Title page

Chapter 12

**Developing high-fidelity in vitro models of traumatic brain injury to test therapeutic biomaterials**

Christopher Adams, Bushra Kabiri, Raja Haseeb Basit, Jessica Wiseman and Divya Maitreyi Chari

Affiliations

C. Adams

Neural Tissue Engineering Group, School of Life Sciences, Keele University, Staffordshire, UK  
c.adams@keele.ac.uk

R. H. Basit

Bradford Royal infirmary, Bradford, West Yorkshire, UK  
haseebbasit.hb@gmail.com

J. Wiseman

Sheffield Institute for Translational Neuroscience, University of Sheffield, Sheffield, UK  
jessica.wiseman@sheffield.ac.uk

B. Kabiri, D. Chari

Neural Tissue Engineering Group, School of Medicine, Keele University, Staffordshire, UK  
BK - b.kabiri@keele.ac.uk; DC – d.chari@keele.ac.uk

**Abstract**  
**Introduction**  
Traumatic brain injury (TBI) can cause significant disability and is a leading cause of death amongst under 40s. There is a clinical need to develop new and effective therapies for TBI but this represents a significant challenge given the complexity of the injury and pathological barriers to regeneration. Development of combinatorial therapeutic biomaterials, capable of achieving multiple clinical goals such as promoting nerve growth, disrupting scarring tissue and modulating neuroinflammation holds promise to augment repair. Fine tuning material properties during developmental screening to optimise efficacy requires complex and pathology mimetic models of TBI. Animal models are not appropriate for initial optimisation given their expense, requirement for infrastructure and expertise and associated ethical hurdles, requiring appropriate alternatives to simulate TBI. Our goal in this chapter is to review the current state of the art in modelling TBI using in vitro model systems which replicate complex pathological processes of TBI, can be interfaced with biomaterials, provide detailed histological and functional readouts, whilst offering ease of use in the laboratory.

**Methods**  
Searches of literature in PubMed and Web of Science were conducted using combinations of “traumatic brain injury”, “neurological injury”, “in vitro model”, “3D model”, “stem cells”, “organoids”, “organotypic”, “biomaterials”, “nanomaterials” and “tissue engineering”. Primary research articles, literature reviews and book chapters were used to inform the writing of the article.

**Results**  
Many in vitro models of TBI exist, ranging in complexity from monocellular cultures to 3D self-assembling organoids and 3D organotypic slices. Each system has advantages and disadvantages for testing therapeutic biomaterials in TBI. The primary drawbacks of the most common models are lack of complex modelling of pathology (especially the neuroimmune component), limited capacity for biomaterial interfacing and limited functional readouts. However, we identify promising in vitro models and new strategies to address these challenges.

**Conclusions**  
Novel advances in in vitro TBI modelling can allow for a translational pipeline of therapeutic biomaterial development in pathology simulating, yet facile benchtop systems.

**Keywords**  
traumatic brain injury, neurological injury, in vitro model, 3D model, stem cells, organoids, organotypic, biomaterials, nanomaterials, tissue engineering

**12.1 Introduction**

Clinical biomaterials are of high interest to regenerative neurology as they have the potential to modulate multiple pathological processes in sites of neurological injury. For example, biomaterials can integrate into sites of injury, provide topographical guidance for regenerating tissue, modulate pathological responses and allow controlled and targeted delivery of therapeutic factors. Accordingly, they are under investigation in multiple neurological diseases and injuries (Führmann et al., 2017; Martinez and Peplow, 2022). Penetrating traumatic brain injury (pTBI) is of particular interest given the clinical severity of the injury, the need for combinatorial treatments and the generation of a cystic cavity within which therapeutic biomaterials can be implanted. Several novel biomaterials are currently in development to treat pTBI (Hu et al., 2023; Tan et al., 2020). Animal models have long been the gold standard for testing new TBI therapies. However, these models are not suitable for exploratory testing and development of biomaterials due to ethical issues, expense and requirement for extensive technical training, specialist infrastructure and staff. There has been a global drive in the past decade to develop high fidelity in vitro models of pTBI within which biomaterial properties can be optimised. In this chapter, we will explore pTBI pathology, highlight new therapeutic biomaterial development, outline the need and ideal parameters for an in vitro test bed and discuss some of the recent advances in this field.

**12.1.1 pTBI has a profound socioeconomic effect**

pTBI is defined as head injury that occurs when a foreign object traversing the skull causes direct parenchymal injury. It has the worst prognostic outcomes of all TBIs and is the commonest cause of death in under 40s in the UK population as of 2015 (Davis et al., 2015). Typical mechanisms of injury include road traffic accidents, sports related injuries, abuse/assault cases and, less commonly in the UK, gun-shot injuries (Lawrence et al., 2016). Generally, patients die before reaching a hospital with initial mortality rates of up to 70% reported (Günther et al., 2021; Gennarelli et al., 1989; Okidi et al., 2020). The morbidity of patients that survive but typically do not recover fully to pre-injury functionality represents a significant socioeconomic burden to society. For example, TBI is estimated to cost the UK around £15 billion per year, which includes providing treatment and ongoing care and the subsequent loss of earnings of patients (Parsonage, 2016). There are also substantial social impacts on the patients and their support networks, with head injuries doubling the risk of developing mental health problems compared to the rest of the population (Parsonage, 2016). Mainstay clinical management focusses on supportive measures, and occasionally decompressive surgery that aims to preserve life, but does little to mitigate the long-term clinical sequelae of severe injury. **Currently, with the brain’s limited internal capacity for repair and lack of approved targeted neuroregenerative therapy administered in early injury, there is a great clinical urgency for more research in this area.** A major challenge for experimental research is to simulate the complex pathophysiology and refractory nature of the injury.

**12.1.2 The pathophysiology of pTBI is complex with many barriers to regeneration**

Description of pTBI pathology is commonly split into primary and secondary injury processes. Primary injury occurs within the first few seconds to minutes of injury and is attributed to the direct impact of the object entering the brain (Ng & Lee, 2019). This can be described anatomically, depending on the characteristics, velocity and pathway of a foreign object travelling through the brain. Penetration results in shearing, rotation, compression, lacerations and ricocheting (typically in gunshot wounds) forces. This causes haemorrhage and haematomas, contusions, vascular injuries, disruption of the blood brain barrier (BBB) and focal or diffuse axonal injury (Vakil & Singh, 2017). Axonal shearing usually results in permanent loss of the associated neuronal circuity and focal neurological deficits. The impact can also cause crush injury and necrosis of cells at the lesion edge (Santiago et al., 2012). High velocity injuries will also trigger wave forces that cyclically stretch and compress the surrounding tissue, forming cavitations and oedema. The irreversible nature of primary injury means prevention is key, through public health education and safety training to prevent common mechanisms of injury (falls, road traffic accidents, gunshot wounds etc.) (Lawrence et al., 2016). Secondary injury is described as the expanded, longer-term effects of the initial injury. It is the result of complex inflammatory cascades, excitotoxicity, mitochondrial dysfunction and oxidative stress which leads to chronic pathology.

Excitotoxicity is the accumulation of high levels of neurotransmitters causing cytotoxic overactivation of their associated receptors, usually due to high glutamate levels (Baracaldo-Santamaría et al., 2022), but also high aspartate (Cho et al., 2003) and gamma-aminobutyric acid (GABA) (Choi et al., 1989; Ogata et al., 1996; Tehse & Taghibiglou, 2019). This eventually results in loss of neuronal membrane integrity, necrosis, oedema and BBB damage (Ankarcrona et al., 1995; Xhima et al., 2016). Excitotoxicity triggers rises in intracellular calcium through many mechanisms (Faden et al., 1989; Baker et al., 1993; Chamoun et al., 2010), and affects both neurons and glia (Floyd et al., 2005). N-methyl D-aspartate receptors (NMDARs), an ionic glutamate receptor, are the primary glutamate receptors to mediate intracellular influx of calcium (Wang & Gao, 2010; Guo & Ma, 2021) and explains the beneficial effects of NMDAR antagonists, including MK-801 (Phillips et al., 1998; Cho et al., 2003), memantine (Xia et al., 2010) and ketamine (Wang et al., 2017) for treating excitotoxicity associated injuries. Once an intracellular rise in calcium occurs, catabolic intracellular cascades mediated by lipid peroxidases, proteases and phospholipases, result in early neuronal swelling, gradual neuronal disintegration and eventually irreversible neuronal injury (Choi, 1987). Mitochondrial dysfunction is one of the main pathways leading to cell death and is triggered by intracellular rises in calcium, as mitochondria harbour many Ca2+-sensitive enzymes triggered in excitotoxicity (Graier et al., 2007). Once this occurs, apoptosis, necrosis, and necroptosis are triggered through caspase-dependent and independent pathways (Galluzzi & Kroemer, 2008; Cheng et al., 2012).

Oxidative stress is another primary pathophysiological mechanism of brain injury (Fesharaki-Zadeh, 2022). The effect of TBI on cerebral circulation by either directly tearing arteries, via primary injury, or through inflammatory changes in the microvascular environment, via secondary injury cascades, results in hypoxia and oxidative stress. Reperfusion injury, defined as the paradoxical rise in cellular injury after blood circulation is restored, also further increases free radical and reactive oxygen species (ROS) production. Free oxygen, nitrite and superoxide radicals (e.g., the formation of peroxynitrite from nitric oxide) further impair cerebrovascular function (DeWitt & Prough, 2009; Rodríguez-Rodríguez et al., 2014). Together, they collectively tip the oxidant-antioxidant balance and further exacerbate the pro-inflammatory state.

At a cellular level, the surrounding chemotactic debris (Liesz et al., 2015), pro-inflammatory cascades and cytokines trigger the mobilisation of immunocompetent cells, microglia, astrocytes and peripheral immune cells (Semple et al., 2010) to the lesion site (Shi et al., 2019). Astrogliosis, the process of astrocytes and microglia forming a glial scar, begins as astrocytes ‘palisading’ into the injury and extending long, fibrillary processes to bridge the injury site (Burda et al., 2016; Zhou et al., 2020). The release of myelin fragments exposes inhibitory molecules such as neurite outgrowth inhibitors (NOGO) which have been shown to mitigate axonal repair and neuroregeneration at the injury site in the long-term (Bovolenta et al., 1993; Karnezis et al., 2004; Granpré et al., 2000). Microglia adopt hyperreactive, amoeboid morphologies and trigger a positive feedback cycle exacerbating the anti-repair and pro-inflammatory state (Donat et al., 2017; DiSabato et al., 2016). Collectively, this translates clinically to the initial parenchymal oedema, cerebral vasospasm and raised intracranial pressures causing high initial mortality levels.

