3D Neural Microtissues as a Platform to Study Neuropathology of Mechanical Injury and Glioblastoma

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This dissertation by Dominick J. Calvao is accepted in its present form by the Institute of Biology, Engineering, and Medicine as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

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Master of Science, Brown University, Providence, RI **May 2017**
Biomedical Engineering, GPA 4.0/4.0

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PUBLICATIONS

Calvao, D.J., Vecchio, F.B., Ocitti, M., Zein-Sabatto, A., Schmidt, A., Shub, M., Viapiano, M., Lawler, S., Hoffman-Kim, D. "A Brain Cancer Microtissue for Studying Tumor Cell Behavior and Cancer-Neural Cell Interactions." Cell Press [In Preparation].

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RESEARCH EXPERIENCE

Doctoral Research Assistant **August 2020 – Present**
Institute for Biology, Engineering, and Medicine, Brown University, Providence, RI
Advisor: Dr. Diane Hoffman-Kim

- Developed a traumatic brain injury model using primary rodent *in vitro* 3D cortical microtissues, and evaluated cellular, molecular, and functional responses as a function of strain and strain rate.
- Developed a cortical glioblastoma model using primary rodent *in vitro* 3D cortical microtissues co-cultured with multiple glioma cell lines; used model to screen cortical and cancer response to chemotherapeutics; evaluated cellular response and cancer infiltration as a function of microtissue age and time of exposure.
- Conducted a multi-lab collaboration to develop an *in vitro* model of pulmonary candidiasis to study *Candida albicans* infection and a novel antifungal liposome treatment.

Graduate Research Assistant **August 2015 – May 2017**
Institute for Biology, Engineering, and Medicine, Brown University, Providence RI
Advisor: Dr. Edith Mathiowitz

- Fabricated insulin loaded polylactic acid nanoparticles at varying loading formulations using phase inversion nanoencapsulation. Characterized nanoparticle size, shape, and composition with DLS, SEM, and AFM.
- Performed in vitro and in vivo (rat model) release experiments and analyzed results using BCA Assay and HPLC.
- Synthesized drug loaded nanoparticles at varying concentrations on an early-stage research and development project for Takeda contracted to graduate school lab.

Undergraduate Research Assistant **August 2014 – May 2015**
Department of Biomedical Engineering, WPI, Worcester, MA
Advisor: Dr. Sakthikumar Ambady

- Developed a biomorphic tissue scaffold for cell seeding to generate living avascular cartilage tissue for implantation.

- Specifically, worked in a team of three students to design the tissue scaffold, generate a hydrogel for enhanced nutrient perfusion through the scaffold, and create protocols for differentiation of progenitor cells into chondrocytes in the 3D environment. The design was able to sustain cell growth and differentiation throughout the depth of the scaffold significantly past the physiological perfusion limit.
- Worked with graduate students on the fabrication of tissue rings for cardiovascular applications. Conducted analytical processes of the research such as imaging and comparing measurements. Performed various histological stains including H+E, among others and Matlab analysis of engineered blood vessel strains.

RESEARCH PRESENTATIONS

Vecchio, F., Gutiérrez, H. B., **Calvao, D**., Kesari, H., & Hoffman-Kim, D. "Development and optimization of a device for simulating traumatic brain injury impacts using an in vitro 3D cortical spheroid model." Poster Presentation: New England Science Symposium, 2025, March 30; Boston, MA.

Calvao, D.J.; Vecchio, F.; Ocitti, M.; Schmidt, A.; Viapiano, M.; Lawler, S.; Hoffman-Kim, D.; "A 3D multi-cellular cortical microtissue model for the study of glioblastoma multiforme pathology and therapeutic screening" Oral presentation: Keystone Symposium Conference, 2025, January 21; Banff, AB, Canada

Calvao, D.J.; Schmidt, A.; Lawler, S.; Hoffman-Kim, D. "A 3D Cortical Microtissue Model for the Study of Glioblastoma Pathology" Poster presentation: Cancer Neuroscience Symposia, 2024, February 28; MD Anderson Cancer Center, Houston, TX

Calvao, D.J.; Vecchio, F.; Stueber, D.; Hoffman-Kim, D. "Cortical Neurospheroid Microtissues in a Custom Designed Impacting Device to Model Blunt Traumatic Brain Injury" Poster presentation: Office of Naval Research PANTHER workshop, 2023, September 20; Madison, WI

Gonzalez-Cruz, R.D.; Wan, Y.; **Calvao, D.;** Alam El Din, D.; Burgess, A.; Vecchio, F.; Renken, W.K.; Franck, C.; Kesari, H.; Hoffman-Kim, D. "Cortical Spheroids Show Strain-Dependent Astrocytic Reactivity & Microglial Activation Following Sustained Compression Injury" Poster presentation: International Brain Research Organization World Congress of Neuroscience, 2023, September 10; Granada, Spain

McLaughlin, R.M.; **Calvao, D.;** Hoffman-Kim, D*. "Biomimetic Neural Microtissue for Study of Diving and Submarine-Associated Gas and Atmosphere Effects" Oral presentation: Military Health System Research Symposium, 2023 August 15; Kissimmee, FL.

Calvao, D.J., and Hoffman-Kim, D. "Optimization of Cortical Neurospheroid Sample Preparation in a Blunt Traumatic Brain Injury Model" Poster presentation: Office of Naval Research PANTHER Workshop, 2022, September 27; Madison, WI.

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LEADERSHIP EXPERIENCES

Student Mentor, Hoffman-Kim Lab, Brown University **2021 – Present**

- Trained and mentored three undergraduate/master's students through year-long and summer projects on traumatic brain injury in the 3D cortical microtissue model.
- Four of my mentees are on my first author publication and two additional mentees are on manuscripts in progress.

Representative, Graduate Student Council, Brown University **2023 – Present**

- Represented the biomedical engineering program on the graduate student council
- Served as a member of the nominations committee, responsible for recognizing new student groups and selecting university award winners.

Teaching Assistant, Biomedical Engineering, Brown University **Fall 2022**

- Held office hours and assisted with final projects for a tissue engineering class of 20 students.
- Managed online resources on Canvas webpage

Supplementary Teaching Assistant, Biomedical Engineering, Brown University **Fall 2016**

- Assisted with the teaching of a drug and gene delivery course of 50+ students
- Held regular office hours, created and graded weekly homework, midterms, and finals.

Peer Teaching Assistant, Biomedical Engineering, Worcester Polytechnic Institute
Spring 2015
- Assisted with the instruction of the Cellular Engineering Lab course of 50+ students.
- Instructed students in hands on cell culture techniques, cell cryopreservation, cell staining, immunocytochemistry, and 3D cell culture.

Residence Hall Council, President **August 2013 – May 2015**
- Managed a 9-member executive board of an organization with over 60 members that executed 50+ campus-wide events with attendances upwards of 800+ students and advocated for the residential community over 1000 students.
- Pitched and secured funding for many new campus wide events, increasing organization budget by ~240% over three years.
- Selected 2015 President of the Year among ~30 schools in the northeastern regional conference of the national organization.

Student Government Association, Committee Chairman **January 2011 – December 2013**
- Elected as a senator by the student body, appointed chairman (1 of 6) of Committee on Student Life Issues by SGA President.
- Managed and mentored committee of 14 students, lead 46 projects (87% successful), and passed one WPI policy resolution.

INDUSTRY EXPERIENCE

MilliporeSigma (Merck KGaA) **July 2017 – August 2020**
Technical Marketing Specialist – Diagnostic Solutions **February 2020 – August 2020**
- Conducted virtual training for 100+ sales professionals globally to increase sales of critical raw materials portfolio.
- Authored 10+ pamphlets, brochures, manuals, and marketing campaigns for critical portfolio targeting scientists.

Business Analyst – CEO Office **June 2019 – February 2020**
- Synthesized quarterly financial reports from 20+ business leaders for CEO communications internally and externally.
- Executed competitive analyses on top 10 market competitors, driving insights on internal strengths and weaknesses.
- Designed market competitor dashboard incorporating C-suite needs and generated target subsector SWOT analyses.

Global Applied Strategy Development – GOglobal Rotational Program **December 2018 – June 2019**
- Conducted internal and external financial analysis for the 2019 global strategy of the Applied business sector.
- Assessed current market size and forecasted growth in the medium-term for an emerging portfolio.
- Delivered key data analysis of the competitive landscape for several portfolios, assessing acquisition/licensing targets.

Asia Pacific Diagnostics Field Marketing – GOglobal Rotational Program **April 2018 – December 2018**
- Led the APAC regional go-to-market strategy for contract manufacturing, establishing new processes and workflow.
- Aided in developing a €1M pipeline of contract projects in three countries, established dedicated internal division.

Global Diagnostics Segment Marketing – GOglobal Rotational Program **July 2017 – April 2018**

- Pitched a one-year trial of new marketing strategy to management, secured funding, and implemented with extensive training for marketing and account managers, resulting in significant increases in reach and impressions.
- Generated a 22% response rate with 47% opportunity conversion by developing a new targeted marketing strategy.

Bard Davol (Becton Dickinson) **January 2016 – August 2016**
BioSurgery Development Engineer Co-op

- Lead activities in the development of five separate test methods from preliminary testing through operator training and test method validation for a surgical hemostatic agent.
- Authored multiple Design Control documents related to the development of new medical device.
- Generated and presented data to set product specifications.
- Conducted various benchtop testing including Karl Fischer titration and fluorescent and chromogenic assays for material characterization and product development.
- Assisted with pre-clinical animal testing of products for preparation for regulatory submission.

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Chapter 1: Introduction

1.1 Background

The central nervous system (CNS) is inherently complex, composed of diverse cell populations working in concert to govern perception, movement, and higher cognitive functions. The CNS may be divided into two major components: the brain which is responsible for the higher functions, and the spinal cord, which is responsible for sending motor commands from the brain to the rest of the body (Thau et al., 2025). The brain may be further divided into several distinct subregions including the cerebral cortex, the thalamus, hypothalamus, medulla oblongata, and cerebellum, many of which can be even further segmented into different lobes, sections, and structures responsible for specific functions (Thau et al., 2025).

There are five primary cell types in the brain: neurons, astrocytes, oligodendrocytes, microglia, and endothelial cells, though each of these major archetypes contains many sub-cell types such as precursor cells, ependymal cells and pericytes (McKenzie et al., 2018). Neurons are the basic building block cell of the brain, responsible for transmitting electrical signals throughout the brain and to the rest of the body. They can be excitatory, inhibitory, or modulatory in nature while both sensing and secreting a variety of neurotransmitters to communicate with other cells in their microenvironment. Neuron morphology can vary greatly between sub-types, but they are classically denoted as having a cell body, or soma, a long neurite extension called an axon responsible for outgoing signals, and several shorter

neurites called dendrites responsible for sensing local chemicals and transmitters in the environment (Raine, 1999).

Astrocytes are star-shaped supporter cells in the CNS that are primarily responsible for maintaining a homeostatic environment, upregulating and downregulating ions, neurotransmitters, and neurotrophic factors in their local microenvironment as needed (Rahman & Suk, 2020). Oligodendrocytes serve the all-important purpose of myelinating the axons of the neurons, integral components to maintaining the complex electrical circuitry of the CNS (Salzer & Zalc, 2016). Microglia serve as the immune system of the brain and function similarly to macrophages found in the rest of the body. They are responsible for scavenging throughout the CNS looking for amyloid plaques, damaged cells, and infectious agents, and thus are the primary line of defense for the CNS (Miron, 2017).

The complexity of the CNS and the diversity of the intercellular interactions therein complicate the study of neuropathology of different diseases and must be studied with a model that fully captures that complexity. As such, the development of neural microtissues has emerged as a promising strategy to model the CNS with increased fidelity. Among these microtissue models, brain organoids are perhaps the most widely adopted approach (Chiaradia & Lancaster, 2020). Typically derived from induced pluripotent stem cells (iPSCs), organoids aim to recapitulate key aspects of neurogenesis and regional brain features (Di Lullo & Kriegstein, 2017). Organoid models are commonly generated by seeding iPSCs into bioscaffolds such as Matrigel to promote three-dimensional growth and

recent advances have enabled the generation of region-specific models mimicking the cortical plate, forebrain, midbrain, and hypothalamus (Jo et al., 2016; X. Qian et al., 2016; Zamproni et al., 2021). Notably, organoid development exhibits many similarities to the development of the fetal brain, supporting their application in modeling early neurodevelopmental processes (Chiaradia & Lancaster, 2020). Additionally, organoids support gliogenic differentiation. Astrocytes are found in abundance, demonstrating region-specific morphologies and spatial distributions similar to the in vivo environment (X. Qian et al., 2020). These astrocytes have been shown to contribute actively to neuronal function, including depolarization, synapse formation, and synaptic pruning (Giandomenico et al., 2019; Sloan et al., 2017). Similarly, oligodendrocyte lineage cells have been observed in organoids, with some studies reporting evidence of partial axonal myelination (Madhavan et al., 2018; Marton et al., 2019; Tanaka et al., 2020). Furthermore, both excitatory and inhibitory synapses have been detected, and calcium imaging studies have revealed spontaneous, long-range electrical activity, indicating functional chemical synaptic communication between neurons (Giandomenico et al., 2019; Quadrato et al., 2017).

Despite these advantages, organoids also present several limitations. Following extended in vitro culture—typically beyond six months—organoids often develop necrotic cores due to insufficient diffusion of nutrients and oxygen to the center of the tissue mass (Giandomenico et al., 2019; X. Qian et al., 2020). Consequently, organoids remain limited in the extent of maturation they can achieve, restricting their utility for modeling late-stage

or neurodegenerative disorders (Lancaster et al., 2013). In addition, although some oligodendrocytes are present, full myelination and formation of nodes of Ranvier have not been consistently observed (Chiaradia & Lancaster, 2020). Microglia, the resident immune cells of the CNS, are also typically absent or only sporadically present (Ormel et al., 2018). Another concern is the well-documented 'batch effect,' wherein organoids generated within a single experimental batch tend to exhibit consistent features, but considerable variability arises across independent batches in terms of differentiation, morphology, and cellular composition (Lancaster & Knoblich, 2014).

While organoid models have proven valuable for studying early neurodevelopment and disease, their inherent limitations in maturation, cellular diversity, and reproducibility have necessitated the development of alternative models. The Hoffman-Kim lab has developed an established, primary tissue-derived microtissue model to study the neuropathology of various diseases.

1.2 Primary Tissue-Derived Cortical Microtissue Model

The Hoffman-Kim lab has previously established a robust, high-throughput cortical microtissue model capable of biomimetically recapitulating the in vivo microenvironment or the cortex with a higher fidelity than many spheroid and organoid models (Dingle et al., 2015). These microtissues are composed of primary cells derived from the cortices of post-natal rat pups, granting them in vivo-like cellular composition and tissue stiffness (Dingle et al., 2015). Furthermore, like organoids, these microtissues are electrically active,

forming complex neuronal networks and spontaneously form capillary-like networks (Boutin et al., 2018; Sevetson et al., 2021). Hundreds of microtissues can be derived from each animal dissected, allowing us to test more conditions with fewer animals and at a fraction of the cost of in vivo models. This also represents a significant advantage over organoid models, which require much more time to reach maturation. While the work described in this thesis is limited to microtissues derived from rat cortices, we have also demonstrated that these microtissues may be derived from other regions of the brain such as the thalamus and hippocampus and may be generated from mouse tissues as well as human induced pluripotent stem cells. A key advantage our microtissue model holds over alternative 3D co-culture models lies in the presence of microglia in our model which are notably absent in most alternatives. As the immune cells of the CNS, microglia play critical roles in the neuropathology of CNS diseases, especially in the case of glioblastoma as previously described. Our cortical microtissue model has previously been used to successfully study various disease states including ischemia and sustained compression injury (González-Cruz et al., 2024; McLaughlin et al., 2023). The robustness, versatility, biomimetic fidelity, and high throughput nature of these cortical microtissues have made them a powerful tool for the study of neuropathology. In this work we will leverage this cortical microtissue model to explore the neuropathology of two diverse disease states in the brain: traumatic brain injury and glioblastoma.

1.3 Traumatic Brain Injury

Traumatic brain injury (TBI) is a leading cause of death and long-term disability worldwide (Maas et al., 2017). TBIs occur whenever normal brain function is disrupted by external forces (Khellaf et al., 2019). TBIs are divided into three primary classifications: mild (concussion, full recovery expected), moderate (decreased consciousness and some cognitive impairment), and severe (coma and/or any penetration of the dura matter) (Pavlovic et al., 2019).

Over 50 million people per year experience a TBI worldwide, resulting in over 70,000 deaths in United States alone (CDC, 2024; Maas et al., 2017). These injuries often lead to persistent physical and psychological injuries including post-traumatic stress disorder, headaches, amnesia, concentration deficits, insomnia, depression, and even suicidality (Ng & Lee, 2019). TBIs are notoriously difficult to study and diagnose because patients rarely exhibit symptoms that can be detected by biological means and are instead diagnosed through psychological and behavioral measures which are highly subjective, prone to human error, and variable in presentation across patients (Turner et al., 2013).

Even more insidiously, TBIs often progress to the fatal chronic traumatic encephalopathy (CTE), a disease even less well understood than TBI. Often associated with repetitive TBIs, CTE is most common among athlete and military populations as groups of people incentivized to return to high-risk conditions as soon as possible (Moszczynski et al., 2018; Phipps et al., 2020). Like TBI, there is no biological mode of diagnosis for CTE with the

added confound that it can take upwards of 14.5 years for outwards symptoms to present, often resulting in diagnosis only in post-mortem analysis (Goldstein et al., 2012). In fact, one 2019 study found that 80% of TBI positive cadavers also exhibited markers of CTE (Phipps et al., 2020). To address the complexity of studying both TBI and CTE a variety of biological models have been developed.

