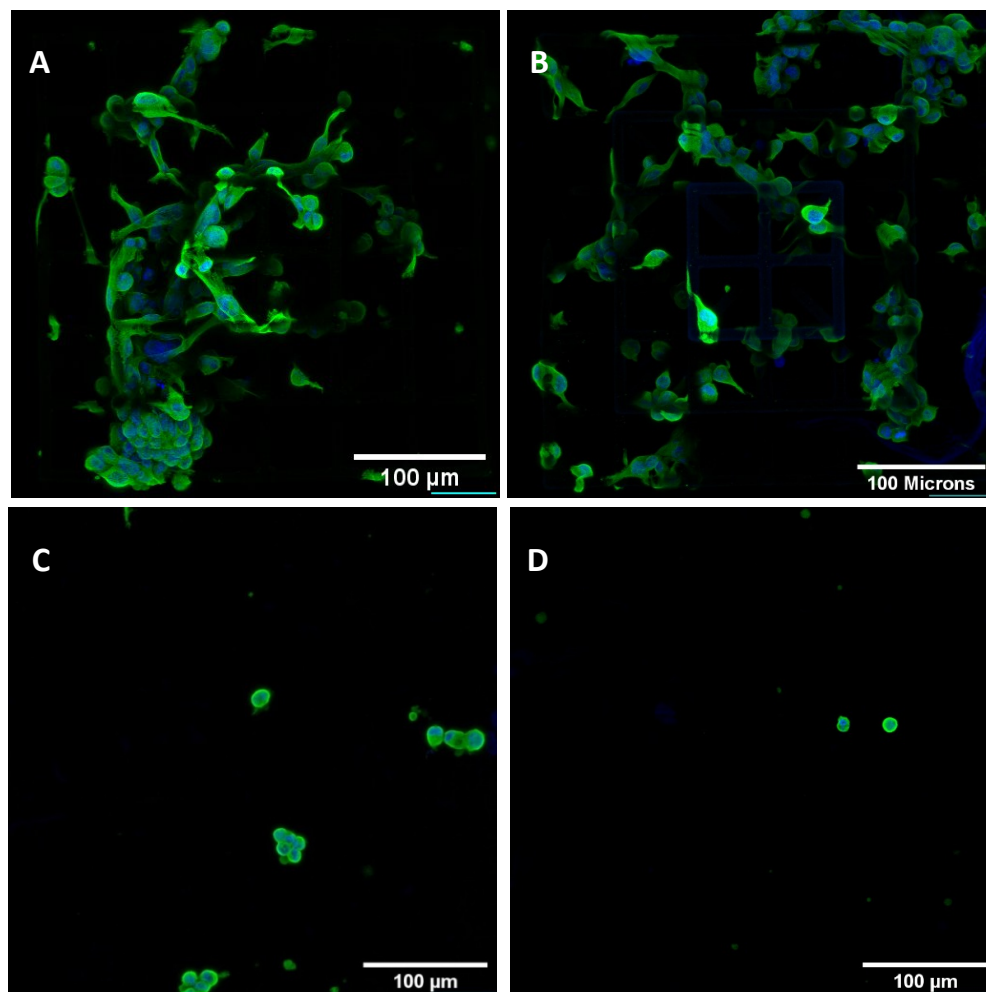


Figure A- 4: Confocal images of GS-830 showing cell colonization on the 3D scaffolds and on the 2D plates. A,B) GS-830 cells on two different scaffold. C) GS-830 cells on the 2D flat plates. D) GS-830 cells on the surrounding ITO-coated glass