In the longer-term, astrogliosis, triggered in early injury, results in glial scar formation, made up of hypertrophic astrocytes, hyperreactive microglia and oligodendrocyte precursor cells (OPCs) (Adams & Gallo, 2018) that limits axonal regeneration across the injury site - a critical barrier to neurorepair. Once inflammation triggers astrogliosis and long-term glial scarring, targeted neurotherapy options become of limited efficacy (Windle et al., 1952; Silver & Miller, 2004). Hence modulating the early anti-repair, pro-inflammatory response in a time-specific window after injury is critical to reduce long-term functional disability.

**12.1.3 Biomaterials as a pro-regenerative therapy for pTBI**

The complex pathophysiology of pTBI necessitates multifaceted and combinatorial therapies; combinations of biomaterials, nanoparticles and stem cell transplantation show significant promise. Several biomaterials have been proposed as suitable therapeutic candidates and are both naturally and synthetically derived. Hydrogels, 3D polymeric scaffold materials, polymers and nanomaterials are the main categories of biomaterials that are commonly tested in neural tissue and will be explored here. Many studies also commonly use different biomaterials in combination to create ‘hybrid materials’, such as combining naturally and synthetically derived scaffolds in hydrogel form, or functionalising nanomaterials using naturally derived polymers (Table 12.1).

**Table 12.1 Table summarising various types of biomaterials and their application in repairing pTBI.**

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Biomaterial** | | **Description** | | **Advantages** | | **Disadvantages** | | **References** | |
| *Naturally derived 3D scaffold biomaterials* | | | | | | | | | |
| Collagen (*rodent, marine and bovine based*) | | Key naturally occurring structural protein  Usually type 1 fibrillary collagen forming major component of connective tissue  Can be used as 2D, 3D scaffold or hydrogel  Frequently combined with other polymers (e.g., Collagen-PGA tubes, collagen-laminin bio-copolymers) | | *All collagen types* -Abundant -Easily sourced  -Extensively researched  -Non-toxic, low immunogenicity -Degradation products non-toxic -Biomodulatory effects (natural cell binding sites, guides natural neural growth and proliferation)  -Can be magnetically aligned for directed axonal growth  *Marine -*Low antigenicity  -High levels of cellular adhesion -High biodegradability -Theoretical decreased risk of cross-species disease transmission (zoonosis)  *Bovine* -Some products (e.g., DuraGenTM) FDA approved for clinical testing and use in neural tissue  -Clinically already widely used as dural graft | | -Low mechanical and tensile strength  -Batch to batch variability  -Risk of immune reactivity (cross species implantation)  -Sterilisation decreases integrity and/or biomodulatory effects | | (Kim, et al., 2015)  (Suri & Schmidt, 2010)  (Jang, et al., 2015)  (Iwashita, et al., 2019)  (Yuan, et al., 2014)  (Liu & Sun, 2014)  (Basit, et al., 2021)  (Wong et al., 2010)  (Xu et al., 2015) | |
| Chitosan | | Linear polysaccharide derived from chitin, abundant polysaccharide typically sourced from crustaceans and shellfish, also from insects and fungi  Gel forming capabilities  Can be processed into sponges, membranes, beads, scaffolds, nanofibers | | -High adsorption capacity  -High biodegradability  -Non-cytotoxic, low-immunogenic  -Antibacterial, antifungal, anti-tumour and anti-inflammatory properties  -Immunoadjuvant, anti-thrombogenic and anti-cholesteremic agent  -Often used to enhance biocompatibility with other synthetic polymers with better mechanical properties  -Can be used as 3D printing bioink for neural constructs | | -Poor mechanical properties when moist  -Low solubility at pH >7  -Cationic polymer (risk of neurotoxicity) | | (Ahmadi et al., 2015)  (Tan et al., 2018)  (Gao et al., 2014)  (Han & Hsu et al., 2017)  (Wang et al., 2017) | |
| Gelatin | | Denatured linear protein obtained by acidic or alkaline hydrolysis of collagen | | -Long history of safe use in pharmaceuticals, cosmetics, food products  -Low cost, high availability  -High biocompatibility and biodegradability  -Less antigenic than collagen (denatured)  -Chemically modifiable for increased cell adhesion and proliferation  -Absorbs water, can be used to form hydrogels | | -Poor printability  -Batch variability  -Lack of thermostability  -Poor mechanical stability  -Linear, need to crosslink, attempting to crosslink can lead to cytotoxic byproducts  -Complex crosslinking process difficult to scale up | | (Yang et al., 2022)  (Hu et al., 2022)  (Gao et al., 2014)  (Sadeghi-Avalshahr et al., 2020) | |
| Elastin; elastin-like polypeptides (ELPs) | | ECM protein that provides elasticity to tissue and organs, consists mainly of tropoelastin and microfibrils  ELPs, a synthetic protein-like material based on repetition of conserved motifs found in native tropoelastin, more commonly used to enhance biocompatibility and stability | | -Inherent biological recognition properties, presence of receptor-binding ligands  -Sensitive to proteolytic destruction (biodegradable)  *ELPs:*  -Greater control in targeted drug/neurotrophin delivery  -Can be used to obtain microfibers, nanofibers, hydrogels, scaffolds  -ELR hydrogels can be used as bioink for 3D printed scaffolds | | -Low mechanical/tensile strength  -Batch variability  -Immunogenicity  *ELPs:*  -High polydispersity  -Long, complex process, high cost | | (McMurtrey, 2014)  (Hearst et al., 2011)  (Nelson & Gilbert, 2021)  (Santos et al., 2019) | |
| Hyaluronic acid | | Glycosaminoglycan found in extracellular matrix, crucial role as lubricant | | -Biodegradable, biocompatible, natural so inherent biological recognition properties  -Support neurite outgrowth, differentiation and proliferation  -Mechanical properties induce differentiation of neural progenitors  -Can be used to form nanoparticles, hydrogels, granular hydrogels or microgels  -Combinable with other biodegradable synthetic polymers (PLGA, poly-L-lysine)  -Can decrease inflammatory response to electroconductive polymers  -Dynamic cross links with shear-thinning and self-healing capabilities allows for injectability, where removal of the force allows cross-links to reform the HA materials | | -Cells not as adherent to surface of hydrogels, commonly blended/cross-linked with other materials to improve cell adherence | | (McMurtrey, 2014)  (Shin et al., 2017)  (Skardal et al., 2010)  (Suri et al., 2010)  (Wang et al., 2012)  (Lin et al., 2009) | |
| Alginate | | Naturally occurring anionic biopolymer obtained from brown seaweed, can also be obtained from bacteria  Can form hydrogels | | -Biocompatible, low toxicity, low cost  -Drug and protein delivery, wound healing, cell transplantation  -Relatively high stiffness | | -Natural presence of impurities (e.g., heavy metals, endotoxins, proteins etc.)  -Needs to be purified in multi-step extraction procedure  -Limited long-term stability of cross-linked alginate gels as they can be dissolved by surrounding media | | (Lee & Mooney, 2012)  (Li et al., 2006)  (Prang et al., 2006)  (Suzuki et al., 2000)  (Wang et al., 2017) | |
| Keratin | | Polypeptide with intermolecular bonding of disulphide cysteine amino acid | | -Biodegradable, biocompatibility and non-immunogenicity  -Good cellular adhesion and proliferation  -Can be formed into hydrogels and scaffolds | | -Limited data in the context of neural tissue engineering | | (Rouse & Van Dyke, 2010)  (Zhu et al., 2019)  (Fearing et al., 2014) | |
| *Synthetic polymeric scaffolds* | | | | | | | | | |
| Polylactic acid (PLA), polyglycolic acid (PGA), Poly-lactoglycolic acid (PLGA) | | Thermoplastic polymers, polyester links of lactic or glycolic acid respectively  Can be used to generate scaffolds or multi-channel conduits, microparticles | | -Can be absorbed or hydrolysed in vivo, biodegradable  -Successfully shown to support neuronal growth and proliferation, as well as neural stem cell differentiation and maturation  -PLA/PGA can be easily combined to form PLGA for superior mechanical properties  -Synthetic, therefore lower risk of immunogenicity  -Can be combined with natural polymers, e.g., gelatin wrap  -Specific ratio of PLA:PGA in PGLA can be altered for specialised functions e.g. drug delivery microparticles  -PLGA microspheres shown effective at transporting molecules across the BBB | | -PLA scaffolds can be structurally unstable, and easily shatter and crumble  -PGA scaffolds good mechanical strength tends to fall months after implantation  -Non-biological material, lack of biomodulatory effects seen in naturally occurring polymers | | (Wong et al., 2007)  (Nguyen et al., 2017) | |
| Poly-ethylene glycol (PEG) | | Poly-ethylene glycol polymer, well suited for hydrogel formation due to hydrophilic properties | | -Highly biocompatible, hydrophilic  -Biochemically inert, versatile  -Non-immunogenic, resist to protein absorption, non-toxic  -Shown to improve neural cell survival, proliferation, differentiation  -PEG hydrogels closely matched stiffness of native brain parenchyma | | -Biologically inactive (synthetic), hence typically used in combination with other polymers | | (McMurtrey, 2014)  (Bjugstad et al., 2010)  (Xian-bin et al., 2017)  (Skardal et al., 2010) | |
| Poly-caprolactone (PCL) | | Synthetic polyester polymer of caprolactone, semicrystalline polymer | | -Controllable hydrolytic degradability rate  -Hydrophobic, slow degradation rate than other synthetic polymers, rate of roughly 24-36 months, useful for long term implants/drug delivery  -Non-toxic  -Can be combined with other polymers (altering degradation rate etc.)  -Can be developed to form calcium phosphate gradient, can lead to spatial gradients in mechanical properties, altering cell proliferation  -Versatile processing, can be 3D printed, heat moulded, good shape memory  -Good solubility in organic solvents  -Can be electrospun to generate sheets, tubes, sponges, parallel oriented fibres | | -Cell adhesion typically poor (due to hydrophobic properties)  -Needs coating with another molecule (e.g., collagen) to promote cell attachment | | (McMurtrey, 2014)  (Nga et al., 2015)  (Wong et al., 2007) | |
| Polypyrrole (PPy) | | Organic polymer of pyrrole monomer, commonly used conductive polymer for neural tissue engineering  Usually used in combination with other synthetic and non-synthetic polymers (PPy-PLA, PPy-PLGA, PPy-PCL, PPy-HA etc.) to enhance biocompatibility | | -Electrically conductive material  -PPy-PLA: enhance neural adhesion, alignment and elongation  -PPy-PCL: support cell proliferation  -Can be electrospun into nanofibers, cells can be thus electrically stimulated to encourage topographical neurite growth  -Can be combined with hyaluronic acid: construct electroconductive 3D hydrogels | | -On its own, poor biocompatibility and cell adhesion | | (Shin et al., 2017)  (Xu et al., 2014)  (Wang et al., 2004) | |
| *Nanomaterials* | | | | | | | | | |
| Carbon nanotubes (CNTs) | | Allotropes of carbon with cylindrical structures  Can be single walled nanotubes (SWCNTs) or multi-walled (MWCNTs) | | -High thermal conductivity and stability  -Optimal mechanical and electrical properties  -Biocompatible and conductive  -SWCNTs modulate and stimulate neurite growth  -MWCNTs have greater stability, have been applied to 3D printing scaffolds for nerve regeneration or targeted drug delivery devices | | -Generally non-biodegradable (can be useful instead as long-term implants)  -In vivo long-term accumulation not established | | (Mattson et al., 2000)  (Hu et al., 2011)  (Nho et al., 2010)  (Shin et al., 2017)  (Bokara et al., 2013) | |
| Graphene | | Allotrope of carbon as a 2D layers in a hexagonal lattice.  Can form 3D scaffolds, foams, graphene nanogrids  Typically, chemically functionalised further to enhance biocompatibility | | -Bactericidal, antiviral, highly biocompatible with generally low cell toxicity  -Electrically conductive (enabling stimulation, differentiation and proliferation of neural stem cells)  -3D scaffold can encapsulate other nanoparticles (e.g. gold) encouraging neuronal differentiation and axonal growth and alignment | | -Reports of 2D graphene causing matrix cellular toxicity (3D scaffold foams reported more effective, biocompatible)  -Higher toxicity in smaller forms | | (Song et al., 2014)  (Li et al., 2013)  (Akhavan & Ghaderi, 2013)  (Akhavan & Ghaderi, 2014)  (Akhavan et al., 2015) | |

