

**Design and Implementation of a Device to Simulate Blunt Traumatic Brain Injury
Using a 3D In Vitro Cortical Spheroid Model**

By

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Thesis

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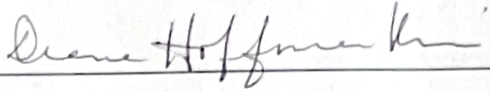
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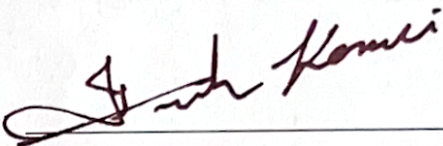
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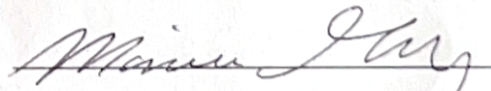
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Table of Contents

<i>Table of Contents</i>	4
<i>Acknowledgements</i>	7
<i>Chapter 1: Introduction</i>	10
1.1 TBI Diagnosis and Treatment	10
1.2 Modes of TBI	12
1.3 Cell Types Involved in TBI	13
1.4 <i>In Vitro</i> Models of TBI	16
1.5 Bistable Compliant Mechanisms	17
1.6 Research Aims	18
Aim 1: Develop a high-throughput indenter array for delivering targeted impacts to cortical spheroids in a 96-well format.....	18
Aim 2: Design and integrate a mechanical impact mechanism for reproducible simulation of blunt-force TBI.	19
Aim 3: Characterize the biomechanical and pathophysiological responses to simulated TBI.	19
<i>Chapter 2: Methods</i>	20
2.1 Agarose Gel Preparation	20
2.2 Cortical Cell Isolation	21
2.3 Preparation of Silicone Indenters	23
2.4 Impactor Device Design	24

2.5 Viability Assay	24
2.6 Immunohistochemistry	25
2.7 Confocal Imaging.....	26
2.8 ATP Assay	27
2.9 Data Analysis	28
2.9.1 Viability Analysis.....	28
2.9.2 TUBB3 Analysis.....	29
2.9.3 GFAP Analysis	29
2.10 Statistical Analysis.....	30
2.11 Mechanical Testing	30
<i>Chapter 3: Results</i>	<i>33</i>
3.1 Silicone indenters were successfully designed and implemented for use in direct impacts to cortical spheroids	33
3.1.1 9-well agarose gel and silicone indenter generation and implementation.....	33
3.1.2 96-well silicone indenter generation and implementation.....	34
3.2 Manual pilot experiments resulted in significant breakdown of neurite networks and increases in microglial volume at 0 and 24 hours post-injury	36
3.3 Initial Impactor Device Design	42
3.4 Initial experiments with the silicone indenter and BiCM showed various viability effects.....	45
3.5 Impactor device designs were improved following preliminary testing.....	50
3.5 BiCM-induced injury leads to significant cellular disruption at 0 and 24 hours post-impact.	52

<i>Chapter 4: Discussion and Conclusions</i>	<i>74</i>
<i>References</i>	<i>80</i>
<i>Appendix</i>	<i>88</i>

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Abstract of “Design and Implementation of a Device to Simulate Blunt Traumatic Brain Injury Using a 3D *In Vitro* Cortical Spheroid Model” by Francesca Vecchio, Brown University, May 2025.

Objective: Traumatic brain injury (TBI) results when external forces exert strain on the brain, resulting in cell death, inflammation, and long-term neurological effects. To screen therapeutics for TBI, there is a need for biomimetic, high-throughput models that simulate blunt TBI. A previously established 3D *in vitro* cortical spheroid model containing the cells present in the brain and exhibiting brain-like stiffness and electrical activity was employed to address this need. Here, we present a novel silicone indenter and 3D-printed mechanical assembly to simulate blunt TBI in cortical spheroids, enabling reproducible and scalable injury delivery.

Methods: Primary rat cortical cells (8000 cells/well) were seeded into 96-well agarose micro-molds to generate spheroids. Silicone indenters with 96 pegs were designed to impact spheroids at 13-15 days *in vitro*. Impacting devices were designed in Fusion360 and 3D printed with polylactic acid on a Prusa i3 MK3S+ printer. Viability was assessed via ATP quantification and Ethidium Homodimer-1 staining. Neuronal and glial responses were evaluated through β -III Tubulin (neurites), Glial Fibrillary Acidic Protein (astrocytes), and Isolectin-B4 (microglia), followed by confocal imaging, ImageJ and graph theory analyses, and statistical evaluation.

Results: Silicone indenters were integrated with one of two custom-designed bistable compliant mechanisms (BiCM1 or BiCM2), both of which successfully delivered mechanical impacts to cortical spheroids. Silicone indenters induced strain levels ranging from 25% to 50%. The approximate force required to deploy the BiCMs were 9N for BiCM1 and 25N for BiCM2. BiCM2 exhibited a high strain rate of approximately 400 to 600 s⁻¹. Spheroids subjected to

impact exhibited immediate and sustained astrocyte activation, neurite degeneration, and reduced viability lasting at least 72 hours, with BiCM2-impacted spheroids experiencing more pronounced cellular damage. Repeated impacts also resulted in cell death.

Conclusions: This study demonstrates a novel platform for simulating blunt TBI in cortical spheroids. Impacted spheroids resulted in reduced viability and increased neuroinflammation, with higher severity injuries resulting in increased spheroid damage. Ongoing work includes mechanical optimization to improve experimental reproducibility. This platform provides a promising tool for mechanistic studies and therapeutic screening in TBI research.

Chapter 1: Introduction

Traumatic brain injury (TBI) affects over 2.5 million people in the United States and leads to the hospitalization of over 200,000 Americans annually.¹ TBIs occur when external forces exert strain on the brain, causing structural damage through either an open, penetrative injury or a closed injury, such as a blunt or blast impact.²

TBIs can be classified as mild, moderate, or severe, with approximately 90% of TBIs being characterized as mild to moderate.³ Common symptoms of a TBI include headaches, dizziness, nausea, and challenges with concentration, but severe TBIs can result in various cognitive and behavioral abnormalities as well as comas.^{4,5} Nationally, TBIs are a major personal and economic burden, with the cost of TBI to healthcare estimated at over \$40 billion annually.⁶ There is a critical need for research to address the physical, mental, and economic burden of TBI by better understanding the pathophysiology of the disease and developing effective treatments for those who have been diagnosed with TBIs.

1.1 TBI Diagnosis and Treatment

Clinically, the Glasgow Coma Scale (GCS) is the standard tool for assessing TBI severity. The GCS evaluates eye-opening, verbal responses, and motor responses to score a patient on a scale of 3 to 15, with lower values indicating more severe TBI and higher values representing milder TBIs.⁷ The GCS values are used as a guide for TBI severity rather than a strict assessment, but they nonetheless assist in clinical management decisions.⁸ Abnormalities from TBI such as focal contusions, hemorrhages, and hematomas can be detected with computed tomography (CT) images or magnetic resonance imaging (MRI).⁹ Cranial CT scans are

commonly ordered in the emergency department following injuries with GCS values lower than 15 or for patients with various risk factors.¹⁰ Typically, MRIs are ordered within a few weeks after mild TBI if additional risk factors, such as chronic symptoms or concerning clinical findings, are present.⁹ Assessment of various biomarkers in the blood serum, such as glial fibrillary acidic protein (GFAP), are also an option for assessing TBI.^{9,11} The clinical workflow for TBI diagnosis can be visualized in **Figure 1**.¹⁰

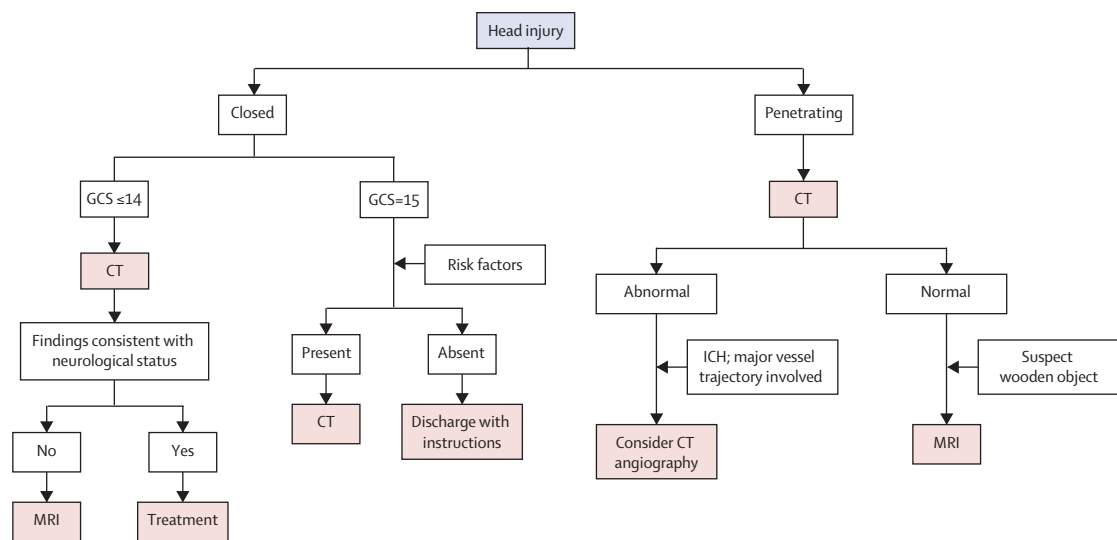


Figure 1: Clinical diagnosis workflow for penetrating and closed TBIs as described by Maas et al.¹⁰ ICH = intracerebral haematoma.

TBIs vary greatly in individuals depending on the type of injury obtained. In general, following an external impact, swelling, hemorrhages, hematomas, and contusions can be aspects of the primary injury response.¹⁰ The secondary injury response begins minutes to days after injury and continues for weeks to years.¹² This response is largely cellular and involves generation of free radicals, inflammation, metabolic dysfunction, apoptosis, and release of various neurotransmitters such as glutamate.¹⁰

Emergency care post-injury involves stabilizing patients as well as avoiding hypoxia and hypotension, which are associated with secondary injury.¹³ Lesions detected on CT scans are operated on as needed. Intensive care, such as ensuring proper oxygenation, is provided to avoid harmful secondary damages and promote recovery of brain tissue.¹⁰ Currently, several pharmacological therapies have been explored for use during the acute phase post-injury and in the post-acute stage. For example, current evidence recommends the antifibrinolytic tranexamic acid to patients 3 hours after injury to treat some forms of TBI, which moderately decreases risk of mortality but is not associated with improvement in neurological function.¹⁴ However, there is a lack of evidence-based treatments for TBI that improve neurological function, inhibiting rehabilitation efforts in patients worldwide.^{14,15} In addition, while many patients recover fully, especially those with mild TBIs, others face severe disability or death.^{9,10}

1.2 Modes of TBI

Understanding both the biomechanics and pathology of a TBI are essential for characterization of injury. The mechanical impact in a TBI can be described in terms of strain and strain rates.¹⁶ While there is little research on the specific values associated with injury severities, several animal studies and finite element analysis models on human tissues have found that injury occurs at strains as low as 10% and strain rates as low as 10 s^{-1} .^{17,18} In a review of *in vivo* controlled cortical impact (CCI) models, the depth of the cortical compression and the velocity of the impact have been used to characterize injury severity; however, there is not complete consensus on what values characterize TBI as mild, moderate, or severe.¹⁹ **Table 1** shows the general parameters that determine TBI severity from *in vivo* CCI models as described by Siebold et al.¹⁹ Therefore, to better understand the biomechanics of injury, we aim to assess

the severity compression upon impacts by calculating strain and strain rates and recapitulating TBI conditions.

Table 1: Guiding principles for classification of TBI severity in the mouse CCI model¹⁹

Criteria	Mild	Moderate	Severe
Tissue loss	None or confined to cortical layer	Overt cortical lesion extending to subcortical tissue	Overt cortical and hippocampal lesion
Neurological severity score	Minor deficits < 24 h	Substantial deficits < 72 h followed by complete recovery or sustained minor deficits	Substantial deficits > 72 h
Cognitive deficits	Minor deficits < 1 week with complete recovery	Substantial deficits < 1 week followed by minor deficits or complete recovery	Substantial deficits > 1 week without complete recovery
Surgical parameters ^a	Cortical depression < 0.5 mm, velocities < 4.0 m/s. Closed-head recommended.	Cortical depression 1.0–1.5 mm, velocities 4.0–5.0 m/s	Cortical depression > 2.0 mm, velocities > 5.0 m/s

1.3 Cell Types Involved in TBI

The secondary response in TBI is characterized by neuroinflammation, altering the neural vascular unit which contains neurons, astrocytes, microglia, endothelial cells, pericytes, and smooth muscle cells.²⁰ Neuronal cell death is a critical aspect of TBI pathology. Neurons are particularly vulnerable to the primary and secondary injury responses. Endothelial cell and smooth muscle cells may also harm cerebral blood flow regulation which can contribute to neurodegeneration.²⁰ While microglia and astrocytes release cytokines that clear debris, they also compromise the blood-brain barrier (BBB) and lead to apoptosis of neurons.²¹ Neuroinflammation has been described as a double-edged sword due to its beneficial and harmful effects.²¹

Neuronal loss has been attributed to physical damage, necrotic cell death from high levels of excitatory neurotransmitter release, and delayed cell death associated with secondary injuries.²² This death begins immediately after injury and at delayed timepoints.²³ Key to neuronal integrity is the cytoskeleton, which is supported by microtubules that maintain axonal

structure.²⁴ Axonal damage can contribute to loss of cytoskeletal structure after an injury.²⁵ The mechanical damage of a TBI has been shown to decrease levels of microtubule-associated proteins in hippocampal tissue.²⁶ Class III β -tubulin proteins have been shown to decrease after a TBI.^{27,28} Researchers have found that some axonal microtubules are able to recover after an injury while others degrade.²⁹ Similarly, myelin degradation and demyelination is characteristic of TBI, but studies have shown remyelination can occur during TBI repair with the generation of new oligodendrocytes.³⁰

The two general types of cell death occurring in TBI include necrosis, an inflammatory process that involves cell swelling and release of harmful cell contents, and apoptosis, a programmed, non-inflammatory process that involves cell volume reduction and removal of damaged cells.³¹ These two types of cell death often coexist.³² Cell death begins in the injured location immediately after an impact, and it often continues in surrounding areas during the secondary response period.³¹ Apoptosis is a common result of cell death in TBI and is associated with oxidative stress, inflammation, and excitotoxicity following injury.³³ There are multiple pathways and factors that induce DNA damage and apoptosis, including increased intracellular calcium, release of free radicals, caspase activation, and release of excitatory amino acids.³³ The metabolic activity within cells is significantly altered following a TBI, and ATP, the primary energy source of the cell, plays a central role in these changes. Immediately after injury, mitochondrial dysfunction involves both an increased demand for ATP and a decreased production of ATP. This energy crisis increases cell death, inhibits cellular repair, and exacerbates the secondary injury response.³⁴⁻³⁷

Astrocytes are the most abundant cell type in the central nervous system (CNS), interacting with neurons and various other cell types present in the CNS.^{38,39} GFAP, a marker for

astrocytes, is one of the most studied TBI biomarkers. Measurements of patient blood serum indicate that levels of GFAP are highly sensitive in assessing whether a patient has a TBI, as GFAP can cross a compromised BBB. GFAP levels also accurately assess the presence of intracranial lesions following a TBI.⁴⁰ Patients with worse outcomes one year after injury have been shown to exhibit higher serum GFAP values.⁴¹ Therefore, it is expected that the increase in GFAP aligns with the clinical severity of TBI. While astrocytes have some protective effects, studies suggest that the presence of reactive astrocytes also contributes to neuronal degeneration following TBI.⁴²

Microglia are the resident immune cell of the CNS and are dynamic cells involved in cytokine generation and phagocytosis.⁴² They extend and retract their branches to detect potential threats, and their morphology often determines their function.⁴³ Healthy, resting microglia are described as ramified, but their morphology changes in response to disruption or injury.⁴² Following a TBI, their retractions may increase as they target the injured site, phagocytose molecules, and release various pro-inflammatory factors.⁴³ Microglia are activated following TBI and may remain so for years following an insult.⁴⁴ The effects of microglia have been described as a double-edged sword due to the pro-inflammatory effects of M1 microglia and the anti-inflammatory effects of M2 microglia. M1 microglia release pro-inflammatory cytokines and reactive oxygen species that lead to cell death and worsening of the injury.⁴⁵ However, M2 microglia have advantages such as removing cell debris and aiding with tissue repair following an injury by releasing anti-inflammatory factors such as interleukin-10 and transforming growth factor- β .^{45,46}

These interconnected processes suggest the need to understand and target cell viability, microglial and astrocytic responses, and neuronal breakdown to promote recovery from TBI.

1.4 *In Vitro* Models of TBI

More research is necessary to understand the pathophysiology of TBI and assess potential treatments for the disease.⁴⁷ Thus, there is a critical need for high-throughput, biomimetic 3D *in vitro* models that simulate blunt TBI impacts. In contrast to *in vivo* models, *in vitro* models offer the benefit of simultaneously assessing the biomechanical and pathophysiological effects of injury in a cost-effective, high-throughput manner.⁴⁸ 3D models have high complexity, near-realistically mimic the brain microenvironment, and display physiologically relevant responses to mechanical and biological stimuli.⁴⁹

There are multiple *in vitro* spheroid and organoid models in existence that have been successfully implemented for neurodegenerative and TBI-related studies. Previously, 3D organoids that mimicked the brain architecture lacked cell type diversity and reproducibility.⁵⁰ While newer induced pluripotent stem cell-derived brain organoids are more reproducible and contain more diversity of cells present in the brain microenvironment, organoids often take months to fabricate, and their geometry is challenging to control.⁵¹

In contrast to organoids, spheroids have more consistent sizes and are highly reproducible.⁴⁹ They mimic *in vivo*-like cellular architecture and behavior and can be manufactured quickly.⁴⁹ Recent developments using the 3D spheroid structure have helped researchers better understand cellular interactions, such as neuronal-glia communication, long-term effects of apoptosis and inflammation, and screening neuroprotective treatments.⁴⁸ Further, new models have been used to study repetitive low-strain injuries, such as those occurring in blast impacts.⁵² Spheroids have also been used to study the diverse types of biomechanical trauma that occur in a TBI, such as biaxial stretch, uniaxial stretch, shear deformation, shock

wave exposure, and compression.⁴⁸ Thus, spheroids serve as an accessible, diverse, and reproducible tool for investigating the cellular and molecular consequences of neurodegenerative diseases.

Previously, researchers at the Hoffman-Kim lab have engineered 3D *in vitro* cortical spheroids to simulate various neurological diseases and disorders.⁵³ Using postnatal rat cortical cells, the 3D spheroids spontaneously self-assemble in 96-well agarose hydrogels. The spheroids contain the diversity of cell types present in the brain, including neurons, microglia, astrocytes, oligodendrocytes, and neural progenitor cells, and display brain-like mechanical properties and tissue stiffness.⁵³ They also exhibit brain-like electrical activity and mature synapses after 14 days *in vitro* (DIV). Further, they form capillary-like networks in culture and contain extracellular matrix proteins and endothelial cells.^{54,55} Previously, neurological diseases and injuries, including sustained compression injury and ischemic injury, have been successfully characterized within the cortical spheroids, and presently, efforts are being made to characterize blunt TBI pathology in the model.^{56,57} This model offers a high-throughput, cost-effective method for the study of neurological diseases and therapeutic screening.

1.5 Bistable Compliant Mechanisms

Previously, the Brigham Young University Compliant Mechanisms and Robotics Group has developed multiple open-source bistable compliant mechanisms (BiCMs). The design for the project described henceforth was inspired by their previous work.⁵⁸ BiCMs exhibit bistable behavior that can be compared to the behavior of a switch. There is power applied only when switching between two stable equilibrium states, and the precise switching makes them easy to control.⁵⁸ Once a force is exerted at the first stable position, the mechanism reaches a critical

point and snaps into the second stable position. The structure of a general BiCM can be seen in Figure 2.⁵⁹ The BiCM has not yet been applied to biological applications, but it is a promising avenue for TBI studies. It is optimized for use the following study since it is simple to manufacture and optimize with 3D printing. Further, it offers cost-effective and compact method to study TBI in a laboratory setting.

