# Simulating traumatic brain injury *in vitro*: developing high throughput models to test biomaterial based therapies

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### *From the Contents*



## Abstract

Traumatic brain injuries are serious clinical incidents associated with some of the poorest outcomes in neurological practice. Coupled with the limited regenerative capacity of the brain, this has significant implications for patients, carers, and healthcare systems, and the requirement for life-long care in some cases. Clinical treatment currently focuses on limiting the initial neural damage with long-term care/support from multidisciplinary teams. Therapies targeting neuroprotection and neural regeneration are not currently available but are the focus of intensive research. Biomaterial-based interventions are gaining popularity for a range of applications including biomolecule and drug delivery, and to function as cellular scaffolds. Experimental investigations into the development of such novel therapeutics for traumatic brain injury will be critically underpinned by the availability of appropriate high throughput, facile, ethically viable, and pathomimetic biological model systems. This represents a significant challenge for researchers given the pathological complexity of traumatic brain injury. Specifically, there is a concerted post-injury response mounted by multiple neural cell types which includes microglial activation and astroglial scarring with the expression of a range of growth inhibitory molecules and cytokines in the lesion environment. Here, we review common models used for the study of traumatic brain injury (ranging from live animal models to *in vitro* systems), focusing on penetrating traumatic brain injury models. We discuss their relative advantages and drawbacks for the developmental testing of biomaterial-based therapies.

**Key Words:** astroglial scar; biomaterial; cortical culture; *in vitro* model; microglial infiltration; multicellular model; penetrating injury; scaffold; traumatic brain injury

## Introduction

The total global annual burden of traumatic brain injuries (TBIs) is an estimated US $400 billion (van Dijick et al., 2019). Such injuries can arise from blunt (closed) or penetrating trauma (open/pTBI). These are prevalent in civilian/military personnel in areas of a high incidence of terrorism/violence and are associated with the worst clinical outcome in head injury cases. An injury track created by a foreign body (e.g. fragments or gunshot rounds) causes cavitation, shearing, and compression of nerve fibers and blood vessels, with damage to neurons and glia including myelin damage (Oehmichen et al., 2001). There is focal and diffuse neuronal apoptosis/necrosis, during the primary and secondary injury phases, and cellular debris leads to a high concentration of damage-associated molecular patterns. Microglial infiltration of lesions and release of pro-inflammatory cytokines such as interferon-γ and tumor necrosis factor-α drive acute inflammation (Lively et al., 2018). Oligodendrocyte precursor cells and fibroblasts infiltrate the lesion and proliferate with early (within 24 hours) astrocyte activation. The latter extend palisading processes into the lesion sealing the lesion core which contains cellular debris and molecules inhibitory to neurite outgrowth (such as myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein; Filbin et al., 2003). The subsequent failure of regeneration, chronic inflammation, and atrophy are suggested to underpin the poor clinical outcomes post-pTBI. Treatments have been refined over the years and include early debridement with or without craniotomy and supportive therapies such as anti-seizure medications, antibiotics, and intracranial pressure monitoring (Vakil et al., 2017). Such injuries have significant contamination (approximately 43% infection risk) so early broad-spectrum antibiotics use is key. Cerebrospinal fluid leaks are also encountered increasing contamination risk and requiring dural repair. Long-term management focuses on neurorehabilitation requiring multidisciplinary input including teams from neurosurgery, neurology, physiotherapy, speech, and language therapy, in addition to input from allied specialties depending on the systemic manifestation of clinical injury. Current clinical interventions are therefore supportive and truly regenerative/neuroprotective therapies post-injury do not exist, remaining a key goal for research in regenerative neurology.

