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Master Thesis

Extraction of Mouse Brain Extracellular Matrix by Decellularization for Porous Scaffold Fabrication

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Abstract

The extracellular matrix (ECM) is a complex of proteins and proteoglycans that contributes to normal physiology of tissues. The ECM of the Central Nervous System (CNS) is important for multiple processes, including basal lamina formation, neural communication and synaptic plasticity stabilization. Alterations to ECM due to injury (e.g. spinal cord injury, stroke) or disease (e.g. Alzheimer's disease) can have dramatic consequences. Since ECM can simulate cells, acellular biologic scaffolds are used for tissue remodeling after damage and have promising applications in CNS regenerative medicine. In this master thesis a new decellularized process was developed in order to purify decellularized brain ECM from adult mouse brain tissue. Decellularized tissue was broken in pieces and lyophilized in order to produce ECM powder, which is a raw material appropriate for scaffold fabrication. The obtained decellularized brain ECM was characterized for its bio-compability and toxicity and demonstrated neuroprotective actions when used as a medium supplement in PC12 and NSC34 cell lines *in vitro*. Furthermore, the obtained decellularized brain ECM was able to regulate the adhesion of primary Embryonic Cortical Neural Stem Primary Cells (NSCs) without affecting their survival or differentiation. These results suggest that the obtained decellularized brain ECM provides neuro-protective ECM components, which can be used for fabricating porous scaffolds and potentially advance NSC-based grafts for CNS regenerative medicine applications.

Ελληνικό Abstract:

Η εξωκυττάρια μήτρα (ECM) είναι ένα σύμπλεγμα πρωτεϊνών και πρωτεογλυκανών που συμβάλλει στη φυσιολογική φυσιολογία των ιστών. Το ECM του Κεντρικού Νευρικού Συστήματος (ΚΝΣ) είναι σημαντικό για πολλαπλές διεργασίες, συμπεριλαμβανομένου του βασικού σχηματισμού λαμινών, της νευρικής επικοινωνίας και της σταθεροποίησης της συναπτικής πλαστικότητας. Οι μεταβολές του ECM λόγω τραυματισμού (π.χ. κάκωσης νωτιαίου μυελού, εγκεφαλικού επεισοδίου) ή ασθένειας (π.χ. νόσος Αλτσχάιμερ) μπορεί να έχουν δραματικές συνέπειες. Δεδομένου ότι ECM μπορεί να προσομοιώσει τα κύτταρα, αποκυτταροποιημένα βιολογικά ικριώματα χρησιμοποιούνται για την αναδιαμόρφωση ιστών μετά από βλάβη και έχουν πολλά υποσχόμενες εφαρμογές στην αναγεννητική ιατρική του ΚΝΣ. Σε αυτή την μεταπτυχιακή διατριβή αναπτύχθηκε μια νέα διαδικασία αποκκυταροποίησης, προκειμένου να απομονωθεί η εξωκυττάρια μήτρα του εγκεφάλου ποντικού από ενήλικο ιστό. Ο αποκυτταροποιημένος ιστός ήταν σπασμένα σε κομμάτια και λυοφιλοποιημένα, προκειμένου να παραχθεί σκόνη ECM, η οποία είναι μια πρώτη ύλη κατάλληλη για την κατασκευή ικριωμάτων. Η ληφθέντα αποκυτταροποιημένη μήτρα (ECM) του εγκεφάλου χαρακτηρίστηκε για τη βιο-ανταγωνιστικότητα και την τοξικότητά του και έδειξε νευροπροστατευτικές δράσεις όταν χρησιμοποιείται ως μέσο συμπλήρωμα σε PC12 και NSC34 κυτταρικές σειρές in vitro. Επιπλέον, η αποκυτταροποημένη εξωκυττάρια μήτρα του εγκέφαλου (ECM) ήταν σε θέση να ρυθμίσει την προσκόλληση των πρωτοπαθών εμβρυϊκών νευρικών βλαστικών πρωτογενών κυττάρων (NSC) χωρίς να επηρεάσει την επιβίωση ή τη διαφοροποίησή τους. Τα αποτελέσματα αυτά δείχνουν ότι η ληφθείσα αποκυτταροποιημένη μήτρα του εγκεφάλου παρέχει νευρο-προστατευτικά συστατικά ECM, τα οποία μπορούν να χρησιμοποιηθούν για την κατασκευή πορωδών ικριωμάτων και ενδεχομένως εκ των προτέρων NSC με βάση μοσχεύματα για εφαρμογές αναγεννητικής ιατρικής του ΚΝΣ.

Table of Contents

1. Introduction

1.1 Introduction

The Extracellular Matrix (ECM) is a complex of biopolymers (including fibrous proteins and proteoglycans) that forms the insoluble part of tissues that surrounds cells. In the CNS, ECM corresponds to 20% of the total central nervous system (CNS) volume (Lau et al. 2013). CNS ECM has been shown to stimulate neural cell growth, survival or activation of molecular pathways (Waele et al. 2015). ECM components are secreted by cells. ECM remodeling is carefully regulated as alterations in the ECM are key aspects of the pathophysiology of several diseases.

Brain ECM has been shown to regulate the phenotypes of multiple kinds of neuronal cells, including Neural Stem Cells (NSCs), neurons and glia. Accordingly, brain ECM is considered a promising raw material for fabricating biomaterials for important applications including 3D cell culture and grafts for the treatment of CNS injuries.

This thesis focuses on developing a decellularization protocol for isolating mouse brain ECM tissue, which subsequently can be used as raw material for fabricating porous collagen-based scaffolds doped with brain ECM components. Chapter 1 presents the physiology and pathophysiology of brain ECM (section 1.2), an overview of published protocols for decellularization of brain tissues (section 1.3), key applications of decellularized brain tissue (section 1.4) and existing methods for porous collagen scaffold fabrication (section 1.5). Finally, section 1.6 summarizes the key results and findings described in the following chapters.

1.2 Physiology and Pathophysiology of Brain Extracellular Matrix

1.2.1 CNS ECM in physiology

ECM is a supportive structure in CNS, providing a functional and beneficial environment for synaptic plasticity, neuron myelination, and cell survival. ECM proteins are widely expressed in the developing and adult nervous system. ECM proteins in the brain are localized in three kinds of structures: the basement membrane (basal lamina), perineuronal nets and the neural interstitial matrix (Lau et al. 2013). **Basal lamina** is the first layer after the brain-blood-barrier (BBB) which also interacts with and supports BBB. Located between endothelial cells and parenchyma, basal lamina provides special molecular cues for cell maturation and surrounds the outer boundary of CNS. Basal lamina consists of collagen IV, fibronectin, laminin, dystroglycan and perlecan (Wang et al. 2007). **Perineuronal nets** (PNs) are mesh-like complexes that surround cell bodies and dendrites. PNs consist of tenascins, proteoglycans (PGs) and link proteins. PNs regulate synaptic plasticity, support neural cells, stabilize the formation of synapses and connections and regulate cell signaling (Kwok et al. 2011; Dauth et al. 2016). The **neural interstitial matrix** consists of molecules (PGs, hyaluronan, tenascins, link proteins and small amounts of fibrous proteins) spread out in the parenchyma tissue (Lau et al. 2013).