Hydrogels

Hydrogels are some of the most common biomaterials tested in in vitro neural studies. They are cross-linked, water-enriched macromolecular networks that can be derived from natural polymers such as collagen (Kornev et al., 2018; Suri et al., 2010), gelatin (Jaipan et al., 2017; Yang et al., 2022), hyaluronic acid (Gerecht et al., 2007), chitosan (Ahmadi et al., 2015) and alginates (Zhou et al., 2022; Prang et al., 2006)) and synthetic polymers, such as polyethylene glycol (Bjugstad et al., 2010). The macroscopic, moisture-rich 3D architecture of hydrogels means they are ideal for biological interfacing, and can also be used to deliver cell therapies, creating a ‘live biomaterial’ for implantation. The primary advantage of hydrogels is the ability to inject the material as a liquid which then gelates in situ, to fill the injury cavity. This facilitates less invasive delivery than implants, whilst still allowing formation of a scaffold for promoting tissue regeneration. Further, delivery can be directly into the parenchyma bypassing the BBB for direct interaction with surrounding neurological cells (Chvatal et al., 2008; Chen et al., 2022; Hu et al., 2022; Nguyen et al., 2017). A similar effect has been shown with delivery of neurotrophic factors (Iannotti et al, 2003; Willerth et al., 2007). Mechanical properties such as compressive modulus, porosity and biodegradation can be altered, usually by altering the density and nature of crosslinks. The ideal hydrogel compressive modulus has been shown to vary depending on cell type, with neurons typically preferring softer surfaces (0.1-1.0 kPa) (Flanagan et al., 2002; Leipzig & Shoichet, 2009) and astrocytes and oligodendrocytes preferring stiffer surfaces (0.5-10 kPa) (Georges et al., 2006). This modulus may also vary depending on the length of time after transplantation, as biodegradation can lead to decreased structural integrity of the material, making it slowly more conducive to neuronal growth and infiltration over time (Hawkins et al., 2011). Biodegradation can therefore be controlled, not just to synergise with the natural proliferation and infiltration of endogenous cells, but also to be slowly replaced by the body’s endogenous extracellular matrix (ECM), which is an important long-term goal. Biodegradation should also be slow enough to mitigate the long-term neuroinflammatory effects after injury. Synthetic oligopeptides have been made into polymers using crosslinkers that can be targeted by endogenous hydrolysing enzymes, such as matrix metalloproteases (MMPs), collagenases, or plasmin (Wang et al., 2018; Lao et al., 2011).

Polymeric implants

Other types of protein scaffolds that can be wet or dry, have also been trialled as biomaterials for neural interfacing after injury. These polymeric implants have similar advantages to hydrogels for transplantation into injury. They are malleable/mouldable to injury sites, support endogenous infiltration/proliferation and/or can house exogenous biological therapies (cell transplants, antibodies etc.) (Boni et al., 2018). Their 3D microarchitecture aims to mimic lost ECM, while being biodegradable to slowly be replaced with hosts ECM. They can be made from similar materials as hydrogels. Frequently, several polymers can be combined to form co-polymers such as poly-lactic-co-glycolic acid (PGLA) or poly-ε-caprolactone-co-ethyl-ethylene phosphate (PCLEEP), PCL-PGLA etc. These materials can be formed through electrospinning, freeze-drying, water-emulsion, 3D printing, microfluidic fabrication, and can be adapted to different mechanical and physical parameters.

A key advance in polymeric scaffold design is the development of electrically conductive polymers (Song et al, 2017; Nezakati et al., 2018) that can potentially restore the electrical activity lost within injury sites (Xu et al, 2014). They have been shown to enhance the migration or proliferation of neural cells to facilitate the reconstruction of lost neuronal circuitry post-injury (Hu et al., 2022). Notably, their conductivity can also be used for controlled, electrostimulation mediated release of encapsulated neurotherapeutic molecules (Jian et al., 2018). These includes materials such as polypyrrole (PPy) (Wang et al., 2004) or polyaniline (PANi) (Thrivikraman et al., 2014). The key disadvantage with conductive polymers is their enhanced stability mitigating their ability to degrade *in vivo*, which may potentially cause further inflammation or immunogenic reactions. Hence these polymers are frequently crosslinked with other biodegradable natural or synthetic polymers to augment biological compatibility.

Nanomaterials

Nanoparticles have also shown great promise for neurotherapy following injury, with various types and formulations tested. They can be formulated for specific biomolecule encapsulation and targeted delivery to injury sites. Nanoparticles are also often incorporated into 3D scaffold materials or hydrogels to enhance their functionality (Bokara et al., 2013; Alam Bony & Kievit, 2019), or have been used to engineer cell transplant populations to increase their therapeutic efficacy (Fernandes & Chari, 2016). Their adaptability has many advantages in a wide range of therapeutic applications, depending on the characteristics of the nanoparticle. Specific nanomaterials that have been developed for neurological injury include carbon nanotubes (CNTs) and graphene.

CNTs have the benefits of being biocompatible, electrically and thermally conductive with optimal mechanical properties. They are made exclusively of carbon atoms forming sheets which wrap into small tubes, either single-walled (SWCNTs) or multi-walled (MWCNTs). Pure CNTs are typically insoluble in water, however this has been resolved by chemical functionalisation of their surfaces, leading to increased solubility and application in biological environments. Studies have demonstrated that CNTs can enhance neuronal electrical signalling (Lovat et al., 2005), decrease astrocyte proliferation and reduce microglial density, mitigating glial scar formation and encouraging regeneration (McKenzie et al., 2004; Bokara et al., 2013). These findings are attributed to their surface conductivity and molecular interactions, resulting in adsorption of endogenous proteins mediating cell adhesion. Neuronal adherence has also been shown to be susceptible to changes in the surface roughness using electron microscopy analyses, where neuronal growth cones preferred surface roughness which matched the diameter of the neuronal processes (Sorkin et al., 2009).

Like the previously mentioned biomaterials, CNTs can also be combined with other polymers, forming mixtures to create a desired blend of mechanical, biological and electric characteristics. They have also been demonstrated to be biocompatible with stem cells offering combined multifactorial therapy (Mattson, et al., 2000). One disadvantage to their use is that they are non-biodegradable. However, this can be used as an advantage when designing implants for long term recovery, providing molecular, electric and topographical cues for longer term regeneration.

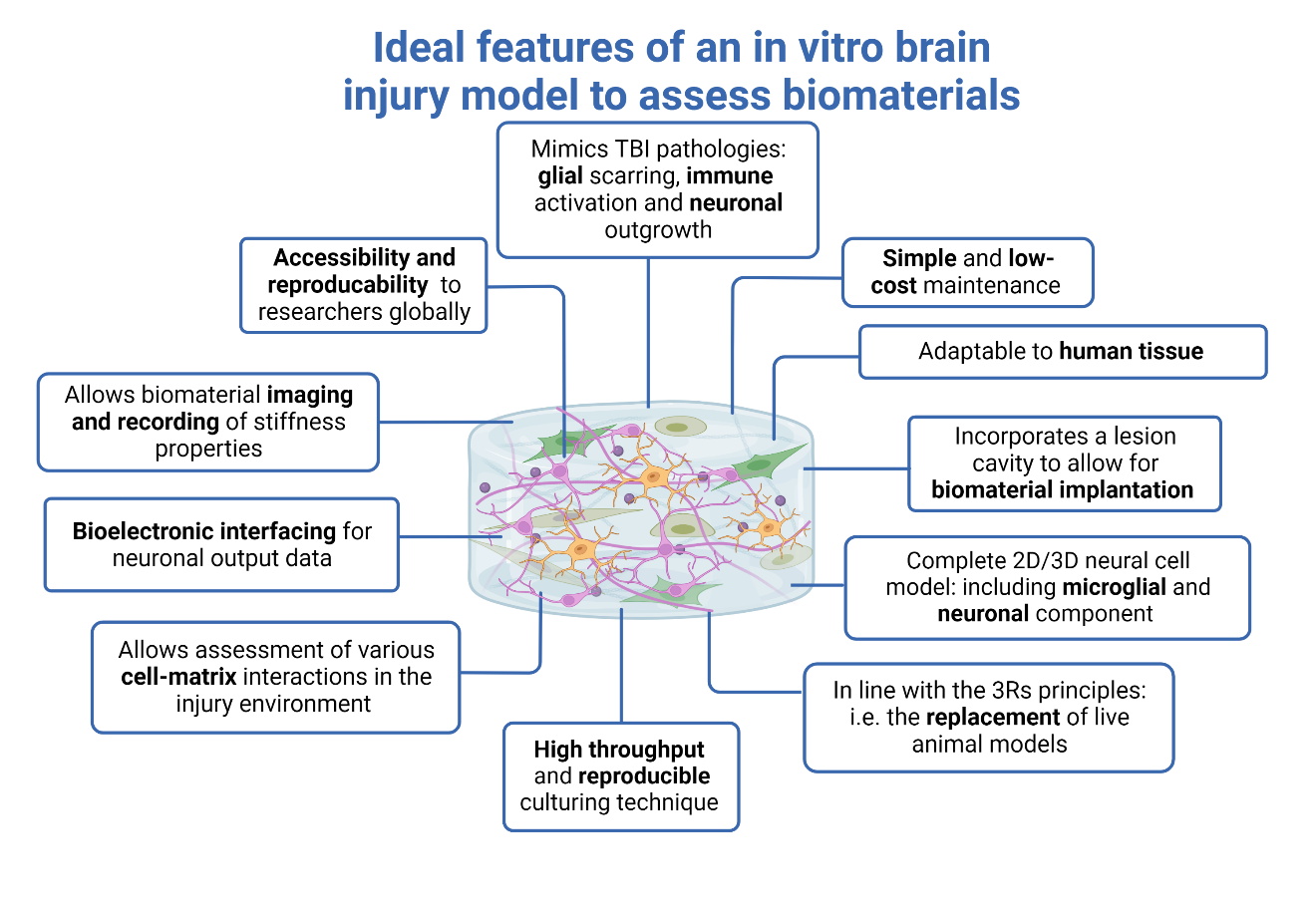
Graphene is an alternative carbon-based allotrope composed of a hexagonal 2D lattice of carbon atoms. It is one of the thinnest, strongest and lightest materials known. It is highly thermally and electrically conductive and known for antibacterial and anti-inflammatory activity. Graphene can be fabricated into sheets, foam, substrates and nanogrids, and has been widely used in in vitro studies (Song et al., 2014; Li et al., 2013; Akhavan & Ghaderi, 2013; Akhavan & Ghaderi, 2014; Akhavan et al., 2015). The disadvantages to graphene include being highly susceptible to an oxidative environment and presence of jagged edges that can pierce cell membranes. Graphene is generally considered a more unstable allotrope, with insufficient active sites, limiting its interaction with other biomolecules. Typically, for this reason, graphene is chemically functionalised by adding an oxide or amine group. Collectively, these functionalised forms of graphene are called graphene-based materials (GBMs) which are stronger and more uniform, although more expensive to produce. Toxicity has also been reported with GBM use, although this is affected by various factors, including size, shape, impurities, functionalisation groups and combination with other therapies (e.g., other nanoparticles). The consensus is that smaller forms of GBMs are associated with high toxicity levels (Devasena et al., 2021). Finally, there is a lack of in vivo studies demonstrating efficacy and patterns of accumulation, degradation and/or excretion of the nanoparticles from the body.