In this context, the use of implantable biomaterials as therapeutic scaffolds to promote repair has been a major recent advance in regenerative medicine. Such matrices can be prepared from many different biomolecules, including numerous proteins/polysaccharides. These are highly versatile for neurological injuries, given their extracellular matrix-like structures, high porosity, and ease of fluid/nutrient movement, supporting the 3-D growth of cells including axon/blood vessel ingrowth from 'host' tissue into implants (Weightman et al., 2014). They offer benefits as drug/cell delivery devices enabling local, controlled release of a therapeutic and, significantly, the ability to modify the post-injury extracellular microenvironment. For example, there is evidence that biomaterials *in situ* reduce the expression of mRNA for inflammatory- and glial cell scarring-related genes in injury sites, limit immune cell infiltration, attenuate glial scarring, and reduce cystic cavitation post-injury (Krings et al., 2016; Basit et al., 2021). They have tissue-mimetic mechanical properties, relatively rapid biodegradability (within about 3 months allowing for gradual replacement with nascent tissue), and mouldability for surgical delivery (Chen et al., 2019). Using a pTBI model, Hou et al. (2005) found neurite outgrowth and angiogenesis into hyaluronic acid hydrogels modified with laminin, which had been implanted into the cortices of Sprague-Dawley rats, with decreased glial fibrillary acidic protein upregulation in areas of biomaterial contact. Similarly, Chen et al. (2019) demonstrated reduced infiltration of activated microglia in a collagen-glycosaminoglycan matrix hydrogel implanted into a surgical rat brain injury, with a modified inflammatory signaling environment (interleukin-6, tumor necrosis factor-α, and interleukin-10 expression).

Such research is encouraging and requires suitable preclinical experimental models to identify and test the most promising interventions for future medicine. Given the pathological complexity of pTBI, this does present a highly challenging goal. An appropriate model, in our view, should offer the following features: wide availability across experimental facilities; facile methods to facilitate researcher training, inexpensive, high throughput nature to test multiple experimental conditions simultaneously, patho-mimicry to accurately simulate *in vivo* pTBI; compatibility with a range of microscopic methods (including, light, fluorescence, time-lapse and electron microscopy);

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and applicability to a wide range of species including genetically modified animals. Importantly, any model should provide an ethically viable approach in line with the 3 R's (Reduction, Replacement, and Refinement) of animal experimentation. Here, we review the literature on experimental models of pTBI and their utility for the study of biomaterial based therapeutics.

## Search Strategy and Selection Criteria

All years were chosen in the search. These searches were performed between June and December 2021 using the PubMed and Web of Science databases. Broad search terms such as traumatic brain injury, penetrating traumatic brain injury, *in vitro* models, neurological injury, 3D models, biomaterials, organoids, organotypic, and neuronal culture were used in various combinations.

## Models of Penetrating Traumatic Brain Injury Vary in Complexity

A variety of pTBI models have been deployed in experimental neurology. Large animal models typically involve researchers introducing penetrating brain lesions through gunshot/stab wounds in anesthetized sheep or monkeys (Finnie et al., 1993), with the evaluation of gross pTBI pathology. However, the considerable ethical implications and rarity in procuring such animals within common research facilities have resulted in these models largely falling out of favor. Small animal rodent models deemed 'less sentient' have been developed (such as a penetrating ballistic-like brain injury -rifle pellet injury) (Plantman et al., 2015). These are relatively inexpensive (versus live animal models), widely available in research facilities and offer ease of handling and standardized neurological/behavioral tests to evaluate the regenerative benefits of any therapy. However, all live animal models of neurological injury require extensive training and monitoring, given that these are some of the most invasive models in experimental research. As such, there is a need for stringent regulation of such work, often requiring experimental licenses and specialist infrastructure and staff for the housing and care of animals. These models are inherently lower throughput, and technically challenging compared to *in vitro* alternatives. The significant ethical implications surrounding live animal use in medical research also continue to be a matter of extensive public and scientific debate.