1.3.1 Traditional models of TBI

Traumatic brain injury has been studied in three primary modes: human post-mortem tissue analysis, in vivo animal studies, and in vitro cellular studies. Each of these approaches offers unique advantages and limitations, offering insights into the broader picture of TBI neuropathology.

Due to the absence of any reliable biological marker, there is no way to study the neuropathology of the brain in humans living with TBI. Therefore, any studies performed on human tissue must by necessity performed post-mortem. These studies have yielded many very valuable insights into the pathology of the brain tissue including release profiles for various cytokines and chemokines, the role of neuroinflammation and both pro- and anti-inflammatory factors, and the deterioration of macro-structures throughout the brain (Frugier et al., 2010; Goldstein et al., 2012; McKee et al., 2015). Currently, these post-mortem analyses are the only method researchers have to study human tissue undergoing TBI-induce neuropathology, making the insights gained from these analyses truly invaluable. It must be noted, however, that this approach is inherently limited, as

researchers have no control over the TBI stimuli the patient received and, as the patient is already deceased, they can only provide snapshot of the terminal stages of the neuropathological progression. Additionally, the time elapsed between the patient's death and tissue analysis can lead to molecular and structural degradation in the tissue, especially in RNA, compromising the quality and integrity (Frugier et al., 2010). For these reasons, in vivo and in vitro models of TBI are the primary methods of studying TBI in a controlled environment.

1.3.1.1 In vivo animal studies

Animal models of TBI have several strengths including the ability to control the age of the animal, the severity and mode of injury, and the mechanical forces introduced to the brain (Xiong et al., 2013). Additionally, due to the relatively short lifespan of many of the animals used, long term neurodegenerative effects of TBI can also be studied (S. J. Tang et al., 2020). These studies are often carried out in rodents, though some larger animals such as pigs, primates, and sheep have also been used. Common models include the fluid percussion injury, weight drop injury, controlled cortical impact , and blast injury (Fesharaki-Zadeh & Datta, 2024). The variety of approaches to modeling TBI in these animal models speaks to the versatility of in vivo studies in this field of research as well as the depth of work required to develop several well characterized injury modalities. The data generated by these models have made huge contributions to our understanding of TBI pathology. Despite the advances these models have contributed to the field, several limitations must also be considered. Many of these models lack standardized parameters,

carry risks for rebound injuries or infection, have high animal fatality rates, and offer limited throughput as each animal tested provides data for only one specific TBI parameter (Fesharaki-Zadeh & Datta, 2024; Zhao et al., 2023).

1.3.1.2 In vitro cellular studies

In vitro cell models of TBI fall within five major modes of injury: stretch, compression, blast, scratch, and shear. These models provide valuable insights into the cellular response to very tightly controlled specific TBI stimuli with a high degree of granularity. Except for compression injuries, these models are typically conducted in a 2D cell culture environment with stretch injuries the most prevalent injury mode by far (Wu et al., 2021a). These models benefit from the speed and lost cost with which they can be performed, quickly generating new data and insights. In vitro models have been instrumental in elucidating aspects of TBI neuropathology including ion dysregulation, inflammatory cascades, microtubule and axon injuries, and cell death in response to these TBI stimuli (Wu et al., 2021a).

However, these models also have several limitations. In a systematic review of in vitro TBI models, Wu et al. found that over two-thirds of all studies were performed using the stretch method which, while useful, does not fully replicate the more complex forces acting upon the brain in vivo. Most in vitro models are also performed specifically on neurons alone (Bar-Kochba et al., 2016; Wu et al., 2021a); homogeneity of this cell population, in addition

to the lack of 3D architecture, misses many of the nuances of the complex intercellular interactions that occur in vivo.

In parallel to the challenges faced in studying TBI, brain cancers such as gliomas present a distinct but equally complex set of obstacles. Like TBI, gliomas impact a wide variety of neural cell types and are notoriously difficult to model due to the inaccessibility and heterogeneity of the brain environment. The following section will focus on glioblastoma, the most aggressive form of brain cancer, and the experimental models used to investigate its progression and interaction with the neural microenvironment.

1.4 Brain Cancer

Gliomas, the most common form of brain cancer, account for approximately 23% of all cancers, with over 25,000 new cases per year in the United States (Price et al., 2024). Gliomas are classified into four grades: grade I, which are very slow growing and potentially curable; grade II, consisting of astrocytomas, oligodendrogliomas, and oligoastrocytomas; grade III, consisting of anaplastic variations of astrocytoma, oligodendrogliomas, and oligoastrocytomas; and grade IV, glioblastoma, the most aggressive form of brain cancer with a median survival of only 14.5 – 16.6 months (Wen & Reardon, 2016). In most cases gliomas initiate at lower grades and will progress to higher grades over time if left untreated.

Glioblastoma, the most severe form of brain cancer, is notorious for exhibiting a high degree of heterogeneity, both between tumors and even within an individual tumor (Rybin

et al., 2022). Part of the severity of glioblastoma lies its prolifically invasive nature, often infiltrating into surrounding brain parenchyma, though rarely metastasizing beyond the CNS (Omuro & DeAngelis, 2013). The current gold standard of treatment for glioblastoma involves surgical resection of the tumor(s), localized irradiation, and a course of the chemotherapeutic temozolomide (Jacob et al., 2020a). However, this treatment is often ineffective, largely due to the poorly delineated edges and diffuse nature of glioblastoma, making it nearly impossible to fully resect the cancerous mass (Jacob et al., 2020a; Rybin et al., 2022; Wen & Reardon, 2016).

While glioblastoma interacts with all neural cell types, its interactions with microglia are among the most critical. In the absence of injury or pathogen, microglia surveil their local microenvironment, activating in response to insult. When activated, they polarize into either a pro-inflammatory state (M1) or anti-inflammatory phenotype (M2). Morphologically, these two phenotypes are indistinguishable, however they express different cytokines and chemokines. Specifically, glioblastoma cells secrete monocyte chemotactic protein-1 (MCP-1/CCL2) which both stimulates tumor growth and chemotactically recruits M2 microglia. These M2 microglia migrate to the tumor site and further secrete CCL2, synergistically creating a pro-tumor microenvironment (Geribaldi-Doldán et al., 2021). Due to the complex interactions between cancer and the tumor microenvironment significant effort has gone into designing robust models of glioblastoma.

1.4.1 Traditional models of glioblastoma

GBM research relies heavily on the use of in vivo and in vitro models. In vivo GBM models consist primarily of murine transgenic models and patient derived xenografts, each with their advantages and disadvantages (Rybin et al., 2021). Transgenic models are excellent for studying tumorigenesis but lack the heterogeneity commonly found in clinical settings (Marumoto et al., 2009; Moquin-Beaudry et al., 2022). Xenografts represent the current gold standard as they model actual patient tumors, but xenografts are a very slow and costly approach to GBM research (Wang et al., 2009). The limitations of these in vivo models have led to an increased interest in the development of in vitro models. Initial forays into traditional cell culture of primary tumor cells by Linkous et al in 2019 revealed that when cultured in homogenous 2D environments the cells exhibited less tumorgenicity and invasiveness. A change in cellular morphology and phenotypes was also observed when culturing the cells for an extended period of time. Additionally, these 2D homogenous culture approaches failed to recapitulate the complex cellular tumor microenvironment (TME) (Linkous et al., 2019a). For these reasons, organoid GBM models have been on the rise in recent years.

Organoid models of GBM have gone through several iterations in the last decade. In 2016, Hubert et al developed a GBM organoid (GBO) consisting of multiple types of patient derived cells, representing an advancement from more homogenous models that preceded it. These GBOs were able to capture more complex cellular morphologies, spatial distribution, and hypoxic gradients than 2D in vitro models (Hubert et al., 2016). This work

was further improved upon in 2020 and tested with common GBM treatments of irradiation, temozolomide, a well characterized alkylating chemotherapeutic commonly used for both astrocytoma and glioblastoma (Jacob et al., 2020a). This model has continued to be studied, with a recent paper finding that despite lacking microglia, GBOs still exhibit an "immune-like molecular program," allowing the interrogation of the active molecular pathways that contribute to neural immune response (Watanabe et al., 2024).

While these models represented a promising advancement in studying the cancer microenvironment, they lacked the ability to interrogate the interactions between healthy cells and cancer cell. In 2018, two new models of GBM organoids were developed to overcome this limitation. Neoplastic cerebral organoids (neoCORs) were developed through CRISPR genetic manipulation to induce GBM tumor growth in cerebral organoids, allowing for the study of the earliest stages of tumorigenesis and brain-tumor interactions. However, this approach is restricted to only predefined, well-characterized mutations and oncogenes, making it less suitable to investigating poorly understood or novel genetic drivers of glioblastoma (Bian et al., 2018; Ogawa et al., 2018).

The most promising of these organoid approaches was the Glioblastoma-Cerebral Organoid Co-culture (GLICO) model, combining the benefits of the GBO model and neoCOR models by leveraging patient derived GBM cells and cerebral organoids. The GLICO model shows the invasive patterns of GBM cells as well as microtubule networks within the organoid, a critical component of brain-tumor interactions as microtubules provide routes of invasion and proliferation to the GBM cells, intercellular communication,

and contribute to radiation resistance. The GLICO model has demonstrated a transcriptome similar to primary tumors, significantly higher expression of known GBM-related genes, a similar distribution of cellular states to primary tumors, and differential sensitives to chemotherapy and radiation. For all its strengths, the GLICO model has two critical shortcomings: a lack of vascularization and the absence of immune cells (Linkous et al., 2019a; Silva et al., 2018). As previously discussed, microglia play a crucial role in glioblastoma tumorigenesis and therapy resistance and as such are a critical component to be able to study in vitro.

1.5 Conclusion

Together, traumatic brain injury and glioblastoma represent two highly distinct, yet biologically complex neuropathologies: one driven by mechanical disruption, the other by biological means. Despite their differences, both conditions suffer from a lack of in vitro models that can faithfully capture the cellular diversity, architecture, and signaling dynamics of the CNS. This dissertation aims to address that shared gap by leveraging a biomimetic cortical microtissue model to study the neuropathology of both mechanical and biological disruption. This work contributes to the growing body of research seeking more physiologically relevant, high-throughput tools for modeling brain disorders. The following chapters describe the development of two microtissue-based models: one focused on replicating features of traumatic injury, and the other on capturing glioblastoma infiltration and the tumor microenvironment. The ability of the cortical microtissue model

to capture the spectrum of biological responses to these insults highlights the versatility and translational potential of cortical microtissues as a tool for advancing our understanding of CNS pathology.

1.6 References

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Chapter 2: Cortical Spheroid Model for the Study of Traumatic Brain Injury

Work by Dominick Calvao and Francesca Vecchio.

2.1 Abstract

Traumatic brain injury (TBI) is difficult to study due to the complexity of the brain and the limitations of existing models, including low throughput and ethical constraints of animal models, and the lack of cellular and structural complexity in 2D in vitro models. In this work, we leverage a cortical microtissue platform that retains in vivo-like structure and cell composition, combined with a custom impactor system to deliver controlled mechanical strain. We first evaluated embedding the microtissues in hydrogels as a medium of force transmission, but found that collagen, PEG, and alginate each introduced cell viability loss or technical challenges. As an alternative, we developed a shear impact method using the agarose hydrogel the microtissues were formed in. This approach successfully delivered biologically relevant strains and strain rates, inducing cell death and cytoskeletal disruption, though with some variability. These findings support cortical microtissues as a high-throughput, biomimetic model for studying TBI and informed the development of next-generation impact systems.

2.2 Introduction

TBIs are medically complex phenomena induced in three primary modalities: blunt impacts, penetration injuries, and blast waves (Ng & Lee, 2019). Blunt impact (iTBI) injuries are the most common, especially within civilian populations, accounting for 88-

95% of all TBIs. Blunt impacts are primarily caused by falls and motor vehicle accidents (Santiago et al., 2012). They also often accompany other forms of TBI as a secondary injury. Penetrating (pTBI) occur when the skull and dura matter are punctured by a foreign object, such as a bullet or shrapnel. These injuries are much less prevalent among civilian populations, accounting for only 5-12% of TBIs but, they have a much higher mortality rate of approximately 40% (Santiago et al., 2012). Blast wave (wTBI) injuries occur when the brain is exposed to a non-impact shockwave and occur most commonly in military service members. While rarely seen in civilian populations, wTBI account for more than 70% of TBI among the military (Phipps et al., 2020). In addition, these injuries often occur multi-modally, for example a soldier experiencing a wTBI from a blast followed by an iTBI as they land on their back and hit their head.

As the modes of TBI vary, so too have approaches to the study of TBI pathology. Blunt traumatic brain injury is often conducted in terms of velocity (m/s), acceleration (m/s^2) and force (N) while wTBI research is commonly conducted in terms of pressure (kPa) and energy transfer (J/s) (Doorly & Gilchrist, 2006; Mishra et al., 2016; Nolan, 2005). More recent research has sought to unify the lens through which this work is conducted by shifting to working in terms of strain (%) and strain rate (s^{-1}), regardless of injury modality. Simply put, strain is the measure of percent compression or elongation (Equation 1), and strain rate refers to the rate at which compression or elongation occurs (Equation 2).

$$\varepsilon = \frac{l_0 - l}{l_0} \qquad 1$$

$$\dot{\varepsilon} = \frac{\varepsilon}{\Delta t} \quad 2$$

Strain and strain rate can be viewed as an underlying unifying mechanism between the different injury modalities as the effect that energy transfer has on the system can be described in terms of strain and strain rate regardless of how the energy is transferred to the system. For example, victims of motor vehicle accidents tend to experience strains between 8 – 14% on their brains while concussed sports players were found to experience between 14 – 40% strain, both iTBIs (Hosseini-Farid et al., 2020; McAllister et al., 2012a; L. Qian et al., 2018; J. Zhang et al., 2006a). Conversely, military service members exposed to wTBIs experience relatively low strains, 10% or lower, but at extremely high strain rates with some papers reporting figures as high as 960 s^{-1} (Hosseini-Farid et al., 2020; Shafieian et al., 2011; Vogel et al., 2020).

TBI has been studied in three primary ways: human post-mortem analysis, in vivo animal studies, and in vitro cell studies, each with their own advantages and limitations. Post-mortem analysis has revealed how different regions of the brain are affected, where different markers accumulate, and a snapshot of which cell types are most impacted by TBI. However, this method is inherently limited to looking at a single snapshot of the pathology, precluding any study of the temporal component of the pathology (Alaylioglu et al., 2020). In vivo animal studies allow for more complete control of the TBI inputs, such as the forces acting on the brain, their mode of delivery, and the timing of the injury. The main limitation of these studies lies in their limited throughput as each animal can only

be used to study a single condition. In an injury landscape with as many variables to consider as TBI, this is a particularly limiting factor as researchers must balance the number of conditions they wish to study with the ethics of animal sacrifice and high financial costs (Kumaria, 2017).

In vitro cell based models of TBI overcome many of the cost and throughput limitations associated with in vivo studies while retaining the ability to tightly control the TBI inputs. However, this higher throughput comes with a trade-off in the complexity of the system. The brain is an inherently complex system composed of neurons, oligodendrocytes, astrocytes, microglia, and neural stem cells all working in concert to conduct the myriad functions of the brain. In vitro models often sacrifice this complexity by using neuron-only monocultures which, while still highly informative, miss many of the nuances of these cellular interactions and cascades. There are many 2D co-culture models where combinations of these cell types are mixed at biologically relevant ratios, but even these more advanced models fail to recapitulate the three dimensionality, tissue stiffness, and extracellular architecture present in vivo (Kanagasabapathi et al., 2011). Recent advances in organoid cultures have begun to address some of these limitations by providing a more physiologically relevant microenvironment that recapitulates in vivo structures, creating a particularly powerful tool for studying neurodegenerative processes and identifying neuropathological markers (Jgamadze et al., 2020; Lai et al., 2024). However, as mentioned previously, these models often lack more advanced structures like vascularization and

myelination, some glial cells, in particular microglia, and have high variability between batches (Jgamadze et al., 2020).

The Hoffman-Kim cortical microtissue model overcomes many of these limitations. Because they are derived from primary cortical tissue, they inherently contain the full complement of cortical cells at biologically relevant ratios, while also providing the three dimensionality, tissue stiffness, and extracellular architecture required to model more complex intercellular interactions found in vivo (Boutin et al., 2018; Dingle et al., 2015; McLaughlin et al., 2023; Sevetson et al., 2021). Additionally, hundreds of microtissues may be generated from each animal, offering a high throughput modeling option well-suited to studying the wide variety of potential TBI inputs.

In this work, I develop and evaluate two distinct TBI injury models using the cortical microtissue platform. I first explored embedding microtissues in various hydrogels to facilitate force delivery through a custom impactor system. However, I found that collagen, PEG, and alginate all significantly reduced microtissue viability, even in the absence of applied injury. These findings led to the development of a novel “shear impact” model, where microtissues are injured directly within their agarose mold. This method enabled the delivery of biologically relevant strains and strain rates, resulting in measurable neuropathological effects, including increased cell death and cytoskeletal disruption. Together, these results establish a new in vitro platform for studying TBI mechanics in a biomimetic, high-throughput context.

2.3 Materials and Methods

2.3.1 Cell isolation and 3D microtissue culture

All animal procedures were conducted in accordance with the guidelines established by Brown University's Institutional Animal Care and Use Committee. 96 well agarose hydrogels were created by dissolving UltraPure agarose (Invitrogen) in heated phosphate-buffered saline (PBS) (Gibco) at a concentration of 2% wt/vol. The molten agarose was poured into 96 peg spheroid micromolds (MicroTissues, Inc.) and scraped to remove excess agarose and ensure uniform shape. After cooling, the agarose hydrogels were removed from the molds and placed in 24 well plates where they were equilibrated in cortical media- (CM-) composed of Neurobasal+ (Invitrogen), 0.5 mM GlutaMAX (Gibco), and 1X Penicillin-Streptomycin (Invitrogen) for two days prior to dissection, with daily CM- changes. On the day of dissection, CM- was exchanged for cortical media+ (CM+), CM- that had been supplemented with B27+ (Invitrogen).