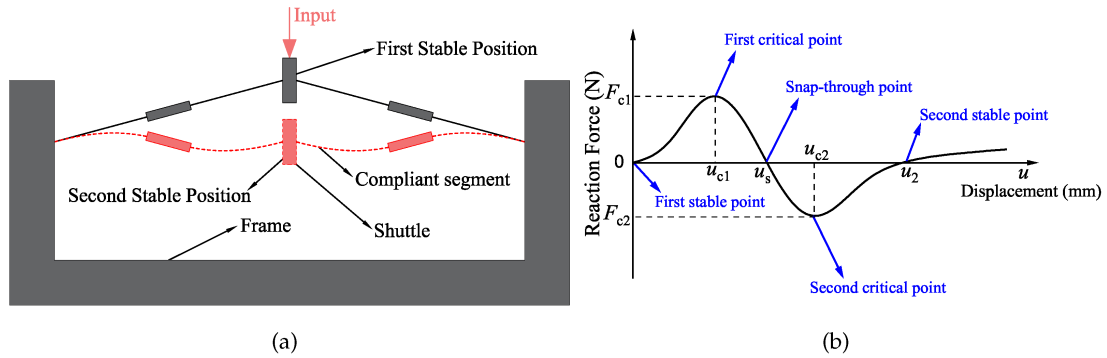


Figure 2: Traditional compliant bistable mechanism. (a) The general design of a bistable mechanism consists of a frame, moving shuttle, and compliant segment. A force is exerted on the moving shuttle at the first stable position, triggering a switch into the second stable position. and (b) The force–displacement curve associated with the equilibrium states of a bistable mechanism consists of two stable points, a snap-through point, and two critical points.⁵⁹

1.6 Research Aims

Here, we present a combined silicone indenter and 3D-printed mechanical mechanism to deliver impacts. We aim to replicate high strain rate impacts on previously established 3D *in vitro* cortical spheroids, creating a cost-effective, sterile, reproducible, and high-throughput TBI injury model.

Aim 1: Develop a high-throughput indenter array for delivering targeted impacts to cortical spheroids in a 96-well format.

To model blunt TBI, we developed silicone indenters to impact individual cortical spheroids in 96-well agarose gels. We evaluated the indenters for their precision in entering wells and compressing spheroids without disrupting gel integrity. Strain delivered by the indenters was approximated.

Aim 2: *Design and integrate a mechanical impact mechanism for reproducible simulation of blunt-force TBI.*

To achieve consistent and repeatable injury, we adapted open-source BiCMs for controlled mechanical impacts. These mechanisms were 3D-printed using PLA and engineered to be compatible with the 96-peg silicone indenters. The design allows for operation in sterile environments and is compatible with live imaging platforms such as confocal microscopy, enabling real-time observation of injury dynamics.

Aim 3: *Characterize the biomechanical and pathophysiological responses to simulated TBI.*

We evaluated the force-displacement profiles of two BiCMs delivering different force magnitudes, resulting in distinct injury severities. Cortical spheroids impacted within 96-well gels were analyzed for cell viability and structural integrity. Post-injury responses were assessed through immunostaining of astrocytes (GFAP), microglia (Isolectin-B4), and neurite networks (β -III Tubulin), allowing quantification of neuroinflammation and degeneration following single and repeated impacts.

Chapter 2: Methods

2.1 Agarose Gel Preparation

Micro-molds were used to generate 2% w/v agarose gels with 96 microwells, each with a diameter of 400 μm and a microwell length of 800 μm (MicroTissues, Inc.) (Figure 3A).⁶⁰ The rectangular chamber, measuring 5 mm by 7 mm, used for cell seeding (Figure 3B), holds 75 μL of media. The overall thickness of the gel is approximately 3.75 to 4 mm. A 2% w/v agarose solution was prepared in phosphate-buffered saline (PBS) and microwaved for a total of 2 minutes, with swirling every 10 to 15 seconds to prevent boiling and ensure uniform mixing.

Using a 10 mL pipette, the agarose solution was dispensed into the 96-well agarose molds. Excess material was removed with a spatula to ensure a flat bottom surface. After solidification, the agarose gels were removed from the molds by inverting the mold and gently squeezing the sides of the mold. The gels were inspected under a microscope to confirm the absence of defects within and around the microwells. The gels were then transferred to a 24-well plate using a metal spatula. Incomplete cortical media (ICM+) containing Neurobasal A Medium (Invitrogen 10888022), 0.5 mM Gluta-Max (Thermo Scientific 35050061), and 1X Penicillin—Streptomycin (Thermo Scientific 15140122) was added to each gel. ICM+ was changed twice prior to dissection.

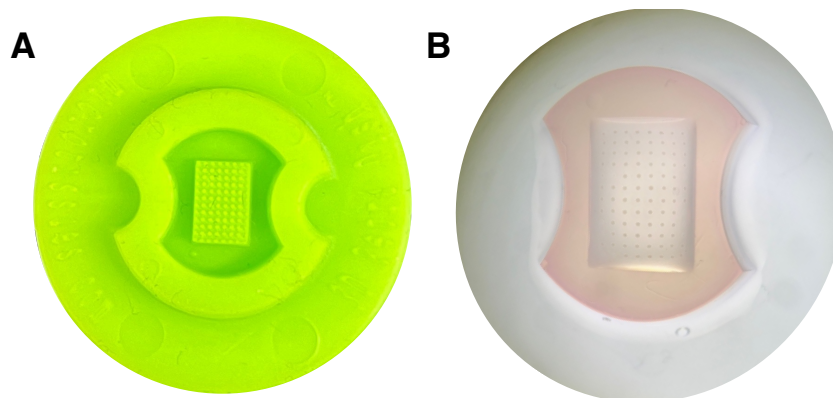


Figure 3: A) Image of the micro-mold designed by Microtissues, Inc. and used to generate agarose gels with 96 wells.⁶⁰ B) Image of 96 well agarose gel generated from the micro-molds.

2.2 Cortical Cell Isolation

A summary of the spheroid formation process can be seen in Figure 4. Primary rat cortical tissues from CD postnatal day 0-2 (P0-2) male and female rat pups (Charles River) were used to engineer cortical spheroids. Complete Cortical Media Plus (CCM+) was prepared using 98% v/v Incomplete Cortical Media Plus (ICM+) and 2% v/v B27+ (Thermo Scientific A3582801). CCM+ was added to each agarose gel on the morning of the dissection.

Between 3 and 20 hours before seeding, a poly-D-lysine (PDL) solution was prepared using 10% v/v 10X PDL (Sigma-Aldrich P6407) in sterile water. A volume of this solution was used to coat the substrate in a 12-well plate with 1 mL per well. The surface was washed twice with water and allowed to dry prior to seeding.

Postnatal day 0-2 mixed-sex pups were collected in accordance with Institutional Animal Use and Care Committee (IACUC) protocols. The pups were anesthetized on ice for 45 minutes, and a paw pinch was performed to confirm proper anesthesia. Using a pair of sharpened scissors, the pups were decapitated between the ears and shoulders. The skin and skull flaps were cut from the neck to the nose to expose the brain. The brain was then extracted and transferred to a 35 mm

petri dish placed on ice. Using bent forceps, an incision was made from the midline of the cortex to the posterior corners. The cortical flap was separated from the remaining tissue, and the meninges and blood vessels were carefully removed. The cortex was placed in a 35 mm dish containing chilled Hibernate A/B27+ buffer solution (BrainBits) and kept on ice. This procedure was repeated for each cortex and pup.

Next, bent forceps were used to break up each cortex. To dissociate the tissue, cortices from two pups were combined in a papain/Hibernate A without CaCl_2 solution (BrainBits) and incubated in a 30°C water bath for 30 minutes, with gentle shaking every 5 to 10 minutes. Next, most of the papain solution was removed using a 5 mL pipette. Hibernate A/B27+ buffer solution was then added, and the tissue was dissociated by pipetting in and out with a fire-polished Pasteur pipette until the solution appeared homogenous.

The cell suspension was filtered through a 40 μm strainer into a 50 mL tube, with an additional volume of Hibernate A/B27+ buffer solution added to assist with filtration. The solution was transferred to a 15 mL Falcon tube and centrifuged at 150G for 5 minutes. After removing the supernatant, the cell pellet was resuspended in warmed CCM+. The solution was filtered, centrifuged again, and resuspended in CCM+. A final centrifugation was performed, followed by supernatant removal, and the pellet was resuspended in 10 mL CCM+.

Cell counting was performed by mixing equal volumes of the cell suspension and Trypan Blue. A hemocytometer was used to count the cells under a microscope using a handheld counter. The number of cells was determined to calculate the appropriate cell densities for the 3D and 2D cultures.

After counting, the media was removed from all gels. The cell solution was mixed thoroughly, and 75 μL was added to each 96-well chamber at a density of 8000 cells per

microwell. The plate was incubated for 20 minutes and shaken twice. CCM+ was then added to the side of each well, using a volume sufficient to cover the gel. The cell solution was also added to wells designated for 2D cultures. Plates were monitored, and CCM+ was changed every other day.

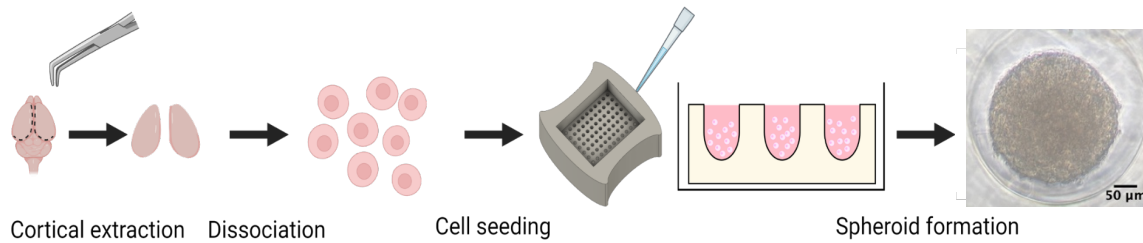


Figure 4: Cortical cell isolation and spheroid formation process. In short, the cortices are extracted from the postnatal rat tissue. The cells comprising the cortices are dissociated and seeded into 96-well agarose gels. Cells self-assemble in the microwells and form 96 cortical spheroids. Figure created with Biorender.com.

2.3 Preparation of Silicone Indenters

Silicone indenters were developed by the Applied Mechanics Laboratory. Indenters consisted of 96 pegs with a diameter of 200 μm and a length of 600 μm , 650 μm , or 800 μm . Various silicones were used to create indenters with different hardnesses. The silicones that resulted in autoclavable indenters with the proper included the following: Mold Star™ 15 SLOW, Dragon Skin™ 30, and Smooth-Sil™ 960. Table 2 specifies the mix ratio and shore hardness of each silicone used. The silicone was carefully washed with a 1% w/v Alcanox solution in distilled water, then autoclaved in a sterile container before impacts.

Table 2: Technical data for the products used for the silicone indenters from SmoothOn, Inc.

Product	Mix Ratio	Shore Hardness	Cure Time
Mold Star™ 15 SLOW	1A:1B by weight or volume	15A	4 hours
Dragon Skin™ 30	1A:1B by weight or volume	30A	16 hours
Smooth-Sil™ 960	100A:10B by weight	60A	16 hours

2.4 Impactor Device Design

The components of the device were designed using the Fusion360 Computer-Aided Design (CAD) software. The open-source BiCM STL files shared by Brigham Young University Compliant Mechanisms and Robotics Group were used and modified for the preparation of the impactor device.^{61,62} Additional parts were designed and assembled within the CAD software.

Following the design of the impactor components within Fusion360, the STL files were imported into the PrusaSlicer application and converted to G-code. The device parts were prototyped using the Original Prusa i3 MK3S+ 3D Printer with 1.75 mm poly-lactic acid (PLA) filament. A 0.15 mm layer height and an infill density of 15% were used. Build plate supports were applied as needed.

For the final design, a 22 x 22 mm cover glass (Ted Pella 260156) or 12 x 12 mm cover glass (BIPEE B00Y21QTT0) were used at the base of the impact devices, on top of which the gels were placed during impacts.

2.5 Viability Assay

Viability assays were conducted using Hoechst and Ethidium Homodimer-1 (EthD) dyes to stain all cell nuclei and dead cells, respectively. A staining solution containing CCM+ with 0.1% v/v Hoechst (Thermo Scientific H3570) and 0.4% v/v EthD (Thermo Scientific E1169)

was prepared. Each gel received 1 mL of this solution, followed by incubation for 40 minutes. After incubation, the stain was removed, and the gels were washed twice with warmed CCM+. Following the washes, spheroids were carefully flushed out of their wells using a P1000 pipette and transferred to a 35 mm glass-bottomed dish (World Precision Instruments FD35-100) for imaging via confocal microscopy.

2.6 Immunohistochemistry

Immunohistochemistry (IHC) was performed to visualize the responses of various cell types involved in TBI. The procedure is described as follows. The medium was removed from each well, and a fixation solution containing 4% v/v paraformaldehyde and 8% w/v sucrose in PBS was added to each well. The gels were incubated at room temperature overnight. The following day, the solution was removed, and the gels were washed three times with PBS for 30 minutes each. Spheroids were then flushed out of each well with a 1000 μ L pipette tip, transferred to 2.0 mL labeled Eppendorf tubes, and stored at 4°C. Some spheroids were isolated as a negative secondary antibody control.

Blocking solution was prepared with 10% v/v normal goat serum (Jackson ImmunoResearch 005-000-121), 4% w/v bovine serum albumin (Sigma-Aldrich A7030-100G), and 1% v/v triton-X 100 (Sigma-Aldrich 9036-19-5) in PBS. PBS was carefully removed from all tubes and replaced with 1 mL blocking solution per tube. Samples were shaken at 90 rpm at room temperature for 2 hours. Next, the primary antibody diluted in blocking solution was added to each tube, except for the negative control, to which only blocking solution was added. The primary antibodies used were mouse anti- β -3-Tubulin (TUBB3) (BioLegend 801202) and rabbit

anti-glial fibrillary acidic protein (GFAP) (Agilent Technologies Z033429-2). The samples were shaken at 90 rpm overnight at room temperature

The following day, each sample was washed twice with 0.2% v/v Triton X-100 in PBS (PBT) for 2 hours per wash, followed by a single wash with blocking solution for 2 hours. The secondary antibodies were subsequently diluted in blocking solution, added to each sample, and shaken overnight. The secondary antibodies used were Cy3 goat anti-mouse (Jackson ImmunoResearch 115-165-068) and Alexa Fluor 488 goat anti-rabbit (Jackson ImmunoResearch 111-545-144). In addition, Isolectin-B4, Alexa Fluor 647 Conjugate (Thermo Scientific I32450) was added to the secondary solution at a 1:100 v/v dilution to visualize microglia. The primary and secondary antibodies and their dilution factors can be seen in Table 3.

On the following day, samples were washed three times with PBT for 30 minutes each. Samples were then shaken for 1 hour in PBT containing 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) (Thermo Scientific D1306). After staining, the samples were returned to PBS and stored at 4°C until imaging using confocal microscopy.

Table 3: Primary antibodies and secondary antibodies used alongside their corresponding dilution factors used during the IHC staining procedure.

Primary Antibody	Dilution Factor	Secondary Antibody	Dilution Factor
Mouse anti-B3Tub	1:50	Cy3 Goat anti-Mouse	1:50
Rabbit anti-GFAP	1:200	AF488 goat anti-rabbit	1:200

2.7 Confocal Imaging

The Nikon AX-R confocal microscope was used for imaging with the NIS Elements software. Samples were placed in a 35 mm glass-bottomed dish (World Precision Instruments FD35-100). A Z-plane stack of 50 slices was taken for each image. Galvano scanning was used

for viability and IHC stains. The laser intensity and gain for each stain was set at the beginning of each imaging session.

2.7.1 Live Imaging and Fixed Imaging of Spheroids

Before imaging, spheroids were flushed out of the agarose gels. Imaging was performed using a 20X air objective (Nikon) with a scan area of 1024 pixels and a pinhole size of 1.0 AU. For live imaging, the microscope's environmental control system maintained the samples at 37°C with 5% CO₂.

2.7.2 Within-Gel Live Imaging of Spheroids

Spheroids were kept in agarose gels for within-gel live imaging. The environmental control aspect of the microscope was used to keep samples incubated at 37°C and 5% CO₂. The 10X air objective (Nikon) was used. A scan area of 1024 pixels and a pinhole size of 1.0 AU were used.

2.7.3 Live Imaging During Simulated Impacts

To image live spheroids within a gel during impacts, the impactor device was positioned inside the confocal imaging chamber. A Hoechst-stained gel was placed on a glass coverslip, which was mounted on the impactor device and aligned above the 10X air objective (Nikon). A time-lapse video was captured to visualize the impacts. A pinhole size of 1.0 AU and scan area of 512 pixels was used.

2.8 ATP Assay

The CellTiter-Glo® Luminescent Cell Viability Assay (Promega Life Sciences) was used to quantify cell viability as a measure of ATP content. Spheroids were transferred to a 6-well plate containing 2 mL of warmed CCM+ and gently pipetted out of the agarose gels with a 1000 µL pipette. Individual spheroids immersed in CCM+ were then transferred to an opaque, clear-bottomed, white 96-well plate (Costar) using a 200 µL pipette with a cut tip, with a final volume of 100 µL per well. Additionally, 100 µL of CCM+ was added to several wells to serve as blank controls.

The CellTiter-Glo® Buffer was prepared by combining the CellTiter-Glo® Substrate with the CellTiter-Glo® Reagent. A multichannel pipette was used to add 100 µL of the reagent to each well. The plate was wrapped in aluminum foil to protect from light and shaken on an orbital shaker at 90 rpm for 2 minutes. It was then incubated at room temperature for 10 minutes. Relative cell luminescence was assessed using a Cytation3 Cell Imaging Multimode Reader instrument (Biotek) and the Gen5 Microplate Reader and Imager Software. A gain of 135 and an integration time of 1 second were used. The plate was read three times, and the average of the three reads was used for statistical analysis.

2.9 Data Analysis

2.9.1 Viability Analysis

The 3D Objects Counter function in ImageJ was used to analyze images from spheroids stained with Hoechst and EthD. A cropped, 8-bit image was opened in ImageJ, and the Hoechst and EthD channels were split. The spheroid was circled, and the 3D Objects Counter was run. A threshold value and minimum value were determined for each stain. The numerical output of the

3D Objects Counter was saved into a spreadsheet. The percentage of death by spheroid volume were determined by Equation 1 below.

$$\% \text{ Death by Volume} = \frac{\text{EthD Total Volume}}{\text{Hoechst Total Volume}} * 100 \quad (\text{Equation 1})$$

2.9.2 TUBB3 Analysis

TUBB3 was analyzed using a neurite network graph theory analysis tool developed by Professor Haneesh Kesari, PhD and Amina Burgess, MS (Burgess, A. Z. 2024. Master's thesis, Brown University; Calvao, D. et al. 2025. Under review.) Images were pre-processed in ImageJ by isolating the TUBB3 channel and cropping individual spheroids. The background was subtracted using a rolling ball radius between 1 to 50, with values around 25 being optimal for most networks. After pre-processing, the TIFF file was saved, and the Mathematica analysis program was opened. The stack thickness was specified. The pixel width values were adjusted based on the properties of the images used, and the voxel depth of 1.5 μm was used. The filter size parameter was also adjusted to a value between 1 and 3. Lastly, the images were inputted into the specified portion of the algorithm, and the code was run. The analysis tool converted the neurite network into graphical representations, and the graphical and numerical outputs were recorded. Various outputs can be obtained from this analysis such as the number of nodes for statistical analysis included total number of nodes, total network length, number of edges, normalized main network length, and number of disconnected subgraphs.

2.9.3 GFAP Analysis

GFAP expression was assessed in ImageJ by measuring the average voxel intensity of each spheroid. A threshold was set within the 3D objects counter and used for each 16-bit

spheroid image. The sum of the integrated density divided by the sum of the number of object voxels was used to determine the average voxel intensity (Equation 2).

$$\text{Average Voxel Intensity} = \frac{\Sigma (\text{Integrated Density})}{\Sigma (\text{Number of Object Voxels})} \quad (\text{Equation 2})$$

2.10 Statistical Analysis

Statistical analysis was conducted using Prism 10. One-way or two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to evaluate statistical significance, with a significance threshold of $p < 0.05$. Values were reported using a box and whisker plot. The significance levels were reported as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

2.11 Mechanical Testing

Given the challenge of assessing spheroid deformation, we assessed the force generated by the two mechanisms using an Instron 5942 machine, a universal testing machine, courtesy of the Brown Design Workshop. The Instron machine applies forces and displacements, allowing users to assess mechanical properties of a material or device.

The Bluehill Universal software is used to program the Instron machine. In brief, a new method was created in the software by selecting a compressive strain test. The test control panel was selected, and displacement was chosen as the primary source of compressive strain. Under the pre-test panel, auto-balance was selected. Under the test panel, a low displacement rate of 5.00 mm/min was inputted. An end of test measurement level, or the total displacement of the Instron machine, was selected depending on the distance between the base of the device and the

bottom of the moving shuttle. Displacement and force were obtained every 0.02 s. Data was collected in a csv format and included force at each displacement and time elapsed at each measurement.