*In vitro* organotypic (organ-like) brain slices provide a 3D alternative. These offer the controllable environment of *in vitro* preparations providing an 'interface' between high throughput screening and pre-clinical animal models. Neural cytoarchitecture, cell inter-relationships, a vascular network, and the extracellular matrix are maintained in such tissue, allowing for neural plasticity, cell migration, and axonal regeneration to be easily examined. Rodents, including transgenic models and higher species (rabbits, pigs, dogs, and humans) can be used as donor sources, and the application of advanced microscopy, electrophysiology, molecular and genomic methods to these models has greatly expanded their practical utility, including for the study of TBI. Our lab previously developed an *in vitro* spinal cord organotypic slice culture model with a penetrating (transecting) injury (Weightman et al., 2014). We showed that the model replicates stereotypical pathological responses seen after neurological injury *in vivo*, namely: (a) reactive gliosis with astrocytes forming a scar, a major barrier to nerve fiber regeneration; (b) gradual infiltration of lesions by microglia; and (c) decreasing nerve fiber outgrowth with increasing donor tissue age, in conjunction with glial scarring and reactive microgliosis in lesions. Whilst offering significant patho-mimicry, such models are moderate throughput at best, and can be technically challenging to establish and maintain.

The most basic *in vitro* models use immortalized cell lines, which are robust, inexpensive, and widely available but are often resistant to cell death and prone to cryptic contamination. These offer major advantages in terms of a more facile and controlled yet high throughput approach, for multiple experimental manipulations or measurements. Systems offering greater complexity have been developed from dissociated cortices which typically take the form of monolayer cultures. For example, an astrocyte scratch wound model (using primary cultures of astrocytes) has been used to simulate traumatic neural injury with glial fibrillary acidic protein upregulation, hypertrophy of reactive astrocytes, and injury triggered calcium waves observed in the injury foci (Gao et al., 2013). In general, such models are high-throughput, facile, inexpensive, and widely available and contribute significantly to the reduction and refinement of animal experimentation, with far less ethical implications than *in vivo* systems. However, the drive for reductionism has often meant these models are overly simplistic, not containing the major neural cell types (notably the immune cells) to simulate the complex pathological responses observed in neural pathology. Therefore advanced *in vitro* systems are still urgently needed. **Table 1** shows a comparison of *in vitro* neural cell models potentially adaptable to injury mechanisms.

## Developing a New Multicellular Model of Penetrating Traumatic Brain Injury *In Vitro*

We recently developed a reliable, technically simple, and high throughput pTBI model, using rodent cortices, to evaluate the effects of implantation of biomaterial scaffolds on neural cells (unpublished data). The model is a variant of a widely used mixed glial culture system and contains the two major immunocompetent neural cell types in the central nervous system- namely the astrocytes and microglia in addition to oligodendrocyte precursor cells (Basit et al., 2021). We found evidence of both microglial invasion of lesions and reactive astrogliosis in peri-lesional astrocytes (hypertrophic palisading astrocytes at the lesion edge) with glial scarring responses and glial fibrillary acidic protein upregulation at the lesion edge. Implantation of a surgical grade matrix Duragen Plus™ into the lesion resulted in microglial and astrocytic infiltration into the biomaterial. Therefore, the model broadly replicates *in vivo* features of TBI pathology. It also highlights the need for experimental models that can predict the responses of major neuroglial phenotypes to introduced biomaterials. This is of high importance given the critical role these cells play in determining the intra-central nervous system fate of therapeutic scaffold materials. Astrocytes, for example, can dramatically remodel biomaterials, and fibrillar contraction in these cells alters their biomechanical properties. Microglia- the intrinsic immune cells of the central nervous system, have roles in biomaterial clearance and digestion which impacts their biodegradability and potential toxicity of the materials. This in turn exerts a critical impact on the pro-regenerative properties of the implant. Accordingly, we consider the model can be used to investigate materials varying in their chemistry, stiffness, and porosity to identify those with the most promise prior to preclinical testing. A major limitation of the model is the lack of a neuronal component, meaning axonal outgrowth– a key aspect of neuroregeneration, cannot be assessed. To address this, we have recently developed an advanced version of the multi-glial model using a simple chemical switch to maintain the neural cells *in vitro* (unpublished data; **Figure 1**). Briefly, cortices from neonatal rodents were extracted and enzymatically dissociated, using a specialized chemical medium found to support the growth of both neurons and all major glial subpopulations, including the microglia. In terms of the cellular constitution, the culture consists of approximately 50% neurons with glial populations making up the remaining cells. 70% of the neurons were found to be gamma-aminobutyric acid (GABA) positive; the remaining neurons were not found to be glutamatergic, in our hands and their identity, remains to be established (**Figure 2**).