Dissections were performed on postnatal (days 0-2) rat pups (Charles River). Mixed sex rat pups were immersed in ice for 45 minutes and decapitated prior to extracting the cortices as described in Dingle et al., 2015. Dissections were performed in Hibernate A (BrainBits) supplemented with 1X B27+ and 0.5 mM GlutaMAX. Extracted cortices were cut into small pieces and digested in 2 mg/mL papain (BrainBits) dissolved in Hibernate A without calcium chloride (BrainBits) for 30 minutes at 30°C with occasional perturbation. Papain was carefully removed and replaced with Hibernate A/B27+ while the cortices were

mechanically triturated with fire polished Pasteur pipettes to homogenize the tissues. The homogenized cell suspension was filtered through a 40 μm cell strainer (CELLTREAT) and centrifuged at 150G for 5 minutes. The cell pellet was resuspended in CM+, filtered again, centrifuged again, resuspended, and centrifuged a third time. The cell suspension was counted using a trypan blue exclusion assay (Invitrogen) and seeded into the agarose hydrogel microwells at a cell density of 8,000 cells/microwell or 768,000 cells/hydrogel. The cells were allowed to settle to the bottom of the wells with occasional perturbations before filling each well with 1 mL of CM+. Additionally, 2D cortical cell controls were plated in wells coated with poly-D-lysine (Sigma-Aldrich) at a density of 250,000 cells per well to ensure general cell health. CM+ was exchanged the day after dissection, and every 2-3 days thereafter.

2.3.2 Hydrogel embedding

To deliver impacts to the microtissues, initial work focused on embedding the microtissues into a variety of hydrogels to serve as a force transduction medium. Irrespective of the hydrogel the microtissues were embedded within directly, an initial preparatory step was taken. An agarose mold with two empty spaces for the impactor's arms and an empty central chamber for the embedded microtissue-hydrogel mix to gelate was created. First, 1.5 mL of molten 2% agarose was poured into a 35 mm FluoroDish (WPI) and a prefabricated stamp was applied for 30 seconds with consistent downward pressure until the agarose cooled. The stamp was carefully removed, leaving behind the necessary agarose mold adhered to the bottom of the 35 mm FluoroDish.

2.3.2.1 Collagen embedding

A collagen mixture was made by mixing to 10X PBS (Gibco), ice-cold 2 mg/mL collagen (Corning), and the microtissues suspended in CM+. Using a pH strip (Fisher Scientific), 1M NaOH was added to the mixture until a pH of 7.4 was achieved. All steps were conducted with reagents at ice-cold temperatures as collagen will begin to gelate at room temperatures. The collagen solution was thoroughly mixed and 200 µL added to the central reservoir of the premade agarose mold in the FluoroDish. The mixture was incubated at 37°C for one hour to allow the collagen to gelate before adding 2 mL CM+ to the FluoroDish. All microtissues were given 24 hours to acclimate to the hydrogel before any impacts were performed. A variation of this procedure was performed to mitigate the cold shock of introducing the microtissues to the ice-cold collagen wherein the microtissues were allowed to cool in CM+ from 37° to room temperature over the course of 20 minutes before being cooled further down by placing the microtissue suspension on ice to more gradually cool the microtissue before mixing them with the ice-cold collagen.

2.3.2.2 Polyethylene glycol embedding

A proprietary (Stem Pharm, Inc.) polyethylene glycol (PEG) was used to embed microtissues. A 1:5 dilution of PEG was mixed with microtissues suspended in CM+ before 200 µL of the mixture was added to the central reservoir of the premade agarose mold. This proprietary PEG formulation had been engineered to crosslink under UV light at a wavelength of 350 nm which is generally considered safe for cells. The PEG-microtissue

was placed under a UV lamp with 350 nm bulbs for up to 30 minutes to crosslink the hydrogel.

2.3.2.3 Alginate embedding

Microtissues were embedded in 2% alginate (Sigma-Aldrich) by first making a 4% alginate solution in water and mixing a 1:1 dilution with microtissues suspended in CM+ before 200 µL of the mixture was added to the central reservoir of the premade agarose mold. The mixture was crosslinked by the addition of either 100 mM calcium chloride (Sigma-Aldrich) or 100 mM zinc chloride (MilliporeSigma) for one minute. The crosslinker was removed and the hydrogels washed twice with warmed CM+ before returning them to the incubator.

2.3.3 Impactor injury

Our impactor device was custom designed and built by members of the laboratory of Christian Franck, PhD at University of Wisconsin-Madison. The main driving component of the impactor device was PIMag High-Load Linear Actuator (PI USA) which moved the impacting arm of the device. Embedded microtissues in 35 mm FluoroDishes were loaded into the sample reservoir and the z-stage lowered until the arms were just above the glass bottom of the FluoroDish. The stationary arm of the impactor was manually adjusted to just press against the agarose wall of the central reservoir while the impacting arm was controlled using PiMikroMove software to just press against the agarose wall on the other side of the central reservoir. The desired arm distance (mm), movement speed (mm/s) and

number of impacts was programmed into the software and impacts delivered to the embedded microtissues. The hydrogels were then returned to the incubator before final characterization.

A variation of this technique, which we dubbed shear impacts, was implemented by delivering impacts to the microtissues in the 96 well agarose hydrogel in which they were originally formed, without first embedding the microtissues into a secondary hydrogel. In this case the seeded agarose hydrogel was placed in the center of a 35 mm FluoroDish and the two arms aligned with the indented edges of the hydrogel before proceeding as previously described. Impacts were visualized by mounting the impactor device above a custom-built inverted microscope apparatus with either a 4x or 10x objective (Nikon) and a high-speed camera (Thorlabs).

2.3.4 Strain and strain rate measurements

Strain measurements were collected using ImageJ FIJI to manually measure the diameter of the microtissues before impact and during impact. To calculate the strain rate with Equation 2, first the Δt had to be determined as:

$$\Delta t = \frac{diameter_{impacted} - diameter_{initial}}{velocity} \quad 3$$

The strain rate was then calculated using Equation 2 for each impact delivered.

2.3.5 Cell viability assay

Cell viability was assessed by staining the microtissues with a mixture of 1 mL CM+, 1 µL Hoechst 33342 (Invitrogen) and 4 µL Ethidium Homodimer-1 (Invitrogen) per hydrogel for one hour. They were washed twice with fresh CM+ and either imaged in their embedded hydrogel or flushed out of the 96 well agarose hydrogel using a cut-off P1000 micropipette tip. Cell viability was modeled by counting the number of cell nuclei (Hoechst 33342) and the number of dead/damaged nuclei (Ethidium Homodimer-1) where:

$$Cell\ viability\ (\%) = \frac{Total\ Cell\ Nuclei - Dead\ Cells}{Total\ Cell\ Nuclei} \times 100 \qquad 4$$

2.3.6 Immunohistochemical staining

At the desired time point, microtissues were fixed in a 4% v/v paraformaldehyde and 8% wt/vol sucrose solution overnight. The next day they were washed three times with PBS for 30 minutes each. The microtissues were flushed out of the agarose hydrogels and transferred to 1.5 mL Eppendorf tubes for storage at 4°C to await immunohistochemical (IHC) staining.

When performing IHC, the microtissues were permeabilized and blocked with a blocking solution consisting of 4% bovine serum albumin (Sigma-Aldrich), 10% normal goat serum (Jackson ImmunoResearch), and 1% Triton X-100 (Sigma-Aldrich) in PBS for two hours at room temperature on a shaker plate. Blocked microtissues were then incubated in primary antibody solutions consisting of either mouse anti-β-iii-tubulin (Biolegend) or

rabbit anti-glial fibrillary acidic protein (GFAP, DAKO), diluted in blocking solution overnight at room temperature on a shaker plate. An additional sample of non-primary control microtissues was set aside and incubated in blocking solution without primary antibodies instead. The next day, microtissues were washed twice for 2 hours with 0.2% Triton in PBS (PBT) and blocked for an additional 2 hours. They were then incubated in secondary antibody solutions consisting of either CY3 goat anti-mouse (Jackson ImmunoResearch), Alexa488 goat anti-rabbit (Jackson ImmunoResearch) or CY3 goat anti-rabbit (Jackson ImmunoResearch) antibodies diluted in blocking solution overnight. On the final day, microtissues were washed twice more for 2 hours in PBT and then incubated in 1 mg/mL of 4',6-diamidino-2-phenylindole (DAPI) in PBT for an additional hour before being returned to PBS for storage at 4°C until imaging.

2.3.7 Calcium imaging

Microtissues were incubated in 5.2 μM Oregon Green 488 BAPTA-1AM (OGB-1, ThermoFisher) and 0.02% Pluronic F-127 acid (ThermoFisher) in CM+ for 25 minutes at 37°C. The microtissues were washed twice with fresh CM+ and transferred to a 35 mm FluoroDish for imaging on the confocal microscope. 1-minute videos of calcium activity were collected at 25-30 frames per second for each microtissue.

2.3.8 Confocal imaging

All imaging was conducted using either an Olympus FV3000RS confocal microscope with Olympus Fluoview FV3000 software or a Nikon AX-R confocal microscope using NIS-

Elements software. All images were captured using either a 10X air objective (Olympus, Nikon respectively) or a 20X air objective (Olympus, Nikon respectively) and laser channels of 405, 488, and 561 nm. Imaging settings were kept consistent between samples for all conditions within the same experiment. All z-stacks were collected in Galvano mode except for calcium imaging which was performed using resonant scanning mode. Live cell imaging was conducted inside environment control chambers which kept microtissues at 37°C. Images were converted from .oir or .ND2 formats to TIFF in ImageJ FIJI.

2.3.9 Image analysis

Viability assayed microtissues were analyzed in ImageJ FIJI using the "3D Objects Counter" plugin to quantify the number of discrete objects present in each channel of the z-stacks. Object size and intensity thresholds were established in each experiment to include all pixels positive for either Hoechst 33342 or Ethidium Homodimer-1 while excluding any background fluorescence or noise. These settings were selected by finding values that worked appropriately across both control and injury groups, then kept consistent for the entire experimental data set.

GFAP-stained microtissues were analyzed using Sholl analysis, a neuroanatomy plugin for ImageJ FIJI to quantify GFAP branch complexity. GFAP channels were filtered to reduce background noise and subsequently binarized. Concentric circles radiating outwards from the center of each microtissue at 10 μm intervals were drawn and the number of GFAP

intersection points counted to serve as a metric for the complexity of GFAP branching behavior.

Calcium imaged microtissues were analyzed using a custom MATLAB script developed by laboratory alumnae Jessica Sevetson, PhD. Regions of interest were manually selected by circling cell soma and fluorescence within each region was monitored. Fluorescence over time was measured and normalized against the first 500 frames of analysis while background noise was filtered out. Normalized fluorescence over time was graphed on a per-cell basis using the same custom MATLAB tool.

2.3.10 Statistics and data visualization

Data visualization and statistical analysis were both performed in GraphPad Prism 10. Data was reported as box-and-whisker plots with all individual data points shown. Center lines of the boxes represent the median while the box boundaries represent the 25^{th} and 75^{th} percentiles. Upper and lower whiskers represent the highest and lowest individual values recorded, respectively. All data analysis performed consisted of either a one-way or two-way ANOVA, depending on if the study contained groups from two time points or not. A p-value of less than 0.05 was considered significant.

2.4 Results

Previous work in the Hoffman-Kim lab has established the cortical microtissue model as a robust, high throughput platform that recapitulates many of the nuances of the in vivo

biological environment(Boutin et al., 2018; Dingle et al., 2015; González-Cruz et al., 2024; McLaughlin et al., 2023; Sevetson et al., 2021). The goal of this work was to design an experimental system that leverages the inherent biological strengths of the cortical microtissue model for the study of TBI using a custom designed impactor device and a hydrogel force transduction medium.

2.4.1 Embedding microtissues in collagen leads to significant cell death

Collagen was chosen initially as our force transduction medium to adapt the methods of our collaborator where 2D cortical cells were grown on top of collagen hydrogels (Bar-Kochba et al., 2016; Franck et al., 2011a; Toyjanova et al., 2014). This method was adapted to allow the entire cortical microtissue to be embedded within the collagen hydrogel so that the impactor device may enact a force on the microtissues and induce strain. However, we found that microtissues embedded in collagen hydrogels experienced significant cell death, even in the absence of an impact being delivered. Using our cell viability assay, microtissues were stained to identify all the cell nuclei and the cell nuclei of dead/damaged cells. From these two values we were able to quantify the cell viability as a percentage of the total number of cells that were not stained with our dead/damaged marker.

All cortical microtissues demonstrate some amount of cell death, even when healthy. Control microtissues exhibited 82.2% ± 8.9% cell viability at DIV15. When DIV14 microtissues were embedded in ice-cold collagen and allowed to acclimate for 24 hours cell viability was found to have fallen to 27.3% ± 14.3%. To try to counteract the cold

shock effects of embedding the microtissues in ice-cold collagen, we employed a gradual cooling technique as previously described. However, we did not observe any recovery in cell viability as rates remained at 25.3% ± 15.5% with this technique. These cell viability results represent a significant ($p<0.0001$) reduction in the health of our microtissues (Figure 1), even in the absence of a mechanical insult, making this embedding approach unsuitable for TBI studies.

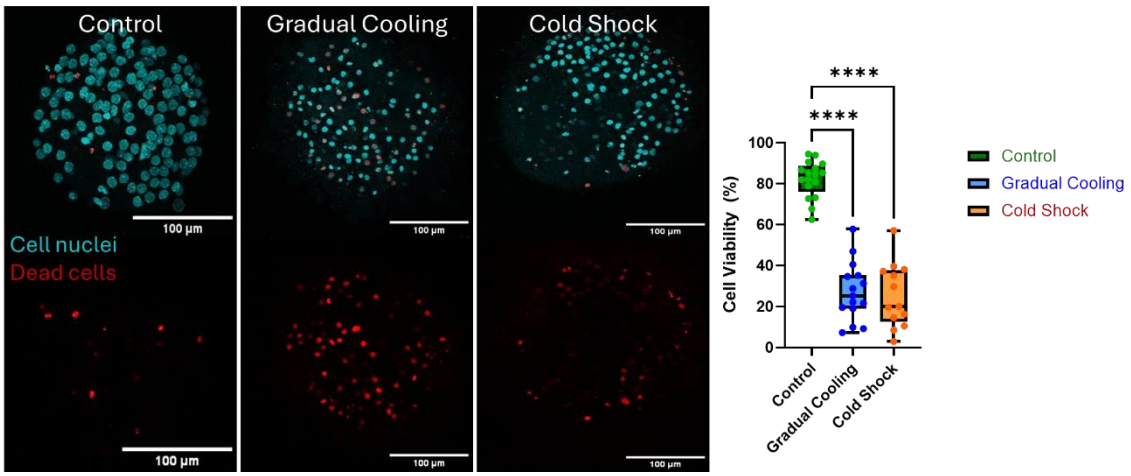


Figure 1: Collagen embedding led to significant cell death.

Microtissues were embedded into collagen using either the gradual cooling method or the standard cold shock technique at DIV14. Microtissues were stained and imaged after 24 hours of acclimation. The total number of cell nuclei (blue) and dead cell nuclei (red) were counted and used to calculate the cell viability (%) of each microtissue. Both gradual cooling (25.34%) and cold shock (27.29%) collagen embedding lead to a significant decrease in cell viability compared to controls (82.22%). n = 13-17 per condition. **** $p<0.0001$.

2.4.2 Alternative hydrogels exhibited a variety of limitations that were unsuitable as force transduction mediums in our impactor system

A proprietary formulation of PEG engineered to crosslink under a biologically safe wavelength of UV light (350 nm) was tested as an alternative to collagen as a medium to

embed the microtissues in. Preliminary tests with the PEG formulation in 96 well plates at volumes of 25 μL showed successful crosslinking within 2 minutes; however when used in larger volumes of 200 μL to fit the premade agarose mold, the PEG formulation did not crosslink completely even with 30 minutes of UV exposure (**Error! Reference source not found.**A). Alginate was also tested using two different crosslinking agents, calcium chloride and zinc chloride, with mixed results. While both crosslinking agents successfully caused the alginate to gelate, only the calcium chloride crosslinker did so without causing the alginate to deform. Alginate crosslinked with zinc chloride was deformed and misshapen, making it unsuitable as a medium for delivering impacts to the microtissues (**Error! Reference source not found.**A). The consistent shape achieved and success of crosslinking alginate with calcium chloride warranted further investigation for use in our impacting system. Microtissues embedded in both modes of alginate hydrogel were further tested for cell viability 24 hours after embedding. In both cases the cell viability fell, to 26.8% ± 12.4% in the case of calcium chloride crosslinking, and 40.1% ± 17.1% in the case of zinc chloride crosslinking (Figure 2B). Additionally, when calcium imaging was performed on microtissues embedded in each hydrogel we found that calcium dynamics were either suppressed or not detectable in any of the microtissues (Figure 2C). These findings prompted us to move to a different impacting approach.