An isometric CAD view of the device setup used can be seen in **Figure 5A-B**. A fork-shaped attachment was designed to attach to the Instron's upper grip fixture. An 11 mm diameter, 43.1 mm length cylindrical rod was designed and 3D printed to serve as attachment points to the attachment. A round opening was already present in BiCM1 to which the cylindrical rod could be inserted. An 11.1 mm circle was drawn on BiCM2 and extruded through the device to enable the insertion of the cylindrical rod. The compression of the PLA material itself is considered negligible in obtaining measurements from the Instron machine. The Instron machine setup to induce mechanical impacts can be seen in **Figure 5C-D**.

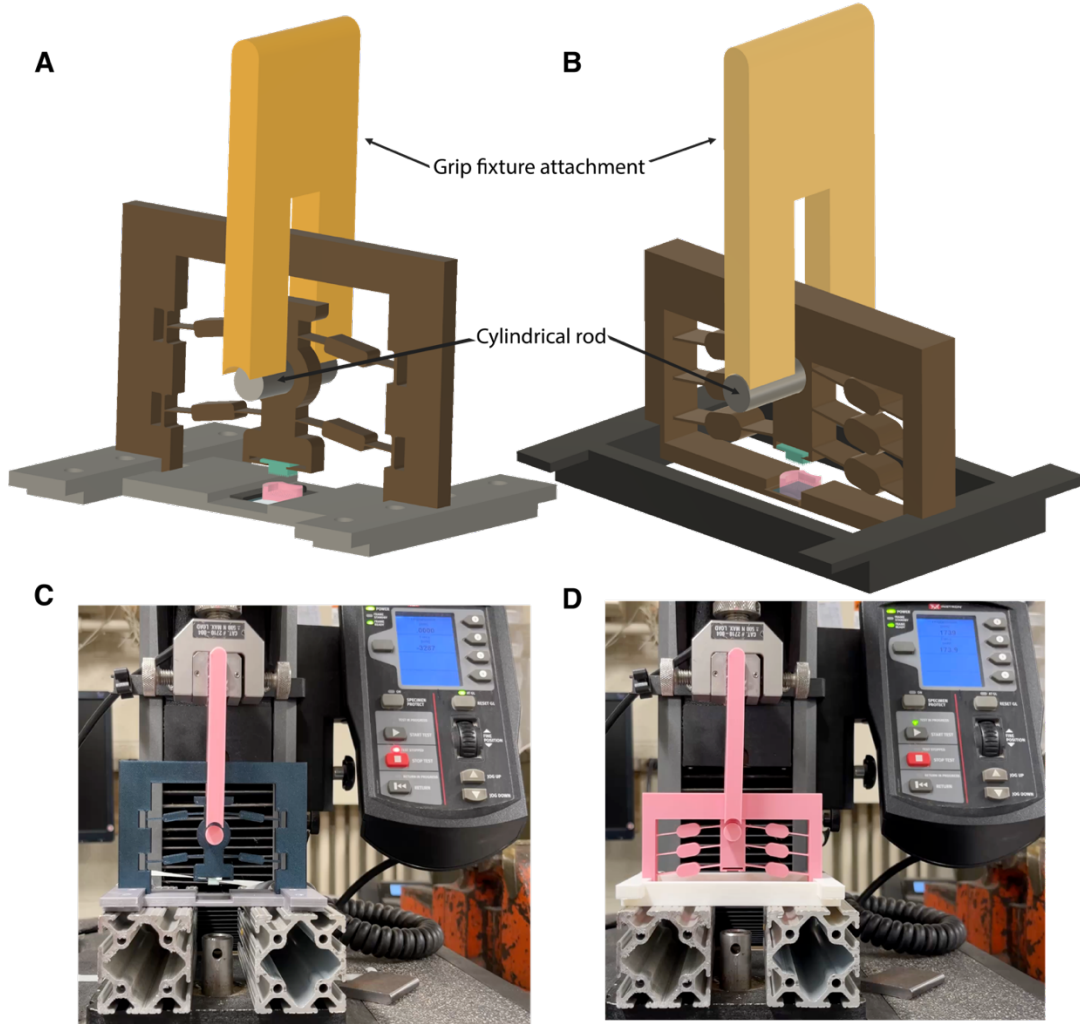


Figure 5: Instron machine design and setup. CAD design images of (A) BiCM1 and (B) BiCM2 with cylindrical rods inserted into the moving shuttle and a grip fixture attachment added to press down on the cylindrical rod, allowing for force-displacement measurements. Images of the Instron setup pre-test for (C) BiCM1 and (D) BiCM2.

Separately, strain and strain rate delivered to individual spheroids was assessed using Equations 3 and 4, respectively.

$$\varepsilon = \frac{\Delta L}{L} \quad (\text{Equation 3})$$

$$\dot{\varepsilon} = \frac{\varepsilon}{t} \quad (\text{Equation 4})$$

Chapter 3: Results

Previous research in the Hoffman-Kim lab has focused on developing an *in vitro* model of TBI using cortical spheroids. This model aims to create a device capable of delivering controlled blunt impacts to induce mechanical deformation. The device is designed to be compact, sterilizable, and operable within a sterile culture hood. The following section details the design of these impact-delivery devices and presents results from preliminary studies evaluating their efficacy.

3.1 Silicone indenters were successfully designed and implemented for use in direct impacts to cortical spheroids

We hypothesized that silicone indenters could be designed to directly impact spheroids within agarose gel wells. Various iterations of the silicone indenters were manufactured by the Applied Mechanics Lab at Brown University.

3.1.1 9-well agarose gel and silicone indenter generation and implementation

Silicone micro-molds were created featuring nine 200 μm pegs. The molds developed contained a 3 $\times$ 3 array of 400 μm pegs, as shown in the CAD model (**Figure 6A**). The cured 15A silicone mold with a 3x3 array is depicted in **Figure 6B**.

Using this mold, agarose gels were fabricated, and cortical cells were seeded at a density of 8000 cells/well. Spheroids were formed in these gels, with their morphology at DIV 14 shown in **Figure 6C**. However, spheroid size varied significantly and did not form in some gels. Additionally, due to the greater spacing between wells compared to standard gel designs, visible

cortical cells were observed on the agarose surface, indicating incomplete seeding into microwells.

To assess the ability of silicone to mechanically deform cortical spheroids in a nine-well gel, a silicone indenter with nine pegs, each 200 μm in diameter and 800 μm in length, was fabricated using 15A silicone. A zoomed-in microscope image of the combined silicone indenter, agarose gel, and cortical spheroids is shown in **Figure 6D**, during manual deformation.

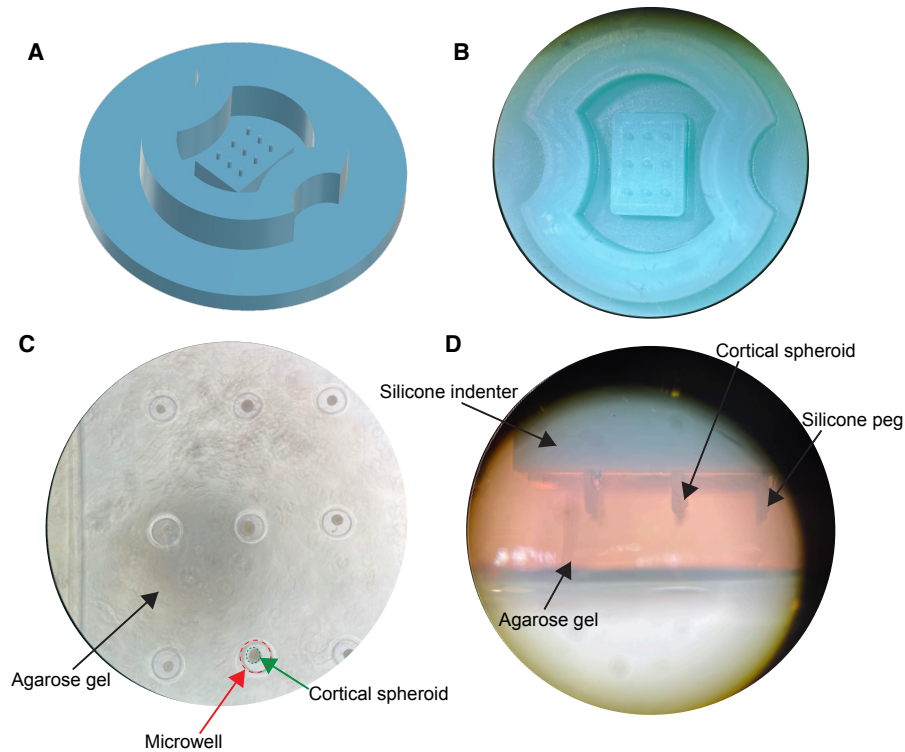


Figure 6: 9-well agarose gel design, spheroid formation, and impact setup. (A) Isometric view of the CAD design for a 9-well silicone mold used to fabricate 9-well agarose gels. (B) Image of the 9-well silicone mold created using 15A silicone for agarose gel generation. (C) Zoomed-in microscope image of a 9-well agarose gel 14 days after cortical spheroid seeding. The black arrow indicates the agarose gel body, the red arrow highlights the microwells (400 μm in diameter), and the green arrow marks a cortical spheroid. (D) Zoomed-in microscope image of an agarose gel with cortical spheroids following impact from a 9-well silicone indenter.

3.1.2 96-well silicone indenter generation and implementation

In the same vein, 96-well silicone indenters aligning with the geometry of the 96-well micro-molds were designed. The wells were separated by 620 μm on the 5 mm length side and 600 μm on the 7 mm length side. The silicone indenters were designed with pegs of 800 μm length, 600 μm length, and 650 μm peg length. The CAD model and essential geometry of the 96-well silicone indenter can be seen in **Figure 7A-C**.

Three silicone types (15A, 30A, and 60A shore hardness) were produced autoclavable indenters with the proper geometry (**Table 2**) (**Figure 7D-F**). The pegs of the silicone indenters were attached to a solid piece of silicone. Initially, this piece was approximately 2 mm in thickness. Later, the piece was altered to 3 mm in thickness to accommodate use with BiCMs. The silicone was tested in agarose gels alone as well as agarose gel containing spheroids by pressing it into the gel manually. Viewed through a traditional microscope, the pegs of the silicone lined up with and entered the agarose gel microwells in response to manual compression (**Figure 7G**).

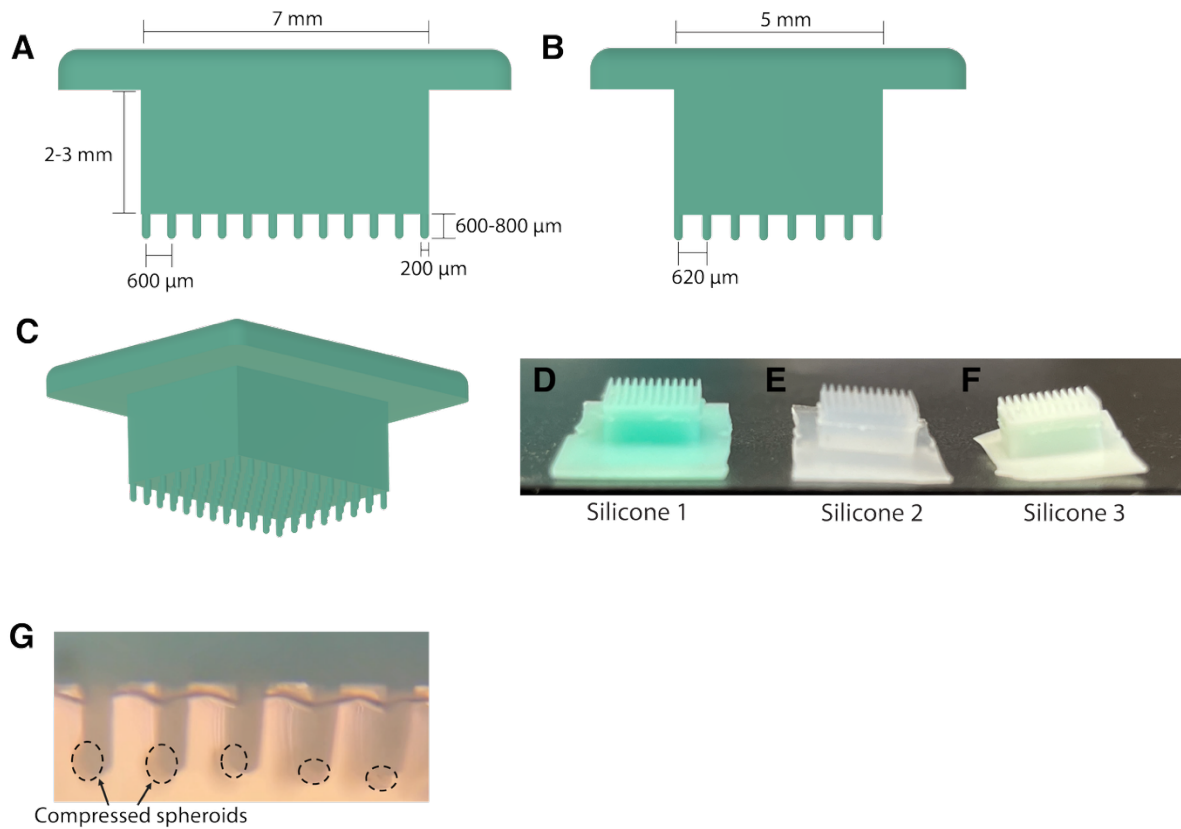


Figure 7: 96-well silicone indenter designs with 200 μm diameter pegs in an 8x12 array. The silicone is 5 mm x 7 mm. (A) CAD view of the 7 mm side of the silicone indenter with 12 pegs, with each peg separated by 600 μm . (B) CAD view of the 5 mm side of the silicone indenter with 8 pegs, with each peg separated by 620 μm . (C) CAD isometric view of the silicone indenter. Cured images are shown of (D) silicone 1, (E) silicone 2, and (F) silicone 3. (G) Microscope-generated image of silicone indenter manually entering agarose gel, with spheroids compressed by the silicone pegs indicated by the dashed circles.

3.2 Manual pilot experiments resulted in significant breakdown of neurite networks and increases in microglial volume at 0 and 24 hours post-injury

To assess the efficacy of the silicone indenters on inducing mechanical deformation and TBI-like responses in cortical spheroids, pilot experiments were performed by hand. A flat metal spatula was used to gently press the indenter into the gel, and the indenter was immediately removed using metal forceps. For this experiment, 15A silicone indenters with peg lengths of

600 μm and 800 μm were both used and evaluated at 0 and 24 hours after impact, with impacts occurring on DIV13. Viability staining and IHC assessing neurite networks and microglia were performed.

Cell death increased in impacted spheroids immediately and 24 hours after injury

To assess cell death, confocal microscopy images of the Hoechst (blue) and EthD (red) stains (**Figure 8A**) and quantification of cell death by volume (**Figure 8B**) at 0 and 24 hours post-injury were recorded. At 0 hours, control spheroids exhibited a baseline cell death of $1.496\% \pm 0.410\%$. However, a significant increase in cell death was observed immediately following impact with a silicone indenter featuring a 600 μm peg length ($7.923\% \pm 2.760\%$, $p < 0.0001$). Spheroids impacted by a silicone indenter with an 800 μm peg length did not exhibit a significant increase in cell death ($3.298\% \pm 1.836\%$, $p = 0.3310$).

At 24 hours post-impact, spheroids impacted by the 800 μm silicone indenter demonstrated a significant increase in cell death ($7.426\% \pm 4.191\%$) compared to controls ($1.754\% \pm 1.055\%$, $p = 0.0001$). In contrast, cell death in the 600 μm indenter condition ($4.458\% \pm 2.827\%$) did not reach statistical significance compared to controls ($p = 0.0906$) but exhibited an upward trend. These results suggest that mechanical deformation induced by silicone indenters leads to significant cell death within the first day post-impact.

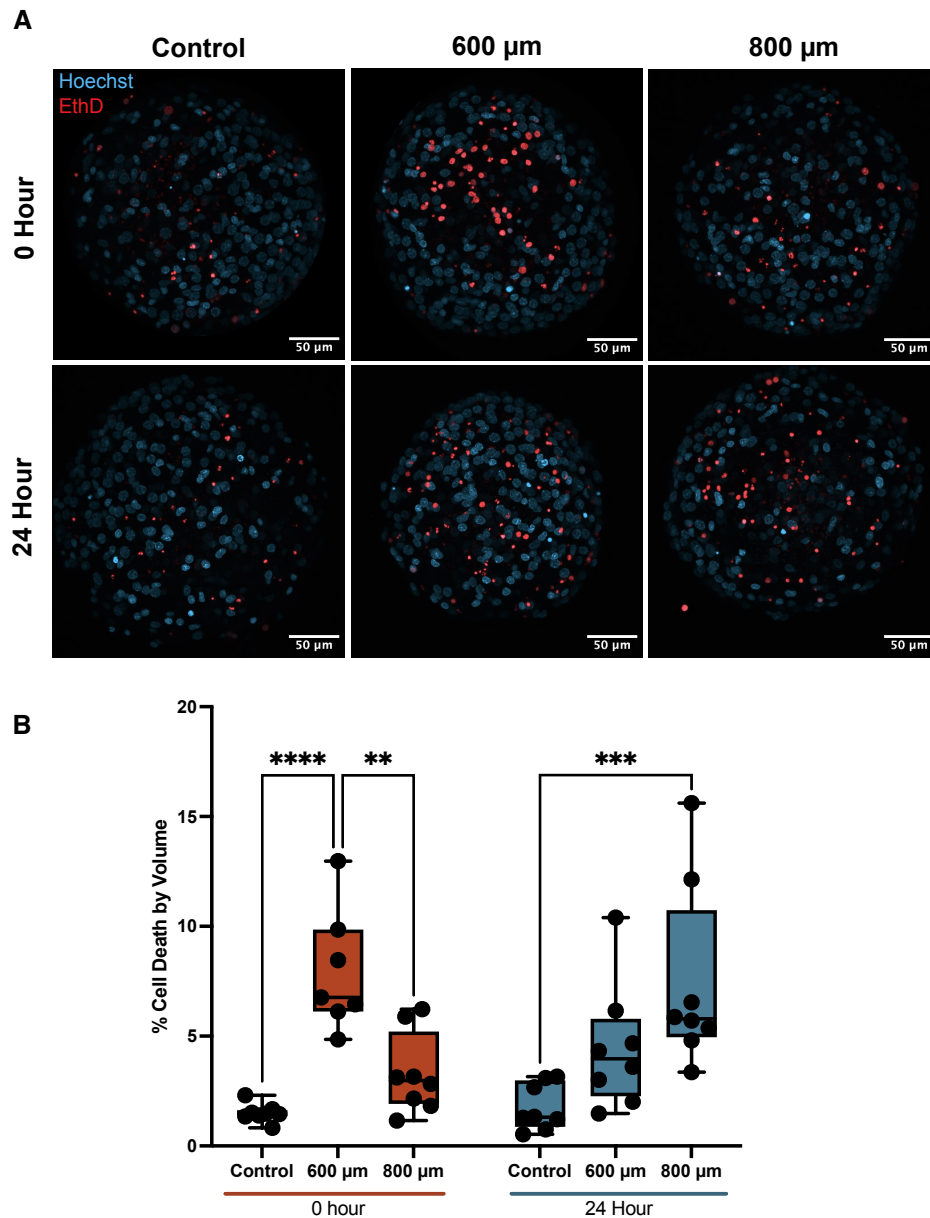


Figure 8: Viability staining results following manual impacts with a 15A silicone indenter with peg lengths of 600 μm or 800 μm . (A) Confocal images of Hoechst and EthD staining for control spheroids and spheroids impacted by 600 μm and 800 μm silicone indenters at 0 and 24 hours post-injury. (B) Quantification of cell death as a percentage of spheroid volume at 0 and 24 hours for both indenter conditions. Statistical analysis was performed using a two-way ANOVA and Tukey post-hoc test ($n=8$). Center lines of box plots represent the median. Boxes extend from the 25th to the 75th percentile. Lower and upper whiskers represent the minimum and maximum values, respectively.

Neurite networks became damaged within 24 hours post-impact

TUBB3 staining revealed neurite network damage and puncta formation in the 600 μm silicone injury condition at 24 hours (**Figure 9A-B**). A custom-built graph theory analysis was employed to assess TUBB3 damage. This method converts the neurite network into a simplified graphs and quantifies the number of subgraphs, or areas of the neurite network that are disconnected from the main network. Number of subgraphs for each condition were recorded (**Figure 9C**). Immediately after injury, the 600 μm condition did not exhibit a significant increase in subgraph count (38.667 ± 25.201) compared to controls (17.167 ± 4.834 , $p=0.3109$). However, at 24 hours post-injury, the number of subgraphs in the 600 μm injury condition (56.167 ± 30.903) was significantly increased compared to the 0 hour control ($p=0.0074$) and showed an increasing trend compared to the 24 hour control spheroids (27.167 ± 7.111 , $p=0.0778$), suggesting microtubule disruption within the neurite network.