CNS ECM proteins regulate CNS cells in diverse ways. They contribute to cell signaling through binding to specific membrane receptors or stimulating intracellular signals in complex with growth factors (Brizzi et al. 2012). Through such mechanisms, ECM can regulate key cell phenotypes including survival, proliferation, differentiation, migration and axon growth (Brizzi et al. 2012, Kwok et al. 2012, Andrews et al. 2009). For example, chondroitin-sulfate proteoglycans (CSPGs) and heparin-sulfate proteoglycans (HSPGs) bind to integrin and arginine receptors, promoting cell signaling and affecting Long Term Potentiation (LTP) in the hippocampus (Bukalo et al. 2001). Each ECM protein has specific roles in supportive CNS, differentiation, regeneration and plasticity in the CNS (Kwok et al., 2012). For example, Tenascin-C (TN-C), the most abundant glycoprotein in the

CNS ECM during development, has various roles in the developing and the mature CNS, acting as a growth-promoting molecule, promotes neural precursor proliferation, migration along with axonal extension, guidance, and growth cone formation (Andrews et al., 2009). Resent results suggest that PGs like aggrecan, brevican and tenascin-R regulate brain development, plasticity and neurite outgrowth (Dauth et al. 2016). ECM proteins are capable of regulating Oligodendrocyte Progenitor Cell (OPC) differentiation and proliferation. Brain ECM proteins from neonatal rats significantly increased proliferation and differentiation in adult rat OPCs compared to adult OPCs seeded in ECM from adult rats or neonatal OPCs cultured in ECM from aged rats (Segel et al. 2019). Furthermore, a variety of ECM proteins (laminin, fibronectin, fibrin, and collagen) has been shown to enhance cell adhesion (Barros et al. 2011).

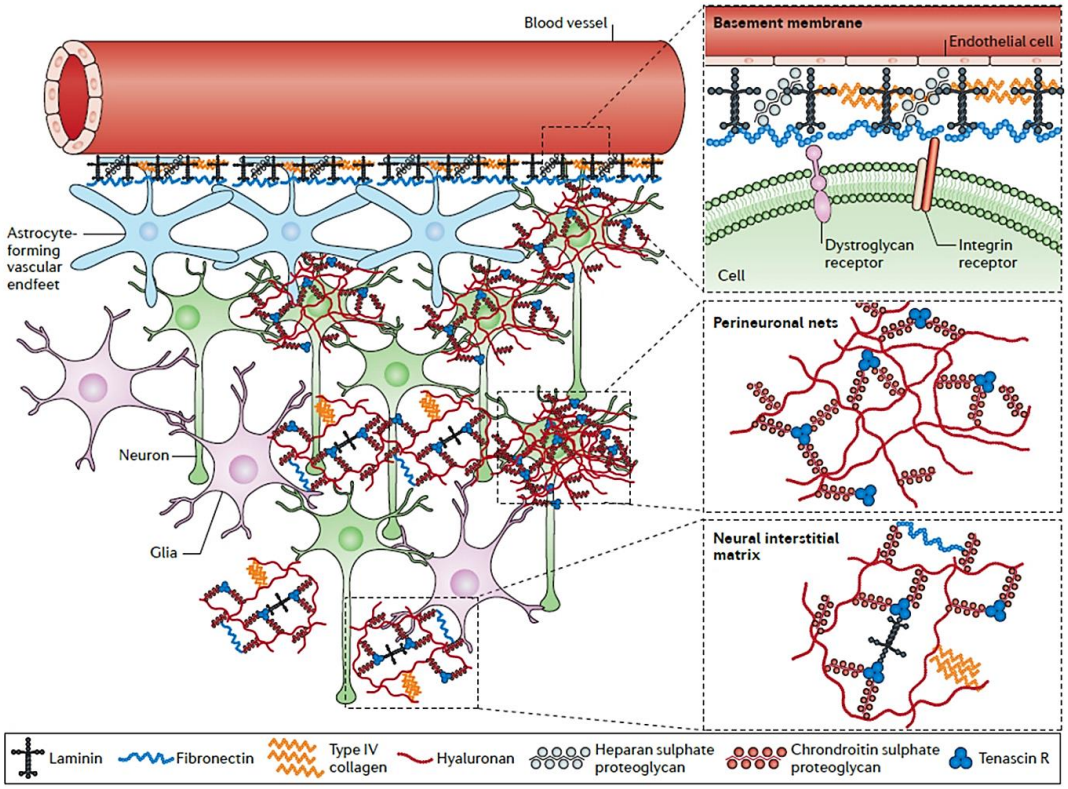


Figure 1.1: Schematic of the three kinds of ECM structure in the CNS: basal membranes, perineuronal nets and neural interstitial matrix. Each ECM layer has a unique composition and structure. Basal membrane communicates with glia, neurons and the BBB. Perineural nets surround neurons and regulate synaptic plasticity. The neural interstitial matrix provides a loose structure in mesenchyme tissue between cells (Lau et al. 2013).

1.2.2 CNS ECM in pathology

There is strong evidence that neurodegenerative diseases (AD, schizophrenia, autism, depression and temporal lobe epilepsy) alters CNS ECM. Alzheimer disease (AD) induces changes in cell surface PGs and/or ECM components. In Dutch people, HSPG composition was associated with β-amyloid senile plaques, neurofibrillary tangles and with vascular deposit of A β plagues (Horssen et al. 2001). Another example is the significant reduction of reelin glycoprotein, important for multiple functions in CNS like synaptic plasticity, and long-term potentiation (LTP) in multiple neurodegenerative diseases (Fatemi et al. 2005). Furthermore, in aged-deficient mice was detected reduced extracellular space in the brain of tenascin-R- and human natural killer 1 (HNK-1)- sulphotransferase (Sykova et al., 2005). The same study proposed that aging is associated with changes in the ECM diffusion parameters that reduce the expression of hippocampal ECM molecules, particularly CSPGs.