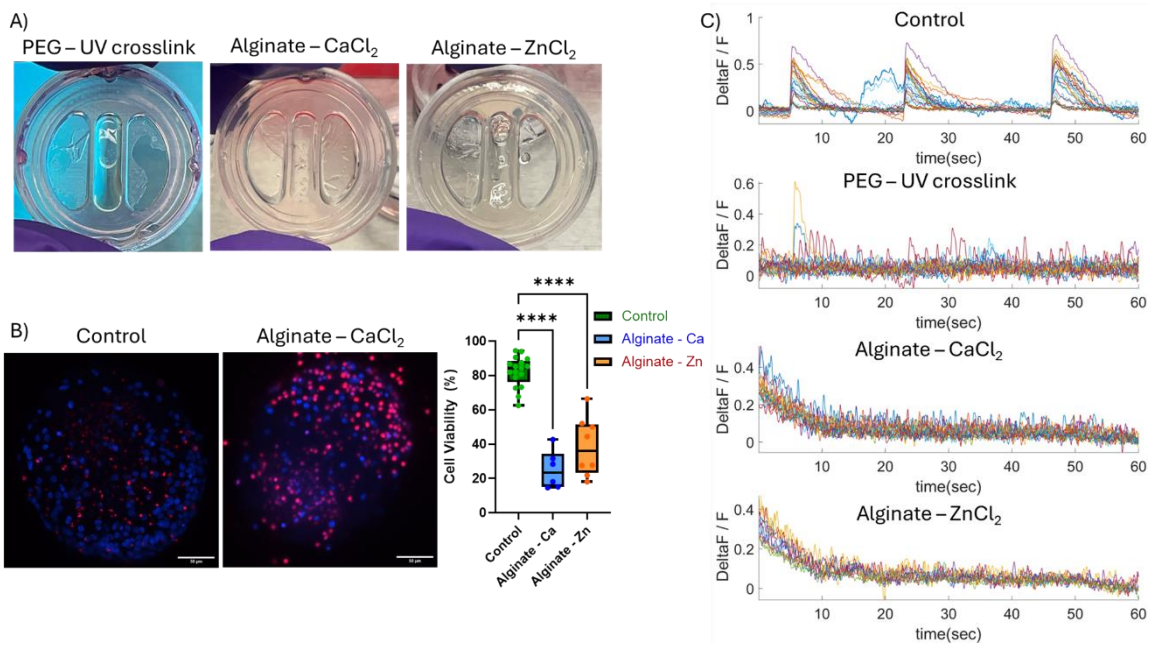


Figure 2: Each alternative hydrogel tested was unsuitable for cortical microtissue embedding.

A) Of the three hydrogels tested, only the 2% alginate crosslinked in calcium chloride successfully formed a suitable hydrogel. The PEG failed to crosslink under UV light at the volumes used and the 2% alginate crosslinked in zinc chloride was misshapen and deformed. B) Cell viability assays were run on microtissues embedded in both alginates after 24 hours of acclimation. Cell viability was significantly lowered in both Alginate – Ca (26.78%) and Alginate – Zn (40.09%) relative to the controls (82.22%). C) Calcium activity was not detected in any microtissue embedded in any of the hydrogel alternatives. n = 6 – 17 for each condition. **** $p<0.0001$. Scale bars = 50 µm.

2.4.3 Shear impacts of the cortical microtissues generated neuropathological effects, but strain was delivered inconsistently.

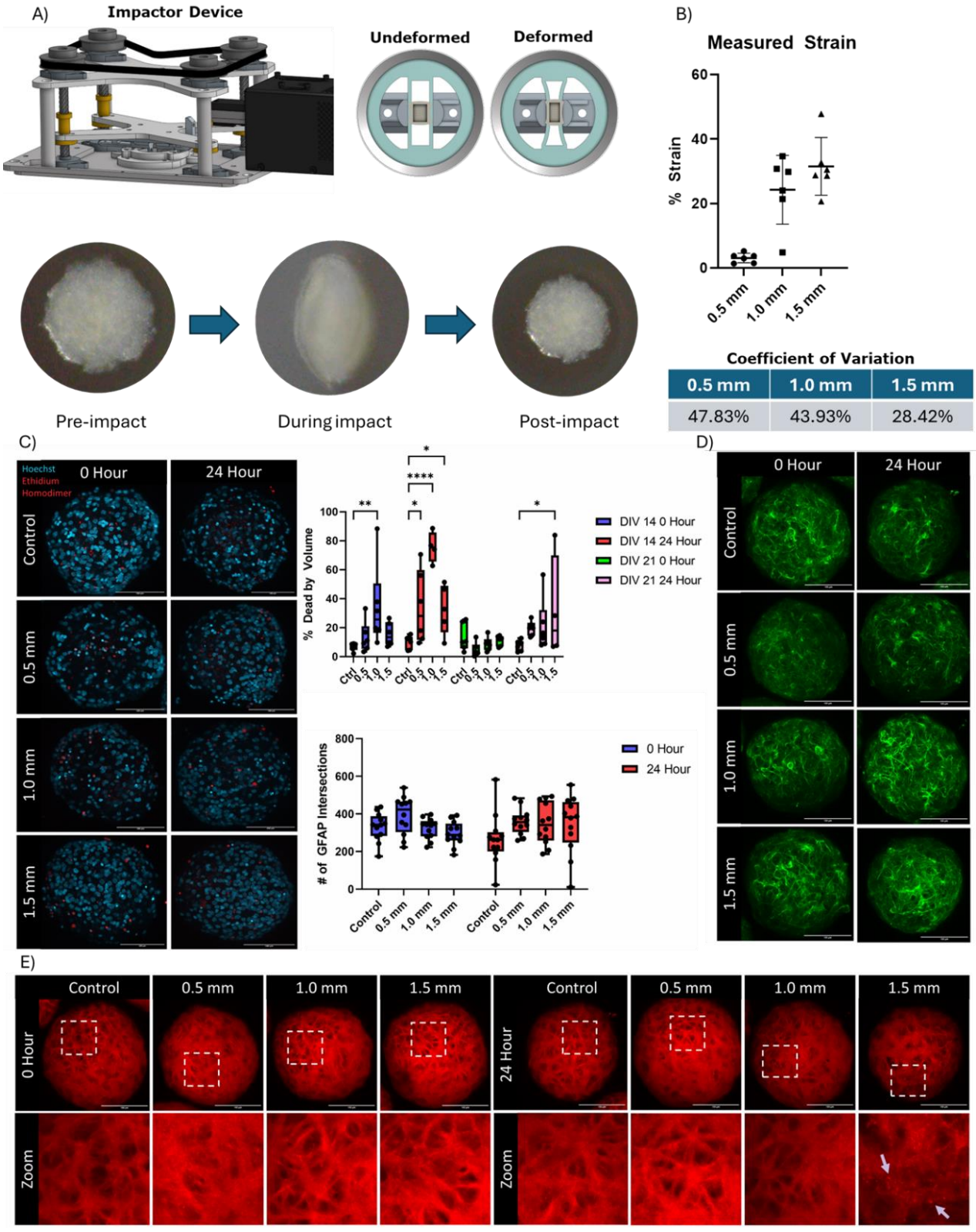
Due to the challenges associated with embedding the microtissues in a hydrogel medium we instead moved to a system of impacting the microtissues in the 96 well agarose hydrogels they were formed in. Using this approach, we were able to successfully compress the microtissues and both visualize and measure that compression using the custom inverted microscope apparatus we built without changing the impactor device set up

(Figure 3A). We conducted a series of experiments where the impacting arm was extended by 0.5 mm, 1.0 mm, and 1.5 mm for three different blunt impact conditions. The speed of movement was kept consistent across the three impact conditions at 600 mm/s. Using this approach we delivered impacts that strained the spheroids by 3.08% ± 1.47% with a calculated strain rate of 37.15 s-1 ± 17.77 s-1 (0.5 mm), 24.25% ± 10.65% with a calculated strain rate of 146.11 s-1 ± 17.77 s-1 (1.0 mm), and 31.52% ± 8.96% with a calculated strain rate of 126.08 s-1 ± 35.83 s-1 (1.5 mm). These strains and strain rates are biologically relevant and within the range of strains and strain rates we are interested in studying. However, they were inconsistent, yielding coefficients of variance of 47.83%, 43.93%, and 28.42%, respectively (Figure 3B).

Cell viability assays were conducted on both DIV14 and DIV21 microtissues impacted with 0.5 mm, 1.0 mm, and 1.5 mm impacting arm extensions immediately after the injury occurred (0 hour) and 24 hours later. Statistically significant increases in cell death were detected in DIV14 0 Hour 1.0 mm (27.79% ± 19.64%), DIV 14 24 Hour 0.5 mm (25.73% ± 19.01%) 1.0 mm (42.04% ± 11.46%), 1.5 mm (26.25% ± 14.04%), and DIV 21 24 Hour 1.5 mm (32.97% ± 30.39%) (Figure 3C). While successfully inducing a detectable injury in our microtissues was a promising sign, again the data was found to be inconsistent with coefficients of variation of 70.68%, 73.89%, 27.25%, 53.48%, and 92.17%, respectively, in each of the statistically significant groups.

Immunohistochemical staining of GFAP and β-iii-tubulin was performed on DIV14 microtissues immediately and 24 hours post-injury for each impact condition. GFAP

network complexity was quantified using Sholl analysis to detect the number of intersections of GFAP with concentric circles radiating from the center of each microtissue examined. No significant change in GFAP expression or network complexity was observed in this model (Figure 3D). Effects on β-iii-tubulin neurite networks were assessed qualitatively and found that 24 hours post-injury the microtissues that had received the 1.5 mm impact experienced the beginnings of cytoskeletal breakdown. In this group bright puncta of β-iii-tubulin are visible (Figure 3E arrows), consistent with indications of β-iii-tubulin breakdown detected in previous work in our lab.



Coefficient of Variation

0.5 mm	1.0 mm	1.5 mm
47.83%	43.93%	28.42%

Figure 3: Shear impacts delivered to the microtissues were inconsistent across the hydrogel but were able to generate neuropathological effects.

A) Instead of embedding the microtissues within a hydrogel the microtissues were impacted inside of the 96 well agarose hydrogels they were generated in. Using this method we can visualize the microtissue compression and measure the delivered strain. B) Three impact conditions were achieved and the strain measured by extending the impacting arm by 0.5 mm (3.08%), 1.0 mm (24.25%), and 1.5 mm (31.52%). The strain delivered by each impact condition was varied, yielding coefficients of variation of 27.83%, 43.93%, and 28.42% respectively. C) Cortical microtissues were impacted at DIV14 and DIV21 and cell viability assays performed immediately after impact and 24 hours after impact. Increases in cell death were detected in DIV14 0 Hour 1.0 mm (27.79%), DIV 14 24 Hour 0.5 mm (25.73%) 1.0 mm (42.04%), 1.5 mm (26.25%), and DIV 21 24 Hour 1.5 mm (32.97%), relative to their respective controls (8.10%, 10.51%, and 9.43%). D) Sholl analysis of DIV14 microtissues that had been impacted 0 hours and 24 hours post injury did not reveal any change in the GFAP network complexity in this model. E) Cortical microtissues that were immunohistochemically stained for β-iii-tubulin revealed a breakdown in cytoskeletal networks after 24 hours in microtissues that were impacted in the 1.5 mm condition as denoted by the formation of puncta (arrows).

2.5 Discussion

Traumatic brain injury pathology is extremely challenging to study due to the difficulty of studying living human patients, the limited throughput of animal models, and the reduced system complexity inherent in cellular models (Alaylioglu et al., 2020; Kanagasabapathi et al., 2011; Kumaria, 2017). The goal of this work was to establish a 3D cortical microtissue model and blunt injury impacting system that would allow us to interrogate TBI through the lens of inflicted strain and strain rate. By leveraging the diverse biological outputs of our cortical microtissue and the versatility of the custom impactor device we set out to map injury-relevant combinations of strain and strain rate to different neuropathologies.

Our initial approach to this study relied on embedding cortical microtissues in collagen hydrogels to serve as a force transduction medium for the impactor device. This approach

was designed to mirror approaches taken with 2D cell culture work where cells are either grown on top of or individually dispersed through collagen hydrogels to study the effects of strain at quasi-static strain rates on a per-cell level by our collaborators in the Christian Franck Lab (Bar-Kochba et al., 2016; Franck et al., 2011b; Yang et al., 2022). However, due to significant reductions in cell viability due to the embedding process, alternative approaches were explored.

Alternative hydrogels of PEG and alginate were selected for their optical clarity, known biocompatibility, and tunable mechanical properties via crosslinking parameters (Aisenbrey & Murphy, 2020; Andersen et al., 2015) with two different crosslinking reagents tested for alginate. While these alternatives were not ultimately suitable for the system we were trying to design, valuable insights were gained from experimenting with them. Beyond causing excess cell death in the cortical microtissues, embedding our model in hydrogels was causing a significant amount of background signal in our fluorescent assays, making quantification difficult and possibly drowning out signal generated by our calcium indicator. We were also limited in our ability to conduct time-course studies, as microtissues would dissociate into the hydrogels after ~72 hours of embedding. Due to these limitations with hydrogel embedding we pivoted to a new approach wherein we delivered impacts to the microtissues while still in the 96 well agarose hydrogels they were formed in.

This new approach, which we call shear impacts, did not require alterations to our existing impacting setup and brought with it several advantages over the hydrogel embedding

approach. By keeping the microtissues in their individual wells we gained the ability to study TBI neuropathology on a longer timescale than was possible with the embedding approach. We were also able to perform IHC on the microtissues, gaining insights into the effects of our delivered impacts on cytoskeletal networks and astrogliosis. Using this approach we successfully delivered impacts to the microtissues in the range of 25 – 30% strain at strain rates of 40 – 140 s^{-1}, injuries that roughly map to the strains and strain rates experienced by the brain in mild car crashes or sports collisions (Hosseini-Farid et al., 2020; McAllister et al., 2012a; J. Zhang et al., 2006a). At these levels of strain and strain rate we detected a significant increase in cell death consistent with previous work conducted in our lab where strains of 18%, 27%, and 35% were found to significantly decrease cell viability as soon as 2 hours post-injury in a sustained compression injury model (González-Cruz et al., 2024). Similarly, we were able to qualitatively determine that β-iii-tubulin networks begin to break down by 24 hours post-injury in at least one of our impacting conditions, which again is consistent with the previous study published by the lab on the sustained compression injury.

Despite these successes achieved, a significant drawback of our shear impact approach lies in the inconsistency of the data generated. The large degree of variation in the data suggests that not all microtissues in the hydrogel are undergoing uniform strain and strain rates with this impacting approach. Due to the shape of the agarose hydrogel and nature of the impactor device having one stationary arm and one moving arm, the microtissues across the 96 well array of the hydrogel are likely experiencing significantly different strains and

strain rates in a single impact. While it is possible to model the gradient of the delivered strains and strain rates across the full array, one of the main goals of this approach is to generate a high-throughput approach capable of generating high n values for rapid screening of tightly controlled TBI inputs.

To overcome the variability inherent in the shear impacting approach developed, initial work has been completed on a top-down impacting approach wherein 96 silicone pegs will enter each well in the agarose hydrogel and impact each cortical microtissue individually. This new approach will leverage a 3D printed bistable compliant mechanism to control the force and speed of impact, allowing the strain and strain rate to be tuned by the parameters input during the printing of the mechanism (Jensen et al., 1998). Initial work with this approach has yielded promising results, similarly detecting increased cell death and the breakdown of β-iii-tubulin networks post-injury. By building off the foundations and learnings established in this body of work, this new top-down impacting paradigm offers a promising future direction in pursuit of a high throughput biomimetic in vitro model for TBI neuropathology.

2.6 Conclusion

Cortical microtissues combine many of the strengths of in vivo models and 2D in vitro models of TBI neuropathology. This model recapitulates the inherent complexity of the neural microenvironment with a higher fidelity than standard 2D cell culture models while offering a robust, high-throughput system for a fraction of the cost, time, and animal

sacrifice of standard in vivo models. We have shown that the model responds to various strain and strain rate stimuli of pathological relevance. While shear impacts are capable of effectively delivering these stimuli, high variability in the data has led us to a shift to a top-down impacting paradigm with promising early results. Overall, this work represents exciting new directions towards the advancement of studying TBI neuropathology.

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Chapter 3: A Brain-Cancer Microtissue Model for Studying Tumor Cell Behavior and Cancer-Neural Cell Interactions

[Under Review]

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3.1 Summary (150 words):

Glioblastoma (GBM) is an aggressive brain cancer with a poor prognosis and is challenging to study due to its high degree of inter- and intra-tumoral heterogeneity and complex tumor microenvironment. Current in vitro models of GBM, including patient-derived organoids and 2D and 3D cell cultures can recapitulate aspects of the GBM biology, but interactions with normal cells of the central nervous system remain challenging to model. Herein, we introduce a new 3D biomimetic brain-cancer microtissue (BCM) developed by co-culturing rat cortical microtissues with rat glioma cell lines. Leveraging this model, we have characterized glioma cell motility, invasiveness, and interactions with neurons, astrocytes, and microglia. GBM cell behavior in the BCM model is consistent with that observed in vivo. This robust and versatile platform will enable future high-throughput therapeutic screening and detailed insights into primary glioma biology and potentially other brain tumors and cancers that metastasize to the brain.

Keywords: glioblastoma, cortical, model, microtissue, cancer, organoid, spheroid, tumor microenvironment, therapeutic screening, infiltration

3.2 Introduction:

Glioblastoma (GBM) is the most common primary brain cancer with over 12,000 new cases per year in the US (Price et al., 2024). Patients with GBM usually decline rapidly, with a 5-year survival rate of just 5% and a 15-month median survival, with the standard

of care comprising surgical resection, temozolomide chemotherapy, and radiation treatment (Alexander & Cloughesy, 2017; Foray et al., 2021; Stupp et al., 2005). GBM is notoriously difficult to treat due to its characteristic vast inter- and intra-tumoral heterogeneity and prolifically invasive behavior (Jacob et al., 2020b; Omuro & DeAngelis, 2013; Rybin et al., 2021). Despite significant efforts over the last 20 years by clinicians and researchers alike, there has been little to no progress in survival expectancy for GBM patients. New therapeutic approaches are desperately needed (Aldape et al., 2019).

GBM has a complex tumor microenvironment (TME) featuring interactions with brain endothelial cells, neurons, and microglia. The tumor cells interact with brain endothelial cells, astrocytes, neurons and microglia – playing key roles in tumor development, progression and therapy resistance (Venkatesh et al., 2019; Winkler et al., 2023). Indeed, over recent years these interactions have been shown to be a critical aspect of GBM pathobiology.