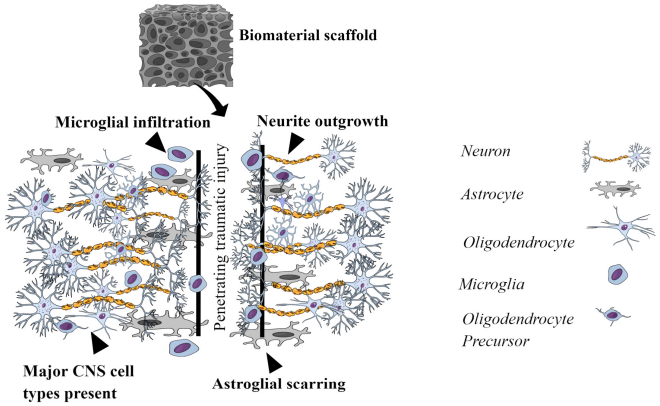


**Figure 1 | The schematic depicts cellular responses of multiple neural cell types to a penetrating lesion *in vitro*, in the experimental model developed by our group.**
Here, microglia infiltrate the intralesional space, with axonal outgrowth and hypertrophic palisading astrocytic processes in the lesion core. Biomaterial scaffolds (and other materials) can be introduced into the lesion gap to evaluate their pro-regenerative properties. CNS: Central neural system.

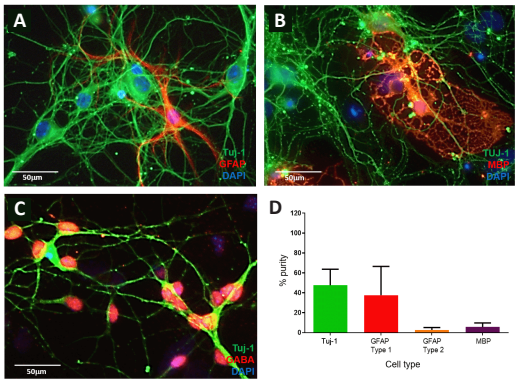


**Figure 2 | Characterization of the multicellular cortical model.**
Representative fluorescent micrographs showing the cellular constitution of the new mixed cortical culture containing neurons (Tuj-1) and astrocytes (GFAP) (A); and neurons (Tuj-1) and oligodendrocytes (MBP) (B). Staining of constituent neurons with GABA is shown in (C). Nuclei shown stained with DAPI. Graphical representation of the relative cell proportions within the culture shown in (D). DAPI: 4′,6-Diamidino-2-phenylindole; GABA: gamma-aminobutyric acid; GFAP: glial fibrillary acidic protein; MBP: myelin basic protein. Unpublished data.

**Table 1** | **Possible *in vitro* systems for modelling brain tissue, traumatic injury mechanisms and biomaterial interventions – advantages and disadvantages, arranged from highest complexity to least complexity**