Alteration of CNS ECM after injury: Brain ECM provides homeostatic pro-survival function after CNS injury. Changes in brain ECM induced by injury (like ischemia, stroke) or aging can damage axons, stop synaptogenesis and increase inflammatory response (inflammatory cells and adhesion molecules in the lesion side). During CNS injury, CNS ECM proteins are digested by enzymes including Metalloproteases (MMP2/9), disintegrin and metalloproteinases (ADAMs) and the serine proteases plasminogen/plasminogen activator (Candelario et al. 2009). MMPs are involved in detachment of basal lamina in cerebral ischemia (Candelario et al. 2009). Brain injury usually leads to degradation of ECM proteins, or blocking ECM proteins from the lesion side, which cause no contribution of ECM in the injury area. Furthermore, after brain injury, CSPGs are actively secreted into the ECM, mainly by reactive astrocytes and neurons (Lau et al. 2013). This leads to an abundance of CSPGs at the injury site, providing an inhibitory environment for neuron regeneration. The main feature contributing to the inhibitory environment is that CSPGs provides their structure and the number of glycosaminoglycan (GAG) side chains. Each CSPG has a various number of side chains (Knock et al. 2011).

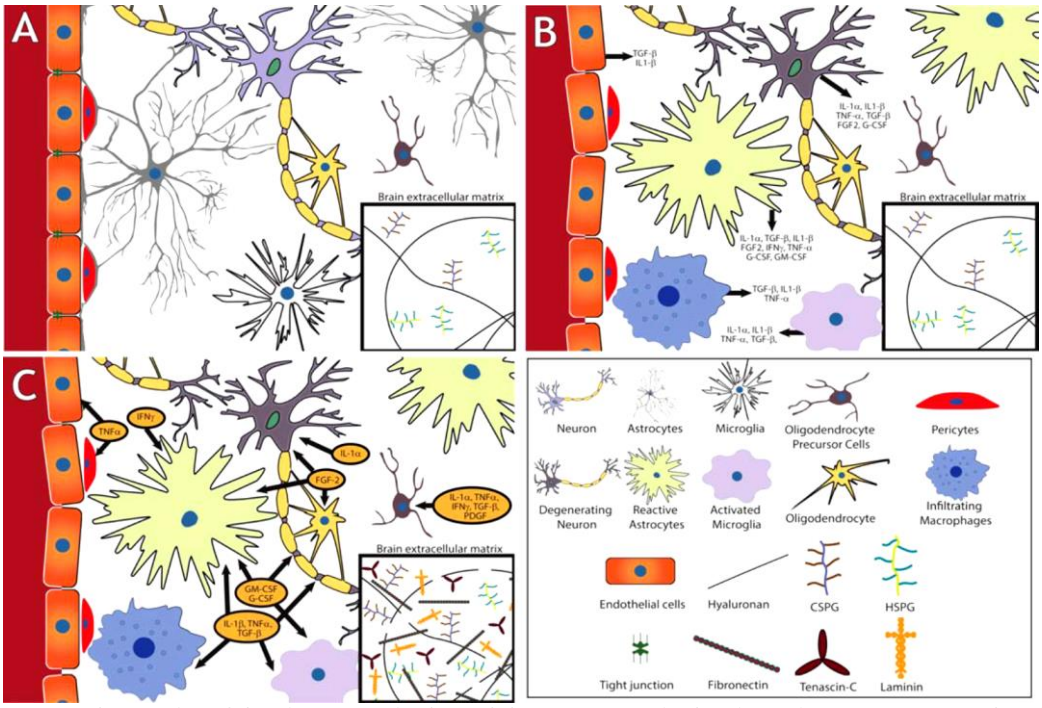


Figure 1.2: Schematic of brain ECM induced by Traumatic brain Injury (TBI). A) Physiological connections of ECM components with glia and neurons. B) Changes of ECM after TBI and the beginning of inflammatory response. C) Changes in cellular communication between cells and ECM proteins (Naijil et al. 2018).

1.3 Decellularization of Brain Tissue

1.3.1 Tissue Decellularization Overview

Decellularization is the process of removing the allogeneic or xenogeneic cellular antigens from a tissue that would initiate an immune response while leaving behind an intact ECM comprising a mixture of structural and functional molecules. The result of decellularization is also referred to as acellular or decellularized matrix (Srokowski et al. 2017).

Multiple decellularization methods have been reported for a variety of tissues (Keane et al. 2015). Published tissue decellularization protocols can be classified in four main categories: physical methods, detergent-based methods, chemical agent-based methods and enzymatic digestion methods.

Physical methods apply freeze-thaw cycles or mechanical osmosis in order to remove cells from the tissue and leave structural proteins (Buckenmeyer et al. 2020). Detergent-based methods remove cells by applying detergents. TritonX-100 (TX100) seems to be the most effective detergent for decellularization compared to Sodium Dodecyl Sulfate (SDS) and Sodium Deoxycholate (SDC) as TritonX-100 doesn't disrupt ECM components (Vavken et al. 2009). Chemical agent methods utilize alcohols (like ethanol), acids (like peracetic acid) and zwitterionic detergents (Keane et al. 2015). Enzymatic digestion methods utilize enzymes like DNases, RNases, trypsin, EDTA, pepsin to remove nucleic residuals and cell contents. EDTA and Trypsin are combined with detergents for more effective cell removal (Buckenmeyer et al. 2020).

Literature reports effective decellularization protocols for several tissues (including heart, spleen, vessels, kidney, brain), which usually utilize combinations of the abovementioned methods. For example, a published protocol for the decellularization of porcine vessels utilized hypotonic breakdown, multiple enzymatic digestions and detergent treatments, mechanical means as well as a heparin treatment in order to prevent clotting (Conklin et al. 2002).

1.3.2 Protocols for Brain Decellularization

This section summarizes published protocols for brain decellularization, some of which are summarized in detail in a recent review (Keane et al. 2015). These protocols formed the basis for the procedures utilized in this study.

Crapo et al. decellularized porcine brain samples by incubating them 1 h in 0.05% trypsin-EDTA in PBS at 37°C, 1h in 3% tritonX-100 in PBS, 15 min in 1 M sucrose, 15 min in dH_2O, 1 h in 4% SDC, 2h in 0.1% peracetic acid 4% ethanol, and finally washed in dH_2O overnight at 4oC. (Crapo et al 2012). A similar method that worked well for fetal brain decellularization includes a 0.2% DNase I treatment and a lyophilization step (Sood et al. 2016).

De Waele et al. decellularized mouse brain samples by treating 1.5mm-thick brain slices (in 48-well plate wells) in three cycles, each cycle consisted of dH_2O, 4% SDC, DNase in 1 M NaCl, dH_2O, 3% TritonX-100, DNase in NaCl and finally dH_2O (De Waele et al. 2015).

Zhu et al. 2015, reported an effective protocol that provides acellular mouse cerebral tissue that was utilized as a scaffold for *in vivo* applications. After transcardial perfusion with a 1% SDS, the cerebellum was isolated and treated with 1% SDS, dH_2O, 0.02% trypsin/0.05% EDTA, dH_2O, 1% Triton X-100, 1 M sucrose and dH_2O.