Modeling these interactions faithfully is challenging. While murine models recapitulate many key features of the disease, they are not high throughput, are costly and are time intensive (Haddad et al., 2021; Liu et al., 2021). Traditional 2D cell cultures, 3D cultures in hydrogels, and neurosphere cultures are useful but do not completely reproduce the complex cellular TME. For these reasons, multicellular models have become increasingly important in the field as recent studies have shown increased similarities and clinical outputs across 3D in vitro models and clinical trials (Liu et al., 2021).

Organoid models of GBM have gone through several iterations in the last decade as researchers have sought methods to capture tumor heterogeneity and preserve cellular morphologies in vitro. Recent co-culture approaches, such as the Glioblastoma-Cerebral Organoid Co-culture, combine patient-derived GBM cells and cerebral organoids to model the invasive patterns of GBM cells within the organoid (Linkous et al., 2019b). Despite advances on this front, these models lack key components of the TME, including vascularization and the presence of innate immune cells, both of which are critical components of GBM progression and therapeutic resistance. These limitations underscore the need for models of a higher level of biomimetic fidelity to gain a more complete understanding of GBM neuropathology.

Herein we present a new in vitro model of GBM/brain interactions, based on biomimetic rat 3D cortical microtissues that replicate many features of the normal brain including electrical signaling, native extracellular matrix secreted by neural cells, and the presence of functional microglia. We show that these microtissues can be readily co-cultured with GBM cells, forming Brain-Cancer Microtissues (BCMs). This model has allowed us to recapitulate the GBM TME with physiologically relevant primary cells including neurons, astrocytes, oligodendrocytes, neural precursor cells, and microglia. The BCM model has allowed us to study GBM infiltration and tumor-neuroimmune interactions in a multicellular context that replicates critical aspects of the GBM TME. This model provides a platform for future studies of tumor invasion and tumor-neuroimmune interactions, useful for both malignant gliomas and potentially for systemic tumors that metastasize to the

brain. Its ease of use and high level of reproducibility with representation of all brain cell types provide a platform for investigation of key biological mechanisms and for efficacious drug screening.

3.3 Results:

3.3.1 Cortical Microtissue and Brain-Cancer Microtissue generation

3D cortical microtissues were generated from primary postnatal (0-2 days) rat cortices as described previously (Dingle et al., 2015). Briefly, cortices were extracted, enzymatically and mechanically dissociated into a single cell suspension, and seeded at 8,000 cells per well into a 96 well 2% agarose hydrogel where they self-assembled into spherical microtissues over the following 24 hours (Figure 1A). To assess the feasibility of this model, 200 CNS1 rat glioma cells (CNS200) were seeded into each microtissue. CNS1 cells infiltrated and incorporated into the cortical microtissue over the first 24 hours of co-culture, resulting in the complete BCM (Figure 1B, Figure S.1).

DIV 14 BCMs were assessed for viability after 7 days of co-culture using Hoechst 33342 to label cell nuclei blue and Ethidium Homodimer-1 to label dead cells red (Figure 1C). There was no significant difference in microtissue viability between BCMs and cortical microtissue controls (Figure 1D). These data indicated the initial feasibility of this modeling approach and led to more extensive optimization and characterization of interactions in the BCMs.

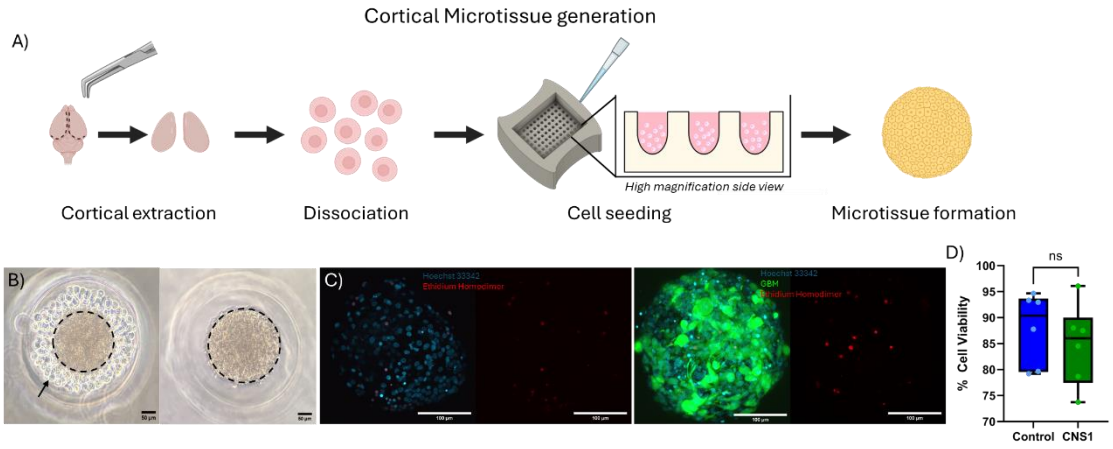

Figure 1: Brain-Cancer Microtissue generation did not affect cell viability.

A) Cortical microtissues were generated by extracting the cortex, dissociating the tissue, and seeding the cell suspension into 96 well agarose hydrogels where they spontaneously self assembled. B) Phase contrast micrographs of BCM formed by seeding CNS1 cells (arrow) into the agarose hydrogel well with DIV2 cortical microtissues (circles). Left: Immediately after seeding. Right: 24 hours after seeding. C) Confocal micrograph z-projections of BCM stained with Hoechst 33342 (blue) and ethidium homodimer-1 (red). CNS1 cells expressed GFP (green). D) Cell viability in microtissues was unaffected by the introduction of CNS1 cells (Control, 87.96% ± 6.99%; BCM, 84.75% ± 7.80%). n = 6. Significance determined by unpaired t-test, ns = $p>0.05$. Center lines of box plots represent median. Lower and upper whiskers represent minimum and maximum values, respectively. Boxes extend from 25th to 75th percentile. Scale bars = 50 µm (B), 100 µm (C).

3.3.2 Infiltrative behavior of glioma cells in BCMs reflect intrinsic cellular and microtissue characteristics

Two GBM cell lines were used to generate BCMs, GFP-expressing 9L rat glioma cells, and GFP-expressing CNS1 rat glioma cells. The 9L gliosarcoma cell line is a widely used model characterized by rapidly growing tumors with clearly delineated margins and little invasion into surrounding brain tissue (Barth & Kaur, 2009; Stojiljkovic et al., 2003). The CNS1 cell line is a useful model of glioma invasion and ECM interactions, with extensive brain infiltration (Barth & Kaur, 2009). BCMs were generated by seeding the glioma cells either at the time of dissection at 100 cells per microtissue, or after 7 days in vitro (DIV) at either 200 or 400 cells per microtissue

We observed a clear difference in the behavior of the 9L and CNS1 cell lines in the BCM model consistent with their reported behavior in vivo. 9L cells aggregated, forming microtumors with clearly delineated edges and little infiltration into the cortical microtissue (Figure 2A). On the other hand, CNS1 cells remained relatively dispersed and infiltrated more deeply into the microtissue than 9L cells and over time invaded throughout the microtissue (Figure 2B). Using custom MATLAB code, we quantified the relative spatial distribution of the GBM cells in each BCM seeding paradigm by defining collections of 4 or more cells within a 30-40 μm perimeter. This analysis revealed that a higher percentage of 9L cells engaged in clustering activity relative to CNS1 cells (Figure 2C, Table 1A). This analysis was conducted to assess the ability of BCMs to preserve the hallmark phenotypic behavior of each of the cell lines examined. 9L cells are well characterized to

form densely packed, non-invasive tumors while CNS1 cells are a standard model of GBM invasiveness in neural tissue. The preservation of these hallmark phenotypes in BCMs is a promising indicator of the model's fidelity.

Our previous work has established that the rat cortical microtissue exhibits in vivo-like stiffness and cellular composition, positioning it as a model to study glioma infiltration (Dingle et al., 2015). To assess the infiltrative behavior of each cell line, infiltration depth was determined by identifying the deepest Z-stack slice in the microtissues that contained green-fluorescent signal from tumor cells. Due to the surface position of the 9L microtumors, this analysis revealed little to no infiltration of 9L cells (data not shown) while CNS1 cells exhibited extensive infiltration into the cortical microtissues. Further analysis of CNS1 infiltration was conducted wherein CNS1 cells were seeded into cortical microtissues at early (DIV2), middle (DIV7, 11, and 13), and late (DIV14) stages and characterized after 1, 3, and 7 days of exposure using confocal microscopy imaging (Figure 2D). CNS1 cells exhibited maturation-dependent infiltrative behavior, with the deepest and fastest infiltration occurring in early and mid-stage microtissues and the shallowest and slowest infiltration occurring in late stage microtissues. After 3 days of co-culture in the early stage microtissues the CNS1 cells had infiltrated to the maximum visualization depth of 75 μm and possibly beyond (Figure 2E, Table 1B). The differences in infiltration depths and rates are likely driven by a higher density and distinct composition of ECM present in

the more mature microtissues, making it more difficult for the CNS1 cells to traverse throughout the microtissue.

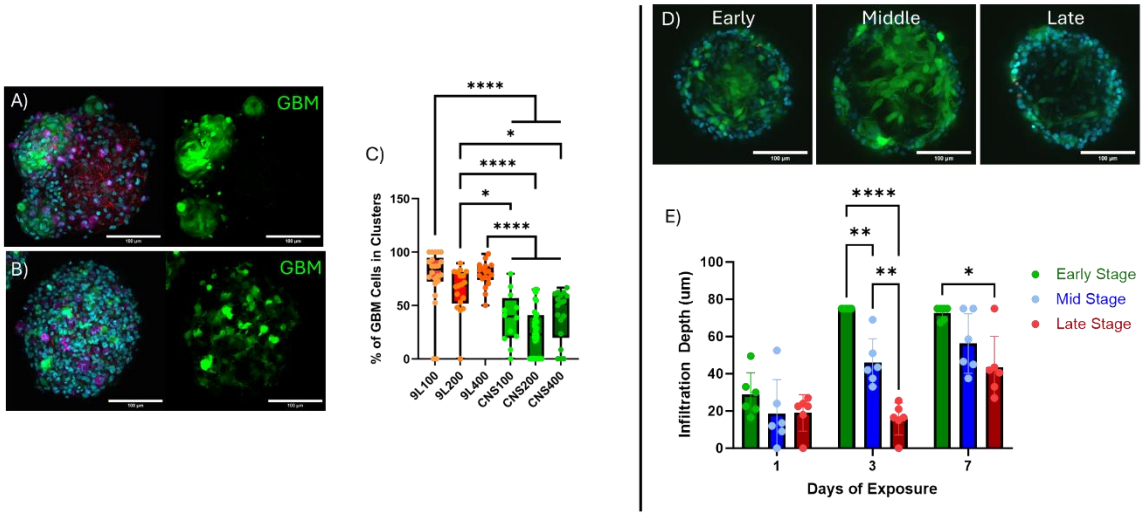

Figure 2: Brain-Cancer Microtissues demonstrated glioblastoma heterogeneity.

A) Confocal micrograph z-projections of 9L BCM. 9L cells formed large, isolated aggregates with little infiltration into the BCM. 9L200 seeded with DIV7 cortical microtissue for 7 days. Green, GBM; Magenta, microglia (IB4); Teal, nuclei (Hoechst). B) Confocal micrograph z-projections of CNS1 BCM. CNS1 cells infiltrated throughout the entirety of the microtissue. CNS200 seeded with DIV7 cortical microtissue for 7 days. Green, GBM; Magenta, microglia (IB4); Teal, nuclei (Hoechst). C) The percentage of cells that engaged in clustering behaviors was higher in 9L groups: 9L100 (76.31%), 9L200 (64.54%), 9L400 (78.74%), than in CNS1 groups: CNS100 (37.88%), CNS200 (25.14%), CNS400 (40.69%). D) Confocal micrograph z-projections of CNS1 BCMs seeded at early stage (DIV2), mid stage (DIV 7), and late stage (DIV14) after 7 days of co-culture. GBM infiltration depth varied with microtissue maturity at time of seeding. E) The infiltration depth of CNS1 in early stage BCMs after 1 day (29 µm), 3 days (75 µm), and 7 days (73 µm), in mid stage BCMs after 1 day (19 µm), 3 days (46 µm), and 7 days (56 µm), and in late stage BCMs after 1 day (19 µm), 3 days (16 µm), and 7 days (44 µm). n = 6 – 36. Center lines of box plots represent median. Lower and upper whiskers represent minimum and maximum values, respectively. Boxes extend from 25th to 75th percentile. * = $p<0.05$, **** = $p<0.0001$. Scale bars = 100 µm.

Table 1A: Summary of Glioblastoma seeding paradigms and clustering behaviors

Condition	GBM Cell Line	Seeding Density	Seeding Age	Analysis Age	Incubation Time (days)	GBM # of Clusters	GBM % Clusters
9L 100	9L	100 cells/BCM	DIV0	DIV14	14	1.83	76.31
9L 200	9L	200 cells/BCM	DIV7	DIV14	7	2.67	64.54
9L 400	9L	400 cells/BCM	DIV7	DIV14	7	4.33	78.74
CNS 100	CNS1	100 cells/BCM	DIV0	DIV14	14	4.17	37.88
CNS 200	CNS1	200 cells/BCM	DIV7	DIV14	7	2.73	25.14
CNS 400	CNS1	400 cells/BCM	DIV7	DIV14	7	7.17	40.69

Table 1B: Summary of CNS1 infiltration study seeding paradigms

Condition	GBM Cell Line	Seeding Density	Seeding Age	Analysis Age	Incubation Time (days)	Infiltrative Depth (µm)
Early Stage	CNS1	200 cells/BCM	DIV2	DIV 3	1	29
				DIV 5	3	75
				DIV 9	7	73
Mid Stage	CNS1	200 cells/BCM	DIV 13	DIV14	1	19
			DIV 11		3	46
			DIV 7		7	56
Late Stage	CNS1	200 cells/BCM	DIV14	DIV 15	1	19
				DIV 17	3	16
				DIV 21	7	44

3.3.3 Tumor-neuron interactions in BCMs showed disruption of tubulin cytoskeletal structures

Tumor-neuron interactions in the BCM model were characterized after 7 days (GBM 200 and 400) and 14 days (GBM 100) of co-culture via immunohistochemistry for β-iii-tubulin,

a marker for the cytoskeletal structures generated by neurons. In a healthy microtissue, this cytoskeletal network is characterized by the formation of strand-like structures and a smooth morphology (Figure 3A). The addition of glioma cells resulted in significant disruption of these neurite networks, with distinct differences between the morphological responses to the two glioma cell lines (Figure 3B-D). The addition of 9L cells resulted in neurite networks that generally appeared healthy, except for the sites of the microtumors, where β-iii-tubulin was absent. Conversely, spheroids seeded with CNS1 cells exhibited a more global cytoskeletal breakdown, characterized by the formation of puncta and cellular blebbing. Quantitative neurite network analysis was conducted using a custom graph theory analysis method to generate morphological graphs of the neurite networks and quantify the neurite network lengths (Figure 3E) (Burgess, 2024). While the neurite changes were morphologically different between the two cell lines, the normalized main network length was significantly decreased across all seeding paradigms (Figure 3F and G).

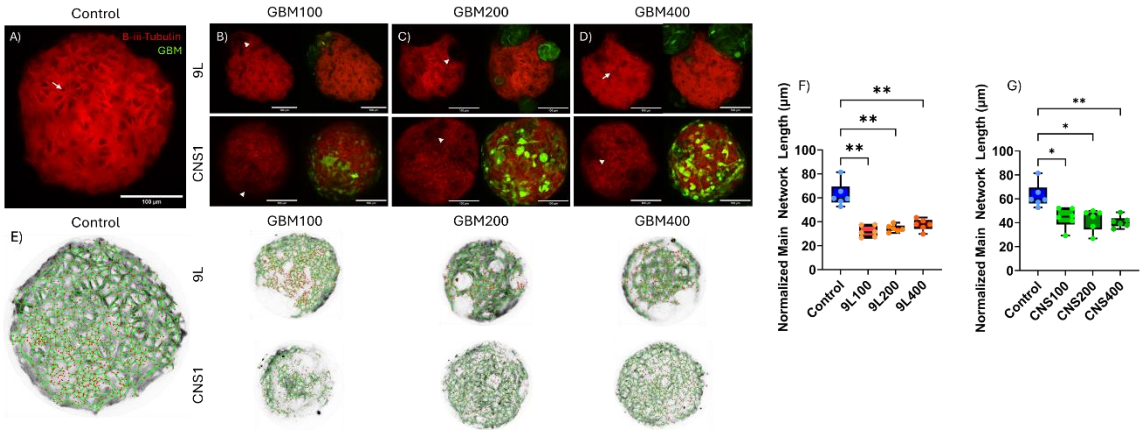

Figure 3: β-iii-Tubulin neurite networks were disrupted in all BCM conditions.

BCMs were generated by co-culturing cortical microtissues with 9L and CNS1 glioblastoma cells (green) for 14 (GBM100) or 7 (GBM200 and GBM400) days. A) Confocal micrograph z-projection of healthy controls, which exhibited β-iii-tubulin networks (red) with clear, interconnected, strand-like structures. B) Confocal micrograph z-projections of cortical microtissues seeded with 9L100 and CNS100 exhibited smaller normalized main network lengths of β-iii-tubulin. C) Confocal micrograph z-projections of cortical microtissues seeded with 9L200 exhibited smaller normalized main network lengths with large regions devoid of β-iii-tubulin (arrow), while CNS200 BCMs exhibited β-iii-tubulin disruption with regional dark spots (arrow). D) Confocal micrograph z-projections 9L400 exhibited smaller normalized main network lengths, large regions devoid of β-iii-tubulin and puncta formation (arrow) while CNS400 exhibited β-iii-tubulin network disruption and regional dark spots (arrow). E) Skeletonized graph network overlay of confocal micrograph z-projects of controls and each GBM seeding paradigm. F and G) Main neurite network length was significantly reduced in all spheroids exposed to cancer cells 9L100 (31.89%), CNS100 (44.19%), 9L200 (34.37%), CNS200 (42.59%), 9L400 (37.53%), CNS400 (40.58%); as compared to controls (62.6%). n = 6. Center lines of box plots represent medians. Lower and upper whiskers represent minimum and maximum values, respectively. Boxes extend from 25th to 75th percentile. * = $p<0.05$, ** = $p<0.01$. Scale bars = 100 µm.