In contrast, neurite network breakdown was not observed after impacts with the 800 μm indenter. There were not significant changes in neurite network subgraphs either immediately ($p=0.9554$) or 24 hours after injury ($p=0.9977$).

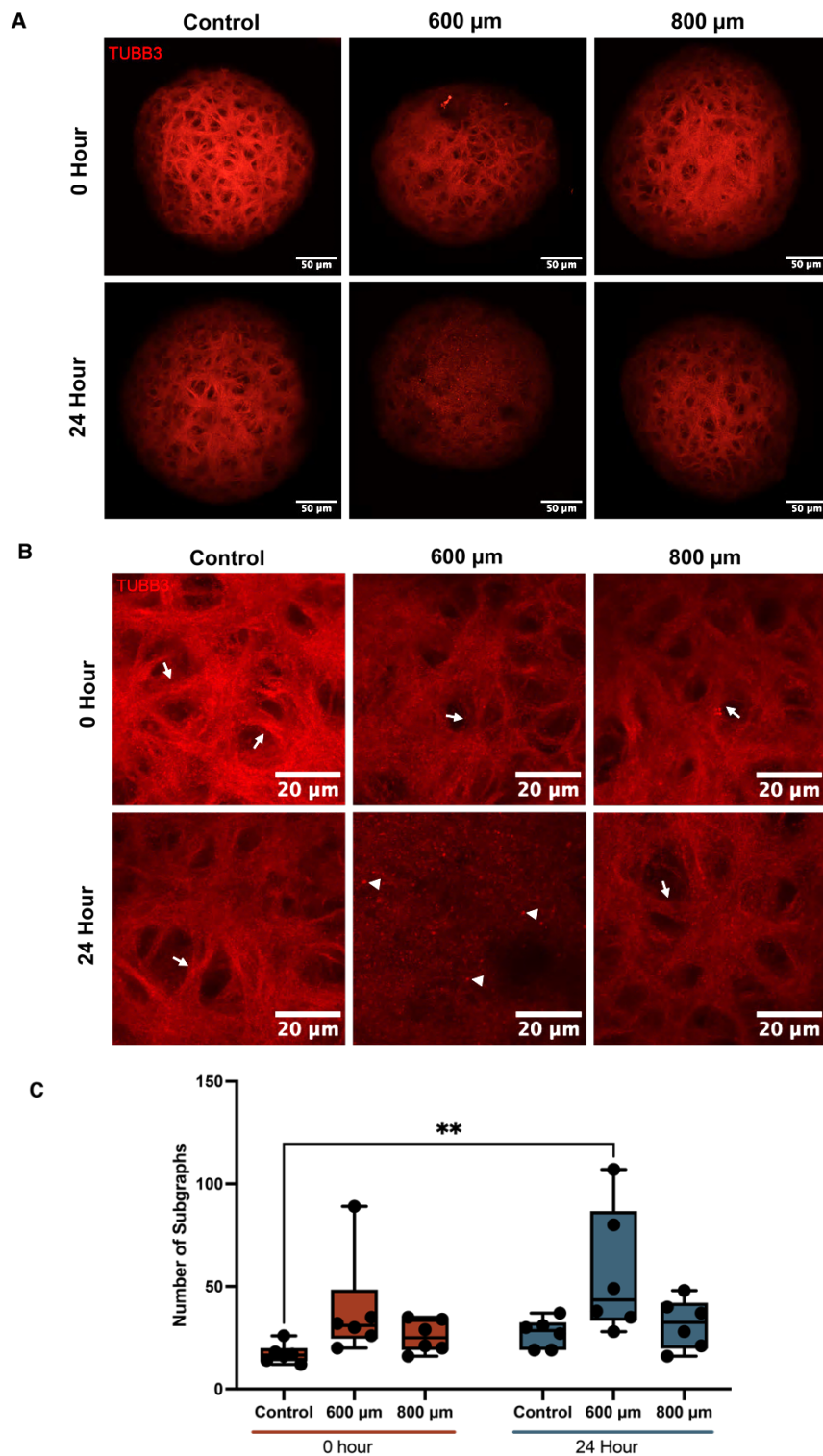


Figure 9: TUBB3 staining results following manual impacts with a 15A silicone indenter with peg lengths of 600 μm or 800 μm . (A) Confocal images and (B) magnified views of TUBB3 staining for control and impacted spheroids at 0 and 24 hours post-injury. White arrows indicate

physiological TUBB3 strands, while white arrowheads designate sites of puncta formation and neurite network damage. (C) Quantification of neurite network disruption using a custom-built TUBB3 Mathematica analysis, measuring the number of subgraphs present for each condition. Statistical analysis was performed using a two-way ANOVA with Tukey's post-hoc test ($n = 6$). Center lines of box plots represent the median. Boxes extend from the 25th to the 75th percentile. Lower and upper whiskers represent the minimum and maximum values, respectively.

IB4 Staining Results

Confocal imaging of IB4 staining for each condition is shown in **Figure 10**. Qualitative observations suggest a potential increase in the number of microglia, which may suggest an increase in microglial activation, in the 600 μm and 800 μm condition immediately post-injury compared to controls. Further characterization will assess microglial motility and morphology following injury.

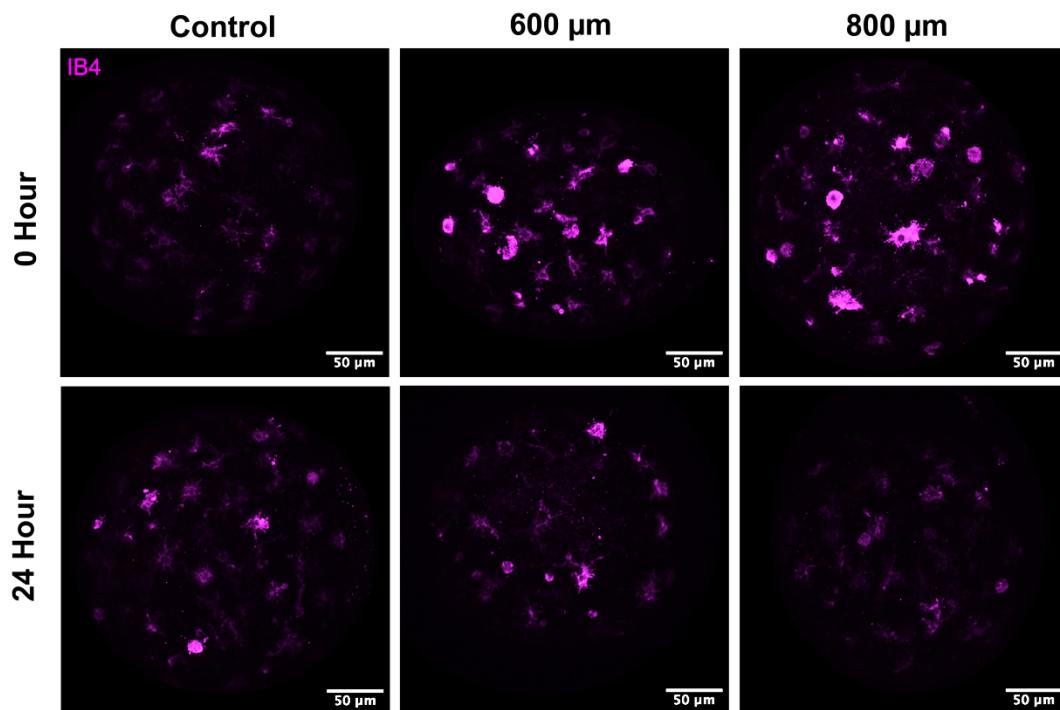


Figure 10: Confocal images from IB4 staining following manual impacts with a 15A silicone indenter with peg lengths of 600 μm or 800 μm at 0 and 24 hours post-injury.

3.3 Initial Impactor Device Design

3.3.1 Description of design of a BiCM as an impactor device

Following manual impact-based experiments, we aimed to develop a reproducible method for delivering controlled forces to cortical spheroids. To achieve this, we designed a compact, 3D-printed BiCM to serve as an impactor device. The design was adapted from a previously described open-source model developed by researchers at Brigham Young University.⁶¹

The BiCM consists of a moving shuttle, which is deployed into one of two stable positions, and two compliant segments attaching the moving shuttle to its frame. For this study, a slot was created at the base of the moving shuttle, allowing the silicone indenter to be slid into the shuttle. A stage was designed to serve as the base of the BiCM and a platform for the agarose gel. A raised platform with dimensions 22x22 mm was added to the center of the stage to allow for placement of a glass coverslip. An agarose gel aligner was designed to position the gel precisely beneath the silicone indenter during impact. Holes were added to the stage legs to securely mount the impactor device to the optical board base. An isometric CAD view of the initial device design can be viewed in **Figure 11A**, and a 3D-printed prototype of the BiCM, stage, silicone indenter, glass coverslip, and agarose gel aligner can be viewed in **Figure 11B**.

The initial structure of the BiCM deployed silicone indenters directly into the agarose gel, but some alterations were performed. Notably, the tolerance between the stage and BiCM frame was reduced, and the agarose gel aligner shape was improved. In addition, stage walls were added to control the distance the BiCM deployed, preventing compression of the agarose gel

itself. A view of these updates can be seen in the isometric CAD image (**Figure 11C**) and in the 3D-printed prototype (**Figure 11D**).

Following prototype review, the agarose gel aligner was adjusted for fit, and the tolerance between the stage and BiCM frame was updated. Further, the device was optimized for confocal microscope compatibility (**Figure 11F**), with regards to the Nikon AX-R stage. To do so, the stage height was decreased such that the glass coverslip could be placed directly above the microscope objective. In addition, the stage legs were altered to fit the assembly on the Nikon stage. Detailed drawings of the mechanism can be found in the Appendix.

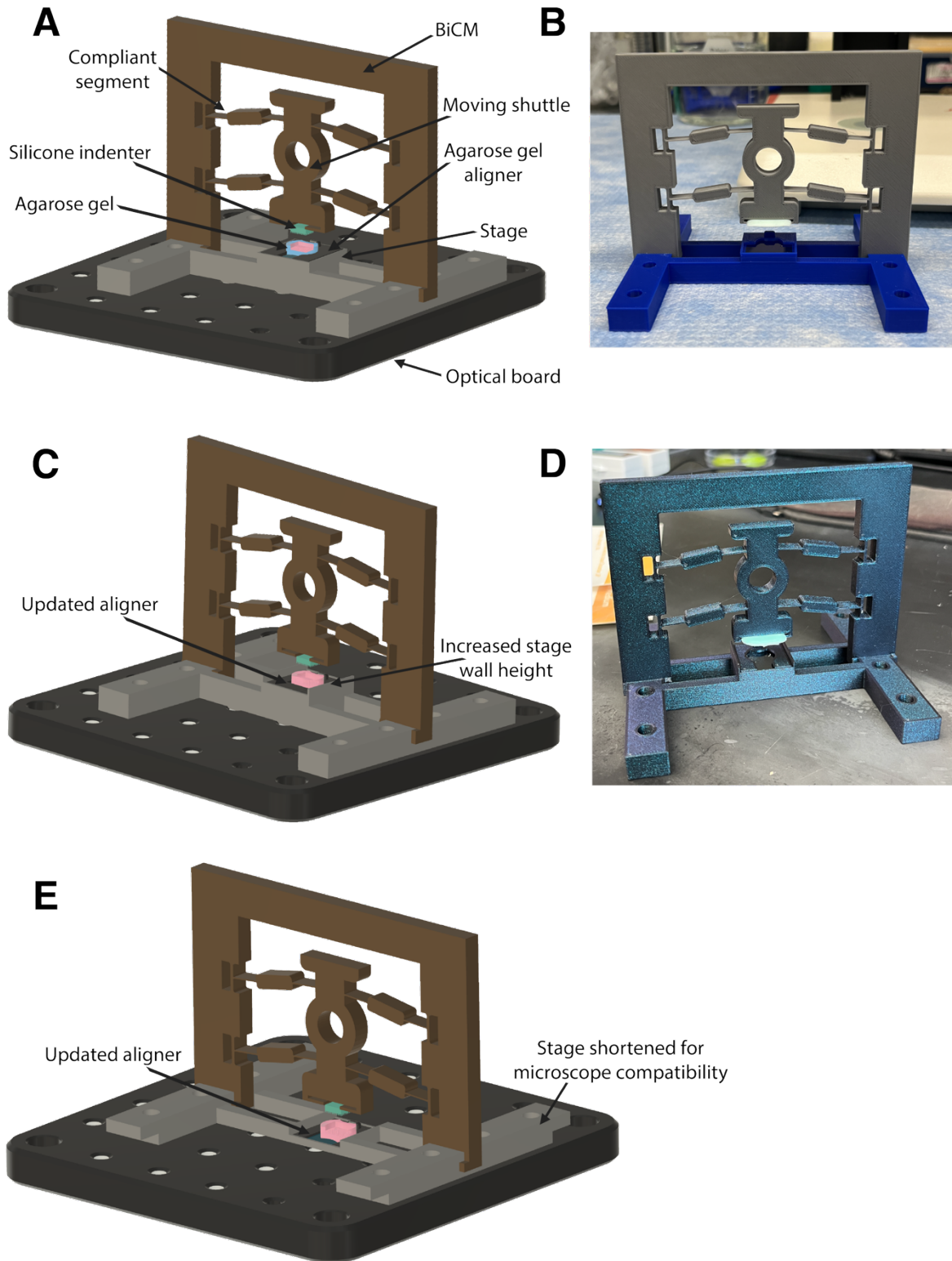


Figure 11: (A) Isometric CAD image of initial impactor device design, consisting of the BiCM itself, base stage, agarose gel, silicone indenter, glass coverslip, and optical board. (B) Initial 3D-printed impactor device setup, consisting of the BiCM, stage, silicone indenter, glass coverslip,

and optical board. (C) Isometric CAD view of the second iteration of the impactor device and (D) corresponding 3D-print of the second iteration, which consists of an improved aligner design and increased stage wall height to control the deployment of the device. (E) Isometric CAD view of the third iteration of the impactor device, consisting of an optimized aligner and a shortened stage allowing for compatibility with a confocal microscope stage.

3.4 Initial experiments with the silicone indenter and BiCM showed various viability effects

Using the optimized, confocal-compatible impactor device iteration setup described in Section 3.3, an initial experiment to assess viability was performed with all three silicone types: the 15A silicone (Silicone 1, S1), the 30A silicone (Silicone 2, S2), and the 60A silicone (Silicone 3, S3). All silicone indenters used had peg lengths of 600 μm . Impacts were conducted on DIV15, and a viability assay and ATP assay were performed 24 hours post-impact. In addition, live impacts were observed using confocal microscopy. The experimental setup for the controls and impacts is outlined below, followed by the results of these pilot experiments using the mechanical assembly.

3.4.1 Control setup demonstrates no cytotoxicity from silicone indenters

To evaluate the cytotoxicity of the silicone types without mechanically deforming the spheroids, a silicone indenter with a thickness of 2 mm and 600 μm pegs was slid into the BiCM slot. Due to the smaller thickness, the silicone piece entered the gel between 1.6 and 2.0 mm, which ensured that it could not reach the microwell entrance and did not impact spheroids directly (**Figure 12A**). This setup served as a control to assess whether any of the silicone types alone could induce cell death, as well as to serve as a baseline for the impact experiments.

Viability staining with Hoechst and EthD showed no significant change in cell death compared to the control condition not exposed to a silicone indenter across all silicone types at

24 hours post-injury (**Figure 12B**). The non-impacted controls had an average cell death of $7.333\% \pm 3.559\%$. Spheroids using a control setup containing S1, S2, and S3 had average cell deaths of $8.667\% \pm 7.421\%$ ($p=0.9959$), $16.17\% \pm 18.45\%$ ($p=0.4629$), and $8.333\% \pm 3.830\%$ ($p=0.9982$), respectively. These results suggest that the silicone indenters themselves do not induce cytotoxicity in the spheroids. Consequently, this control setup was used as a baseline in all subsequent studies.

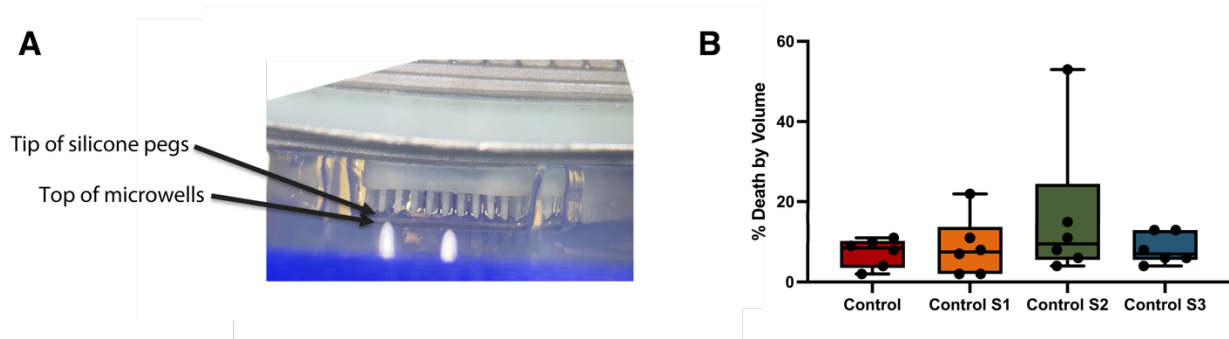


Figure 12: A control impact setup does not result in significant changes in spheroid viability. (A) Control impact setup consisting of a silicone indenter deployed into an agarose gel without entering individual microwells. (B) Quantification of control impact setup using non-impacted controls and controls with three types of silicone (S1, S2, and S3) deployed into the agarose gel using the control impact setup. Quantification shows the percentage of death by volume. A one-way ANOVA and Tukey post-hoc test was performed ($n=6$).

3.4.2 Impact Setup

In contrast to the control impact setup, for the impact conditions, the silicones had a thickness of 3 mm with pegs of length 600 μm . These indenters were designed to directly enter the wells upon BiCM deployment and induce mechanical deformation in cortical spheroids.

3.4.3 Viability staining and ATP assays showed no changes in viability after one impact and a decrease in viability after multiple impacts.

Two gels each were impacted with each of the three silicone types to assess differences between silicone impacts and investigate impact reproducibility using the mechanical mechanism. Controls were also used to assess cytotoxicity, as described previously, and were exposed to all three silicone types. Viability staining was performed using Hoechst and EthD to quantify the extent of cell death. Quantification was performed to assess the percentage of cell death by volume. Qualitatively, no major difference in cell death was seen between the control and impacted spheroids (**Figure 13A**). Mean percentage of cell death for the control, S1 impacted, S2 impacted, and S3 impacted spheroids was 7.333 ± 3.559 , 14.75 ± 15.33 , 42.42 ± 56.29 , and 23.42 ± 29.68 , respectively. Based on a one-way ANOVA with a Tukey post-hoc test, there were no significant differences in cell death across conditions (**Figure 13B**).

An ATP assay assessed the viability of impacted spheroids 24 hours post-injury from S1, S2, and S3 indenters slid into the impacting assembly. Controls were exposed to S3 for the ATP assay. Compared to controls (1.000 ± 0.1124), there was a significant decrease in relative ATP content in S1 impacted spheroids (0.7781 ± 0.1030 , $p=0.0394$), but there was no change in S2 (0.9853 ± 0.1484 , $p=0.9978$) or S3 (0.8454 ± 0.2363 , $p=0.2449$) impacted spheroids (**Figure 13C**). Next, to investigate the effects of multiple impacts, relative ATP content was recorded for spheroids were impacted once (0.8454 ± 0.2363 , $p=0.3126$), twice (0.6885 ± 0.1099 , $p=0.0054$), or three times (0.6185 ± 0.1954 , $p=0.0006$) by S3 at 24 hours post-impact (One-way ANOVA, Tukey post-hoc test) (**Figure 13D**). ATP content was significantly reduced for spheroids impacted twice or three times. The effects of silicones 1 and 2 following multiple impacts were not assessed.