**12.1.4 Requirements for alternatives to in vivo TBI models for developmental testing of biomaterial based therapies**

Clearly there are a wide range of therapeutic biomaterials under development, all with the capability to modulate different aspects of neuropathology. Optimising biomaterial mode of action therefore requires complex neurological injury models to provide multifaceted, mechanistic readouts. Live animal, in vivo models of pTBI provide some of the most useful scientific data on the efficacy and applicability of a neuroregenerative therapy. These models typically involve either large animals, such as monkeys, pigs, sheep etc., or small animals, such as rats, mice and other rodents, to evaluate the efficacy, applicability and side-effects of introduced biomaterials, either in living or post-mortem tissue. These studies provide valuable research data, relevant to human neuropathology. For instance, features such as an intact, functional circulatory system and BBB are difficult to replicate ex vivo, but significantly influence the delivery, mechanism of action and efficacy of a proposed neurotherapy. However, live adult animal models have considerable drawbacks with respect to developmental testing of therapeutic biomaterials. For example, significant costs are associated with housing and rearing animals and in training research staff in animal handling and surgical skills. Further, several animals are typically required for statistical analysis, with the numbers of animals required increasing exponentially with the number of biomaterial variables to be tested. Animal experiments are also low throughput given the time needed to rear the animal, administer an injury and therapeutic biomaterial, monitor behavioural changes and analyse large volumes of tissue post-experiment. The current scientific consensus is to limit the use of animals, with a focus on the 3Rs (replacement, reduction and refinement). Therefore, researchers should aim to reduce animal usage and limit the suffering and distress associated with in vivo models. Overall, these models are poor options in the *initial* testing of an emerging neurotherapy and may be considered a later option, if a therapy has sufficient in vitro evidence to warrant further testing.

**12.1.5 Outlining important parameters for in vitro pTBI modelling to test therapeutic biomaterials**

Given the complexity of pTBI and combinatorial nature of biomaterial mediated repair, there is a key requirement in the field to develop in vitro models of pTBI with greater relevance to in vivo injury for development and testing. Ideally, such models would be simple to use but with high predictability for clinical use. A summary of the ideal properties of a pTBI model for testing therapeutic biomaterials is shown in figure 12.1 and will be discussed further in this section.



***Figure 12.1. Schematic highlighting ideal features of an in vitro neurotrauma model to test biomaterial therapeutics utility.***

Of high importance is the model’s relevance to in vivo and human pTBI. For this, we believe models should replicate major pathological processes of pTBI such as limited nerve fibre growth at injury sites, astrogliosis and glial scarring and neuroinflammation. Whilst relatively simple cellular systems can model these processes in isolation, few model systems are described which can model them *simultaneously*. Further, very few in vitro models contain the neural immune component – the microglia. This is a major omission given the role of microglia in modulation of nervous tissue responses to injury and implanted/injected biomaterials.

Alongside replicability of injury/bioimplant modulation, the model should be compatible with standard and advanced analytical procedures. Immunocytochemical evaluation of in vitro systems is widely reported. Advanced imaging techniques, including time-lapse microscopy, should also be able to be used to investigate real time cellular responses to biomaterials, offering advantages over in vivo systems where this is challenging. Interfacing in vitro injury models with bioelectronics allows for the understanding of electrophysiological characteristics before and after injury, which is critical for functional readouts post treatment. There has been little focus on this aspect to date. When recapitulating brain physiology and the mechanisms of injury within in vitro models, it is vital to understand the maturation of neuronal networks and the developmental changes in electrophysiological activity. To achieve this, multi electrode arrays (MEAs) have been developed in two-dimensional (2D) and three-dimensional (3D) designs for the monitoring of neuronal firing activity. These systems record culture wide dynamics by recording changes in extracellular ionic concentrations generated by endogenous action potentials and provide a potential system to achieve this.

Finally, any in vitro model generated should be scalable, high throughput and adaptable by neuroregenerative laboratories globally. Whilst there will always be a trade-off between simplicity and replicability, new approaches may mean enhanced orders of replicability can be achieved by using relatively simple techniques. For widespread use, an in vitro model should also be cost-effective. Costs associated with in vitro culture are not always obvious, but include funding requirements for animal facilities, initial cost of commercially available cells, licencing and management costs for animal and particularly human cells and the expense of specialised media.

**12.2 Current in vitro models of pTBI and the extent to which they meet idealised criteria**

To address these challenges researchers have developed in vitro models of the brain which range in complexity from the simplest, 2D monolayer cell cultures to the highly complex 3D organotypic slice or organoid cultures. Different in vitro models have specific associated features which determine their research utility i.e., pathological relevance, technical difficulty and the ease of maintenance. An overview of in vitro models for modelling central nervous system (CNS) tissue, pathology and testing of therapeutic biomaterials is highlighted in table 12.2. In this section, we will discuss these different systems in detail and how they compare with an idealised pTBI model.

**Table 12.2. *In vitro* systems for modelling brain tissue, traumatic injury mechanisms and biomaterial interventions.** The table is split into culture model systems and cell sources with advantages and disadvantages to each system described. Adapted and updated from Basit et al., 2023.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Description** | **Advantages** | **Disadvantages** | **References** |
| ***In vitro* models** |  |  |  |  |
| **3D ‘organotypic’ slices** | *Ex vivo* brain tissue slices | -Retain *in vivo* cytoarchitecture  -Retain major brain cell types (including microglia)  -Ease of mechanical manipulation  -Moderate difficulty to induce mechanical injury  -Can be interfaced with biomaterials  -Display complex injury responses  -Adaptable for excitotoxicity/hypoxia studies | -Moderate throughput  -Can be technically difficult to isolate and maintain slices  -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  -Can be human/patient specific  -Closely simulate *in vivo* cellular communication  -Adaptable for excitotoxicity studies  -Ideal for disease and development studies | -Moderate throughput  -Little uniformity between aggregates  -Largely immature in cellular development  -Long culture periods  -Few injury systems reported  -Lack vascular and immune components  -Problematic for mechanical manipulation due to free floating nature  -Spheroid centres can become hypoxic due to lack of nutrient access  -Difficult cellular analysis  -Complicated for biomaterial interfacing | Birey et al., 2017; Jgamadze et al., 2020; Ramirez et al., 2021; Pamies et al., 2017; Shi et al. 2021; Shoemaker et al. 2021 |
| **Brain-on-a-chip** | Microfluidic culture systems of 3D iPSC derived cultures | -Tissue-like physiology  -Perfusion system of 3D tissue  -Adaptable for disease/toxicity mechanisms  -Axonal strain injury attempted | -Low throughput  -Scalability limitations  -Lack immune and vascular components  -Lack cellular maturity  -Difficulty with mechanical injury induction  -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  -Physiologically relevant cellular morphology  -Simple maintenance  -Moderate to high-throughput  -Moderate technical difficulty for injury mechanisms  -Biomaterial interfacing feasible | -Difficult analysis of 3D environment  -Can lack complexity if not multicellular constructs e.g., lack immune component if cells are NSC derived  -Not currently documented with all the major cell types of primary brain cell dissociates  -Lack vascular component (but feasible with tissue engineered blood vessels) | Haycock, 2010;  Antoni et al., 2015;  Raimondi et al., 2020 |
| **2D cultures** | Single or multicellular neural cultures grown as a monolayer | -Can encompass major brain cell types (including microglia and neurons)  -Simple injury mechanisms  -High throughput  -Low technical difficulty  -Simple maintenance and analysis  -Biomaterial interfacing feasible | -2D environment -Cells undergo artificial responses to adapt to the flat, stiff substrate -Lack vascular component | Goshi et al., 2020;  Basit et al., 2021;  Gao et al., 2013 |
| ***Cell source*** |  |  |  |  |
| **Primary multicellular models** | Complex multicellular cultures of brain dissociates | -Can encompass all major brain cell types (including microglia and neurons)  -High throughput  -Low technical difficulty  -Simple maintenance and analysis | -Lack vascular component | Goshi et al., 2020;  Basit et al., 2021; Basit et al., 2023 |
| **Primary neural stem cell cultures** | Cultures of differentiated stem cells isolated from neurogenic regions e.g., subventricular zone (SVZ) | -High throughput  -Low technical difficulty  -Multicellular cultures | -Lack immune component  -Moderate length differentiation protocols -Preferential differentiation to astrocytes  -Lack vascular components | Gao et al., 2013;  Vagaska et al., 2020; Fernandes and Chari, 2013; Lööv et al., 2012; Reubel-Gallasch et al., 2021 |
| **Induced pluripotent stem cells (iPSCs)** | Stem cells genetically reprogrammed from adult cells | -Indefinite propagation  -Can be of human origin  -Patient specific (retain genetic identity)  -Beneficial for patient specific disease modelling | -Moderate technical difficulty -Moderate throughput (long differentiation protocols)  -Differ genetically/phenotypically from endogenous counterparts – altered morphology  -Heterogeneity of cells  -Resistant to cell death  -Risk of mycoplasma contamination -Biomaterial-injury interface not reported  -Lack immune and vascular components | Ulrich et al., 2001;  Kang et al., 2017;  Pistollato et al., 2017; Tukker et al., 2018; Pamies et al., 2017; Gunhanlar et al., 2018; Shi et al. 2021; Ramirez et al. 2021; Shoemaker et al. 2021; Sherman et al., 2016 |
| **Primary pure cell cultures** | Primary cultures from brain dissociates; purified through sequential shaking or specific media components | -High throughput  -Low technical difficulty  -Useful to study specific cell responses | -Overly simplistic model of the brain -Absence of multicellular interactions  -Lack vascular and immune component (if not ‘pure’ microglial cultures) | Geddes et al, 2003;  Chen et al., 2007;  Vellis and Cole, 2011 |
| **Cell lines** | Immortalised cell lines | -Indefinite propagation  -High throughput  -Facile  -Can be of human origin | -Genetically and phenotypically differ from endogenous counterparts  -High risk of mycoplasma contamination  -Cellular heterogeneity  -Resistant to cell death -Lack immune and vascular components | Gordon et al., 2013;  Carter and Shieh, 2015;  Tapia and Scholar, 2016 |