3.3.4 Tumor-astrocyte interactions in BCMs showed GBM type-specific effects

In the BCM, astrocytic response to the addition of GBM cells was visualized by immunohistochemical staining for glial fibrillary acidic protein (GFAP). Astrocytes are highly branched cells with many processes extended in myriad directions as they survey the microenvironment to maintain homeostasis, exhibiting highly complex and intertangled networks when healthy (Figure 4A). The two rat glioma cell lines interacted with the astrocytes in the BCM in distinct ways (Figure 4B-D). GFAP expression was quantified as a function of the measured voxel fluorescent intensity normalized against the microtissue volume.

The addition of 9L cells did not lead to a significant change in levels of GFAP expression in the BCMs (Figure 4E); however, they elicited an interesting morphological response from the astrocytes. Branches of GFAP infiltrated and integrated into the 9L microtumors in all three seeding paradigms, most thoroughly in the 9L100 BCMs where they were co-cultured together for the longest amount of time (Figure 4B-D). This response suggests that astrocytes more readily integrate with 9L GBM microtumor sites, unlike neurons which were characteristically excluded from the microtumors.

BCMs seeded with CNS1 cells exhibited lower GFAP expression. GFAP voxel intensity was significantly lower in all three seeding paradigms of CNS1 relative to control cortical microtissues (Figure 4F). The introduction of CNS1 cells may also have contributed to a partial breakdown of GFAP branches since small detached puncta of GFAP appeared in

CNS1 BCMs, especially in the CNS100 and CNS400 seeding paradigms which also showed the lowest amount of GFAP expression.

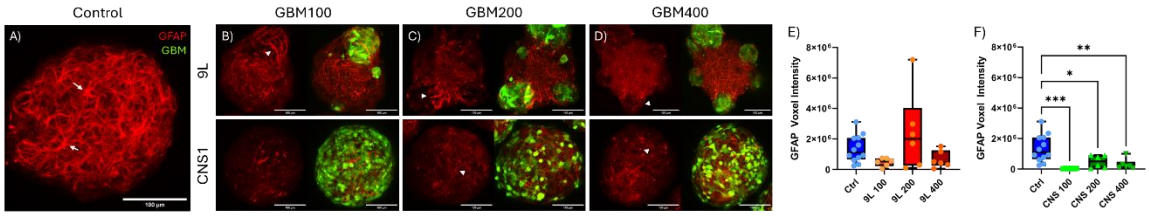

Figure 4: GFAP expression was significantly disrupted in CNS1 BCMs but not in 9L BCMs.

A) Confocal micrograph z-projection of control microtissues, which express GFAP as bright intertangled networks. B) Confocal micrograph z-projections of 9L100 BCMs in which GFAP branches (red) extended and infiltrated into the microtumors (green). CNS100 BCMs showed less GFAP expression. C) Confocal micrograph z-projections of 9L200 BCMs showed a similar, though less complete, tendency for GFAP branches to extend and infiltrate into the microtumors while CNS200 BCMs also exhibited less GFAP expression. D) Confocal micrograph z-projections of 9L400 BCMs also showed an integration of GFAP branching into the microtumors while CNS400 BCMs appeared to form puncta, suggesting a breakdown of existing GFAP branches. E) 9L BCMs showed similar average voxel intensities to control microtissues. Control (1,357,644), 9L100 (473,753), 9L200 (2,430,529), 9L400 (662,942). n = 6-12 per condition. F) Average voxel intensity was significantly lower in all CNS1 BCMs than in control microtissues. CNS100 (31,227), CNS200 (490,232), CNS400 (354,396). n = 6-12 per condition. Center lines of box plots represent median. Lower and upper whiskers represent minimum and maximum values, respectively. Boxes extend from 25th to 75th percentile. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. Scale bars = 100 µm.

3.3.5 Tumor-microglia interactions in BCMs showed higher numbers of microglia

In the BCM, microglia response to the addition of the glioma cells was assessed through immunohistochemical staining of ionized calcium-binding adapter molecule 1 (Iba1). Microglia in their homeostatic surveillant state are characterized by branched and ramified processes and even distribution throughout the microtissue (Figure 5A). 9L BCMs showed significantly higher numbers of microglia than controls, with observable localization to the microtumors (Figure 5B, 5C, 5D, S.2). Interestingly, 9L100 BCMs exhibited significantly higher numbers of microglia than controls, 9L200 and 9L400 BCMs (Figure 5E). 9L

microtumors are noted in literature as particularly immunogenic and prone to microglia infiltration (Barth & Kaur, 2009; Kundu et al., 2024). Our findings suggest that the additional time of co-culture experienced by 9L100 BCMs resulted in a stronger microglial response, even with the lower initial seeding density.

CNS1 BCMs also showed higher numbers of microglia than controls; however, the response appeared to vary by seeding paradigm (Figure 5F). Similar to the 9L seeding conditions, CNS100 BCMs exhibited the greatest microglia numbers, suggesting that the temporal component of co-culture is more important to microglial response than initial seeding density. CNS400 BCMs also showed higher numbers of microglia relative to controls, but CNS200 did not. While 9L cells are noted in literature for their strong immunogenic responses, there is less research on CNS1 immunogenicity (Kundu et al., 2024). These results indicate an overall weaker microglial response to CNS1 cells, though with high enough initial seeding density (CNS400) or long enough co-culture (CNS100), a significant microglial response may still be elicited. Overall, these data reveal that rat brain microtissues can support GBM cell tumor growth and that interactions between tumor and normal cells lead to changes in behavior that may be relevant to human disease.

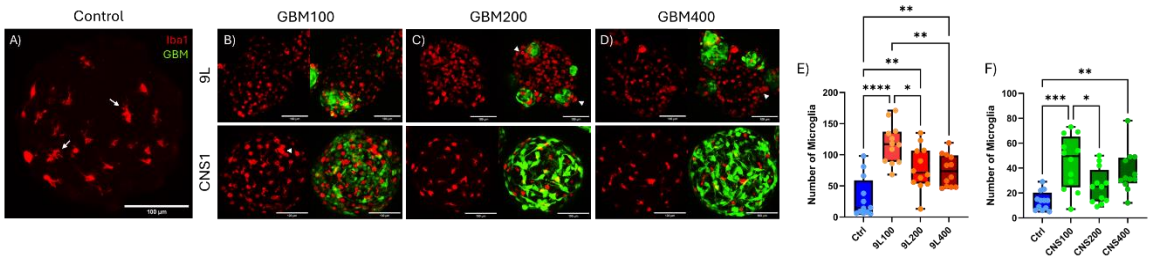

Figure 5: The number of microglia present was higher in microtissues exposed to GBM.

(A) Confocal micrograph z-projection of control microtissues at DIV14, which typically contain microglia (red) with multiple processes as they surveil the environment. (B) Confocal micrograph z-projections of cortical microtissues seeded with GBM cells (green), showing more microglia in both 9L100 and CNS100 BCMs than in control microtissues. C) Confocal micrograph z-projections of 9L200 BCMs, which also exhibited more microglia present with clusters of microglia localized near the microtumors. CNS200 BCMs did not show differences in the number of microglia. D) Confocal micrograph z-projections of 9L400 BCMs, which exhibited more microglia. E) The number of microglia present in all 9L BCMs was significantly higher than controls. 9L100 (117.33) exhibited significantly higher microglia numbers relative to both the control (30.92) and the 9L200 (78.25) and 9L400 (74.75). In CNS1 BCMs, only the CNS100 (44.5) and CNS400 (36.92) had significantly higher numbers of microglia relative to controls. CNS200 (25.58) BCMs did not have significant differences in the numbers of microglia present. n = 12 for each group. Center lines of box plots represent median. Lower and upper whiskers represent minimum and maximum values, respectively. Boxes extend from 25th to 75th percentile. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. Scale bars = 100 µm.

Discussion:

GBM is a challenging cancer that forms a complex array of interactions between tumor cells and local brain tissue that are known to be critical for tumor growth but are not yet well understood. Here, we describe a multicellular BCM model that represents an advancement in the field of in vitro GBM models. The model is easy to use, robust, reproducible, and provides a foundation for future drug screening and discovery efforts. With this approach we have been able to simultaneously study GBM-neuron, GBM-

astrocyte, and GBM-microglia interactions in an environment where the complex brain TME is biomimetically represented, offering an advantage over other approaches.

The BCM model demonstrated effective incorporation of GBM cells within the first 24 hours of co-culture with no adverse effects on cell viability in the microtissue. Both examined glioma cell lines exhibited their characteristic in vivo behaviors in the model, with 9L cells forming clearly delineated microtumors and CNS1 cells infiltrating throughout the entirety of the microtissue (Barth & Kaur, 2009; Stojiljkovic et al., 2003; Viapiano et al., 2008; H. Zhang et al., 1998). We characterized the differences in spatial distribution of each GBM cell line, as well as the impact of cortical microtissue maturity on the infiltrative behavior of CNS1 cells. The BCM model exhibited significant breakdown of neuronal networks in all seeding paradigms while capturing distinct differences in the morphology of that breakdown between each GBM cell line. GFAP expression also showed a cell line specific response, with directed infiltration and integration into 9L microtumors and significantly less GFAP expression when seeded with CNS1 cells. Additionally, the microglial response to each of the GBM cell lines was captured, revealing a difference in immunogenicity between 9L and CNS1 cells.

GBM research relies heavily on the use of in vivo and in vitro models. In vivo GBM models consist primarily of murine transgenic models and patient derived xenografts, each with advantages and disadvantages (Rybin et al., 2021). Transgenic models are excellent for studying tumorigenesis but lack the heterogeneity commonly found in clinical settings (Marumoto et al., 2009; Moquin-Beaudry et al., 2022). Patient-derived xenografts

represent the current gold standard as they model patient tumors, but xenografts often grow slowly and can be costly (Wang et al., 2009). The limitations of these in vivo models have led to an increased interest in the development of in vitro models.

The most advanced current in vitro models, such as the GLICO model, consist of patient-derived glioma stem cell (GSC) organoids co-cultured with iPSC-derived cerebral organoids (Linkous et al., 2019b). These organoids' properties include GSC proliferation, microtubules, and the ability to detect a difference in GBM lethality depending on the invasiveness of the GBM cells (Linkous et al., 2019b; Weth et al., 2022). These models can recapitulate parts of the GBM TME, but have a key drawback of lacking vascularization and innate immune cells (Silva et al., 2018). The interaction of microglial immune cells with GBM cells plays a key role in therapeutic resistance in GBM, making it a key element of interest for the study of GBM response to therapies (Geribaldi-Doldán et al., 2021; Gutmann & Kettenmann, 2019; Hambardzumyan et al., 2016; Pine et al., 2020; Quail & Joyce, 2017). The presence of innate immune cells in the BCM model is therefore an important step forward in the development of representative in vitro models of GBM pathology.

The TME is a highly dynamic environment with many interconnected and complex interactions occurring simultaneously among the myriad normal and cancerous cells surrounded by a tissue scaffold that regulates these interactions. For this reason, it is imperative for a biomimetic in vitro model to successfully recapitulate as much of the TME as possible. By sourcing all the primary cells in the brain region, BCMs capture more fully

the complexity of the in vivo environment. Further, these cells faithfully reproduce the ECM architecture native to the cortex, a feature that is absent in most other 3D models and which plays an important role on modulating GBM cellular motility and invasiveness (Dingle et al., 2015; Viapiano et al., 2008; H. Zhang et al., 1998). BCMs provide the cellular composition and mechanical properties of native cortical tissue scaffolding, resulting in high fidelity to model tumor cell motility and invasion. One limitation of the model at this stage is the lack of a myeloid component, which is a significant component of patient GBM, and which will be investigated in future studies (De Leo et al., 2020). Additionally, while our cortical microtissue model has previously shown the formation of capillary-like networks, investigating GBM interactions with capillary-like networks was outside of the scope of this study and will be investigated thoroughly in our future work (Boutin et al., 2018).

Of note, the versatility of the BCM model lies not only in the diverse cellular make-up of the model but also in its modular design. We can model any region of the brain, having successfully modeled the cortex, cerebellum, thalamus, and hippocampus. We may also incorporate any number of different cancer cell types. Moreover, BCMs are not restricted to only studying GBM cell lines; the model offers a promising avenue for studying the metastatic behavior of other types of cancer, such as breast or small cell lung cancer into the brain. This model can also be used with any number of cancer seeding paradigms. In this work, 200 GBM cells per microtissue was chosen initially to reflect the relative average size of GBM tumors in the average size of the human male brain (DeFelipe, 2011; Palpan

Flores et al., 2020). The GBM 400 groups were chosen to reflect a high cell density seeding paradigm, while the GBM100 groups were chosen to study longer term effects on the cortical cells and to evaluate if there would be adverse effects to BCM formation. It could be argued that the addition of GBM cells at the time of dissection does not fully reflect the dynamics of tumorigenesis since in clinical settings tumors grow on neural tissue that is already organized. Conversely, tumorigenesis propagates from a small initial population of cancer stem cells which are surrounded by neural tissue as captured in our GBM100 seeding paradigms (Del Vecchio et al., 2013). The versatility to study both of these seeding paradigms in BCMs underscores the breadth of research questions they may be used to interrogate.

There are many promising paths forward with BCMs. The current therapeutic gold standard in clinical GBM is surgical resection, followed by courses of irradiation and treatment with the standard of care chemotherapeutic drug temozolomide (Jacob et al., 2020b). Future studies applying this therapeutic course to BCMs will allow us to determine the efficacy on a per-cell-type basis and assess the magnitude and time course of recovery for each cell type. Furthermore, due to the high-throughput nature of BCMs, the model could prove to be critical to quickly and effectively screening new therapeutic approaches. Recent findings in the field of "cancer neuroscience" have also indicated integration of GBM cells into the neuronal circuitry (Amit et al., 2024; Monje et al., 2020). Conducting electrophysiological studies to assess GBM integration into electrochemical signaling pathways may reveal useful insights into the role of neuron-cancer interactions in tumor

growth and chemoresistance. Finally, expanding BCMs as a model of metastasis presents a large swath of possibilities for future studies. Lung, breast, and skin cancer are the most common forms of cancer to metastasize to the brain, with roughly 50% of lung cancers metastasizing to the brain (Achrol et al., 2019; Price et al., 2024). Incorporating cancer cell lines from these lineages into BCMs will yield new insights into how these disparate cell types interact with neural cells while also serving as a high-throughput therapeutic screening platform.

The BCM model offers new opportunities to study GBM-neuroimmune interactions in cellular and molecular detail. The high levels of reproducibility and robustness of the system will provide a platform for drug screening and new insights into the complex biology of GBM in the context of the brain.

Resource availability:

Lead Contact: Diane Hoffman-Kim, Diane_Hoffman-Kim@Brown.edu

Materials Availability: This study did not generate new unique reagents.

Data and Code Availability: Experimental data have been deposited at Open Science Framework and are publicly available as of the date of publication at [DOI]. All original code has been deposited at Open Science Framework and is publicly available at [DOI] as of the date of publication. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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Declaration of Interests: The authors declare no competing interests

3.4 STAR Methods:

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-β-iii-tubulin	Biolegend	801202
Rabbit anti-GFAP	DAKO	Z0334
Rabbit anti-Iba1	Fujifilm	018-28523
CY3 Goat anti-mouse	Jackson ImmunoResearch	115-165-068
CY3 Goat anti-rabbit	Jackson ImmunoReseaerch	111-165-144
Chemicals, peptides, and recombinant proteins		
Neurobasal A +	Invitrogen	A3582901
B27 +	Invitrogen	A3582801
Penicillin-Streptomycin (10,000 U/mL)	Fisher Scientific	15140122
GlutaMAX (100X)	Gibco	35050-061
PBS pH 7.4 (1x)	Gibco	10010-023
Papain	BrainBits	
Trypan Blue, 0.4% Solution in PBS	MP Biomedicals	1691049
Poly-D-Lysine	Sigma-Aldrich	P6407-5MG

RPMI-1640		
FBS	ScienCell Research Laboratories	0025
DMEM	Gibco	11330032
G418	MilliporeSigma	4727878001
Paraformaldehyde 20% Solution, EM Grade	Electron Microscopy Sciences	15713-S
Sucrose	Sigma-Aldrich	S9378-1KG
Normal Goat Serum	Jackson ImmunoResearch	005-000-121
Bovine Serum Albumin	Sigma-Aldrich	A7030-100G
Triton X-100	Sigma-Aldrich	X100-100ML
Hoechst 33342, trihydrochloride, trihydrate	Invitrogen	H3570
Ethidium Homodimer-1	Invitrogen	E1169
4', 6-Diamidino-2-Phenylindole (DAPI) 1000X	Sigma-Aldrich	32670
UltraPure Agarose	Invitrogen	16-500-100
Phosphate Buffer Saline Tablets	MP Biomedicals	2810305
Hibernate A	BrainBits	
Hibernate A -$CaCl_2$	BrainBits	
Ethyl Alcohol 95% (190 Proof)	PHARMCO	111000190
0.25% Trypsin-EDTA	Gibco	25200-056
Critical commercial assays		
Deposited data		
Open Science Framework	This study	DOI:
Experimental models: Cell lines		
CNS1	Hickey (Dartmouth University, NH)	RRID: CVCL 5276
9L	Yamaguchi (Sanford-Burnham Institute, CA)	RRID: CVCL 1928
Experimental models: Organisms/strains		
Rats	Charles River	CD
Software and algorithms		
ImageJ FIJI		
MATLAB		
Mathematica		
NIS-Elements		
Other		
Agarose molds	MicroTissues, Inc	24-96

FluoroDish	WPI	FD35-100
40 um cell strainer	Cell Treat	229481
10 mL Plastic Syringe	Fisher Scientific	20230130
Absorbent Bench Cover	Fisher Scientific	14-206-64
35mm dishes	Corning	353001
24 well plates	Cell Treat	229524
Pasteur Pipettes	Fischer Scientific	13-678-20C
Hemocytometer	Propper Manufacturing	12-519-10
Centrifuge	Eppendorf	5810 R
50 mL Centrifuge Tube	CELLTREAT	229421
15 mL Centrifuge Tube	CELLTREAT	229411
Snap Cap Low Retention Graduated Microcentrifuge Tubes, 2 mL, Sterile, Natural	Fisher Scientific	21-402-905
24 well glass bottom plates	Cellvis	P24-1.5H-N

3.4.1 Cell isolation and 3D microtissue culture

All animal procedures were conducted in accordance with the guidelines established by Brown University's Institutional Animal Care and Use Committee. Two days before dissection, 2% agarose 96-well hydrogels were created using molds from Microtissues, Inc. The hydrogels were allowed to acclimate to the cortical media- (CM-) consisting of neurobasal A+, penicillin/streptomycin, and GlutaMAX for two days, with daily CM- changes. The hydrogels were changed over to cortical media+ (CM+) consisting of neurobasal A+, 1X penicillin/streptomycin, 0.5 mM GlutaMAX, and 1X B27+ on the morning of dissection.