Beachley et al. decellularized bovine brain samples. After an initial 3 h incubation in peracetic acid, samples were cut into 5 mm-thick slices. Slices were incubated 18 h in 1% TritonX-100 2 mM EDTA, 18 h in 600 U/ml DNase 10 mM Mg_2Cl at 37°C, washed in dH_2O and lyophilized. Particles were further broken in a cryo-mill, sonicated, filtered and suspended in 100 mg/ml HEPES (Beachley et al. 2017).

Lam et al. reported decellularization of rat brain samples. Tissue samples were snap-frozen, incubated 72 h in MiliQ H_2O, 60 min in 1% TritonX-100, 30 min in dH_2O, 60 min in 4% SDC, 30 min in dH_2O, 60 min in 0.2% DNAse I in 1 M NaCl, and 30 min in dH_2O. The resulting tissue was lyophilized and then incubated in 1 mg/ml pepsin in 0.1M hydrochloric acid for 24-48 h at RT. Primary neuron basal medium was added to the resulting ECM (1:1 ratio), neutralized with 1 M NaOH and then diluted with basal media in order to obtain a final stock of 1–1.2 mg/ml (Lam et al. 2017).

Beachley et al. decellularization porcine brain in order to fabricate 2D and 3D ECM tissue microarrays via four different decellularization protocols: 1) 10 min peracetic acid & 18 h DNAse, 2) 3 h peracetic acid, 18 h Triton X-100, 18 h DNAse, 3) 3 h peracetic acid, 18 h Triton X-100, 18 h

DNAse, a 16 h SDS treatment and 4) 3 h peracetic acid, 18 h Triton X-100, 18 h DNAse plus pepsin digestion (Beachley et al. 2015).

Granato et al. decellularized mouse brain using SDS and freezing/thawing cycles. The resulting tissue was used as a 3D scaffold for neuronal cell culture (Granato et al. 2019). In each freezing/thawing cycle, mouse brain was soaked in liquid nitrogen for 10 min and then thawed in room temperature in PBS for 5 min. After 3 cycles, the resulting tissue was soaked in 1% SDS agitated for 24 h.

	Tissue	Physical methods	Detergents	Chemical agents	Enzymatic digestion	Applications
Guo et al. 2010	Rat Spinal Cord		1% TX100 1% SDC	dH_2O		
Crapo et al. 2012	Porcine brain		3% TX100 4% SDC	dH_2O 1 M sucrose 0.1% peracetic acid 4% ethanol	0.05% trypsin-EDTA	
Baiguera et al. 2014	Rat brain	Freeze-thaw	1% TX100 4% SDC	dH_2O	2000kU DNase	Hydrogel fabrication for ECM recellularization
De Waele et al. 2015	Mouse brain		4% SDC 3% TX100	1 M NaCl	DNase	
Zhu et al. 2015	Mouse cerebrum		1% SDS 1% TX100	1 M sucrose	0.02% trypsin 0.05% EDTA	Scaffolds for *in vivo* applications
Sood et al. 2016	Fetal porcine brain		3% TX100 4% SDC	dH_2O 1 M sucrose 0.1% peracetic acid 4% ethanol	0.2% DNase	3D ECM-Collagen gel
Beachley et al. 2015	Porcine brain		1% TX100 SDS	peracetic acid	DNAse pepsin	2D & 3D ECM tissue microarrays
Beachley et al. 2017	Bovine brain		1% TX100	dH_2O peracetic acid	600U DNase 2mM EDTA 10mM Mg_2Cl	ECM-glycosaminoglycan composite hydrogels
Granato et al. 2019	Mouse brain	Freeze-thaw	1% SDS			Scaffolds for neuronal cell culture
Lam et al. 2019	Postnatal Rat brain		1% TX100 4%SDC	dH_2O	0.1% DNase I	ECM coating for cell culture

Table 1.1: Overview of published research on CNS decellularization protocols.

1.3.3 Characterization of Decellularized Brain Tissue

Characterization of decellularized brain tissue focuses on two major questions. First, provide evidence that all cellular content has been removed. Second, quantify the chemical composition of the resulting acellular tissue.

Most published studies characterize the extent of tissue decellularization via immunohistochemistry (IHC). Results in decellularized samples are compared against results in untreated (non-decellularized) control brain samples. Most studies use histology stains [e.g. Hematoxylin-Eosin (H&E)] or fluorescence dyes (e.g. DAPI) to detect cell nuclei in thin slices of decellularized brain samples (Guo et al. 2010, Yuan et al. 2011, Crapo et al. 2012, Zhu et al. 2015, Waele et al. 2015, Beachley et al. 2017, Granato et al. 2019). Other studies characterize the extent of tissue decellularization by extracting mRNA extracted from decellularized brain and quantifying DNA content by PCR (Waele et al. 2015; Lam et al. 2019). Granato et al. 2019 isolated DNA and RNA from decellularized tissues and performed nucleic acid amount. Finally, certain papers quantify dsDNA content in decellularized tissues using dyes that fluorescence upon binding to dsDNA, including the PicoGreen fldsDNA nucleic acid stain (Zhu et al. 2015).

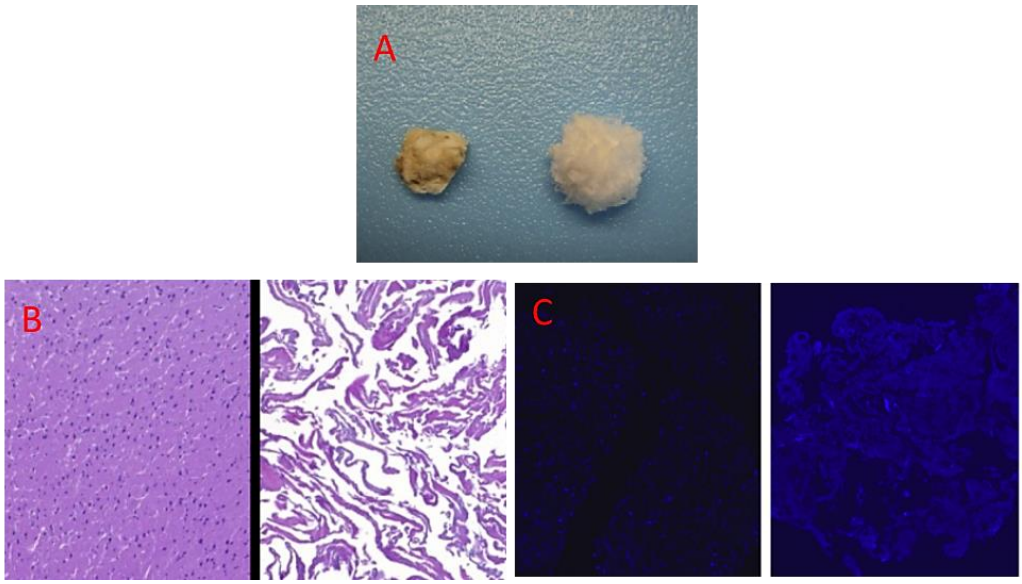


Figure 1.3: Characterization of decellularized porcine brain samples. A) Native brain tissue (left) and decellularized brain (right). B) Representative images of native brain tissue (left) and decellularized brain tissue (right) sections after H&E staining. Nuclei are detected in native brain tissue but not in decellularized brain. C) Representative fluorescence images of native brain tissue (left) and decellularized brain (right) after DAPI staining. (Crapo et al. 2012)