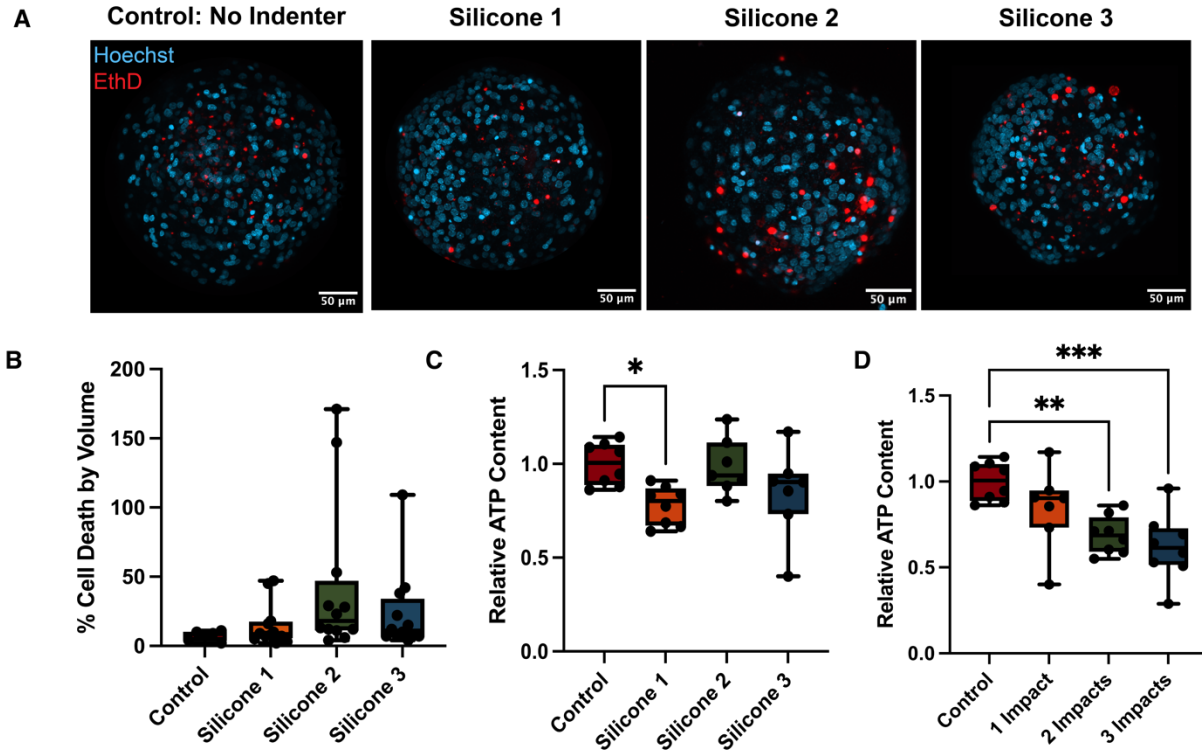


Figure 13: Viability results from initial experiments with the BiCM impacting device loaded with one of three types of silicone (Silicone 1, Silicone 2, or Silicone 3). (A) Confocal images from viability staining of Hoechst and EthD 24 hours following impacts from the initial BiCM device and tested using three types of silicone. (B) Quantification of percentage of cell death by volume for confocal imaged spheroids impacted by a BiCM and three silicone types. One-way ANOVA with Tukey post-hoc test (n=6-12). (C) Relative ATP content quantified from an ATP assay 24 hours post-injury after impacts with three types of silicone. Controls were exposed to S3. One-way ANOVA with Tukey post-hoc test (n=8). (D) Relative ATP content for a control gel impacted with S3, alongside gels that underwent with 1, 2, or 3 impacts using a BiCM and S3. Center lines of box plots represent the median. Boxes extend from the 25th to the 75th percentile. Lower and upper whiskers represent the minimum and maximum values, respectively.

Live Impacts via Confocal Microscopy

As previously described, the 3D printed device was designed for compatibility on a confocal microscope. Hoechst-stained spheroids within a 96-well agarose gel were visualized upon deployment of the mechanical assembly during live imaging on the confocal microscope,

and high frame-rate videos were taken. As can be seen in **Figure 14**, likely areas of spheroid compression by silicone indenters are represented by the dashed circles.

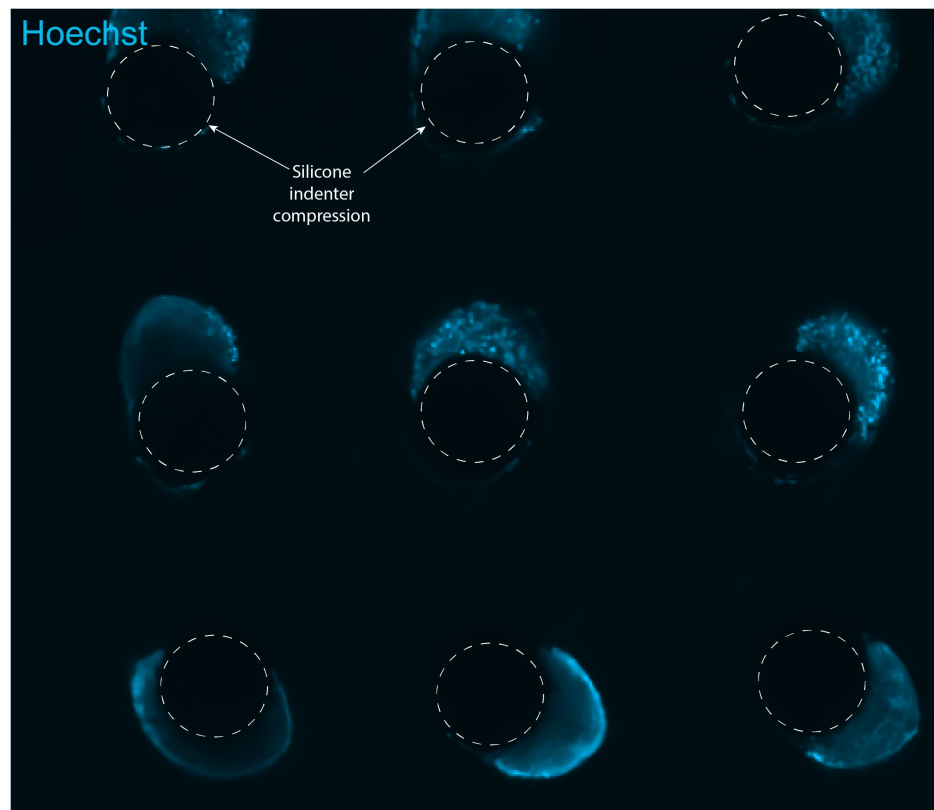


Figure 14: Confocal image generated of spheroids impacted live by a BiCM impacting device and a Silicone 3 indenter. Spheroids are stained with Hoechst (blue). Dashed circles represent areas of likely compression by silicone indenters.

Limitations

The designs showed promise, but there were a few minor issues that warranted improvement. For instance, the gel tended to be pulled up along with the silicone when returning the structure to its initial stable position after impact. Additionally, the glass coverslip often shifted out of position despite the designated slot. Nevertheless, the structure proved effective for initial testing. It is worth noting that, upon deployment, the structure shifts into the second stable

position but does not remain there without additional force. Following deployment, the structure is released and returns to the first stable position.

3.5 Impactor device designs were improved following preliminary testing.

Following the initial BiCM studies, modifications were made to the BiCM. In addition, a new BiCM design and a new silicone indenter peg length were explored.

BiCM1 Modifications

Modifications were performed to optimize the impactor device setup. Slots were originally used to connect the BiCM to the stage of the mechanical assembly, which induced movement within the frame of the mechanism. Material was then added to the stage base to tightly fix the frame of the BiCM into the stage and increase its strength (**Figure 15A**). However, at this point, the compliant segments broke following printing at their thinnest point. PLA was dried prior to printing, but the product remained unstable. Therefore, the BiCM connections were thickened by 0.25mm on each side, which prevented subsequent print failing. More material was added the anterior and posterior sides of the stage to decrease pliability (**Figure 15B**). Henceforth, this structure will be referred to as BiCM1.

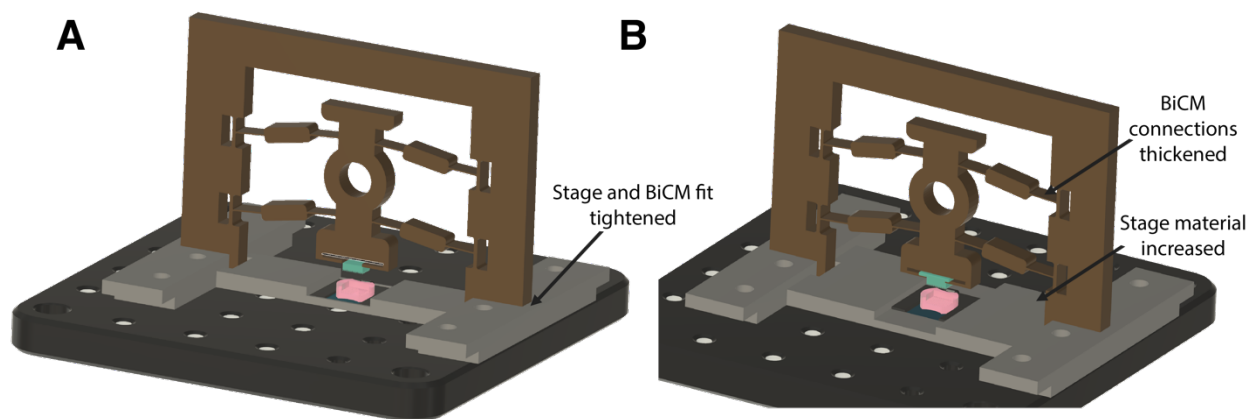


Figure 15: BiCM1 mechanical assembly with isometric CAD images of (A) prototype with stage and BiCM fit tightened; and (B) BiCM connections thickened alongside an increase in stage material volume.

BiCM2 Generation

Simultaneously, a second BiCM was generated using an open-source resource, which will be referred to as BiCM2 (**Figure 16**).⁶² Like BiCM1, BiCM2 consists of a BiCM frame, a moving shuttle, and compliant segments. The structure deploys when a force pushes the moving shuttle (which is separated from the BiCM frame by a small gap) downward. A slot was added onto the base of the moving shuttle to slide in the silicone indenter. The base of the frame from the original design was optimized to generate a stage for the agarose gel by developing a 12x12 mm platform below the moving shuttle where a glass coverslip could be placed. An agarose gel aligner was developed to center the agarose gel. A base was also designed to allow BiCM2 to be tightened into an optical board.

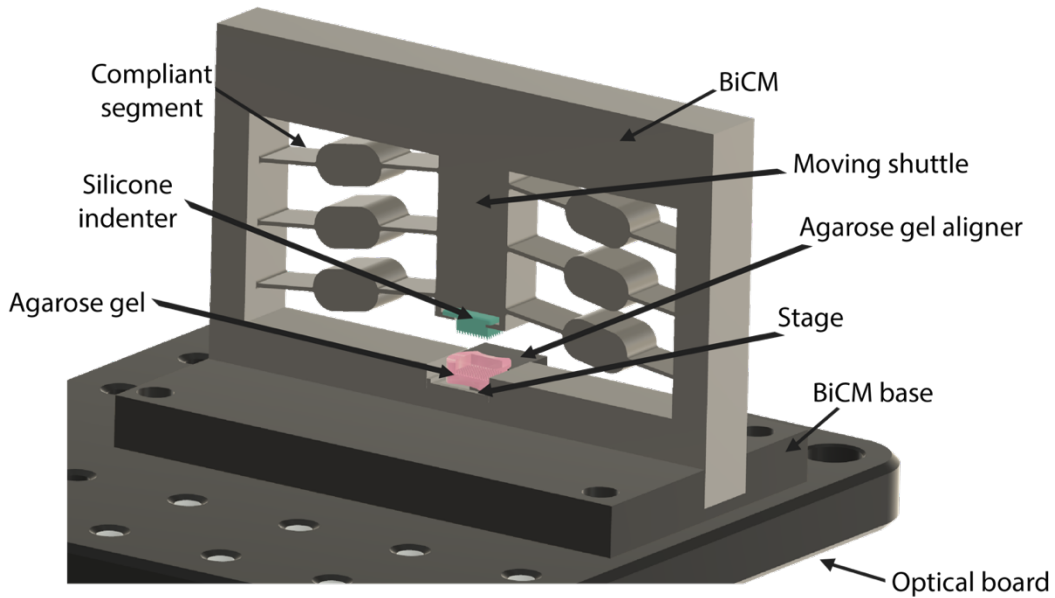


Figure 16: Isometric CAD view of BiCM2. This device consists of a frame, compliant segment, and moving shuttle. A stage was generated to center the agarose gel on a 12x12 mm glass coverslip. A slot was generated in the moving shuttle for silicone indenter placement. A base was also generated to secure the BiCM, which can be tightened into a compact optical board.

Updated Silicone Indenters

In addition to mechanical assembly optimizations, we designed silicone indenters with 650 μm length pegs in contrast to the original 600 μm length. In forthcoming studies, the most recent prototype of BiCM1, the new BiCM2 prototype, and the 650 μm silicone indenter were examined.

3.5 BiCM-induced injury leads to significant cellular disruption at 0 and 24 hours post-impact.

Using BiCM1 and BiCM2 as described in section 3.4 as well as S3 indenters with 650 μm length pegs, viability was assessed using an ATP assay, and responses of neurite networks,

microglia, and astrocytes were tested via IHC. Spheroids were assessed at 0 and 24 hours post-injury. These experiments were performed immediately after impact (DIV14) and 24 hours post-injury (DIV15). Control conditions experienced the setup described in Section 3.3 with S3.

3.5.1 ATP content was significantly decreased in BiCM2 at 24H but was not altered in BiCM1

To compare the effects of the two BiCMs, an ATP assay was performed with results assessed immediately and 24 hours post-injury with data was plotted relative to the controls (**Figure 17**). Mean relative ATP content at 0 and 24 hours, respectively, was recorded for controls (1.008 ± 0.2323 , 1.114 ± 0.181), BiCM1 (1.074 ± 0.287 , 1.088 ± 0.213), BiCM2 (1.115 ± 0.393 , 0.554 ± 0.081). Based on a two-way ANOVA, there was no statistically significant difference between controls and BiCM1-impacted spheroids ($p=0.8571$) or BiCM2-impacted spheroids ($p=0.6696$) at 0 hours. Additionally, there was not a significant ATP content difference in control versus BiCM1-impacted spheroids at 24 hours ($p=0.9777$), but ATP content decreased significantly in BiCM2-impacted spheroids ($p=0.0003$).

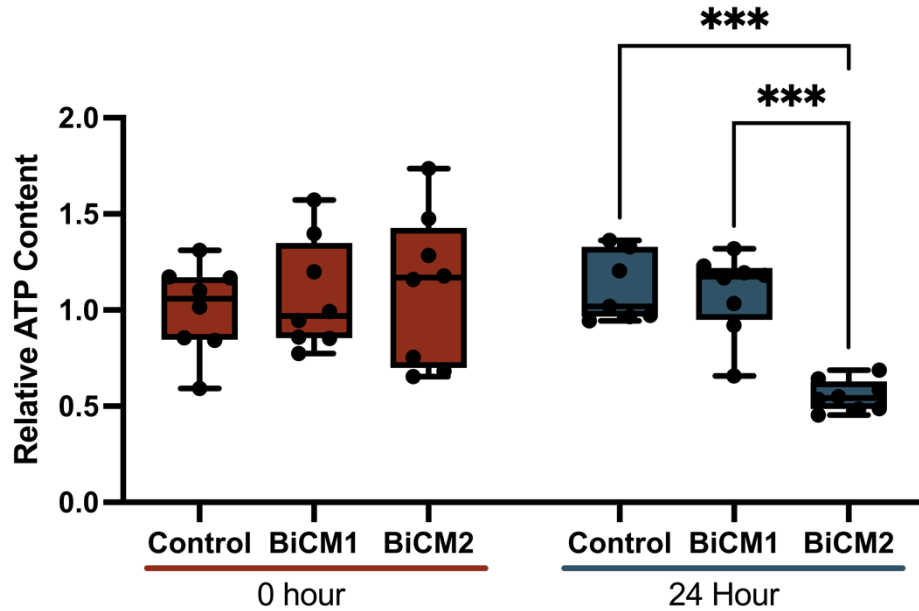


Figure 17: ATP content relative to time-matched control conditions. ATP assessed at 0 and 24 hours post-injury in control spheroids and spheroids impacted by BiCM1 or BiCM2. Two-way ANOVA with Tukey post-hoc test (n=8). Center lines of box plots represent the median. Boxes extend from the 25th to the 75th percentile. Lower and upper whiskers represent the minimum and maximum values, respectively.

3.5.2 Astrocytes exhibited an immediate reactive response following injury in spheroids impacted by BiCM1 and BiCM2 and remained reactive 24 hours later in spheroids impacted by BiCM1

Spheroids were stained with GFAP to assess astrocyte reactivity following injury. Physiological branch morphology (white arrows) was altered by impact, with an increase in reactive morphology (white arrowheads) as evidenced by thicker branching and increased GFAP signal intensity (**Figure 18A-B**). Average GFAP voxel intensity was quantified to assess astrocyte reactivity (**Figure 18C**). Immediately (0 hours) following impact, GFAP voxel intensity increased in spheroids impacted by either BiCM1 (1374.745 ± 94.816) or BiCM2 (1299.407 ± 324.261) compared to controls (989.711 ± 73.916 , $p=0.0013$ and $p=0.01$,

respectively). At 24 hours, voxel intensity was increased in BiCM1 (1455.059 ± 190.249) compared to its time-matched control (1013.073 ± 48.668 , $p=0.0002$), but not for BiCM2 (969.882 ± 108.614 , $p=0.9976$). However, GFAP intensity appeared saturated in spheroids impacted by BiCM1. As a result, the measured intensity may not accurately reflect the true extent of the voxel intensity.

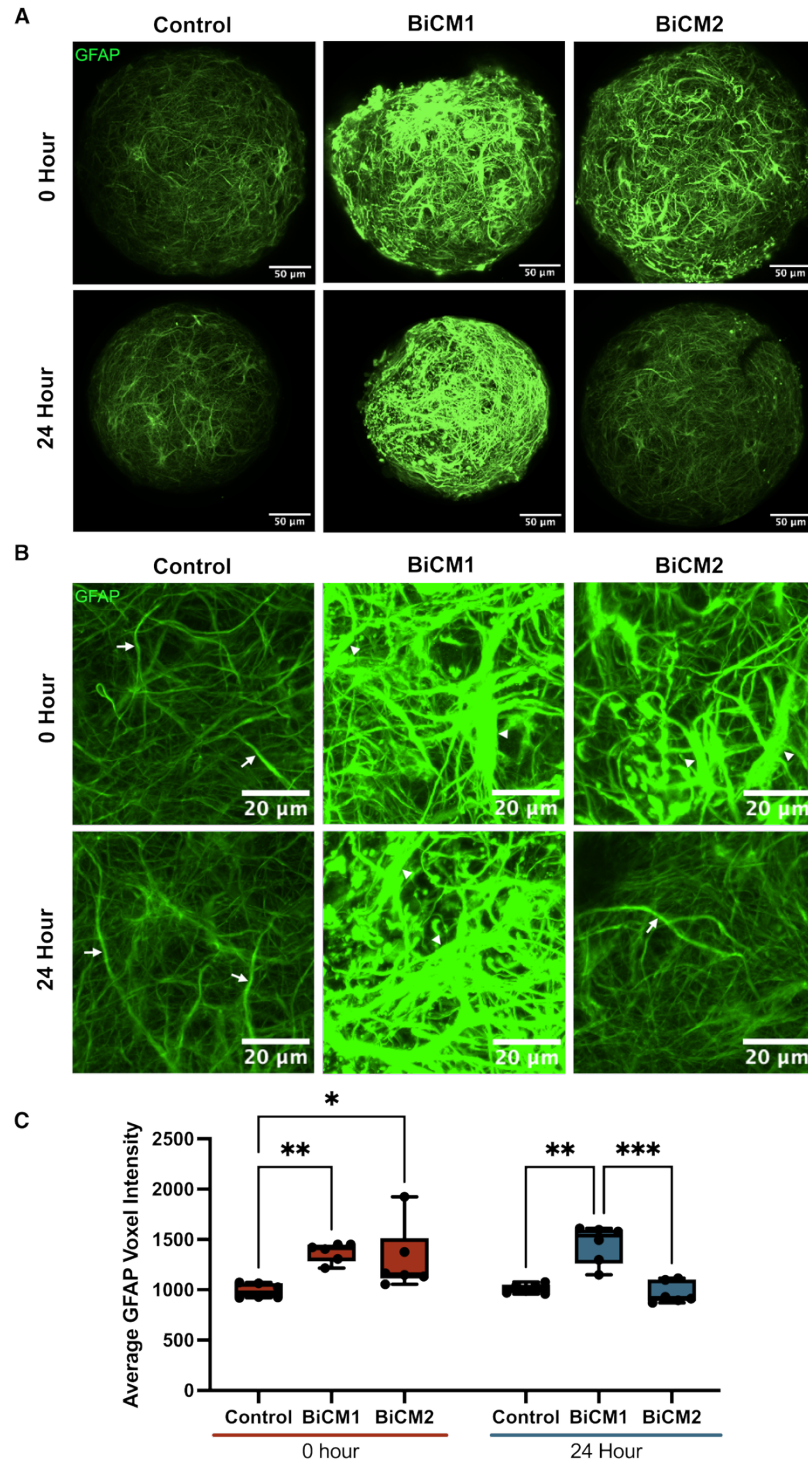


Figure 18: Astrocytes displayed a reactive morphology following injury in spheroids impacted by BiCM1, as well as spheroids impacted by BiCM2 after 24 hours. (A) GFAP at 0- and 24-hours post-injury in control spheroids and spheroids impacted by BiCM1 or BiCM2. White

arrows represent phenotypical branch morphology. White arrowheads represent reactive morphology. (B) Average GFAP Voxel intensity at 0- and 24-hours post-injury in control and impacted spheroids. Two-way ANOVA with Tukey post-hoc test (n=6). Center lines of box plots represent the median. Boxes extend from the 25th to the 75th percentile. Lower and upper whiskers represent the minimum and maximum values, respectively.