Postnatal (days 0-2) rat pups were immersed in ice for 45 minutes and decapitated prior to dissection. Cortices were extracted and homogenized, first with enzyme digestion in 2

mg/mL papain solution at 30°C for 30 min with occasional dissection, and then mechanically with fire-polished Pasteur pipettes. Cortical cells were separated via gentle centrifugation and counted on a glass hemocytometer with Trypan Blue to determine viability. The cells were then diluted to a concentration of 8,000 cells per well with mixed sex cell populations and allowed to settle into the wells for 20 – 40 minutes with plate shaking every 10 minutes. Over the following 24 hours, the microtissues spontaneously form in each of the microwells. They were stored in an incubator at 37°C and 5% CO_2 with changes of CM+ every 2 – 3 days. Additionally, 2D control plates were generated at 250,000 cells per well on poly-D-lysine coated 24 well tissue culture plates to ensure general cell health.

3.4.2 Cancer cell line culture, seeding, and Brain-Cancer Microtissue generation

CNS1 cells were cultured in RPMI-1640 with 2 mM L-glutamine, 10% fetal bovine serum, and 1x pen/strep. 9L cells were cultured DMEM, 10% fetal bovine serum, 1x pen/strep, and 50 μg/mL of G418 antibiotic. Both cell lines were passaged using standard cell culture procedures every 2-3 days. GBM cells were counted on a glass hemocytometer and seeded into the microtissues either at the time of dissection at a seeding density of 100 GBM cells per microtissue (9,600 cells per hydrogel) or after a certain number of days in vitro at either a concentration of 200 GBM cells per microtissue (19,200 cells per hydrogel) or 400 GBM cells per microtissue (38,400 cells per hydrogel).

3.4.3 Viability assay

Viability assays were performed by co-staining microtissues with Hoechst 33342 (all nuclei) and Ethidium homodimer (dead/damaged nuclei). Gels were incubated in a dye mixture consisting of 1 mL CM+, 1 µL Hoechst, and 4 µL Ethidium homodimer while protecting them from light. They were washed twice with warmed PBS and then returned to fresh CM+. Using a cut-off P1000 micropipette tip the spheroids were flushed out the gels and transferred to glass bottomed 35 mm confocal and taken for immediate confocal imaging. Images were analyzed in ImageJ FIJI using the "3D Objects Counter" plugin to quantify the number of discrete objects present in the z-stacks. Cell viability was therefore modeled as:

$$Cell\ viability\ (\%) = \frac{Total\ Nuclei - Dead\ Cell\ Nuclei}{Total\ Nuclei} \times 100$$

3.4.4 Immunohistochemistry

At DIV14 microtissues were fixed by adding 1 mL of 4% v/v paraformaldehyde in an 8% wt/vol sucrose in PBS solution and incubating them at room temperature overnight. Microtissues were washed 3 times for 30 minutes in PBS and then flushed out of the hydrogels and transferred to 1.5 mL Eppendorf tubes. Microtissues were blocked with blocking solution consisting of 10% normal goat serum, 4% bovine serum albumin, and 1% triton-x 100 in PBS on a shaker plate at room temperature for two hours. The microtissues were then moved to a primary antibody solution consisting of either mouse

anti-β-iii-tubulin, rabbit anti-GFAP, or rabbit anti-Iba1 diluted in blocking solution overnight. A non-primary control for each primary antibody used was set aside in fresh blocking solution as well. The following day, after two 2-hour washes with PBT, microtissues were blocked in blocking solution for another 2 hours and then moved to a secondary antibody solution consisting of goat anti-mouse antibody conjugated to CY3, or goat anti-rabbit antibody conjugated to CY3 overnight. After removing the secondary antibody, the microtissues were washed three times for 30 minutes in PBT and then incubated in 1X DAPI for one hour. They were then transferred back to PBS and stored at 4°C until imaged.

3.4.5 Imaging

All microtissues were imaged on a Nikon AX-R confocal microscope controlled by NIS-Elements software using glass bottom plates. Fluorescent images were acquired using a 20X air objective (Nikon, Plan APO, NA 0.75, WD 1.0 mm) or a 40X oil objective (Nikon, Plan Fluor, NA 1.3, WD 0.2 mm) and laser diodes (405, 488, and 532 nm) laser lines with 2 – 3x digital zoom. Imaging settings were consistent between samples for all conditions within the same experiment. ND2 files were converted to TIFF format in ImageJ FIJI and analyzed using several software solutions.

3.4.6 Cancer cell behavior analysis

The spatial distribution of clusters formed by tumor cells within the 3D microtissues was quantified using MATLAB (Mathworks) code (DOI:tbd). Multi-channel fluorescence

images of 50 slices (pixel width and height of 0.2442241 or 0.2849281 pixels/μm and voxel depth of 1.5 μm) were pre-processed using ImageJ FIJI software to split the DAPI (nuclei) and GFP (tumor cell) channels and provide intensity thresholding. Each channel was saved using tiff format, loaded into the code, and binarized. After binarization, images were filtered for size and morphology to shed artifacts. Tumor cells that overlapped with nuclei were segmented, and a second size filter was used to remove puncta. For CNS-1 GBM analysis, a cluster was defined as a collection of at least four objects within a 30 μm perimeter in any direction, while for 9L GBM analysis, the perimeter was defined as 40 μm. Initial clusters were determined using the distances between individual objects that fit the cluster criteria. Objects that could belong to multiple clusters were assigned to a single cluster, and a new cluster center was calculated by taking the average of all the objects' spatial coordinates within that cluster. The distances between each cluster center and object centroid were iteratively checked to update the cluster center coordinates until a steady state was reached.

CNS1 cell infiltration depth into microtissues was assessed early (DIV2), middle (DIV7, 11, and 13), and late (DIV14) stages after 1, 3, and 7 days of exposure. Z-stack images obtained using confocal microscopy were analyzed in ImageJ FIJI. The CNS1 fluorescent channel was isolated and binarized to visualize the cancer cells. A circle was drawn around the perimeter of each microtissue to determine the center point, and a second circle with a diameter of 100 μm was positioned at the center of the microtissue. To determine the infiltration depth, slices from the Z-stack (1 to 50) were examined for the presence of GBM

signal within the 100 μm circle. The slice number corresponding to the deepest detectable GBM cells was recorded. To calculate the depth of CNS1 infiltration, the slice number was multiplied by the 1.5 μm z-step of each slice.

3.4.7 B-iii-tubulin analysis

We show the normalized main network length of the BCMs in Figure 3F and 3G. These metrics were measured from a graph representation of the BCM's β-iii tubulin neurite network.

Graph representation of the BCM neurite network

Z-projections were generated from confocal micrographs collected of the BCMs using the confocal microscope. The images were 512 pixels wide and 512 pixels tall. The intensity at each pixel was recorded as a 16-bit binary number. We converted these binary numbers to decimal numbers, and then in each image, we affinely transformed the pixel intensities so that the maximum pixel intensity became 1.0 and the minimum became 0.0. This was done to increase the contrast in each image. Mathematically, our images can be described as two- dimensional arrays, or matrices, whose elements are real numbers ranging from 0.0 to 1.0.

Let M_k, for $k = 1, 2, 3, \ldots, n_{\text{stack}}$, denote the set of images acquired at different depths.

This set of images is often referred to as the z-stack. Here, n_{stack} denotes the total number of images in the z-stack, which was typically 50 in our case. The intensity of a pixel in the k-th image M_k, located at the i^{th} row and j^{th} column, is denoted by $M_k(i, j)$.

We define a single image called the "z-projection", M_{proj}, using the z-stack as

$$\mathcal{M}_{\text{proj}}(i,j) = \max_{k\in\{1,\ldots,n_{\text{stack}}\}} \mathcal{M}_k(i,j)$$

Creation of skeleton from Z-projection: We thresholded M_{proj} to further increase its

$$\tilde{\mathcal{M}}_{\text{proj}}(i,j) = \begin{cases} 1, & \text{if } \mathcal{M}_{\text{proj}}(i,j) > \mu, \\ 0, & \text{otherwise} \end{cases}$$

contrast. That is, we selected $\mu \in (0,1)$ and defined a binary image $\widetilde{M}_{proj}$ as

such that the contrast of $\widetilde{M}_{proj}$ was greater than that of M_{proj}. We then applied a median filter to $\widetilde{M}_{proj}$. That is, we replaced the value at each pixel with the median value of all the pixels in its r-neighborhood. More precisely, we defined a new image $\widehat{M}_{proj}$ using $\widetilde{M}$proj as

$$\hat{M}_{\text{proj}}(i,j) = \text{median}\left\{\tilde{M}_{\text{proj}}(m,n) \mid (m,n) \in \mathcal{N}_r(i,j)\right\},$$

where the r-neighborhood around pixel (i,j), $N_r(i,j)$, is defined as

$$\mathcal{N}_r(i,j) = \left\{(m,n) \in \mathbb{Z}^2 \mid |m-i| \leq r,\ |n-j| \leq r\right\}.$$

In our processing $r = 2$.

We created a "skeleton" of $\widehat{M}_{proj}$ using the following approach. We first binarized $\widehat{M}_{proj}$ then repeatedly thinned it. A thinning operation turns a white pixel that lies between a white (pixel value =1) and black region (pixel value =0) to black when that operation does not change the topology of the white and black regions in the image. Thinning was repeatedly performed until all regions were a single pixel thick.

Creating a graph from the skeleton: A mathematical graph, G, is defined as a set of vertices/nodes (V) and set of edges (E); where an edge is a pair of nodes. More precisely, E ⊆ {(u, v) | u, v ∈ V, u≠ v}. We created a graph from the binary skeletonized image using the algorithm given in Algorithm 1 (DOI:tbd).

Graph theory-based metrics for the BCM's β-iii tubulin neurite network

Lengths: In a graph corresponding to a BCM β-iii tubulin neurite network, we define the

length of an edge as the Euclidean distance between the pixels corresponding to the two nodes of that edge. We define the length of graph G as the sum of the lengths of all of its edges. We denote that length as ℓ (G).

Connected graph: A graph is called connected if one can reach any of its vertices starting from any other of its vertices and traversing the edges. We found that graphs corresponding to the BCM β-iii tubulin neurite networks were rarely, if ever, connected.

Sub-graph and maximally connected sub-graph: A sub-graph of the graph G = (V, E) is another graph G′ = (E′, V ′) where $V' \subseteq V$, and $E' \subseteq E \cap V' \times V'$. We found that the G corresponding to β-iii-tubulin networks in the BCMs consisted of many connected subgraphs. A graph is called a maximally connected sub-graph of G if it is a sub-graph of G, it is connected, and no new connected sub-graph of G can be created by adding any more vertices from V into V'′ irrespective of how we modify E′. In general, a graph G can always be uniquely decomposed into a collection of maximally connected subgraphs.

Once we have a graph from a z-stack of images of the BCM we decompose it into a collection of its maximally connected subgraphs. Let these maximally denoted subgraphs be Gi, i = 1, 2, ... We computed the lengths of each of those Gi. We define the normalized main network length as

$$\frac{\max_i \ell(G_i)}{\sum_i \ell(G_i)}$$

3.4.8 GFAP analysis

The average voxel intensity of GFAP-stained spheroids was calculated using ImageJ FIJI by importing confocal images of GFAP-stained spheroids. Individual spheroids were cropped, and the background was removed to ensure that only single spheroids were analyzed. The two-dimensional radius (2DR) of each spheroid was then approximated from the area of the circular cross-section using:

$$2DR = \sqrt{\frac{area}{\pi}}$$

The height (h) of each spheroid was determined as:

$$h = number\ of\ slices \times step\ size$$

The three-dimensional radius (3DR) was estimated using a geometric approximation derived from the Pythagorean theorem, using the height and 2D radius of the spheroid cross-section:

$$3DR = \frac{h^2 + 2DR^2}{2h}$$

To quantify GFAP signal intensity, we used the "3D Objects Counter" plugin in ImageJ FIJI to measure the average voxel intensity of the GFAP-positive regions of each spheroid. Thresholding was standardized across all conditions by measuring the pixel width of a representative astrocyte strand and adjusting the threshold to the same pixel width of the selected astrocyte feature. The "3D Objects Counter" plugin was further used to calculate the total integrated density (total pixel intensity) of GFAP-positive regions throughout the spheroid. The average voxel intensity was then obtained by normalizing the total integrated density to the 3D radius of the spheroid as modeled by:

$$Average\ Voxel\ Intensity = \frac{Total\ Integrated\ Density}{3DR}$$

3.4.9 Microglia analysis

The count and spatial distribution of microglia within the 3D microtissues was quantified using the same MATLAB code as previously described to quantify the spatial distribution of GBM cells. The pixel width and height were adjusted to 0.3419137 pixels/voxel while retaining the same voxel depth of 1.5 μm. In this case the ImageJ FIJI pre-processing step split the DAPI (nuclei) and the Cy3 (microglia) channels for intensity thresholding. Microglia that overlapped with nuclei were segmented and counted. For microglia a cluster was defined as a collection of at least four objects within a 30 μm perimeter.

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3.6 Supplementary Materials

S.1 – Timelapse of CNS1 Infiltration

240413_CNS1_Infiltration_72hour_Timelapse.mp4

S.2 – Spatial Analysis Code:

```
clc
clear all

%% Load in data tiffs using full file name inside single quotes

DAPI_raw = tiffreadVolume('DAPI_ADT.tif');
IBA_raw = tiffreadVolume('IBA_ADT.tif');

%% Binarize image and apply filter filter

% Ignore: add a median filter
% DAPI_filt = imboxfilt3(DAPI_raw, [3 3 3]);
% IBA_filt= imboxfilt3(IBA_raw, [3 3 3]);


% Ignore: saturate the top and bottom 1% of the data to improve contrast
% DAPI= imbinarize(imadjustn(DAPI_raw));
% IBA = imbinarize(imadjustn(IBA_raw));

DAPI= imbinarize(DAPI_raw);
IBA = imbinarize(IBA_raw);

% Exclude objects that have pixel lengths less than minSize; apply to IBA
% binary images
minSize = 150;
IBA_bw = bwareaopen(IBA, minSize);


% Set a morphology filter for DAPI to exclude unresolved nuclei
se1= strel('sphere', 3);
%se1 = strel('sphere', 1);
DAPI_bw= imopen(DAPI, se1);

% Overlap DAPI and IBA binary images and remove small objects
combined= DAPI_bw.*IBA_bw;
combined= bwareaopen(combined, 25);

%Ignore
%se1 = strel('disk', 1);
% se2 = strel('sphere', 1);
%se3= offsetstrel('ball', 3, 1);
%combined_bw1 = imopen(combined, se1);
% combined_bw2 = imopen(combined, se2);
%combined_bw3 = imopen(combined, se3);

%Visualize the binary data
volshow(DAPI_bw)
volshow(IBA_bw)
volshow(combined)
```

```
%% Find the center of each object and measure the distance between it and all
other objects
CC= bwconncomp(combined);
stats = regionprops3(CC, "Centroid");

n_centers = height(stats);
center = stats{:,:};

% Empty array to save the distances data
distances = zeros(n_centers, n_centers);

for ii= 1:n_centers
    for jj= 1:n_centers
        distances(ii,jj)= sqrt( (center(jj,1)-center(ii,1)).^2 +
(center(jj,2)-center(ii,2)).^2 + (center(jj,3)-center(ii,3)).^2  );
    end
end

% Plot all the centers of the objects in 3D space
figure(1)
plot3(center(:,1), center(:,2), center(:,3), '.')


% Calculate the means and standard deviation of all the distances for each
% object
for zz=1:n_centers
    Mean_distances(zz)=mean(nonzeros(distances(:,zz)));
    Stdev_distances(zz)=std(nonzeros(distances(:,zz)));
end


%% Generate Histogram to determine distribution of all values
distances_form= squareform(distances);
figure(2)
hold on
title("Distances between all objects")
ylabel("Counts")
xlabel("Distances in Pixels")
histogram(distances_form, 10)
hold off

%% Find the distance of all objects to a center point of the image taken

DimX = size(DAPI,1);
DimY = size(DAPI,2);
DimZ = size(DAPI,3);

core = [DimX/2 DimY/2 DimZ/2];
```

```
for ii= 1:n_centers
        distances_core(1,ii)= sqrt( (core(1)-center(ii,1)).^2 + (core(2)-
center(ii,2)).^2 + (core(3)-center(ii,3)).^2  );
    end

    figure(3)
    hold on
    ylabel("Counts")
    xlabel("Distance in Pixels")
    title("Distances to the center")
    histogram(distances_core,50)
    hold off


%Ignore
% K-means cluster analysis
% evaluation = evalclusters(center,"kmeans","gap","KList",1:6);
% figure(2)
% plot(evaluation)


%% TO DO:
% Add an overlay between IBA and combined image
% Sort through the data to identify certain clumps "using threshold" and
% criteria
```

S.3 - Algorithm 1: Creation of a graph from a binary skeletonized image

Input : Binary image with white pixels
Output: A graph representation of the image, G=(V,E).
1 Set V = E = ∅;
2 Initialize the set U with all white pixels in the image;
3 Choose a white pixel p at random from U ;
4 while U is not empty do
5 Delete p from U ;
6 Let n ← number of white pixels among the 8 nearest neighbors of p;
7 switch n do
8 case 0 do
9 Designate p as an isolated point and include it in V ;
10 Choose a white pixel p at random from U ;
11 end
12 case 1 do
13 Designate p as a node and edge point and include it in V ;
14 if pold exists then

```
15      create a new edge {p, pold}  and include it in E;
16      Delete pold;
 17                  Choose a white pixel p at random from U ;
18      else
19      pold ← p;
20      p ← neighbor of p in U ;
21      end
22      end
23      case 2 do
24      Designate p as an interior point;
25            p ← neighbor of p in U ;
26      end
27      case n > 3 do
28      Designate p as a node and branch point and include it in V ;
29      if pold exists then
30      create a new edge {p, pold} and include it in E;
31      pold ← p
32                p ← neighbor of p in U ;
33      else
34      pold ← p;
35                p ← neighbor of p in U ;
36      end
37      end
38      end
39 end
```

Chapter 4: A Versatile Platform for Modeling Traumatic Brain Injury and Glioblastoma In Vitro

4.1 Modeling brain tissue

The central nervous system is an incredibly complex interconnected network of myriad cells, macro- and micro-scale structures, and intricate functional interactions responsible for regulating sensation, movement, and cognitive functions. This system, composed of highly specialized cells including neurons, astrocytes, oligodendrocytes, and microglia working in concert with one another is particularly difficult to model due to its inherent complexity at the cellular and structural levels (McKenzie et al., 2018; Miron, 2017; Rahman & Suk, 2020; Salzer & Zalc, 2016; Thau et al., 2025). Two particularly challenging disease states of the CNS, traumatic brain injury and glioblastoma, require advanced modeling systems to understand the complex neuropathology they induce (Aldape et al., 2019; Alexander & Cloughesy, 2017; Maas et al., 2017; Ng & Lee, 2019).