The chemical composition of the resulting decellularized tissue can be characterized by IHC and proteomics, including electrophoresis-based methods and mass spectroscopy. IHC can quantify the presence of important ECM components in decellularized CNS tissue, including laminin and CSPGs (Waele et al. 2015; Zhu et al. 2015; Beachley et al. 2017). Several ECM components can be detected using histological stains including sulfated glycosaminoglycans [by Alcian blue / Periodic Acid-Schiff (PAS) staining] and collagen (by Masson's trichrome staining) (Zhu et al. 2015).

Proteomic characterization of decellularization tissue via electrophoresis is challenging because ECM components are cross-linked and form aggregates. Pepsin digestion prior to electrophoresis has been shown to enhance the detection of high molecular-weight ECM proteins (Beachley et al. 2015; Sood et al. 2016; Lam et al. 2019).

In the MIT Matrisome project[1], ECM-enriched fragments from tumors with human melanoma cells, were prepared for mass spectrometry (Naba et al. 2012): Enriched ECM fragments were incubated for 30 min in solution 1 (8 M urea, 100 mM ammonium bicarbonate, 10 mM DTT, pH 8) at 37°C,

[1] (http://web.mit.edu/hyneslab/matrisome)

then in 25 mM iodoacetamide for 30 min, and in 2M urea 100 mM ammonium bicarbonate. Samples were then de-glycosylated for 2 h in 1000U N-glycosidase F at 37°C before being digested twice by Lys-C and trypsin.

Other means for characterizing the chemical composition of decellularized brain tissues include liquid chromatography and mass spectrometry for protein analysis and proteomic analysis and micro-array for DNA analysis (Beachley et al. 2015).

1.3.4 Converting Decellularized Brain Tissue to Powder

Utilizing decellularized CNS tissue as raw material requires converting the decellularized tissue in some sort of powder form. Several protocols for converting decellularized tissue in powder form have been described in the literature. Sawada et al. decellularized aorta tissue using supercritical CO_2 in and obtained purified ECM powder that can used for biomaterial fabrication (Sawada et al. 2008). Similarly, Gilbert et al. decellularized porcine urinary bladder and turned ECM tissue to powder for future tissue engineering application ECM powder was formed by snap frozen in liquid nitrogen and then comminuted in a rotary knife mill, or by saturated decellularized tissue in a NaCl solution prior to snap freezing and grinding. (Gilbert et al. 2005). In another study (Beachley et al. 2017), decellularized samples were washed in deionized water, lyophilized, stored at -20°C under argon and finally were mechanically broken into particles via cryomilling under liquid nitrogen.

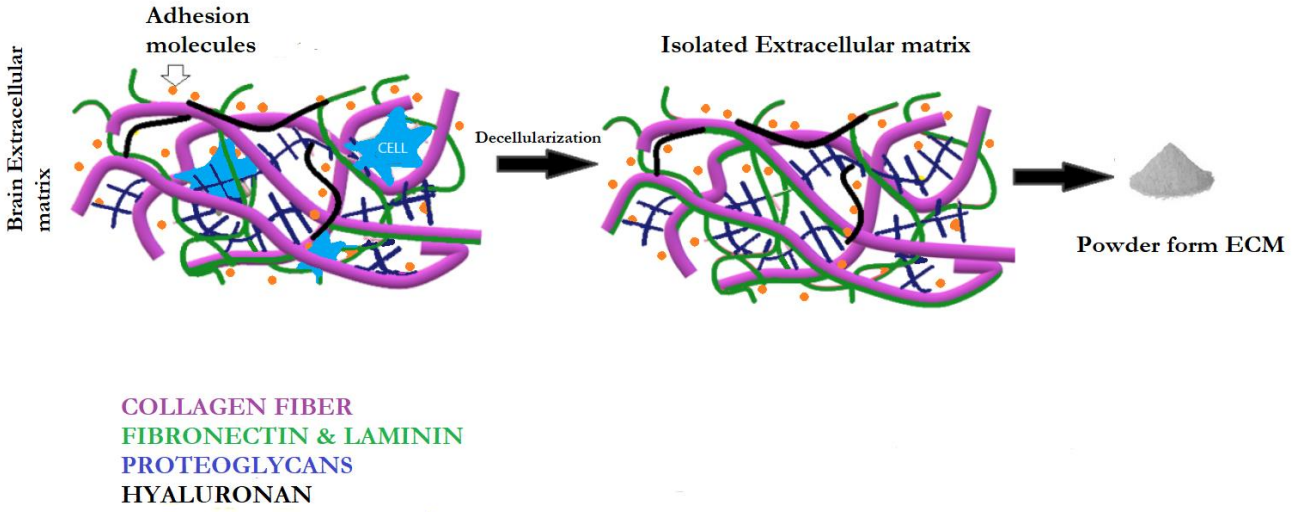


Figure 1.4: Schematic of the 2-step procedure for isolating CNS ECM components in powder form. First, brain tissue is decellularized in order to remove cellular component. Second, decellularized ECM is converted into powder form, appropriate for biomaterial fabrication.

1.4 Applications of Decellularized Brain Tissue

Decellularized brain tissues are utilized in two major applications. First, decellularized tissue pieces can be used as a scaffold for 3D cell culture. Second, purified tissue ECM converted into powder can be utilized as raw material for the fabrication of biomaterials for cell studies or tissue engineering.

Crapo et al. 2012 seeded decellularized porcine brain tissue with PC12 cells and characterized the *in vitro* effects of brain ECM in PC12 cells. After 24h incubation, CNS ECM increased PC12 proliferation and induced chemotaxis, suggesting that cell-free ECM has the ability to interact with cells and regulate them. These results suggest that ECM tissue can provide beneficial environments for cells. Sood et al. 2016 seeded primary rat neurons in a scaffold consisting of decellularized fetal porcine brain tissue and silk. In the resulting scaffold (ECM tissue-silk) cultured with cells they added a mix of decellularized fetal porcine brain tissue and Collagen 1. The 3D ECM-silk-Collagen-scaffolds co-cultured with cells have a morphology similar to white matter and gray matter of brain.

Due to that morphology they considered the scaffold as a 3D bioengineered tissue model of the Cortex.