**12.2.1 Cell lines and primary cell monocultures**

Immortalised single cell monocultures represent the simplest end of the spectrum, in relation to cost and convenience, versus in vivo models. Monocultures of single cell types are often bought commercially and used frequently. These cell lines are derived from cancer biopsies or are artificially immortalised. They have fewer ethical concerns, are easy to set up and maintain, require minimal training and are cost effective. The downside is their over-reductionist and simplistic nature which make them poor models of pTBI. They are neoplastic, showing a high variability in genetic makeup and expression over time and may thus exhibit abnormal physiological responses to injury. At best, they represent the behaviour of a single cell type, lacking the complex inter-cellular behaviours and interactions seen in the pathophysiology of pTBI. Moreover, studies have also shown frequent cross-contamination with cell lines procured from sources not always validated. Thus, evidence from these studies has limited therapeutic applicability.

Primary single cell monocultures have the benefit of being sourced from live animals, typically small rodents, so are considered more physiologically representative than cell lines. Classically for TBI modelling, monocultures of cortical neurons are established by plating cells from the dissociated cortex of embryonic rodents. Typically, this culture produces highly pure neuronal cultures, with less than 5% glial cell populations (Liaudanskaya et al. 2020), in which an injury can be induced. With an adjustment to culturing techniques, individual glial cells can also be isolated and plated out into glial monocultures. Here, the method of culture is adapted from McCarthy and de Vellis (1980) who initially outlined a method for the bulk production of primary glial cells, to be separated out into individual cell type populations. Postnatal rodents are euthanised, cortical tissue is extracted and dissociated before being grown onto 2D plastic or glass. Cells mature to show a typical stratification with astrocytes forming a base layer upon which oligodendroglia and microglia form, allowing for easy isolation of specific cell types using a shaker. Injury induction in the 2D single cell cultures is also facile, using a pipette tip or needle to create an injury, making this a high throughput choice (Gao et al., 2013). However, such systems can only provide data on single cell responses to injury and biomaterial intervention, which are not likely to be reflective of cellular responses in a complex, pathological environment.

**12.2.2 Primary defined co-culture models of pTBI**

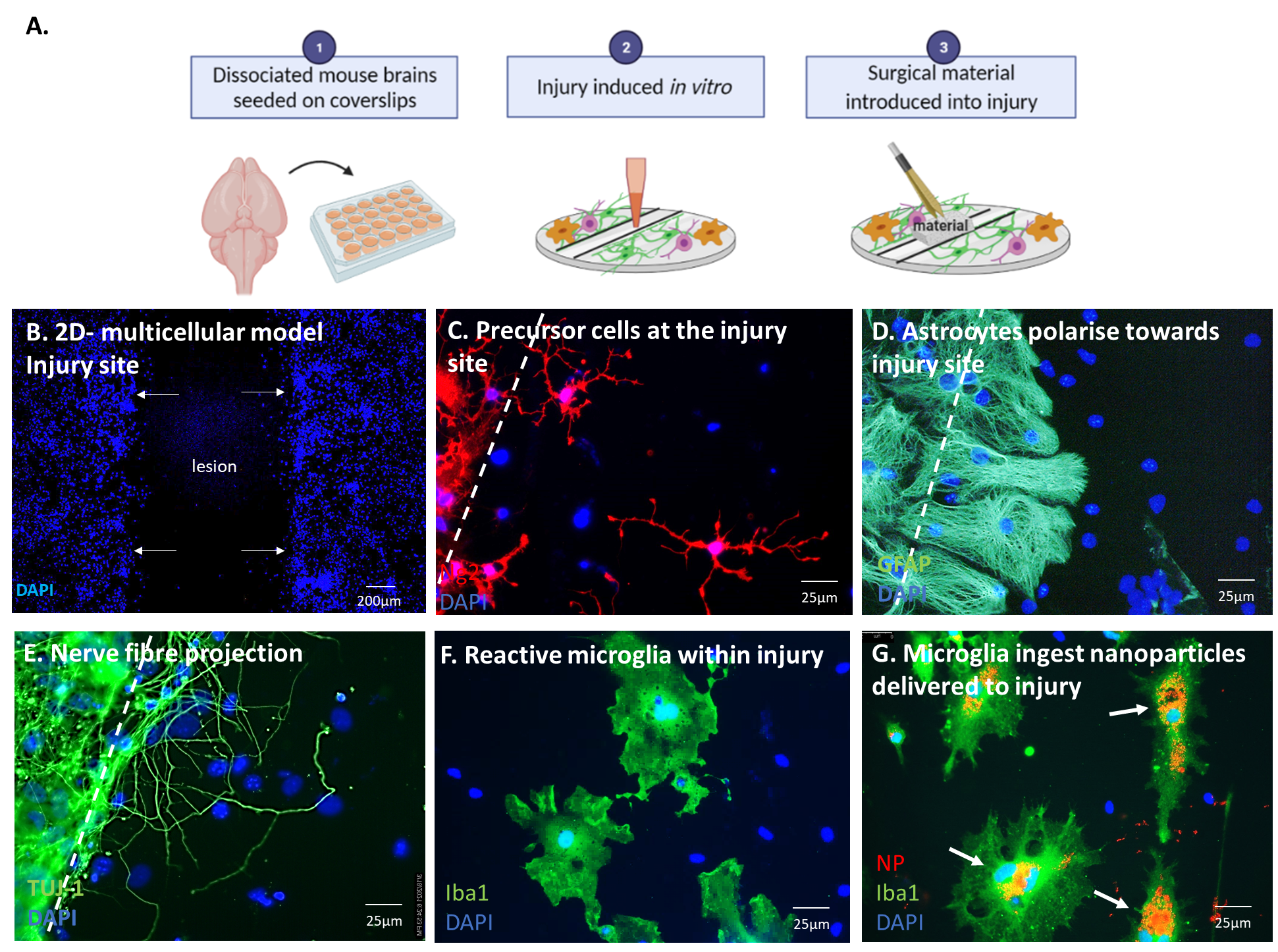
Primary monocultures of neural cells are easy to establish but do not recapitulate the intercellular interactions which occur in complex sites of neurotrauma. A relatively simple way to address this is to combine monocultures to form more complex in vitro cellular systems. Indeed, initial co-culture models were developed with common combinations including astrocyte-microglia (Jenkins et al., 2018) and astrocyte-neurons (Goshi et al., 2020). Such systems were able to simulate key pathological neural responses seen within pTBI, for example palisading astrocytic glial scarring responses to trauma (Bardehle et al., 2013). In addition, these systems have been employed to investigate intercellular differences in biomaterial handling, demonstrating that microglia out-compete other cells paired in the co-culture for uptake of therapeutic nanoparticles (Jenkins et al., 2015).

Co-culture systems provide a useful platform for the study of individual cellular responses to bio/nanomaterials, including complex intercellular interactions. Therapeutic testing for functional recovery in pTBI is often centred around targeting neurons / OPCs (regeneration) or attenuation of glial scarring (reactive astro/micro – gliosis) to create a repair- conducive environment. For such readouts, co-cultures suffice as a facile, repeatable and dependable strategy to study high throughput interactions. However, one drawback is that setting up defined co-culture systems requires multiple culture steps to establish two separate cell types with subsequent mixing at a later point, adding complexity and time to the procedure.

**12.2.3 Primary multicellular models of pTBI**

To address the above issues, complex in vitro systems derived from dissociated cortices have been developed which contain multiple, relevant neural cell types. Using similar protocols to those originally dissociating embryonic cortical tissue, we have shown that two multicellular systems for modelling pTBI can be established from post-natal mice that address many limitations associated with previous models. Using post-natal mice confers additional advantages as cultures do not require sacrifice of the mother. The first system is a mixed glial/polyglial culture system where the stratified mixed glial cultures from McCarthy and de Vellis (1980) are plated directly onto glass coverslips. Here, astrocytes, oligodendrocytes and microglia are present in reproducible ratios. We demonstrated that a reliable scratch injury could be introduced into the cultures with simulation of key pathological responses (including reactive astrocytic scarring and microgliosis- amoeboid microglia infiltration of lesions). Further, a clinical grade collagen bio scaffold, DuraGen Plus, was interfaced into the lesion core and showed microglial infiltration and astrocytic invasion of the material (Basit et al 2021). This model is particularly suited to studying glial responses to therapeutic biomaterials (including glial scarring around materials, glial based material remodelling and microglia-based material breakdown) in injury environments.

We have since evolved the model to include the neuronal population alongside multiple glial cells, through a simple switch in growth medium. Using similar techniques to the mixed glial system, an injury was established and pathological processes were shown to be replicated along with limited nerve fibre growth into the lesion cavity. We have also tested therapeutic biomaterials within this model, indicating high and dominant nanoparticle uptake by microglia in comparison to all other cell populations, when nanoparticles are delivered to the injury site (Figure 12.2). In addition, straightforward interfacing of the model with MEA systems can provide global, network electrophysiological data to be generated at sites of injury and regeneration (Wiseman et al., submitted, 2023). We are currently scaling the mixed neuronal injury model to 3D by encapsulation in soft, collagen hydrogels.