Current approaches to modeling these diseases have generated many invaluable insights into their pathophysiological responses. However, these models are not perfect and several gaps exist in how effectively each of these diseases can be studied. The main approaches to studying TBI consist of post-mortem tissue analysis, in vivo animal studies, and in vitro cellular models (Frugier et al., 2010; Goldstein et al., 2012; McKee et al., 2013, 2015; V. W. Tang, 2020; Wu et al., 2021b; Xiong et al., 2013). Post-mortem tissue analysis has yielded insights into human TBI neuropathology, cytokine and chemokine profiles, neuroinflammatory responses, and macro-structural deterioration (Alaylioglu et al., 2020; Frugier et al., 2010; Goldstein et al., 2012). However, these insights come with the

limitations of being unable to experimentally control the original TBI stimuli, a lack of dynamic disease progression data, and a decay in molecular integrity due to the time between patient death and tissue analysis (Alaylioglu et al., 2020; Frugier et al., 2010; Goldstein et al., 2012). In vivo animal studies allow researchers to control the TBI stimuli and choose which stage of disease progression they want to interrogate more specifically, providing observations into long term neurodegeneration. Despite these strengths, in vivo animal models of TBI suffer from low throughput, high rates of animal fatality, and risks of rebound injury or infection (Kumaria, 2017; Zhao et al., 2023). In vitro cell models overcome many of these limitations due to their high throughput nature, absence of animal-associated complications, and ability to precisely study specific TBI modalities at a cellular level. However, these come at the cost of being conducted primarily in 2D neuron monocultures absent of the complexity of multi-cellular interactions and 3D architecture found in vivo (Kanagasabapathi et al., 2011; Wu et al., 2021b).

These challenges are not unique to the study of TBI, when modeling CNS pathology there is a consistent tradeoff between model complexity and experimental control. The study of glioblastoma, while a very different disease in nature and pathology, suffers from many of the same challenges as TBI when attempting to model neuropathology. Like TBI, glioblastoma affects not just the neurons, but the broader microenvironment and requires a model that capture these intercellular dynamics in three dimensions.

The study of glioblastoma pathology is largely conducted using in vivo models, traditional 2D cell culture models, and organoid models. In vivo models are generally divided into

transgenic models, which provide valuable insight into tumorigenesis, and xenograft models, which generate data that is highly representative of patient tumors. However, these models are limited by low heterogeneity, prolonged experimental timelines, and high associated costs (Marumoto et al., 2009; Moquin-Beaudry et al., 2022; Wang et al., 2009). Traditional 2D cell culture models are highly versatile and facilitate precise investigation of specific cellular responses; however, they fall short in modeling tumor invasiveness and the complexity of the TME, primarily due to the limited ability to successfully co-culture multiple relevant cell types at biomimetic concentrations (Geribaldi-Doldán et al., 2021; Pine et al., 2020). GBM research has successfully leveraged a variety of organoid models including GBOs, neoCORs, and GLICOs to generate key insights into GBM invasiveness, spatial organization, and the TME. While these insights are extremely valuable, these models provide limited control over which GBM-inducing genetic mutations can be studied, lack vascularization, and are absent of microglia, which are of critical importance in GBM pathology (Hubert et al., 2016; Linkous et al., 2019b; Silva et al., 2018).

The limitations of current models of TBI and glioblastoma pathology present an opportunity to develop improved systems capable of more accurately capturing the native complexity of the CNS. In response to this need, we employed the cortical microtissue model developed by the Hoffman-Kim lab. This biomimetic model captures important hallmarks of the in vivo environment, including cellular diversity, functional connectivity, and three-dimensional architecture. This model enables the study of neuropathology with

enhanced fidelity, throughput, and experimental control compared to traditional in vitro and in vivo approaches.

4.2 Cortical microtissues as a model of neuropathology

The cortical microtissue model represents a significant step forward in modeling CNS diseases. This self-assembled 3D model incorporates relevant CNS cell types, including neurons, astrocytes, oligodendrocytes, and microglia at biomimetic densities and contains both structural and functional characteristics of native cortical tissue, including both tissue stiffness and electrochemical signaling (Boutin et al., 2018; Dingle et al., 2015; Sevetson et al., 2021). The reproducibility, versatility, and high throughput of this model position it well to bridge several of the existing gaps in the field and advance the study of TBI and GBM disease progression.

This cortical microtissue model provides several key strengths that enhance its applicability to neuropathological studies of TBI and GBM. Critically, this model retains the complex cellular diversity of native cortical tissue as stated previously, perhaps the most important of which are microglia, which are notably absent in many alternative in vitro models (Abreu et al., 2018; González-Cruz et al., 2024; Gutmann & Kettenmann, 2019; Hambardzumyan et al., 2016; E. Jung et al., 2021). These microtissues also exhibit tissues stiffnesses consistent with native cortical tissue, an essential element to accurately modeling the mechanical stimuli associated with TBI and GBM cell motility through the

system (Dingle et al., 2015). Additionally, the stability and robustness of the model facilitates our ability to conduct temporal analyses of the progression of these diseases.

While we did not focus extensively on electrophysiology in this work, it is worth noting that the model supports neuronal network formation and calcium signaling, offering additional functional readouts for future studies (Sevetson et al., 2021). We also did not explore the proto-vascularization observed in the model previously in the form of capillary-like networks (Boutin et al., 2018). Although these structures do not recapitulate full vascularization or establish a functional blood brain barrier (BBB), their presence does provide the opportunity to study localized tissue responses in proximity to capillary-like networks. While there are models specialized to study nutrient diffusion or BBB dynamics such as BBB-on-a-chip, the cortical microtissue model presented herein is better suited to studying multicellular responses to diseases after crossing the BBB (Jagtiani et al., 2022). Altogether, the cortical microtissue model provides a versatile and powerful platform for the interrogation of TBI and GBM disease states.

4.2.1 Cortical microtissues as a model of TBI pathology

In this thesis we leveraged the biomimetic fidelity and high throughput nature of the cortical microtissue model to establish a novel approach to modeling TBI neuropathology. Our initial goal was to systematically examine TBI pathology by delivering an array of physiologically relevant strains and strain rates reflective of real-world injury scenarios (Hosseini-Farid et al., 2020; McAllister et al., 2012b; J. Zhang et al., 2006b). Using a

custom built impactor device designed by our collaborators at the Christian Franck lab, we successfully delivered blunt impact injuries to the cortical microtissues, achieving strains ranging from 3% - 32% and strain rates up to 146 s^{-1}, aligning with the mechanical stimuli observed most commonly to induce mild TBI, such as sports collisions or vehicle accidents.

Initial efforts to embed the cortical microtissues within collagen revealed significant detrimental effects to cortical cell viability. As we explored alternative hydrogels of alginate and PEG we found that the cell health and assay sensitivity of our microtissues continued to be compromised. We pivoted to a new approach wherein we delivered a shear impact to the microtissues within the agarose hydrogels in which they were initially formed. With this approach we were able circumvent the negative effects on cell viability we uncovered when embedding the cells into hydrogels.

Using this shear impact approach, we detected significant increases in cell death immediately and 24 hours post-injury, as well as disruption to the neuronal cytoskeletal networks as denoted by β-iii-tubulin breakdown 24 hours post-injury. These findings were consistent with known injury responses in prior sustained compression injury studies, thus validating our model's ability to recapitulate key hallmarks of TBI pathology (Kanagasabapathi et al., 2011; Wu et al., 2021b).

However, one significant limitation of this approach was the inconsistency in how strain was distributed across the array of microtissues, likely due to uneven force distribution inherent to the shear impact method. To overcome this variability and increase the precision

of the model we have begun work on a top-down impacting paradigm using a 3D printed bistable compliant mechanism (BiCM) to drive 96 individual silicone pegs into each well of the hydrogel array. Preliminary results demonstrated that this new system can replicate the injury responses seen in shear impacts including decreased cell viability and the breakdown of β-iii-tubulin neuronal networks post injury.

Taken together, these efforts establish the cortical microtissue model as a viable platform for studying TBI at both cellular and structural levels, offering a balance between biomimetic properties and a high degree of experimental control. Having demonstrated the cortical microtissue model's ability to model mechanical injury induced neuropathology, we sought to interrogate the system's ability to model another complex CNS disease: glioblastoma.

4.2.2 Cortical microtissues as a model of glioblastoma pathology

To use the cortical microtissue model in a different neuropathological context we introduced glioblastoma cell lines, developing Brain-Cancer Microtissues (BCMs). This approach allowed us to recapitulate many features of the brain-tumor microenvironment while maintaining our model's strengths in its high fidelity cortical makeup and cellular architecture. Leveraging the presence of all native cortical cells, especially microglia which are notably absent in most current organoid models, we investigated how two different GBM cell lines interacted with neurons, astrocytes, and immune cells in a 3D setting, as well as GBM invasiveness (Geribaldi-Doldán et al., 2021; Miron, 2017).

The BCM model exhibited distinct behaviors between the two GBM cell lines tested, 9L and CNS1. 9L cells formed discrete, compact microtumors with minimal invasion, whereas CNS1 cells displayed infiltration throughout the microtissues, both consistent with their respective in vivo phenotypes (Barth & Kaur, 2009; Viapiano et al., 2008; H. Zhang et al., 1998). In CNS1 cells, infiltrative behavior was moderated by microtissue maturity, suggesting that ECM composition and tissue architecture influence GBM motility.

We also observed distinct effects of GBM co-culture on each neural cell type we examined. Both GBM cell lines disrupted the β-iii-tubulin neuronal networks, though in completely distinct morphologies. Astrocytes exhibited divergent responses to each GBM cell line as well, with CNS1 suppressing GFAP expression while 9L microtumors were infiltrated by astrocyte processes. This interaction in particular was quite interesting as literature indicates that GFAP expression is typically upregulated in glioblastoma patients to such a degree that in severe cases it can be detected in their blood (C. S. Jung et al., 2007). While we saw two distinct effects on the astrocytes in our model, neither resulted in GFAP expression upregulation, raising the question of whether this disparity is due to the particular cell lines we chose to examine or if there are more complex interactions occurring in vivo that BCMs are not capturing. Microglia exhibited a much stronger response to 9L cells than CNS1, consistent with the high degree of immunogenicity of 9L reported in literature, though the time of co-culture with both GBM cell lines appeared to have an even more powerful effect on microglia proliferation (Barth & Kaur, 2009).

These findings position BCMs as a powerful and flexible system for studying both GBM pathology, and screening potential therapeutic approaches. BCMs combine biomimetic cell composition, 3D architecture, and notably, the presence of immune cells, offering a new platform for the investigation of brain cancer.

4.3 Future directions and impact

The work presented in this thesis lays the groundwork for a biomimetic, high throughput in vitro platform for the study of neuropathology. There are several exciting future directions for both the TBI and the GBM projects to go, building upon the findings presented here to further advance our understanding of CNS disease states and therapeutic strategies.

In the near term, we aim to further refine and validate the top-down impact paradigm delivered by the bistable compliant mechanism (Jensen et al., 1998). While impacts are currently delivered to the microtissues with this approach, efforts are underway to quantify the strain and strain rate experienced by each microtissue during impact. We also plan to more fully characterize the responses of individual cortical cell types to injury over time, and to investigate the effects of repetitive impacts, which have been shown in literature to induce synergistic, rather than additive, neuropathological effects (Goldstein et al., 2012; McKee et al., 2013, 2015; Moszczynski et al., 2018). By tuning the mechanical properties of the BiCM to control the strain and strain rate delivered to the microtissues, we will leverage this new paradigm to fulfill our goal at the outset of this work to systematically

interrogate our microtissue's response to an array of physiologically relevant strains and strain rates reflective of real-world injury scenarios. This will play an important role in achieving our ultimate goal of mapping out TBI pathology and progression as a function of strain and strain rate and identifying biomarkers released as the injury progresses. These findings may be used in the future to help diagnose and treat TBI accordingly, overcoming current challenges in TBI diagnosis.

The BCM platform will be expanded upon to include additional glioblastoma cell lines to capture the effects of a more diverse range of GBM phenotypes on cortical cells. Functionally, the BCM model is well suited for screening therapeutic approaches; a clear next step for this model would incorporate standard-of-care treatments. Specifically, courses of irradiation and temozolomide together could be applied to the model to study their effects on both, the GBM cells in the model and the cortical cell response to these interventions (Jacob et al., 2020b). In the long term, this model can also be adapted to explore other modes of brain cancer, notably metastases from breast, lung, or skin cancer origins, each of which commonly metastasizes to the brain (Achrol et al., 2019; Price et al., 2024). While a strength of our model lies in the presence of microglia as the resident immune cells of the brain, we are also interested in incorporating circulating immune cells into the model in the future to mimic the neuroimmune response when the BBB has been compromised in vivo. By introducing T-cells and monocytes, we could begin to explore how peripheral immune infiltration interacts with glioblastoma progression and the tumor microenvironment (Dejaegher et al., 2021).

This cortical microtissue model is well-suited to support a wide range of molecular and functional assays, including RNA sequencing, cytokine profiling, proteomic analysis, and ATP-based viability measurements. These techniques would allow for deeper mechanistic insight into both TBI and glioblastoma-induced changes in cellular behavior, signaling cascades, and tissue-wide responses. This opportunity exists across both the TBI and GBM applications of the model, enabling investigations into inflammatory signatures, neuroprotective pathways, and treatment responses within a single biomimetic model. While the current methodology of producing 96 microtissues per agarose gel generates soluble factors below the detection threshold of many commercially available assays, this is a solvable constraint. Now that foundational parameters have been validated for both disease models, future iterations can increase the number of microtissues generated per gel to produce higher analyte yields. This simple modification will expand the analytical capabilities of the cortical microtissue model and strengthen its role as a versatile platform for molecular screening across diverse CNS pathologies.

These future directions aim to address critical gaps in our understanding of CNS pathology by enabling more nuanced studies of cell-cell interactions, the neuroimmune environment, and treatment responses in a controlled, biomimetic system. As the model continues to evolve, its combination of physiological relevance and high throughput positions these cortical microtissues as a valuable tool for advancing both fundamental research and translational studies in TBI and glioblastoma.

4.4 Contributions of this thesis work

The following list summarizes the primary contributions of this thesis to the field of neural tissue engineering, traumatic brain injury, and glioblastoma research:

- Physiologically relevant strains and strain rates were successfully delivered to 3D cortical microtissues, inducing hallmark TBI phenotypes including increased cell death and cytoskeletal breakdown, establishing the platform as a viable in vitro model of TBI neuropathology
- Glioblastoma cells integrated into cortical microtissues while demonstrating unique phenotypic behaviors including cell-line specific infiltration patterns and distinct interactions with neurons, astrocytes, and microglia, revealing critical aspects of brain-tumor microenvironment dynamics.
- Temozolomide preserved cortical cell viability in the presence of GBM cells, demonstrating the model's resilience and potential as a screening platform for therapeutic approaches.

4.5 Conclusion

This thesis presents the development of a 3D biomimetic in vitro model for the study of the neuropathology of traumatic brain injury and glioblastoma. Through an iterative process of developing and refining mechanical injury delivery methods and the integration of cancer co-cultures, this model has been shown to recapitulate complex cellular responses

across multiple neural cell types. Importantly, this platform opens the door to rapid therapeutic screening in a controlled, reproducible, and physiologically relevant environment. These contributions enhance the broader neuroscience community's ability to model CNS diseases with appropriate nuance and precision, laying the foundation for future investigations into a wide range of neurological pathologies.

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