Zhu et al. 2015 evaluated *in vitro* the effect of decellularized ECM cerebral scaffolds on human NSC proliferation, migration and differentiation. Results were compared against the effects of urinary bladder ECM scaffold. Cerebellar ECM scaffolds increased the rate of NSC mitogenesis by 20% and induced a large chemotactic response. Acellular cerebral scaffolds were implanted subcutaneously and intracranially in rat frontal lobes, which demonstrated their biocompatibility *in vivo*.

The second major application of decellularized brain tissue is to utilize purified brain ECM as raw material for the fabrication of biomaterials including hydrogels and scaffolds.

Multiple research efforts focused on fabricating hydrogels based on purified ECM components.

ECM powder scaffolds can be combined with cells and implanted at injury site of animals. ECM powder provides effective absorption of proteins and efficient seeding of cells in the injury side. Cells can be seeding in powder form of ECM or in a solubilized powder of ECM.

Zhang et al. produced tissue-specific (skin, skeletal muscle, and liver) ECM powder, solubilized it, and used it as a coating substrate for cell culture. Cells from the skin, skeletal muscle, and liver were then cultured in the resulting culture dishes coated with tissue-specific ECM. ECM coating resulted in increased cell proliferation and differentiation compared to cells cultured on uncoated dish (Zhang et al. 2009).

Segel et al. presented the effect of brain ECM hydrogels in functional fate of Olygodendrocyte Progenitor Cells (OPC) isolated from rats. After cultured neonatal ECM (decellularized from rat brain) with neonatal OPC (P7 from rat) they realized an increased proliferation and differentiation compared the OPCs (from rats 3 to 5 months age) seeded in aged ECM, resulting that ECM is implanted the functions of OPCs (Segel et al. 2019).

Beachley et al. cross-lined Chondroitin sulfate and Hyaluronic acid with decellularized porcine brain particles via amine coupling in order to form a GAG–ECM particle hydrogel network. ECM components retained their functional bioactivity and could be utilized for *in vitro* culture of primary mouse ESCs or as implantable grafts (Beachley et al. 2017).

Wu et al. 2017 reported that implantation of a ECM hydrogel (from decellularized porcine brain tissue) in the right parietotemporal area of mouse brain reduced the effects of traumatic brain injury (TBI), including reduced lesion volume, less post-TBI neurodegeneration in the hippocampus, and less microglial pro-inflammatory response (Wu et al. 2017)

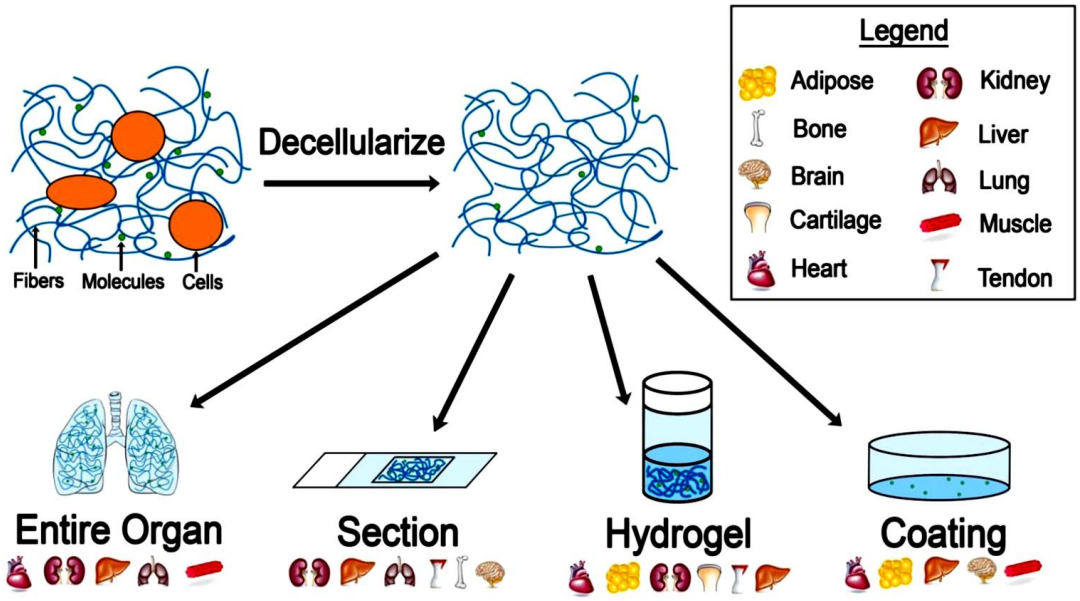


Figure 1.5 Applications of decellularized tissues in tissue engineering. Decellularized ECM can be used in tissue regeneration, or be processed into a section, in a hydrogel or coating. ECM from different tissues are utilized for different application (Among et al. 2016).

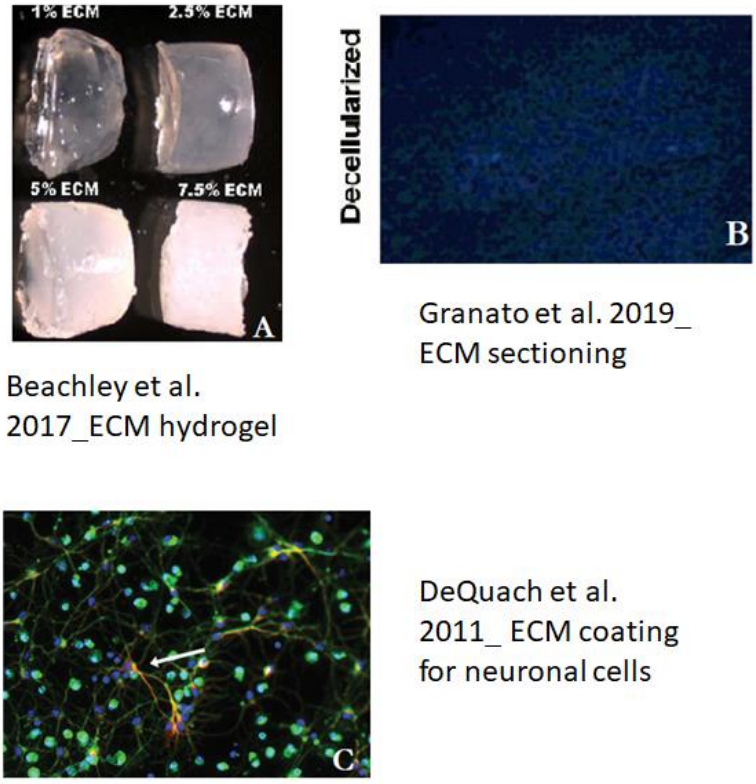


Figure 1.6: Representative images of various applications of decellularized brain tissue.