| *In vitro* models | Description | Advantages | Disadvantages | References |
|---|---|---|---|---|
| **3D 'organotypic' slices** | *Ex vivo* brain tissue slices | -Retain *in vivo* cytoarchitecture<br>-Retain major brain cell types (including microglia)<br>-Ease of mechanical manipulation<br>-Moderate difficulty to induce mechanical injury<br>-Can be interfaced with biomaterials<br>-Display complex injury responses<br>-Adaptable for excitotoxicity/hypoxia studies | -Moderate throughput<br>-Can be technically difficult to isolate and maintain slices<br>-Requires more animals versus other *in vitro* models | Morrison et al., 2000; Di Pietro et al., 2012; Bar-Kochba et al., 2016; Krings et al., 2016; Campos-Pires et al., 2018; Ucar et al., 2021 |
| **3D organoids** | Stem-cell derived self-organising suspension cultures forming brain-like spheroids (iPSC origin) | -Cytoarchitecture recapitulates developing tissues<br>-Can be human/patient specific<br>-Closely simulate *in vivo* cellular communication<br>-Adaptable for excitotoxicity studies<br>-Ideal for disease and development studies | -Moderate throughput<br>-Little uniformity between aggregates<br>-Largely immature in cellular development<br>-Long culture periods<br>-Few injury systems reported<br>-Lack vascular and immune components<br>-Problematic for mechanical manipulation due to free floating nature<br>-Spheroid centres can become hypoxic due to lack of nutrient access<br>-Difficult cellular analysis<br>-Complicated for biomaterial interfacing | Birey et al., 2017; Ogura et al., 2018; Jgamadze et al., 2020; Ramirez et al., 2021 |
| **Brain-on-a-chip** | Microfluidic culture systems of 3D iPSC derived cultures | -Tissue-like physiology<br>-Perfusion system of 3D tissue<br>-Adaptable for disease/toxicity mechanisms<br>-Axonal strain injury attempted | -Low throughput<br>-Scalability limitations<br>-Lack immune and vascular components<br>-Lack cellular maturity<br>-Difficulty with mechanical injury induction<br>-Difficulty with biomaterial interfacing | Dolle et al., 2014; Bang et al., 2019 |
| **3D hydrogel constructs** | Cells encapsulated within a 3D matrix | -3D architecture resembling tissue-like environment<br>-Physiologically relevant cellular morphology<br>-Simple maintenance<br>-Moderate to high-throughput<br>-Moderate technical difficulty for injury mechanisms<br>-Biomaterial interfacing feasible | -Difficult analysis of 3D environment<br>-Can lack complexity if not multicellular constructs i.e., lack immune component if cells are NSC derived<br>-Not currently documented with all the major cell types of primary brain cell dissociates<br>-Lack vascular component (but feasible with tissue engineered blood vessels) | Haycock, 2010; Antoni et al., 2015; Raimondi et al., 2020 |
| **2D primary multicellular models** | Complex multicellular cultures of brain dissociates | -Can encompass major brain cell types (including microglia and neurons)<br>-Simple injury mechanisms<br>-High throughput<br>-Low technical difficulty<br>-Simple maintenance and analysis<br>-Biomaterial interfacing feasible | -2D environment (*Cells undergo artificial responses to adapt to the flat, stiff surface of 2D cultures systems)<br>-Lack vascular component | Kumaria, 2017; Goshi et al., 2020; Basit et al., 2021 |
| **Primary neural stem cell cultures** | Cultures of differentiated stem cells isolated from neurogenic regions e.g. subventricular zone (SVZ) | -High throughput<br>-Low technical difficulty<br>-Multicellular cultures<br>-Simple injury manipulation<br>-Biomaterial interfacing feasible | -Lack immune component<br>-Moderate length differentiation protocols<br>-2D environment *<br>-Preferential differentiation to astrocytes<br>-Lack immune and vascular components | Goa et al., 2013; Barbora et al., 2020; Vagaska et al., 2020; Mogas et al., 2021 |
| **Induced pluripotent stem cells (iPSCs)** | Stem cells genetically reprogrammed from adult cells | -Indefinite propagation<br>-Can be of human origin<br>-Patient specific (retain genetic identity)<br>-Low technical difficulty<br>-Beneficial for patient specific disease modelling<br>-Biomaterial interfacing feasible | -Moderate throughput (long differentiation protocols)<br>-Differ genetically/phenotypically from endogenous counterparts – altered morphology<br>-Heterogeneity of cells<br>-Resistant to cell death<br>-Risk of mycoplasma contamination<br>-2D environment *<br>-Biomaterial-injury interface not reported<br>-Lack immune and vascular components | Ulrich et al., 2001; Kang et al., 2017; Pistollato et al., 2017; Tukker et al., 2018 |
| **2D primary pure cell cultures** | Primary cultures from brain dissociates; purified through sequential shaking or specific media components | -High throughput<br>-Low technical difficulty<br>-Useful to study specific cell responses<br>-Simple injury mechanisms<br>-Biomaterial interfacing feasible | -Overly simplistic model of the brain<br>-2D environment *<br>-Absence of multicellular interactions<br>-Lack vascular and immune component (if not 'pure' microglial cultures) | Geddes et al, 2003; Chen et al., 2007; Vellis and Cole, 2011 |
| **Cell lines: Pure cells, NSCs/ESCs (neural/ embryonic stem cells)** | Immortalised cell lines | -Indefinite propagation<br>-High throughput<br>-Facile<br>-Can be of human origin<br>-Biomaterial interfacing feasible<br>-Simple injury mechanisms | -Genetically and phenotypically differ from endogenous counterparts<br>-High risk of mycoplasma contamination<br>-Cellular heterogeneity<br>-Resistant to cell death<br>-2D environment *<br>-Lack immune and vascular components | Gordon et al., 2013; Carter and Shieh, 2015; Tapia and Scholar, 2016 |