3.5.3 Neurite networks in spheroids impacted by BiCM1 became damaged within 24 hours following injury

Neurite networks were assessed at 0 and 24 hours post-impact using confocal microscopy (**Figure 19A-B**). Typically, TUBB3 appears as a dense, strand-like network in healthy spheroids (white arrows). However, neurite network damage and microtubule degradation were apparent in spheroids impacted by BiCM1 after 24 hours as evidenced by neurite strand breakdown and puncta formation (white arrowheads). Graph theory analysis was performed, and the number of subgraphs present was recorded to assess extent of neurite network damage (**Figure 19C**). At 0 hours post-injury, there were no significant differences between mean subgraphs of controls (31.167 ± 41.340) and spheroids impacted by BiCM1 (16 ± 14.588 , $p=0.9998$) or BiCM2 (88.5 ± 133.269 , $p=0.9048$). At 24 hours, controls (31.333 ± 38.614) had similar subgraph numbers to BiCM2 (43.167 ± 33.343 , $p>0.9999$). However, compared to controls, BiCM1-impacted spheroids displayed a significantly increased number of subgraphs within the neurite networks (217.5 ± 183.609 , $p=0.0250$).

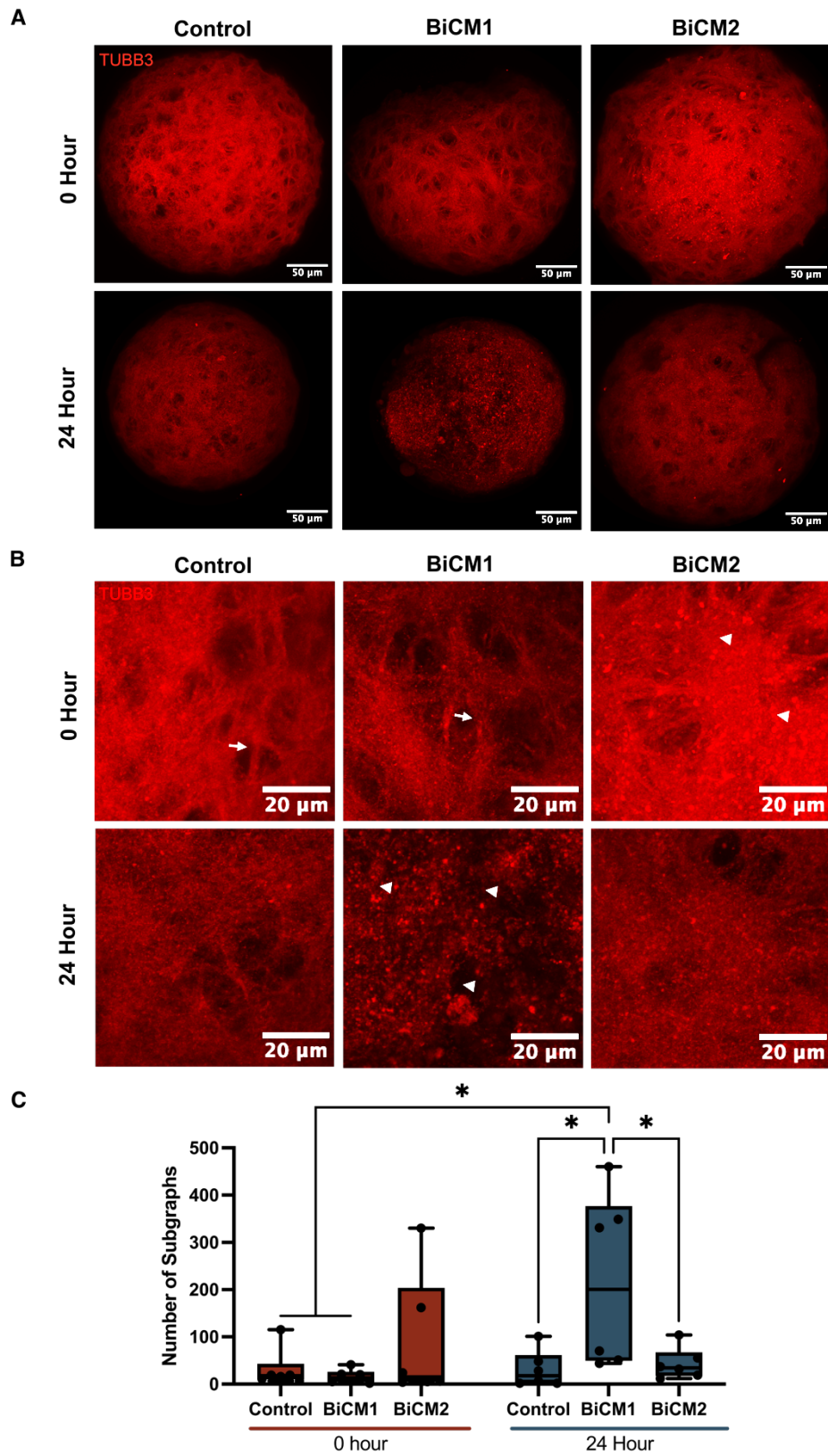


Figure 19: Neurite networks display decreased connectivity, puncta formation, and damage within 24-hours following BiCM1-induced injury. (A) Confocal images and (B) magnified confocal images of TUBB3 networks in control, BiCM1-injured and BiCM2-injured spheroids at 0 and 24 hours post-impact. White arrows represent physiological branch morphology. White arrowheads represent neurite network damage. (C) Subgraph count in control, BiCM1-injured, and BiCM2-injured spheroids at 0 and 24 hours post-injury as assessed by the custom graph theory analysis method previously described. Two-way ANOVA performed (n=6). Center lines of box plots represent the median. Boxes extend from the 25th to the 75th percentile. Lower and upper whiskers represent the minimum and maximum values, respectively.

3.5.4 Quantification of microglial response and morphological evaluation post-injury is ongoing

Qualitatively, 0 hour control spheroids appeared to have more rounded morphologies and increased numbers of microglia (**Figure 20**). Microglial response evaluation is ongoing. Further studies will evaluate microglial morphological characteristics, motility, time-dependent behavior, and object count following injury.

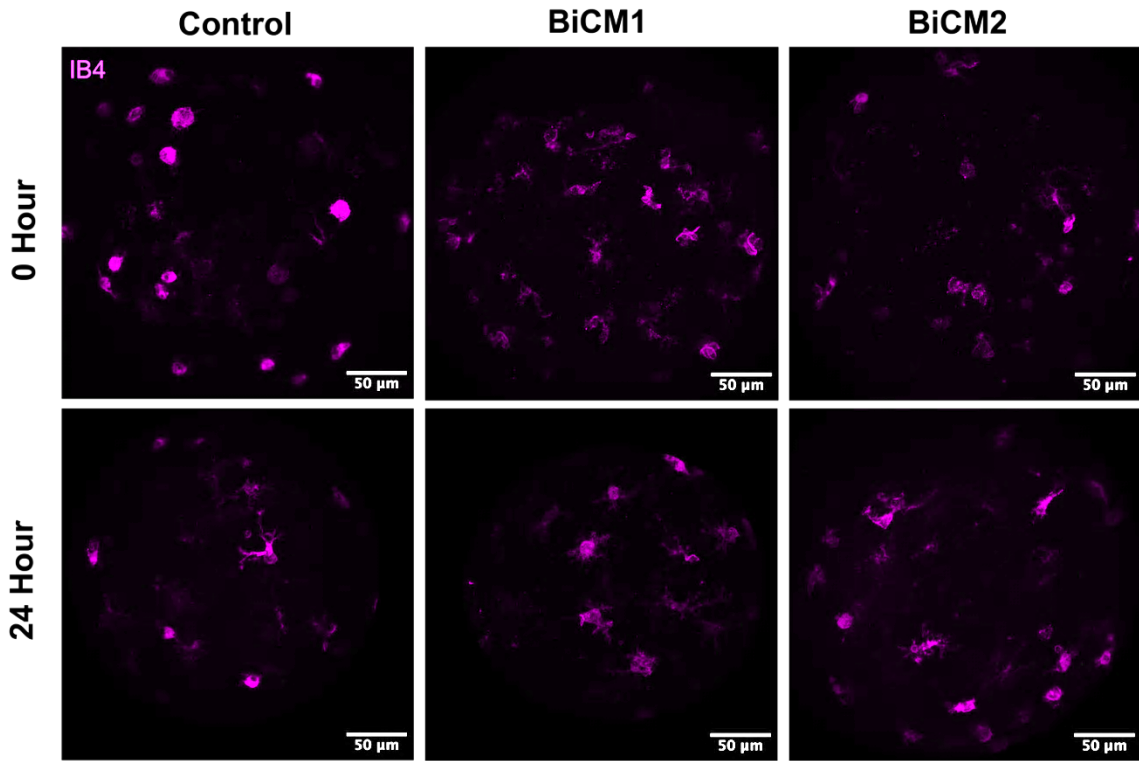


Figure 20: Confocal images of microglia (IB4) labeled spheroids after 0 and 24 hours in controls and spheroids impacted by BiCM1 or BiCM2.

3.5.5 Ongoing design needs from the experiments

Of note, this was the first experiment with BiCM2, thus some areas to optimize were noted during the previously described experiments. BiCM2 was altered prior to additional mechanical testing of both mechanisms.

3.6 BiCM2 Mechanical Assembly Optimization

The structure of BiCM2 was optimized to allow for confocal microscope compatibility and improve experimental accuracy and workflow (**Figure 21**). To improve the structure of BiCM2, tolerance was added to BiCM2 to improve the fit of the glass coverslip. The height was

also optimized with the assumption of a 3.75 mm agarose gel. The agarose gel aligner was also improved, with adjustments allowing it to print directly on the BiCM stage. This improves the accuracy process for alignment of the agarose gel prior to impact. Further, like BiCM1, BiCM2 was optimized for confocal microscopy compatibility by decreasing the stage height and redesigning the base. The optimized BiCM2 was used for future studies.

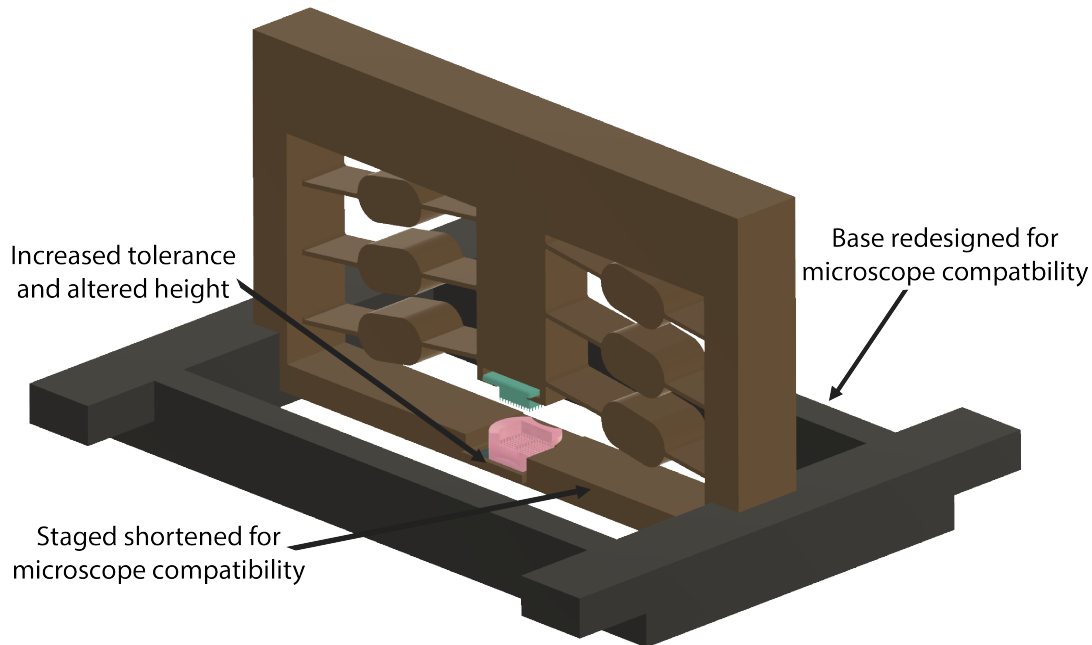


Figure 21: The BiCM2 assembly was optimized. Of note, the base was redesigned, and the stage was shortened to allow for confocal microscope computability. In addition, the height of the agarose gel stage and glass coverslip tolerance were altered. An agarose gel aligner was redesigned to be printed directly on the BiCM2 stage, improving the alignment process.

3.7 Experimental assessment of BiCM1 and BiCM2 over time shows loss of viability, astrogliosis, and neurite network damage, with some recovery present after 72 hours

The effects of the optimized BiCM1 and BiCM2 devices over time were assessed. Using the 650 μm peg length silicone indenter (S3) and the updated BiCM1 and BiCM2 devices, spheroids were impacted on DIV13 and stained through IHC and impacted on DIV14 for ATP

studies. They were analyzed immediately following injury and at various time points up to 3 days post-injury. While BiCM1 spontaneously reverts to its first stable position after an impact, it is important to note that BiCM2 requires manual deployment to its first stable position following impact. BiCM2 was gently deployed out of the agarose gel immediately following impact for these experiments.

3.7.1 ATP content was significantly decreased by BICM2 between 0 to 24 hours

To assess spheroid viability after impacts at various time points, ATP content was assessed at 0 hours, 8 hours, 24 hours, and 72 hours post-injury with BiCM1 or BiCM2 (**Figure 22A, Table 4**). After a single and instantaneous impact, relative ATP content was significantly decreased in BiCM2-impacted spheroids at 0 hours, 8 hours, and 24 hours compared to controls. ATP content from BiCM1-impacted spheroids was significantly decreased at 24 hours following impact but not at other timepoints.

Spheroids were also assessed for ATP content after 2 consecutive impacts at 0, 24, and 72 hours. In BiCM1-impacted spheroids, there was no change in relative ATP content at any timepoint studied after 2 impacts compared to controls (**Figure 22B**). In BiCM2-impacted spheroids, there was a significant decrease in ATP content at 0 hours and 24 hours post-injury (**Figure 22C**). However, ATP content was significantly greater after 2 impacts than after 1 impact at 24 hours for BiCM2-impacted spheroids. There was no significant change in ATP content at 72 hours after 2 impacts.

Table 4: Relative ATP content in spheroids impacted once or twice by BiCM1 and BiCM2 after 0, 8, 24, and 72 hours. Data reported as mean $\pm$ standard deviation. P values reported are compared to time-matched controls. Two-way ANOVA with Tukey post-hoc test (n=7-8)

Time (hours)	Control	BiCM1 (1 impact)	BiCM2 (1 impact)	BiCM1 (2 impacts)	BiCM2 (2 impacts)
0	1.000 $\pm$ 0.123	0.978 $\pm$ 0.089, p = 0.9305	0.565 $\pm$ 0.158, p < 0.0001	0.871 $\pm$ 0.192, p = 0.1562	0.489 $\pm$ 0.117, p < 0.0001
8	1.000 $\pm$ 0.118	0.967 $\pm$ 0.082, p = 0.8637	0.793 $\pm$ 0.073, p = 0.0036	N/A	N/A
24	1.000 $\pm$ 0.139	0.846 $\pm$ 0.119, p = 0.0486	0.458 $\pm$ 0.091, p < 0.0001	0.901 $\pm$ 0.140, p = 0.3748	0.653 $\pm$ 0.135, p = 0.0002
72	1.000 $\pm$ 0.188	1.048 $\pm$ 0.142, p = 0.7320	0.884 $\pm$ 0.123, p = 0.1719	0.944 $\pm$ 0.074, p = 0.7158	0.851 $\pm$ 0.267, p = 0.1669

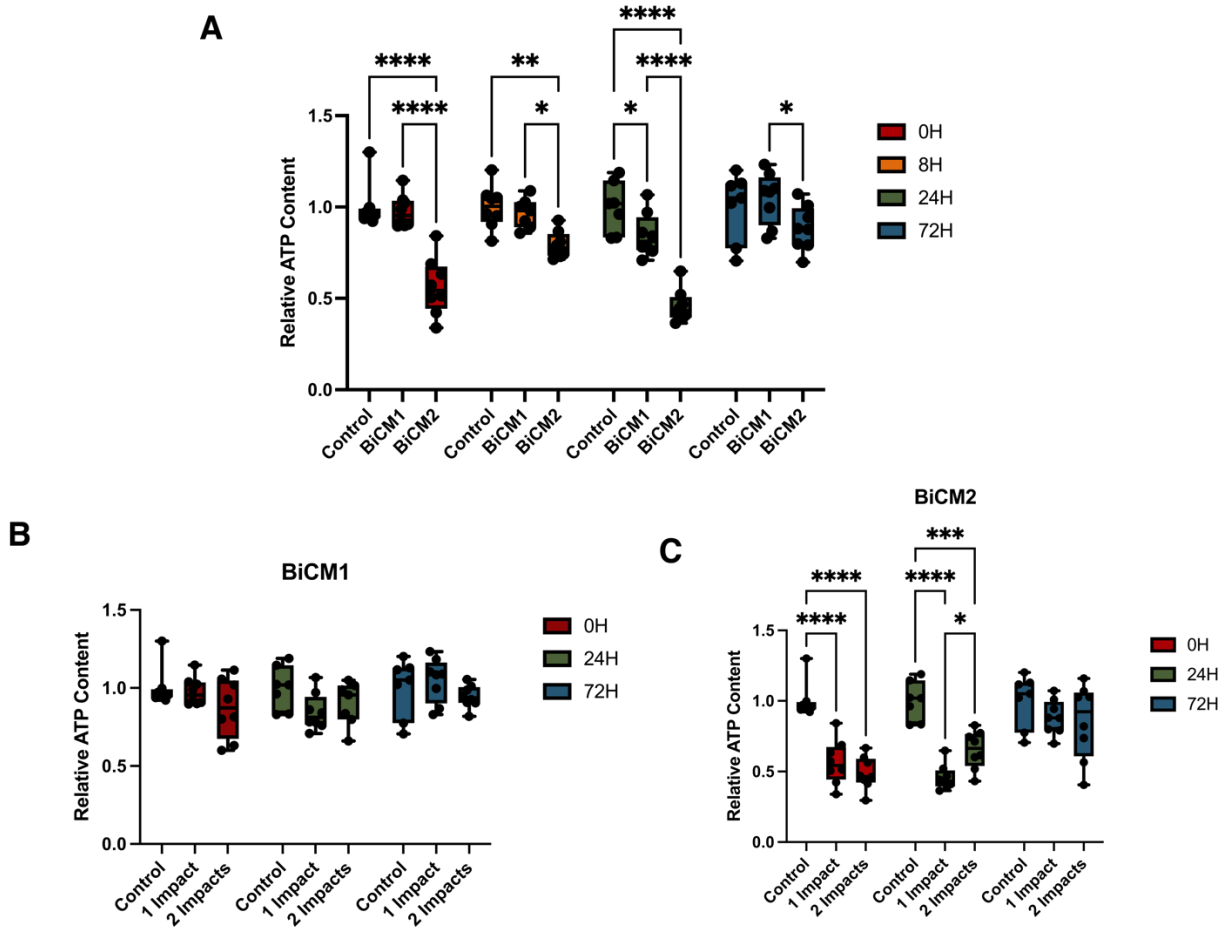
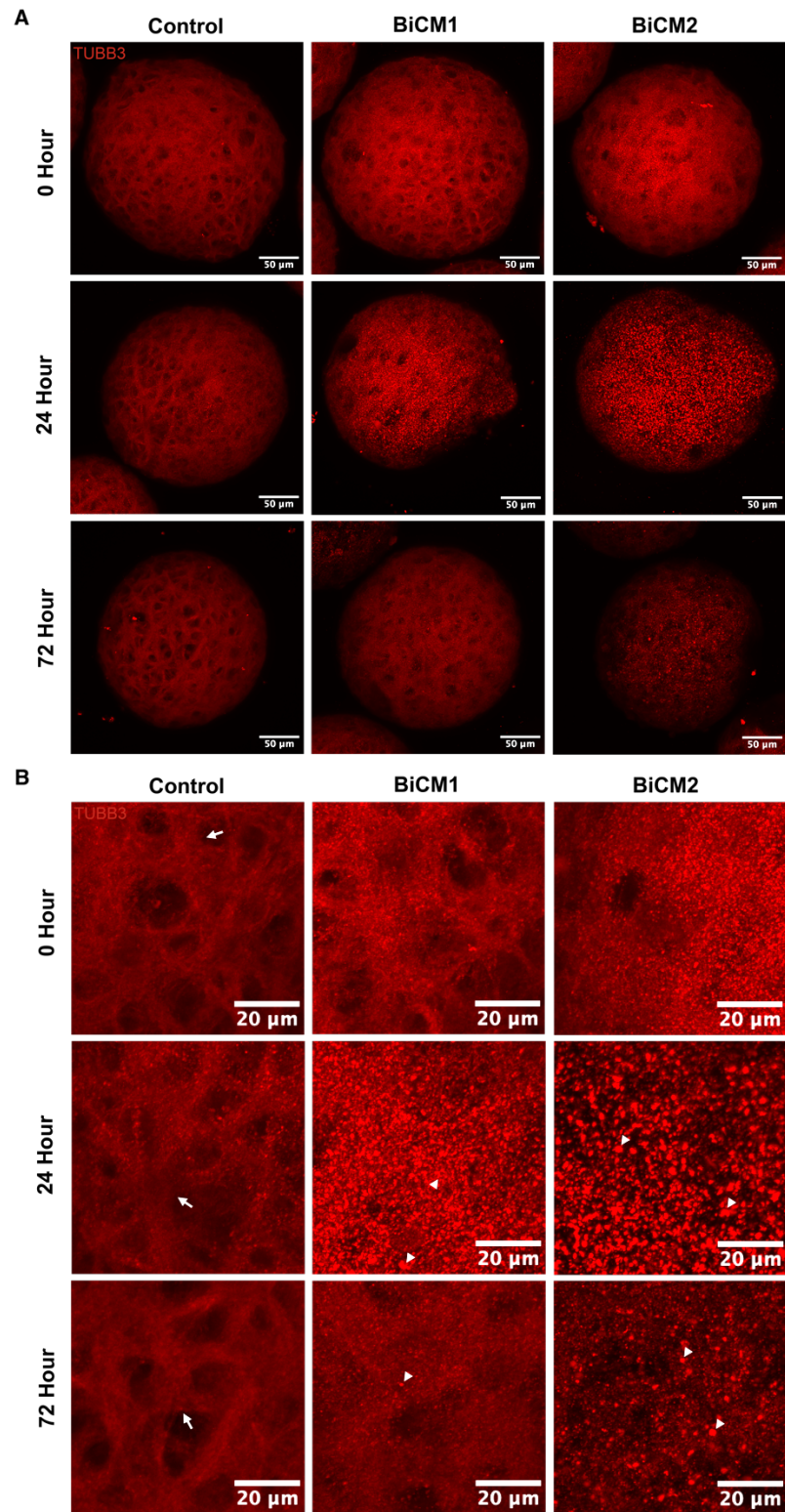