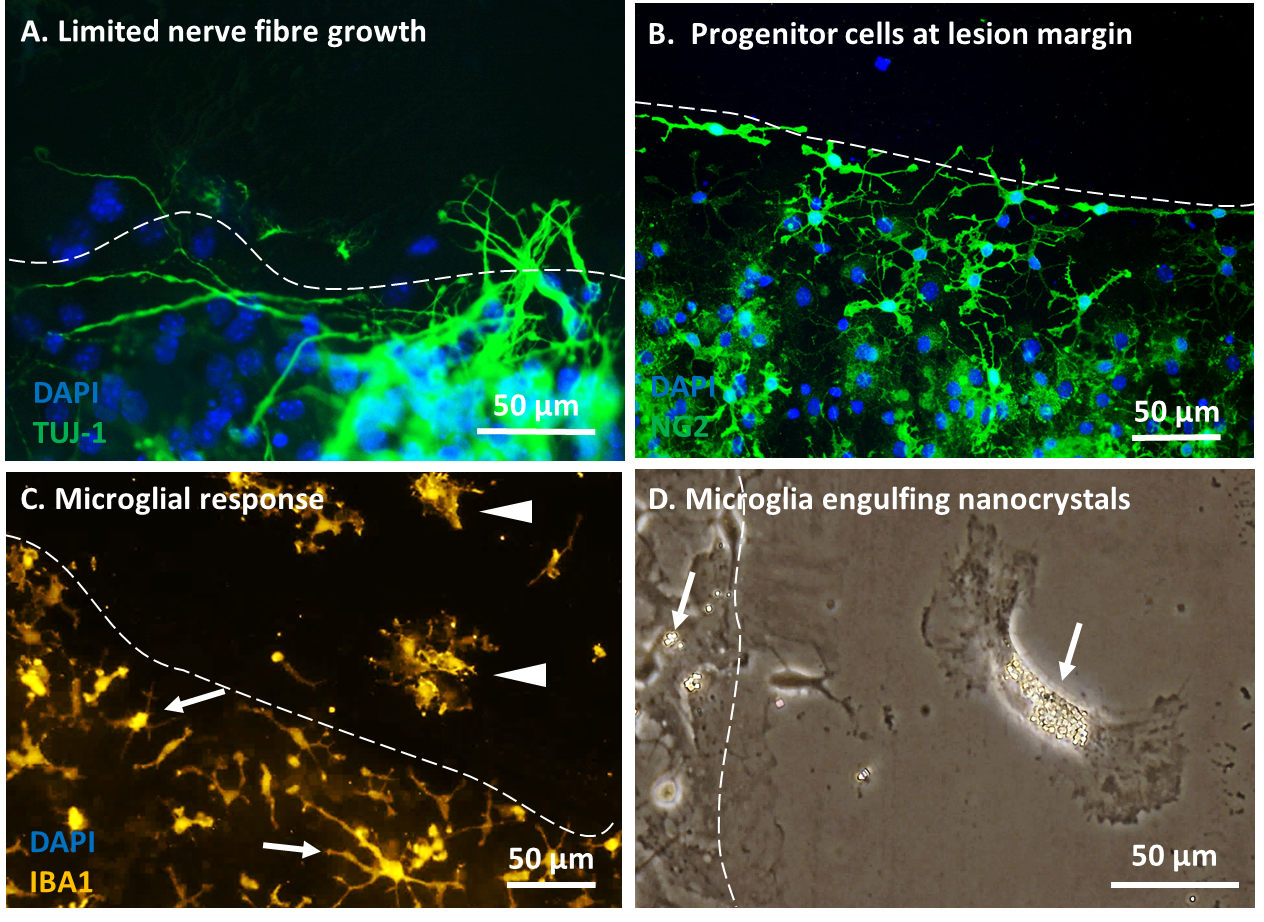


***Figure 12.2. Development of complex 2D multicellular models of pTBI.*** *(A) Schematic demonstrating general protocol for establishing multicellular models of neurotrauma from dissociated cortex tissue.**In summary, (1) cortical tissue is dissected and enzymatically dissociated. Once the dissociated culture is plated it matures into multicellular cultures with a “mixed glial culture” containing glial cells (astrocytes, oligodendrocyte lineage cells and microglia) or a “mixed neuronal culture” containing glial cells and neurons together. (2) An injury can be induced through scratching with a pipette tip with (3) subsequent biomaterial introduction and investigation into pathological responses.**(B-G) Representative images from the mixed neuronal culture showing (B) reproducible injury induction, (C) progenitor cell migration at injury borders, (D) astrocytes hypertrophy and polarise towards the injury site, (E) nerve fibre projection into the lesion and (F) reactive, amoeboid microglia within the lesion. Biomaterials can be delivered to the injury with (G) demonstrating nanoparticle uptake by microglia in the injury. Unpublished data from the NTEK group.*

**12.2.4 Stem cell derived models of TBI**

In vitro TBI models based on the use of stem cells have been extensively reported (Table 12.2). For TBI modelling, stem cells are differentiated into target neural cell populations (e.g., neurons, astrocytes or mixed cultures) prior to induction of injury. Broadly speaking, the advantages of stem cell models are the ability to expand the parent cell to increase experimental replicates, the capability to generate multiple neural cell types simultaneously, the potential to control cell phenotypes (e.g., specific differentiation into cortical neurons) and the ability to replicate more immature injuries e.g. foetal or young adult. Further, with the advent of cerebral organoid, brain-on-a-chip and 3D scaffold based culture, there is now added capability to replicate 3D neuroanatomical areas of the brain using differentiated stem cells.

Numerous sources and classes of stem cells exist and have been reported. To simplify this section, we will refer to three different classes – tissue specific, embryonic and induced pluripotent stem cells. Tissue specific stem cells are found within niches in foetal to adult neural tissue e.g., the subventricular zone, which maintains a population of neural stem cells (NSCs) throughout life in mammals. These stem cells are already committed to a neural fate. Therefore, there are short, simple and cost-effective differentiation protocols to generate neural cells. Robust protocols for NSC derivation from rodents are available, and the culture and differentiation of these cells is extensively characterised within the literature (Zhao and Moore, 2018). We have previously shown differentiated populations of NSCs generate all major neural cell types (astrocytes, neurons and oligodendrocytes) simultaneously and can be used to test biomaterial function. For example, we demonstrated astrocytes out-compete neurons and oligodendrocytes for cellular uptake of nanoparticles in these multicellular cultures, which may be driven by enhanced levels of membrane activity (Fernandes and Chari, 2014; Fernandes et al., 2015). Elegant studies from the Erlandsson laboratory have shown the utility of differentiating NSCs to model TBI. Here, NSCs derived from embryonic mice cortices were differentiated for seven days to generate mixed cell cultures. Scratch injuries were induced with a scalpel and the authors were able to show astrocyte and neuron growth towards sites of injury, autophagy of dying cells by astrocytes and enhanced levels of S100β and glial fibrillary acidic protein (GFAP; markers of astrogliosis) secretion into the medium in injured slices (Lööv et al., 2012; Reubel-Gallasch et al., 2021). Our group has adapted these systems to NSCs derived from post-natal mice to test biomaterials in sites of TBI. Our preliminary data indicate that injured differentiated NSC cultures demonstrate poor nerve fibre growth around injury sites, progenitor cell accumulation at injury margins and microglia invading the injury (Figure 12.3, unpublished data).



***Figure 12.3. pTBI modelling using differentiated populations of NSCs.*** *Representative images showing (A) poor nerve fibre growth into the lesion cavity, (B) progenitor cell accumulation at injury margins, (C) microglial activation and invasion of injury sites, note amoeboid microglia in injury cavity (arrowheads) versus ramified microglia in cell dense areas (arrows). (D) Biomaterials can be introduced into the injury site. In this example, nanocrystal growth factor carriers appear to be ingested by invading microglia (arrow). Unpublished data from the NTEK group.*

In order to generate human-relevant neuromimetic models, NSC derivation from human foetal tissue is also well established (Lu et al., 2011). For more mature models, adult rodent NSC derivation has been reported (Isaksen et al., 2020; Ahmed et al., 2021). Despite these potential advantages, primary NSC-based models of neural tissue injury are rarely reported. An alternative is to use an earlier precursor to generate NSCs. Embryonic stem cells are derived from the inner cell mass of a human blastocyst, normally donated during in vitro fertilisation processes. Many hESC lines are now commercially available, with protocols available to differentiate them into NSCs, then into specific neurons and glial cells. Ordinarily, this process still requires embryo use, raising ethical concerns (Vazin and Freed, 2010).

These concerns have, in part, driven the development of human induced pluripotent stem cells (hiPSCs) - somatic cells reverted to an embryonic stem cell like state through introduction of transcription factors or proteins into the cells. Crucially, hiPSCs avoid embryo use yet are human stem cells capable of generating neural cells. These systems can develop into the three main cell types of the CNS (neurons, astrocytes and oligodendrocytes) under appropriate culture conditions. Further detailed characterisation has indicated establishment of different cortical neuronal subpopulations within a hiPSC preparation (Pamies et al., 2017), showing different cellular responses can potentially be investigated. Electrophysiological recordings have demonstrated that neurons mature over time, with mature, burst action potential signatures found after 8-10 weeks (Gunhanlar et al., 2018). hiPSCs can also be derived in a patient specific manner, for potential testing of personalised therapeutic approaches. There is a recent surge in the use of hiPSCs to model TBI. Sherman et al. (2016) demonstrated hiPSCs grown as 2D monolayers in adapted 96 well plates generated human neurons. The adapted plates allow for a stretch injury to be administered in a controlled manner to the 2D cultures. After injury, cell death and axonal degradation were observed mimicking some aspects of TBI pathology.

Perhaps the most exciting development in terms of hiPSCs are their ability to self-aggregate into 3D CNS organoids. These organoids have been shown to mimic embryonic brain development which results in the formation of a “mini brain” displaying multiple regions of neural tissue comparable to that of the human brain (Qian et al., 2016). Myelination has also been detected within the structures (Pamies et al., 2017) which may allow investigation into myelin pathology following TBI. Using this system, Ramirez et al. (2021) demonstrated organoids with cortical layering could be achieved. Using a controlled cortical impact on the structures, astrogliosis was initiated, with astrocytes elevating GFAP expression and becoming hypertrophic. Further, apoptosis and neuronal damage was elevated in the model. Using a similar system, Shi et al. (2021), also demonstrated astrogliosis and cell death in an impact-based model for mild TBI, indicating such approaches can be reproducibly adapted across laboratories. Shoemaker et al. (2021) showed that compression of organoid-like constructs also resulted in suppressed electrophysiological activity and secretion of neurofilament light chain, a candidate biomarker for indicating neurotrauma, into the culture medium.