*: Cells undergo artificial responses to adapt to the flat, stiff surface of 2D cultures systems. Mechanical injury includes stretch, weight drop and penetrating injuries.

The model is pathomimetic, inexpensive, high throughput and contains all of the central nervous system cell types. Penetrating lesions can easily be induced within the culture system, and potential therapeutic interventions such as biomaterial implantation or nanoparticle delivery assessed. There are very few reports that focus on the response of all major neural cell types simultaneously and thereby overlook the multi-dimensional cellular response, despite the known importance of all glial and neuronal cell functions in neuroinflammation, therapeutic assessment, and regeneration. Our model offers significant benefits in this regard. All the cell types were homogeneously distributed throughout the cultures allowing sufficient evaluation of the multiple cell responses to injury. The potential to study the therapeutic impact on all major neural cell types simultaneously has significant benefits when considering a high throughput, facile brain tissue model. For example, Goshi et al. (2020) reported that astrocytes and neurons respond differently to neuroinflammatory stimulators and neurotrauma in the presence of microglia. This reinforces the importance of the immune component in mixed neural cultures, and in turn the significance of the micro-dynamics between all neural cell types. Accordingly, we consider our new model offers a versatile adaptable platform for the study of a range of TBI mechanisms, therapeutics, and drug testing within the neuro-regenerative field, using a simple but scalable system. This robust brain tissue-modeling platform would be adaptable to multiple injury mechanisms including weight

drop contusions, glutamate-induced excitotoxicity, lipopolysaccharide stimulated neuroinflammation and hypoxia, to understand concurrently the response of all brain cell types.

Interestingly, we have found that the model can be adapted into a 3D format using cell seeding into a hydrogel matrix, thereby expanding its utility to study more complex pTBI, such as pellet simulating ballistic injuries or crush injuries through weight drop (unpublished data). Further refinements in the future could include the addition of endothelial cells to simulate the blood-brain barrier, and the inclusion of peripheral immune cells, to enhance the pathomimetic potential of the approach. A more detailed assessment of the lesion pathology is also needed, for example, the use of proteomic methods to study the spatial and temporal molecular expression profiles in the injuries (for comparison to *in vivo* data), or an assessment of the extent of myelination/demyelination in the injuries, including use of high-resolution electron microscopy. Whilst such pTBI models can never outright replace preclinical testing, we believe they are of high value for the developmental testing and identification of promising biomaterial scaffold-based therapeutic interventions. Their versatility also allows them to be deployed to test other promising neuro therapies such as novel nano-pharmaceuticals and glial scar attenuation/immunomodulatory treatments.

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