1.5 Fabrication and Applications of Porous Collagen Scaffolds

Collagen I is a major component of the ECM of many tissues. Purified collagen I has been utilized extensively as raw material for various kinds of biomaterials, including hydrogels and scaffolds. This work emphasizes on a particular type of biomaterials, porous collagen scaffolds (PCS), similar to

scaffold grafts utilized clinically in the induced regeneration of skin and peripheral nerves (Yannas et al. 1982, 1989; Soller et al. 2012). PCS have also been utilized as ECM *in vitro* studies of ECM-cell interactions (O'Brien et al. 2005; Harley et al. 2007). PCS are fabricated by lyophilizing a suspension of microfibillar collagen type I using specific constant cooling rate protocols (O'Brien et al. 2005). Lyophilization (freeze-drying) is a dehydration procedure that provides a dry scaffold, whose mean pore size can be tuned between approximately 20 and 200 μm. The resulting dry scaffold is cross-linked via the Dehydro-thermal treatment (DHT) method, where the scaffold is treated in high temperature (105 °C to 120 °C) under vacuum for 24 to 48 h (Yannas & Tobolsky 1967). DHT cross-linking significantly increases the scaffold resistance to degradation by cell-secreted proteases, and slightly increases the scaffold mechanical stiffness (Harley et al. 2007). The resulting dry PCS is a highly-permeable sponge-like biomaterial.

While collagen I is the most abundant ECM protein of many tissues (Cen et al. 2008), CNS ECM does not include collagen I. Instead, collagen IV is a major component of CNS basal lamina tissues and a key component of the BBB (Lau et al. 2013). Similarly, published PCS designs have not included components of CNS ECM other than chondroitin-6-sulfate. It is reasonable to hypothesize that doping PCS with CNS ECM molecules can provide molecular clues that will favor the interactions of PCS with neural cells including neural stem cells, neurons and glia. Such PCS designs could lead to novel designs of PCS-based grafts for CNS injuries, or novel *in vitro* tissue models.

1.6 Aim of this Study

This study focuses on developing and characterizing methods for isolating the ECM from mouse brain tissue and convert it in powder form, appropriate for the fabrication of porous scaffolds via freeze drying.

Chapter 2 (materials and methods) presents the developed protocols for mouse brain decellularization, techniques for characterizing the resulting decellularized tissue, and protocols utilized to study the effect of purified mouse brain ECM on three neural cell lines. Chapter 3 (results) presents the acquired experimental data. Chapter 4 (discussion) provides a brief interpretion of the results and suggests ways to exploit them in future experiments.

2. Materials and Methods

2.1 Brain Decellularization

Brain tissue obtained from adult C57BL/6 mice, were cut in pieces and placed inside a 2 mL tube. The complete protocol includes two parts: 1) decellularization of brain tissue and 2) solubilizing the resulting ECM products (Figure 2.1).

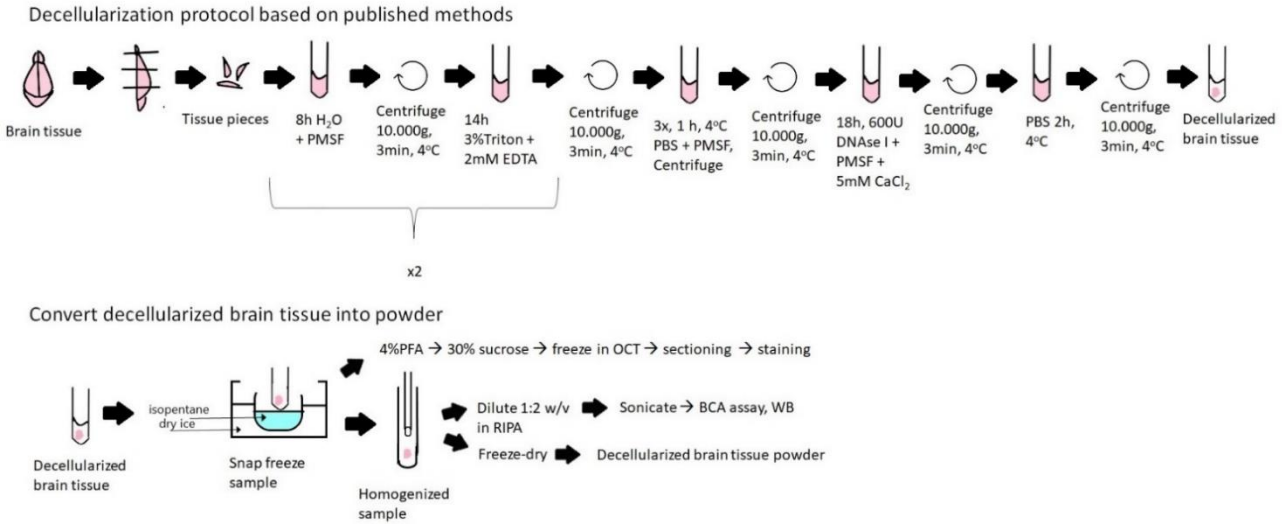


Figure 2.1: Schematic of the protocols utilized in this study in order to prepare and characterize decellularized brain tissue and prepare decellularized brain tissue powder for biomaterial fabrication.

The first part (brain decellularization) started with an 8 hour incubation in H_2O supplemented with 1% PMSF (a serine protease inhibitor) (step 1). Then, samples were incubated for 14 hours in Triton X-100 supplemented with 2 mM EDTA in a cold room (step2). Steps 1 & 2 were repeated once more. Then, samples were washed in PBS 3 times for 1 hour each, treated with DNase at 37°C for 18 hours and finally washed with PBS at 4°C for 2 hours. After each one of the above-mentioned treatments, samples were centrifuged (3 min, 10.000xg at 4°C) and the supernatant was discarded.

The second part aims to solubilize the obtained decellularized brain tissue in order to produce a dry powder that can be used as raw material for scaffold fabrication. Decellularized tissue pieces were immersed and snap frozen in isopentane for 1 min. The resulting frozen tissue pieces were further broken using a dounce homogenizer (50 up and down cycles) placed on dry ice. The resulting powder was transferred in a tube and was lyophilized.

For proteomic characterization (BCA quantification, immunoblotting), decellularized tissue was snap frozen, homogenized and then (instead of lyophilization) was reconstituted in ripa buffer, further homogenized (via a dounce homogenizer; 3 rounds of 2 min up and down) and sonicated (SONICS Virba cell Ultrasonic Processor 72434) for 3 times of 30 seconds at pulsed 40 amplitude. This process (homogenization and sonification) was repeated for 3-4 more times per day, two days in row until samples were fully homogenized (store in between overnight at 4°C). It should be noted that for accurate protein quantification, samples should be totally homogenized and big protein chunks should not be detected via optical microscopy. Only small particle (smaller than the size of a cell) should be detected. Yet, samples should not be sonicated more than 5 times since homogenization leads to significant sample loss. If samples are not fully homogenized and have big protein chunks,

protein quantification of the sample will not be accurate. Control samples for protein quantification were prepared using the same protocol as decellularized samples: control (not decellularized) brain tissue were snap frozen in isopenatane, homogenized in a dounce homogenizer, diluted in ripa buffer and finally further homogenized and sonicated.