Whilst highly promising, some drawbacks exist with the use of hiPSCs. The protocols are time-consuming and result in significant inter-culture variability. For example, Ramirez et al. (2021), applied their injury after 220 days in culture. Even so, these are relatively immature cultures, more akin to foetal or neo-natal lifespans. Further, hiPSCs do not form microglia in these self-patterned systems. The immune component is of critical importance when assessing biomaterial implantation. There has been mechanical disruption induced by high-intensity focused ultrasound (Lai et al., 2020) and controlled cortical impact devices (Jgamadze et al., 2020; Ramirez et al., 2021). However, a clear injury cavity was not defined therefore not readily adaptable for biomaterial implantation. Finally, mechanical manipulation of organoids is likely to be technically challenging given their size (<1mm in diameter) and free-floating nature. Some solutions are being developed with protocols now available for hiPSC derived microglia, which could be incorporated into the system (Sabate-Sole et al., 2022). One study even raises the possibility of microglia developing innately within organoids, cultured under conditions which permit some mesodermal lineage cell differentiation (Ormel et al., 2018). We have not found any examples of hiPSC-based testing of therapeutic biomaterials; therefore this appears an attractive area for research.

**12.2.5 Organotypic slice models of pTBI**

Organotypic slice (OTS) cultures represent a transitional system that preserves the original tissue, and its inherent structural architecture, combining the features of *in vivo* and *in vitro* models (Wiseman, 2023). Whole organs are dissected, e.g., the brain, and the tissue is cut into slices using a tissue vibratome (without any cellular dissociation), and thickness of slices vary depending on experimental need (Gahwiler, 1981). There are two methods of achieving organotypic culture. The first developed was the roller-tube culture, where slices are embedded into a collagen/plasma matrix on glass coverslips and then placed into culture tubes with growth medium which undergo a continuous slow rotating action. This rotation permits the oxygenation of the slice by generating an alternating liquid-gas interface. Via this method, slices can be cultured for several weeks (Gahwiler, 1981). The second technique (and now the most common technique for neural OTS culture), involves a semi-porous membrane supporting the tissue slice above the growth medium creating an air-medium interface. The medium below the membrane allows for appropriate nutrient absorption, facilitated via capillary action and oxygenation is permitted from above the slice (Stoppini et al., 1991; Gahwiler et al., 1997). An advantage of this interface membrane method is that the medium does not submerge the OTS permitting therapeutic agents, materials, stimuli or mechanical injury to be applied directly to the slice at any stage of culture. Before simulating an impact injury upon these slices, time is required after initial culture to allow for slices to adapt to the culturing conditions, attach to the membrane and mature. This period of time can vary; some groups advise 12-14 days (Adamchik et al., 2000) others suggest less than a week or four days for sufficient attachment/stabilisation (Krassioukov et al., 2002; Sypecka et al., 2015). There have been several established brain OTS models derived from various species including mouse, rats, rabbits (Savas et al., 2001) and human (Jeong et al., 2011).

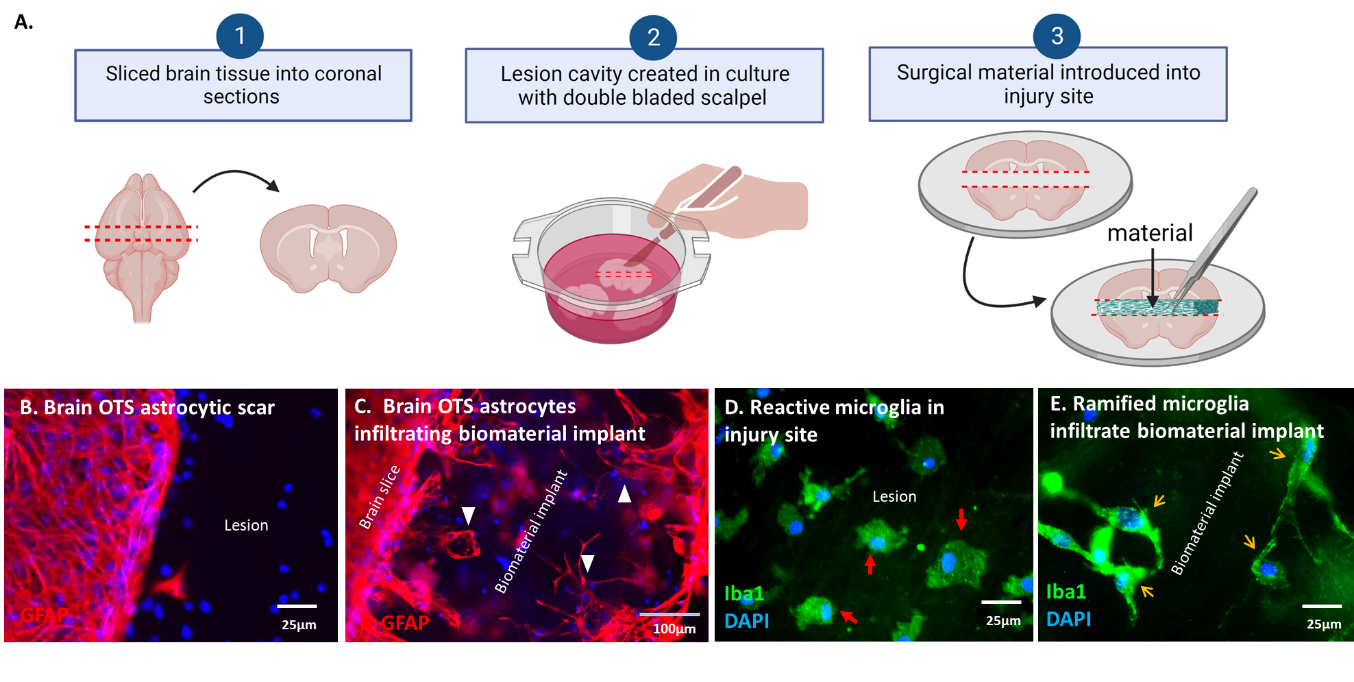
*Ex vivo* tissues in culture retain their native tissue cytoarchitecture and structures, including white and grey matter, neuronal tracts and the central canal. In addition, they continue to develop and mature within culture. In terms of mechanisms for modelling injury, manipulation of the slices is more straightforward with no requirement for the invasive procedures or ethical issues associated with *in vivo* TBI models. Various types of injury can be introduced in the slices; ischemic injury (involves a depletion of oxygen) (Li et al., 2016), injury through over exposure of glutamate (Dorset et al., 2017), LPS-induced neuroinflammation (Lutz et al., 2015), or traumatic injury (involving damage from an external force). Traumatic external force mechanisms include: i) weight drop mechanisms mimicking contusion injuries (Adamchik et al., 2000; Krassioukov et al., 2002; Pansamooz et al., 2019), ii) stretch mechanisms (Morrison et al., 2000; Morrison et al., 2006; Di Pietro et al., 2013), iii) a compressed-air driven shock tube (Campos-Pires et al., 2018), iv) focal injury mechanisms induced by dropping a metal stylus under stereomicroscopic control (Krings et al., 2016) or, v) through a transecting mechanisms where a doubled bladed scalpel is used to create an injury cavity within the organotypic tissue (Weightman et al., 2014; Guijarro-Belmar et al., 2019, Tickle et al., 2022). The primary advantage of transecting lesion injury models over the other models mentioned here, is the generation of an injury cavity which is ideal for the implantation of biomaterials that can fill the lesion space.

Implantation of biomaterials into in vivo models of TBI can be complicated as it includes an intricate secondary procedure after the initial injury with risks of infections and arduous physiological monitoring. In contrast, implantation into organotypic slices is much simpler due to ease of mechanical manipulation within this in vitro setting. Further, slices are easily stained and imaged with standard imaging apparatus. Therefore, OTS offer a moderate throughput platform to simultaneously monitor parameters of neural regeneration (i.e., nerve fibre outgrowth, glial scar formation, remyelination, and immune cell activation) in response to various injury mechanism and therapeutic biomaterial implantation. A general protocol for this process is shown in figure 12.4, alongside preliminary data from our laboratory which indicates the multiple pathological parameters which can be investigated at the material-tissue interface.

It is important to note that most successful organotypic slice models are generated with immature tissue from early postnatal animals, due to greater plasticity than adult tissue (Finley et al., 2004). The majority of traumatic injuries to the CNS will occur at adult age so younger tissue is a limitation when considering translational therapy assessment. However, brain OTS cultures have been successfully maintained for weeks to several months to increase tissue age before injury (Ostergaard et al., 1990; Marksteiner and Humpel, 2008; Sypecka et al., 2015).

In recent years, there has been increasing focus on human organotypic tissue cultures. However, human organotypic TBI models are extremely rare and an efficient and accessible, well characterised human injury model is yet to be developed. In 2014, a group developed a brain OTS model that facilitated *in vitro* culture of human adult brain tissue for up to 3-4 weeks. This tissue was obtained during surgeries in patients with focal epilepsies (Eugene et al., 2014). Another group was able to establish a long-term adult human brain slice culture in 2019 (Schwarz et al., 2019). Lin et al., (2020) achieved the first human spinal injury/TBI organotypic model through culture of human foetal brain stem and spinal cord slices. This tissue was harvested from pregnancy donors within the first trimester and a weight drop injury mechanism was performed on transverse slices (Lin et al., 2020). In 2022, our Keele Neural Tissue Engineering (NTEK) group developed a benchtop brain injury model using resected donor tissue from patients with Chiari malformation. Viable tissue was maintained for two weeks with all major neural cell types detected. After induction of a traumatic transecting injury into the slices, some cardinal pathological features were identified demonstrating potential for biomaterial assessment and development (Tickle et al., 2022). Our group has also developed a human foetal spinal cord injury (SCI) slice model with a direct comparison to an established mouse SCI slice model. Here, a surgical grade biomaterial was successfully implanted into the lesion site with promising readouts (unpublished data).

Although these human OTS studies show proof of concept, the clinical availability of human brain tissue that is not pathological, post-mortem or foetal limits their biological utility and scale-up potential. Additionally, due to the ambiguity of human OTS cultures and the extensive use of rodents for *in vivo* therapeutic testing, rodent organotypic modelling systems may be the most suitable replacement at this point for screening novel pharmacological tools. Although these current rodent TBI OTS cultures offer significant benefits particularly in studying biomaterial implantation, there is still a need to develop robust human specific model systems. This could aid with overcoming the lack of clinical translation that may be associated with cross-species differences (Haring et al., 2017).