Figure 22: ATP content in spheroids impacted by BiCM1 and BiCM2. (A) Relative ATP content at 0, 8, 24, and 72 hours in spheroids impacted by BiCM1 or BiCM2 compared to controls. Two-way ANOVA (n=7-8) Relative ATP content at 0, 24, and 72 hours in spheroids impacted by (B) BiCM1 or (C) BiCM2 either once or twice. Two-way ANOVA (n=7-8)

3.7.2 Neurite networks showed damage 24 and 72 hours post-impact

Spheroids were stained with TUBB3 to assess neurite network damage over time (**Figure 23A-B**). Neurite network damage was quantified, and the number of subgraphs were recorded for each sample (**Figure 23C**). At 0 hours post-injury, there were no significant quantitative or qualitative differences in neurite networks among control, BiCM1-, and BiCM2-impacted spheroids. By 24 hours, significant network degradation was observed in both BiCM1

(242.667 ± 123.537 , $p = 0.0064$) and BiCM2 (578.000 ± 183.280 , $p < 0.0001$) groups compared to controls (22.667 ± 21.078). Qualitatively, both impacted conditions exhibited increased puncta, indicative of microtubule breakdown. At 72 hours post-injury, neurite damage remained significantly elevated in BiCM2 spheroids (167.667 ± 221.101 , $p = 0.0061$) relative to controls (50.833 ± 24.572), though qualitative assessments suggested partial recovery, with fewer puncta and a lower mean subgraph count than at 24 hours. Similarly, BiCM1-impacted spheroids showed signs of recovery by 72 hours (272.000 ± 135.439 , $p = 0.2851$), with no statistically significant difference from time-matched controls. While some puncta remained, they were less prominent than at the earlier timepoint.



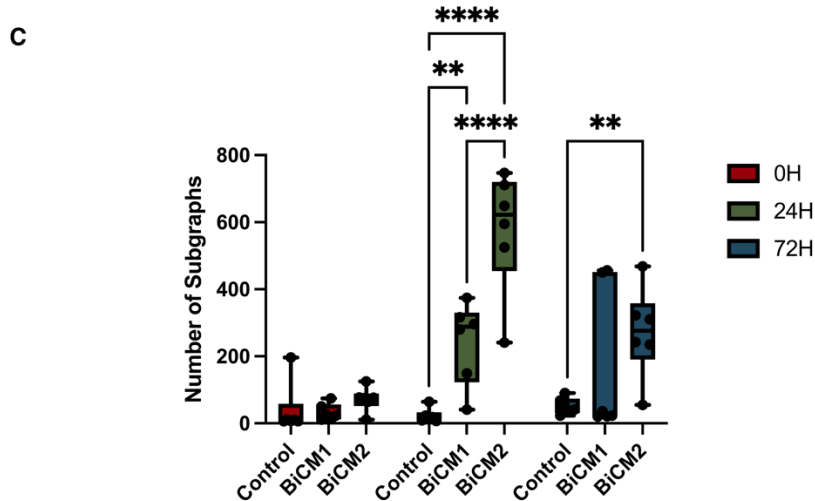
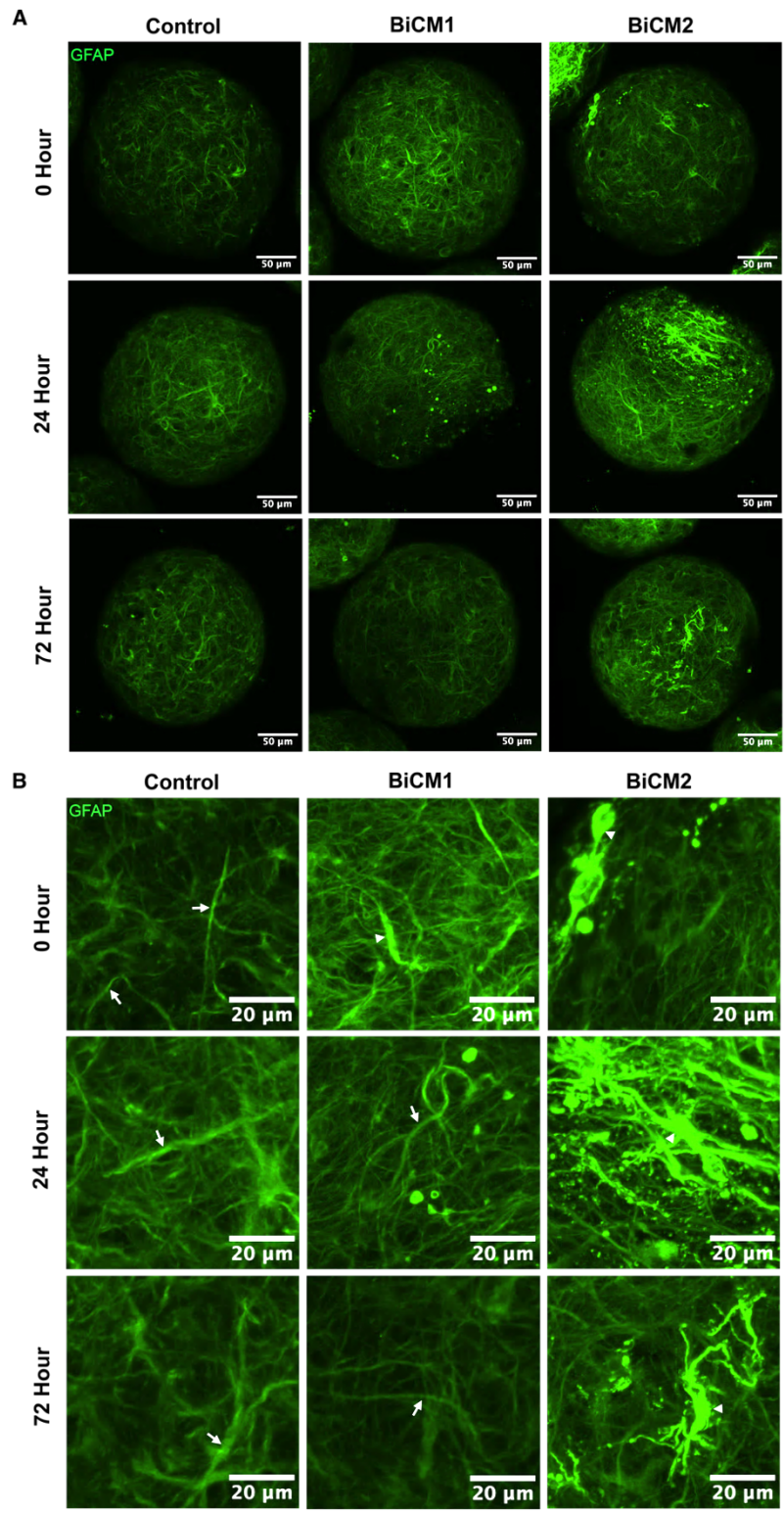


Figure 23: Neurite networks display decreased connectivity, puncta formation, and damage within 24-hours following BiCM1 or BiCM2-induced injury. (A) Confocal images and (B) magnified confocal images of TUBB3-stained neurite networks in control, BiCM1-injured and BiCM2-injured spheroids at 0, 24, and 72 hours post-impact. White arrows represent phenotypical branch morphology. White arrowheads represent sites of neurite network damage. (C) Subgraph count in control, BiCM1-injured, and BiCM2-injured spheroids at 0, 24, and 72 hours post-injury as assessed by the custom graph theory analysis method previously described. Two-way ANOVA performed with Tukey post-hoc test (n=6). Center lines of box plots represent the median. Boxes extend from the 25th to the 75th percentile. Lower and upper whiskers represent the minimum and maximum values, respectively.

3.7.3 Astrocyte showed reactive morphologies at 0 and 72 hours post-impact in spheroids impacted by BiCM2

Astrocyte reactivity was visualized at 0, 24, and 72 hours post-injury using GFAP staining (**Figure 24A-B**). This reactivity was quantified by assessing GFAP voxel intensity at each timepoint studied (**Figure 24C**). In spheroids impacted by BiCM2, voxel intensity increased significantly immediately after injury ($p < 0.0001$). Although reactivity was not significantly elevated at 24 hours ($p = 0.773$), it rose again by 72 hours post-injury ($p = 0.0446$) compared to uninjured controls. Qualitatively, GFAP-positive processes appeared thicker than

those in controls at all post-injury timepoints following BiCM2 impact. In contrast, BiCM1-induced impacts did not result in a significant increase in GFAP voxel intensity at any timepoint.



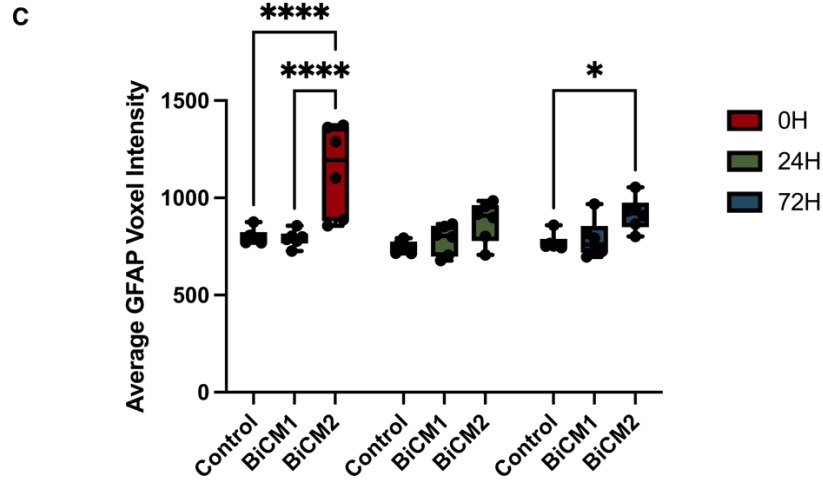


Figure 24: Astrocytes displayed a reactive morphology following injury in BiCM2 post-injury. (A) Confocal images and (B) magnified confocal images of GFAP stained spheroids at 0, 24, and 72 hours post-injury in control spheroids and spheroids impacted by BiCM1 or BiCM2. White arrows represent phenotypical branch morphology. White arrowheads represent reactive astrocytes. (C) Average GFAP voxel intensity at 0, 24, and 72 hours post-injury in control and impacted spheroids. Two-way ANOVA with Tukey post-hoc test (n=6). Center lines of box plots represent the median. Boxes extend from the 25th to the 75th percentile. Lower and upper whiskers represent the minimum and maximum values, respectively.

3.7.4 Microglial responses at 0H, 24H, and 72H

Microglia were visualized at 0, 24, and 72 hours post-injury using the IB4 stain (**Figure 25**). Qualitatively, microglia appeared to be high in number at 0 and 72 hours post-injury in BiCM1 and BiCM2 but not at 24 hours. Quantitative analysis of microglia morphology and motility post-injury has yet to be assessed.

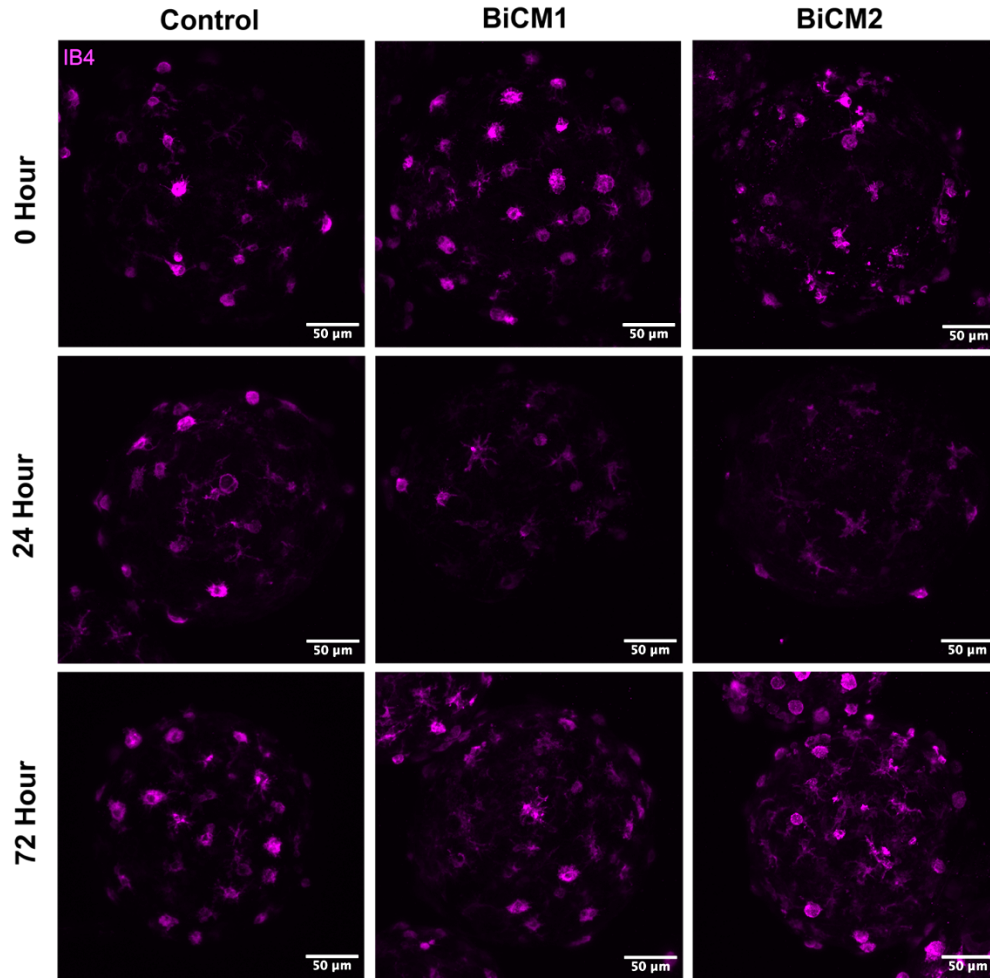


Figure 25: Confocal images of microglia (IB4) labeled spheroids after impacts with BiCM1 and BiCM2 at 0, 24, and 72 hours.

3.8 Biomechanical testing

3.8.1 Compression testing for impacting devices using an Instron machine and customized design

The Instron machine was used to assess the force generated during deployment of the impacting devices. Force-displacement curves were generated for both BiCM1 and BiCM2, as shown in **Figure 26A** and **Figure 26B**, respectively. BiCM1 did not fully deploy into its second

stable position; therefore, its force profile is represented continuously across the displacement range. Two distinct peaks were observed at approximately 8.6 N and 9.5 N, the latter occurring when the device contacted the stage platform. In contrast, BiCM2 successfully transitioned into its second stable position toward the end of testing, producing a characteristic drop-off in its force-displacement curve. The maximum force at the first critical point for BiCM2 was approximately 25 N (higher than BiCM1). The snap-through event occurred after 6 mm of displacement, with forces rapidly decreasing from 12 N to 0 N following deployment.

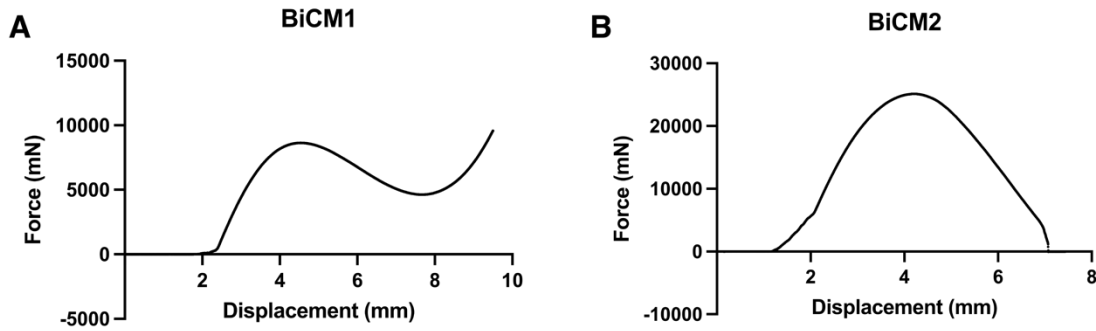


Figure 26: Force-displacement curves generated from Instron testing. (A) BiCM1 and (B) BiCM2 force (mN) -displacement (mm) curves obtained from raw Instron data.