***Figure 12.4. Development of mouse organotypic slice models of pTBI.*** *(A) Generalised schematic for organotypic slice modelling of pTBI and biomaterial implantation. In summary, (1) the appropriate brain region is dissected and sliced using a tissue chopper; (2) These slices can be maintained on culture inserts for months, with traumatic injury induced via a transecting lesion; (3) Lesioning creates an injury cavity within which biomaterials can be implanted/injected with monitoring of complex pathological responses. (B) Representative immunofluorescence image indicating upregulation of GFAP at the injury margin with clearly defined injury border. (C) Representative fluorescence image showing astrocyte processes infiltrate a bioimplant inserted into the lesion, disrupting the injury border. (D) Representative image showing reactive microglia with amoeboid morphologies invading the injury site. (E) In contrast, exploratory microglia invade a bioimplant inserted into the lesion, with a morphology depicting a transitional state from amoeboid to ramified. Unpublished data from NTEK group. OTS – Organotypic slice.*

**12.2.6 Advanced approaches for modelling brain injury in vitro**

2D in vitro monolayer models such as those in culture wells can be beneficial in high throughput drug screening but fail to model the intercellular-matrix intricacies and complexities of cellular signalling that 3D models can offer. Modelling systems which employ human cells (in particular, hiPSCs) such as microfluidic bionetworks, brain-on-a-chip and 3D brain organoid systems have gained considerable popularity lately. Microfluidic systems can mitigate the challenges of long-term maintenance and environmental control presented with some 2D and 3D cultures. Recently, there has been a large focus on combining hiPSCs with microfluidic systems to generate brain-on-a-chipmodels which resemble a tissue-like physiology (Bang et al., 2019). These microfluidic systems are chips that support miniature engineered 2D or 3D cellular constructs, integrating a cellular scaffolding with mechanical, biochemical and topographical cues and a controlled flow of air, blood and nutrients to create physiological conditions and recapitulate specific organs. These chips offer precise control, manipulation and real-time analysis of the tissue through a system of interconnected channels in a microfluidic device (Bang et al., 2019). In recent years, there have been significant technological advancements in the generation of human-specific brain models mimicking complex pathophysiological responses. There are three broad categories of neural systems in microfluidic chips including: microfluidic, compartmentalised and hydrogels chips (Chuankai et al., 2022). These systems are more commonly used to understand neurophysiological mechanisms or to model neurodegenerative disorders and diseases rather than modelling traumatic injury. However, some injury mechanisms have been studied. For example, Hosie et al. (2012) studied TBI related glutamate excitotoxicity in a compartmentalised neural chip which isolated soma and axons. A stretch strain injury model has been developed by Dolle et al. (2014), where a uniaxial strain can be applied through a specific microfluidic device. The model was designed to be capable of applying a range of mechanical strains with the ability to strain individual axons or bundles of axons through the control of microchannels. Siddique and Thakor (2013), developed a model to manually induce neural injury within a compartmentalised device and administer isolated treatments in a biomimetic 3D environment (Siddique and Thakor, 2013). Ghannad-rezaie et al. (2012), generated a microfluidic CNS device that enabled live imaging of the neural response to axonal injury in Drosophila larvae that was induced via application of a UV laser for 5 seconds (Ghannad-rezaie et al, 2012). Other axotomy models within the microfluidic system have used pinching of the nerve fibres in situ with forceps to generate injury, vacuum-assisted axonotomy (Sala-Jarque et al., 2020) and chemical injury (Taylor et al., 2005).

Although neural microfluidic devices can be useful platforms for studying neural injuries within a relatively complex model of neural anatomy compared to other in vitro cellular models, they still lack the cellular microenvironment niches that arise from larger 3D tissue structures. Additionally, these models are predominantly employed for the study of pathomechanisms or drug development purposes rather than interactions with biomaterials. These systems are particularly beneficial in developing personalised medicine, as brain-on-chip devices can be produced using patient specific iPSC cells to generate the neurons. However, with regards to biomaterial development as an implantation therapy, microfluidic devices are not yet suitable for this application.

As an alternative, engineered 3D neural tissues, such as cortical cells embedded in hydrogels or seeded in polymer scaffolds, can be generated with animal or human cells.Hydrogels can be modulated to match the endogenous tissue stiffness of the brain which can change depending on area (3-10 kPa) (Juge et al., 2016) and upon injury. Prager et al. (2020) demonstrated an efficient clinical method to match collagen hydrogel stiffness to the measurable stiffness of an injured canine spinal cord using ultrasound elastography. This would be useful for generating an efficient 3D modelling platform and for matching bioimplant stiffness with target tissue. Liaudanskaya et al. (2020), reported a controlled impact study on a 3D cortical neuronal culture, using a controlled impact device similar to that used in in vivo models. Others involve calcium dependant injury and oxygen/glucose deprivation injury on 3D constructs of primary human NSCs (Vagaska et al., 2020) or the use of a compression device on cortical neuronal 3D hydrogels (Bar-Kochba et al., 2016). These 3D brain-like culture systems can mimic many aspects of in vivo responses, providing evidence that they can be used to study the molecular, cellular, and functional dynamics of TBI, opening up new possibilities for discovery of therapeutics. However, for biomaterial study, a specific lesion cavity within these cellular-hydrogel models must be generated for implantation and further assessment within an injury environment. While there are several injury mechanisms investigated within 3D constructs, a 3D neural brain-like tissue model that studies penetrating injury and biomaterial implantation has not yet been reported.

**12.2.7 Future directions to enhance the translational utility of pTBI models**

Whilst substantial progress has been made in developing 3D models of traumatic injuries, there is still potential to develop more advanced systems for biomaterial testing specifically: (i) introduction of other non-neural but key cellular and tissue components in pathological response and biomaterial handling; (ii) spatial precision in mimicking the cytoarchitecture of the nervous system; (iii) reproducible scale up for high-throughput testing and (iv) developing 3D analytical tools to examine spatiotemporal cellular responses throughout the model. Cutting edge technologies are now being developed which could address some of these issues and further advance in vitro modelling of pTBI processes.

Brain microvasculature and the BBB are key physiological features in the response to neurological insult and injury processes, yet most models lack these. Introduction of vasculature into 3D systems, not only more faithfully replicates native tissue, but also allows perfusion of the tissue with nutrients, enhancing longevity. Further, there is the potential to add circulating immune cells which are vital in pathological progression post-TBI. Studies are starting to demonstrate that vasculature can be introduced in 3D culture systems (both organoid and scaffold-based) using a number of methodologies. Co-culture of pericytes, endothelial cells and astrocytes in a 3D spheroid was demonstrated to spontaneously produce neurovasculature-like structures (Urich et al., 2013). Tubular structures can also be templated into 3D scaffolds and seeded with endothelial cells. The surface of the tubes then mimics the surface of the vasculature with the capability to grow neural cells around these tubes (Galpayage Dona et al., 2021). Templating structures into 3D scaffolds requires subsequent seeding of cells into different areas of the scaffold. As an alternative, advances in 3D bioprinting facilitate bio fabrication of complex cellular structures, including vessel structures for vascularisation (Galpayage Dona et al., 2021).

Building on 3D printing of vasculature, there is a lot of excitement around 3D printing to engineer constructs matching the cytoarchitecture and physicochemical properties of the nervous system. 3D printing allows for precise spatial control over single and/or multiple polymer arrangements in the same structure. Several scaffold stiffnesses can be printed to match the physical properties of target tissue, and different ECM proteins (or mimics) can be used to match tissue-specific chemical properties (Liu et al., 2021). Polymers can be rapidly and reproducibly printed for scalable, high throughput production of scaffolds. Some studies have shown that neural cells can be bio printed with high viability (Liu et al., 2021; Yao et al., 2022; Rouleau et al., 2023). Encapsulation of patterned growth factors in constructs has also allowed control over neural cell differentiation in 3D bioprinter scaffolds (Ilkhanizadeh et al., 2007). The area is still in its infancy with respect to developing neural tissue models, especially neurological injury models, but appears a highly promising area for future research.

As mentioned in section 12.1.5, electrophysiological data provides functional readouts after biomaterial implantation. However, most recording techniques are complex (single cell patch clamping) or developed for 2D systems (MEAs) limiting their utility for high-throughput 3D model systems. A more traditional technique of electrical readout is calcium imaging, but this is limited by optical penetration into 3D structures and field of view for large scale cultures. New bioelectronic platforms are needed for the study of multicellular CNS models and injury-induced pathologies. These must capture network wide neuronal activity within dense mixed cultures of neurons and glia in 2D or 3D formats, to support the assessment of injury development and functional readouts of post injury regeneration after biomaterial implantation. The application of MEAs is considered an important paradigm for detailed mapping of functional damage and time-dependent evolution of injury (Krishna et al., 2020), so we believe this will be essential for assessing recovery of function. There are now reports of 3D MEAs, with electrodes positioned throughout tissue-engineered brain structures (Shin et al., 2021; Soscia et al., 2020). Cutting edge developments in soft, nanoelectronics are demonstrating mesh electrodes that can be integrated with organoid systems for dual recording and stimulation (Li et al., 2022). One example shows a 3D organoid forming around an electrode mesh for generation of recording traces throughout the depth of a hiPSC derived cardiac organoid (Li et al., 2019). In addition, Heidemann et al. (2014) successfully cultured organotypic spinal cord slices on MEAs for at least three weeks by using the roller-tube method. In these experiments, two slices were cultured adjacent to each other. Connections were formed between the two separate organotypic spinal cords which lead to synchronised bursts between the slices. These connections were then severed and this disrupted the synchronised signal. Over time they observed the return of synchronisation of electrophysiological activity as the slices regenerated and re-connected. Kang and Morrison (2015) also developed an advanced MEA system which allowed measurement of electrophysiological activity before and after mechanical deformation (stretch injury) of living organotypic hippocampal brain slice cultures at tissue strains and strain rates relevant to traumatic brain injury. A novel challenge is to combine such systems with 3D pTBI models to allow real-time, functional data generation in the most complex in vitro TBI models currently available.

Advanced proteomic techniques are also now being applied to biomaterial testing. We have employed an unbiased, SWATH (Sequential Window acquisition of All THeoretical mass spectra) mass spectrometry approach to evaluate safety of nanoparticle mediated genetic engineering of neural cell populations. We found only a few proteins demonstrated altered expression in engineered compared to non-engineered neural stem cells, indicating the safety of the procedures (Finch et al., 2020). If adapted to in vitro neural models, such techniques could provide global and unbiased assessments of pathological processes, proteomic disturbances upon application of biomaterials and insights into mechanisms underpinning any regeneration observed upon biomaterial implantation.

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