3.8.2 Estimation of strains delivered by silicone indenters and strain rates delivered by BiCM2

Based on usage of the 650 μm peg, the strain delivered to individual spheroids can be estimated. Measurements assume that each microwell is 800 μm in length. We assume that spheroids of 8k density range in size from around 200 μm to 300 μm and that the spheroids are at the bottom of the well at the onset of an impact. Based on these assumptions, there are 150 μm of additional space in a microwell following an impact. Measurements are estimated for a 200

μm spheroid in Equation 5 and $300\ \mu\text{m}$ spheroid in Equation 6, resulting in approximate strains of 25% and 50%, respectively.

$$\varepsilon = \frac{200\ \mu\text{m} - 150\ \mu\text{m}}{200\ \mu\text{m}} = \frac{50\ \mu\text{m}}{200\ \mu\text{m}} = 25\% \quad (\text{Equation 5})$$

$$\varepsilon = \frac{300\ \mu\text{m} - 150\ \mu\text{m}}{300\ \mu\text{m}} = \frac{150\ \mu\text{m}}{300\ \mu\text{m}} = 50\% \quad (\text{Equation 6})$$

Currently, it is challenging to estimate the strain rate produced by BiCM1. As it has one stable position, there is human error that impacts its velocity. Thus, strain rate has not been calculated for BiCM1. However, BiCM2 displays typical bistable behavior. Thus, its strain rate was estimated. Following Instron testing, the velocity of BiCM2 was estimated at around $337.5\ \text{mm/s}$. The time during which the spheroids were compressed was estimated using Equation 7 and 8 for a smaller and larger spheroid, respectively. The estimated time was calculated by spheroid size divided by BiCM2 velocity (Equation 7 and Equation 8). Strain rates were then calculated for a smaller and larger spheroid, resulting in rates ranging from 422 to $562\ \text{s}^{-1}$ (Equation 9 and Equation 10, respectively). Thus, BiCM2 delivers a high strain rate.

$$t_{\text{compression}_1} = \frac{\text{spheroid size}}{\text{velocity}} = \frac{200\ \mu\text{m}}{337.5\ \text{mm/s}} = 592\ \mu\text{s} \quad (\text{Equation 7})$$

$$t_{\text{compression}_1} = \frac{\text{spheroid size}}{\text{velocity}} = \frac{300\ \mu\text{m}}{337.5\ \text{mm/s}} = 889\ \mu\text{s} \quad (\text{Equation 8})$$

$$\dot{\varepsilon} = \frac{0.25}{592\ \mu\text{s}} = 422\ \text{s}^{-1} \quad (\text{Equation 9})$$

$$\dot{\varepsilon} = \frac{0.5}{889\ \mu\text{s}} = 562\ \text{s}^{-1} \quad (\text{Equation 10})$$

Chapter 4: Discussion and Conclusions

Our study successfully demonstrated the feasibility of using a mechanical assembly to induce blunt TBI in cortical spheroids, providing an innovative model for studying the biomechanical and pathophysiological effects of TBI. The assembly, using silicone indenters and a BiCM, enabled high-throughput impact testing and allowed us to effectively monitor cellular responses to mechanical deformation. This platform enables the use of 3D printing technology, which is a promising tool for rapidly prototyping devices and optimizing experimental conditions, facilitating further exploration of TBI mechanisms.

4.1 Mechanical Assembly of Blunt TBI Assessment

Initially, we manually impacted spheroids using silicone indenters to assess whether direct mechanical deformation induces TBI-like effects. The variability of manual impacts likely contributed to variations in neurite network subgraphs and cell viability. Nevertheless, the mechanical impacts resulted in significant changes in spheroid viability and neurite network integrity within the first 24 hours post-injury, which aligns with previous studies that have reported rapid damage to neural structures after TBI. Our selection of a silicone indenter with a 600 μm peg length for subsequent experiments was based on the observation that this length would compress the spheroid but still leave space for the spheroids at the bottom of the 800 μm wells. Ultimately, we utilized a 650 μm peg length silicone indenter to ensure that sufficient compression could be delivered to smaller spheroids as well as larger ones. Silicone peg length can be varied to assess the effects of various strains at consistent strain rates.

Selecting the BiCM for reproducible impacts offered ease of production, compact size, and flexibility in adjusting impact conditions.^{63,64} The use of two BiCM designs enabled us to investigate impacts of varying severities, as different forces were required for deployment. This allowed us to explore how impact intensity influences the cellular response to TBI. BiCM1-induced impacts likely have a shorter impact duration, as the device does not remain in its second stable position without an external force. In contrast, BiCM2 deploys into its second stable position and requires a manual force to return to its initial stable state. Previous studies have shown that impacts from automobile accidents, concussions, and falls typically last longer than 3 ms, which is consistent with the manual forces exerted by these mechanisms.⁶⁵ While strain has not been extensively characterized in TBI models, studies suggest that diagnosed concussions are associated with strains ranging from 14% to 40%, which is consistent with our estimated strain range of 25% to 50%.^{66,67} However, strain rate is also critical in determining injury severity.⁶⁸ BiCM2 was estimated to deliver strain rates ranging from approximately 400 to 600 s⁻¹. The combined values of strain and strain rate for BiCM2 and a 650 μ m silicone indenter align with a severe TBI which may be typical of a severe car crash or fall from a height.⁶⁹ Although BiCM1 and BiCM2 are expected to deliver similar strains due to the use of the same peg length silicone indenter, their strain rates differ. Based on the forces generated by each mechanism, we hypothesize that BiCM1 represents a milder injury, while BiCM2 reflects a moderate to severe injury. Future studies will aim to quantify strain rates to further validate this assumption.

Furthermore, our optimized BiCM setup enabled us to visualize spheroid compression and assess the effects of mechanical deformation using Hoechst-stained spheroids within the gel. However, we encountered limitations in visualizing the compression through the transmitted

light channel due to obstruction from the BiCM structure. To overcome this, future experiments will incorporate additional light sources, such as fluorescent silicone, glow-in-the-dark PLA, and LED lighting, to enhance visualization and potentially allow for strain rate measurements.

4.2 Pathophysiological Assessment of Simulated Blunt TBI

Viability assessments indicated decreased viability immediately after injury and up to 24 hours post-injury in spheroids impacted by BiCM2, while no significant changes in viability were observed in BiCM1-impacted spheroids. This suggests that BiCM2 delivers a more severe impact than BiCM1. Relative ATP content in BiCM2-impacted spheroids decreased at 0, 8, and 24 hours post-injury, but ATP was no longer significantly altered after 72 hours, suggesting a possible resolving of the injury. This aligns with mild TBI studies, which have reported significantly decreased ATP at 24 hours, followed by recovery to control-like levels at 72 hours post-injury.⁷⁰ *In vivo* studies have similarly found metabolic dysregulation following severe injury within the first 24 hours, while mild injury leads to limited dysregulation that resolves within a few days.⁷¹ Live viability staining has shown no significant impact on BiCM1-impacted spheroids, consistent with our ATP results, while viability staining for BiCM2 is currently under investigation.

In addition, initial experiments with multiple impacts using BiCM1 demonstrated a decrease in spheroid viability as the number of impacts increased from 1 to 3, suggesting that our model has the potential to investigate pathophysiological effects of repeated injuries, such as those seen in chronic traumatic encephalopathy.

In terms of cellular response, neurite damage was commonly observed at 24 hours post-injury in impacted spheroids, as evidenced by microtubule breakdown and the loss of the

characteristic strand-like structure associated with TUBB3. Downregulation of TUBB3 has been reported in the long-term following TBI and in post-mortem samples from individuals with chronic traumatic encephalopathy.⁷⁰ TUBB3 is exclusively expressed in neurons, and its downregulation is linked to axonal degradation.⁷⁰ RNA sequencing studies have suggested that targeting tubulin structures through therapeutics could be a promising strategy for TBI treatment.²⁸ While TUBB3 is not essential for neuronal development, it is important for axonal regeneration and microtubule dynamics.⁷² Therefore, understanding microtubule stability and TUBB3-associated pathways in our model is a promising avenue for assessing therapeutic strategies aimed at mitigating axonal damage and promoting neuronal regeneration following TBI.

Reactive astrogliosis is a hallmark of TBI, and its severity can range from mild to severe. Mild to moderate astrogliosis typically involves reversible astrocyte hypertrophy and increased GFAP expression, whereas severe astrogliosis is characterized by astrocyte proliferation and long-lasting structural changes.⁷³ In our high-severity injuries, we observed a significant increase in astrocyte reactivity, as measured by GFAP expression, immediately after injury, which is consistent with findings from other TBI models.^{74,75} Studies have found presence of astrocytic hypertrophy after mild-moderate TBI within an hour post-injury.^{76,77} Researchers have found astrocyte dysregulation within 30 minutes post-injury *in vivo*.⁷⁸ Other studies have shown more gradual changes in astrocyte reactivity occurring within the first 6 hours post-injury.⁷⁹ In our model, we see a rapid increase in GFAP expression immediately following impact but a less extreme response over time, highlighting the sensitivity of our model to mechanical injury. Our findings align with reports that concussions and other forms of TBI can lead to rapid reactive astrocytic responses, with GFAP being a key biomarker in such conditions.

Through this work, we have successfully simulated blunt TBI in cortical spheroids by inducing mechanical deformation using a combined bistable mechanism and silicone indenter. Neurite damage and increased GFAP expression suggest that our model is sensitive to mechanical deformation and can potentially be used to study neuroinflammation. Our work demonstrates that the higher-force BiCM2 induces more severe cellular responses than BiCM1, as shown by decreased viability and increased astrogliosis and neurite damage. Our model also provides a valuable platform for investigating both single and repeated TBI impacts, allowing us to better understand TBI mechanisms and potential therapeutic strategies.

4.3 Future Directions

Further experiments will aim to alter the strain and strain rates delivered by our model to offer the opportunity to simulate various severities of blunt TBI. Additional work is needed to achieve consistent and reproducible strain rates. The use of high-speed confocal microscopy will be valuable in evaluating experimental strain and strain rates during impact which will add context to our theoretical biomechanic estimations. Silicone indenters can be optimized with varying peg lengths and diameters as well as various silicone hardnesses to alter the extent of impact and strain delivered to spheroids.

A promising avenue for future studies is the evaluation of microglial responses to injury, which will include assessments of their morphology and motility. These cells play a critical role in neuroinflammation following TBI, and we plan to assess their proliferation and activation in response to injury by incorporating markers such as Ki67 and CD68.⁸⁰ The addition of timepoints beyond the 72-hour window will also allow for a more comprehensive understanding of the long-term effects of TBI on the microglial population and other cell types involved in the

injury response. In the future, we also hope to assess calcium dynamics post-injury, which tends to be dysregulated in response to TBI.⁸¹

Currently, BiCM2 acts as a typical bistable compliant mechanism, containing two stable positions. However, a typical TBI would require the force to be released following an injury. Furthermore, manual deployment following an impact introduces human error into the experiments. Thus, future work will optimize the mechanical assembly such that the strain is released following the impact. There are multiple avenues for this method. The base of the mechanism can be slowly released using a dampening system. Alternatively, tension can be used to relieve the strain in the mechanism. Magnetic forces can also be used to remove the mechanism from the stage of the agarose gel. We will evaluate various mechanisms to achieve our goal and will analyze the responses of various cell types as well as overall viability of cortical spheroids.

Our model provides a valuable platform for studying TBI-induced cellular responses and can be adapted to simulate varying injury severities. We expect that this model will contribute to understanding the complex biomechanic and pathophysiological mechanisms underlying TBI and provide an avenue for high-throughput therapeutic testing, leading to improved patient outcomes for TBI.

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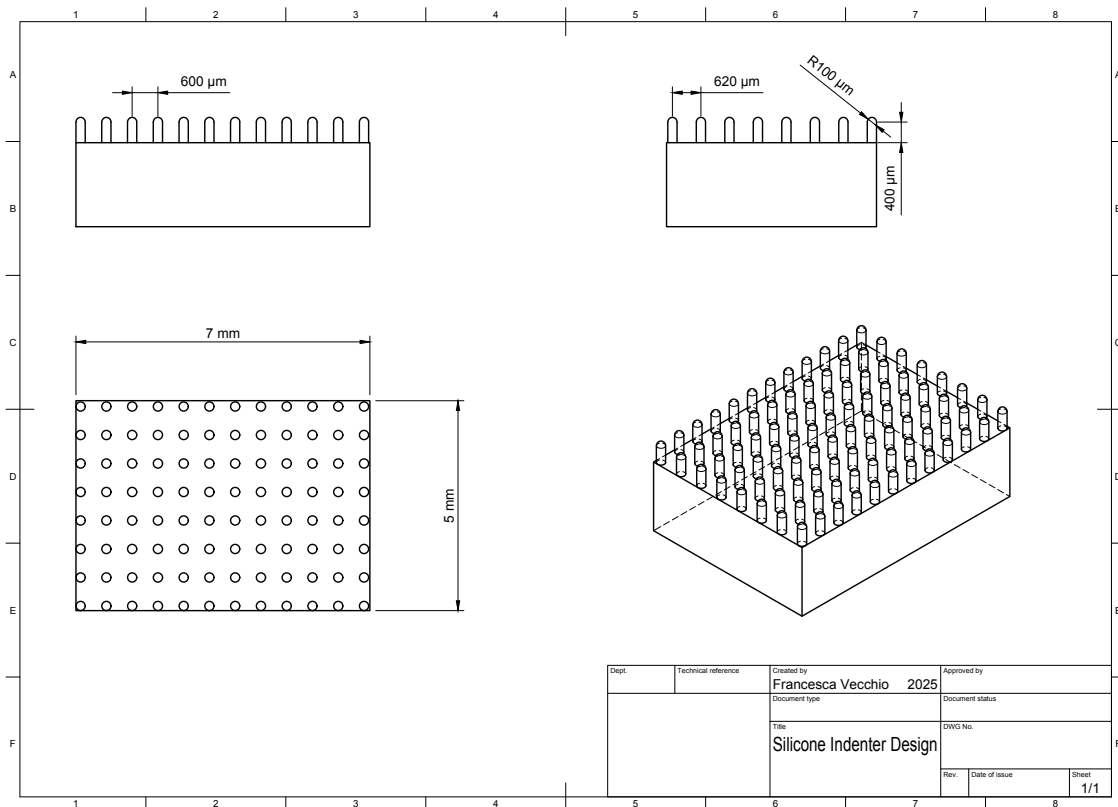
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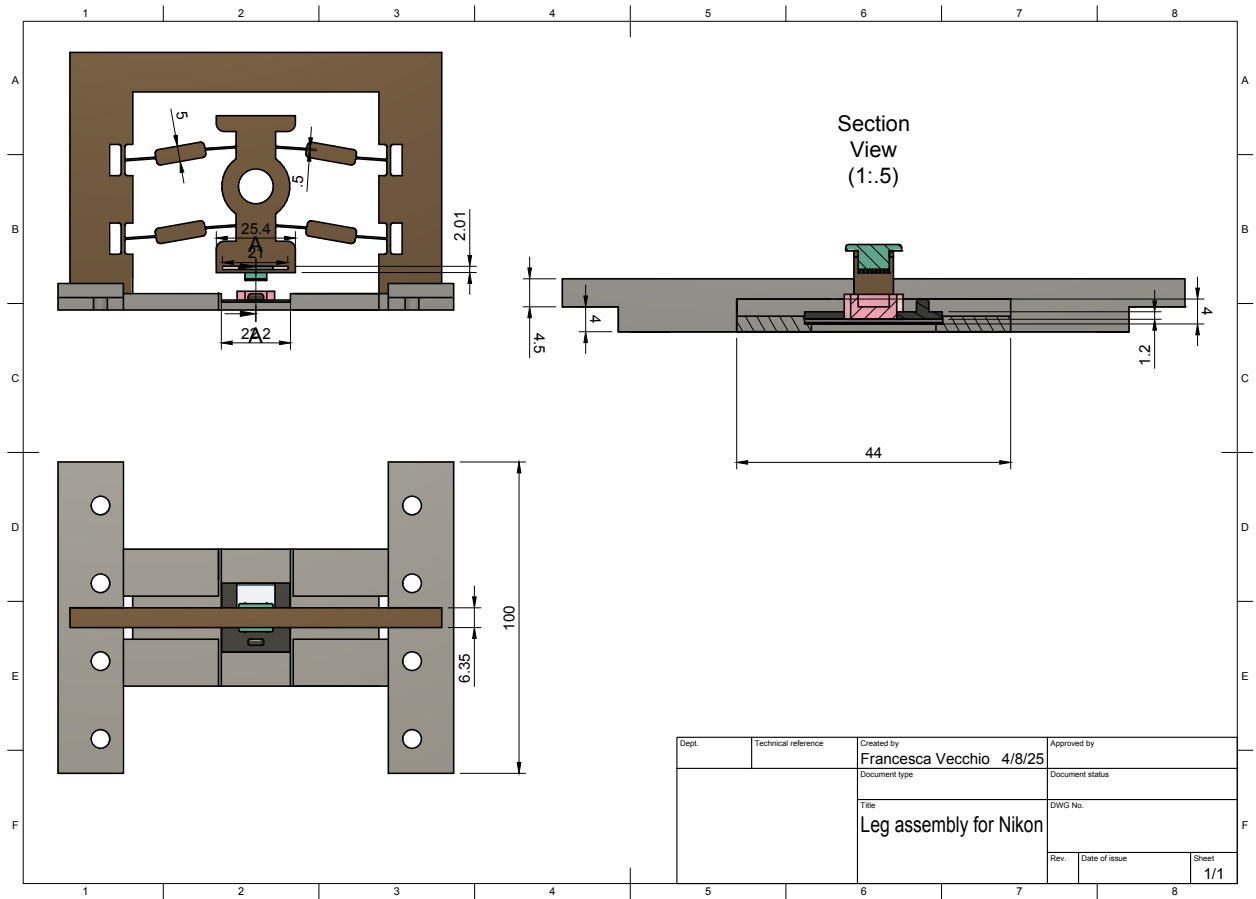
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Appendix

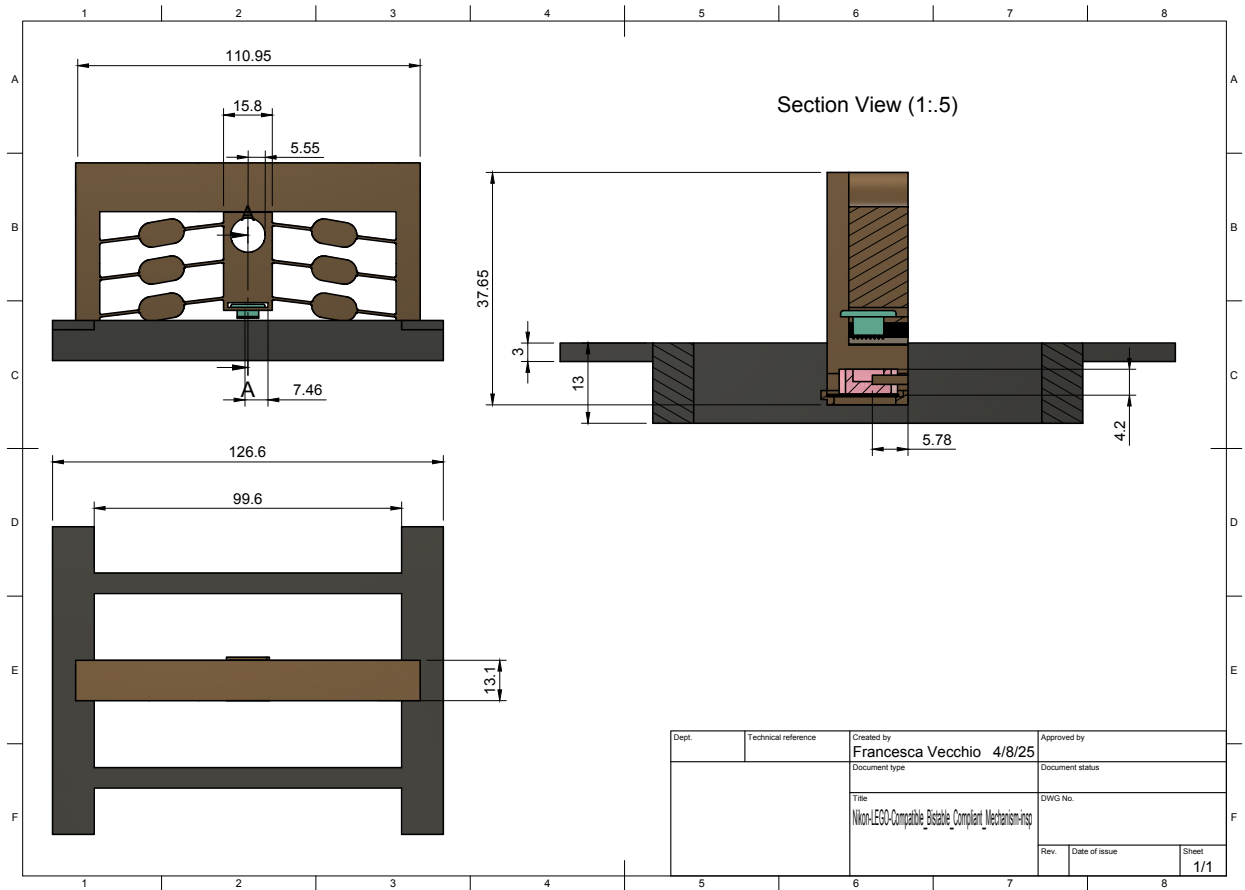
The STL files for the optimized BiCM1 and BiCM2 can be found in the lab's Google Drive [here](#). General CAD drawings developed in Fusion360 can be seen in **Supplementary Figure 1, Supplementary Figure 2, and Supplementary Figure 3.**



Supplementary Figure 1: Silicone indenter drawing with notable dimensions. 600 μm pegs are shown, but silicone indenters with 650 μm and 800 μm pegs were also generated. Created in Fusion360.



Supplementary Figure 2: Optimized BiCM1 assembly drawing generated in Fusion360. Top left panel shows a front view; bottom left panel shows a top view; right panel shows a section view through the side of the assembly.



Supplementary Figure 3: Optimized BiCM2 assembly drawing generated in Fusion360. Top left panel shows a front view; bottom left panel shows a top view; right panel shows a section view through the side of the assembly.