2.2 Protein Quantification

The protein concentration of brain lysate solutions (from untreated control or decellularized samples) was measured via the bicinchoninic acid (BCA) assay. Measurements were calibrated using a standard curve derived by quantifying a series of Bovine Serum Albumin (BSA) solutions of known concentration (0, 0.1, 0.5, 0.75, 1 mg/ml) in 1:10 ripa:PBS buffer. 10 μl of each BSA solution or sample were loaded in duplicates in a 96 well plate. Before loading, each sample was vortexed for 1 min. 200 μl BCA working reagent (1:50 reagent B solution: reagent A solution) was then added per well. The 96 well plate was incubated in a 37°C oven for 30 min while covered with aluminum foil. Then, the optical absorbance of each sample at 545 nm was measured using a platereader (ChroMate Awareness Technology; Garinis lab, IMBB). Sample concentration (μg/μl) was calculated based on absorbance measurements of BSA standards, which were utilized to derive a linear calibration curve.

2.3 Immunoblotting

50 μg of each brain lysate sample was diluted in 5x laemmli buffer (0.25M Tris-HCl; 2,5% v/v 2-mercaptethanol; 10% SDS; 0.3 mM Bromophenol blue; 50% glycerol). Samples were heated at 95°C for 10 min and loaded in the wells of 4-20% gradient acrylamide gels (Bio-rad 4561094). The gel was placed in an electrophoresis tank filled with running buffer (0.25 M Trizma Base; 1.92 M Glycine, 1% SDS). Electrophoresis was performed at 50 mA for 60 min. Afterwards, proteins were transferred to 0.45 μm nitrocellulose membranes (health lifecare sciences) by electrophoresis at 300 mA for 120 minutes in room temperature (for proteins in the 11-75 kDa range), or at 30 V for 16 hours (for proteins in the 75-300 kDa range) at 4°C (IMBB common cold room) in a tank filled with cold transfer buffer (0.25 M Tris-HCl; 1.92 M Glycine; 5% Methanol). Membranes were blocked in blocking buffer [5% BSA diluted in TBS-T (20 mM Tris-Cl, pH 7.6; 150 mM NaCl; 0.1% Tween20)] for 1 hour at Room Temperature (RT) and incubated with primary antibody solution overnight at 4°C in a shaking platform. The primary antibody for anti-fibronectin (rabbit; MIT Hynes Lab) was diluted 1:1000 in blocking buffer. The primary antibody for anti-β actin (β-actin, anti-mouse; Santa Cruz Biotechology; sc-47778) was diluted 1:5000 in blocking buffer. The next day, membranes were washed 3 times for 15 min with TBS-T and then were incubated with secondary antibody solution (anti-rabbit for anti-fibronectin and anti-mouse for anti-β-actin at 1:5000 dilution in blocking buffer for 1 hour at RT. After 3 washes of 15 min each, membranes were treated with ECL Western Blotting Kit (Thermo 34087) and imaged using the Bio-rad Chemi Doc imager (IMBB). Protein bands were manually quantified using the Image J software. The analysis of each blot image follows a standard procedure: 1)The command "Analyze>Gels>Select First Lane" is used to highlight the first blot lane. 2) The remaining lanes are then highlighted using the command "second lane". 3) After lane selection, the command "Analyze>Gels>Plot Lanes" draws a plot of the total intensity of each lane. 4) For every plot, the user draws a line to peak of the part of the plot that will be included. 5) The command "Label Peaks" presents the size of every peak, which corresponds to the signal of a particular band of interest.

2.4 Tissue Preparation

Decellularized brain tissues and untreated (control) brain tissues were post-fixed with 4% Paraformaldehyde (PFA) in PBS overnight at 4°C in a 2 ml Eppendorf tube. Samples were then cryo-protected in 30% sucrose in Phosphate Buffer (PB) for 2 days at 4°C. Next, sucrose solution was discarded and samples were immersed in Optical Cutting Temperature (OCT) compound, frozen in isopentane placed on dry ice at -70°C for 5 min. Samples were cut in 10 μm sections at -25°C using a cryotome. Sections were placed in Superfrost slides and were stored at -80°C until use.

2.5 Immunostaining & Histology

Slides with sectioned brain samples were treated with acetone at -20°C for 5 min, air-dried in chemical hood for 10 min, washed twice in PBS for 10 min at RT , were treated in 0.1% TritonX-100 in PBS for 15 min at RT, and then in 0.3% TritonX-100 in PBS for 30 min at RT. Only immunostaining for anti-fibronectin staining, sections were incubated with 10 mM sodium citrate in PBS, pH=6, in oven at 85°C for 10 min. After cooling down to RT for 3 min, slides were washed in 0.1% PBStx (0.1% TritonX-100 in PBS) 15 min in RT, blocked in blocking solution (10% Normal Serum, 0.1% BSA in PBStx) for 1 hour at RT and then were incubated with the corresponding primary antibody overnight at 4°C (antibodies were diluted in blocking solution). The primary antibody for anti-fibronectin (rabbit; MIT Hynes Lab) was diluted 1:250 in 0.1% PBStx. The primary antibody for anti-laminin (rabbit; Sigma; l3939) was diluted 1:100 in 0.1% PBStx.The next day, slides were washed 3 times with 0.1% PBStx for 15 min, and then were incubated with the corresponding secondary antibody solution (diluted 1:1000 in 0.1% PBStx) for 1 hour at RT. After 1 wash in 0.1% PBStx for 15 min at RT and 1 wash in PBS for 15 min, slides were incubated for 15 min with Hoechst stain (1μg/ml in PBS) at RT. Slides were then washed twice in PBS for 15 min at RT, washed once in PB for 15 min at RT and covered with a glass coverslip after applying Vectashield mounting medium. The coverslip was fixed using nail polish.

The number of nuclei in stained sections were counted using the "Analyze particles" tools of Image J. Prior to counting, the Hoechst channel of acquired images was converted to a binary image using the "Threshold" command and further processed using the "Binaly-> Wathersheet" tool. For each section the average of nuclei number was calculated. For decellularized and control brain 5 tissues were stained with Hoechst and 10 sections were imaged of every group.

2.6 Lyophilization

After homogenization of decellularized tissue in dry ice decellularized tissues were diluted and homogenized in 50mM acetic acid. 100 mg decellularized tissue was reconstituted in 250 μl 50 mM acetic acid. Gentle pipetting was required to disperse decellularized brain in acetic acid. Solubilized decellularized brain sample in acetic acid was transferred in glass vials for freeze-drying, see Fig. 2.2. Reconstituted samples were kept at 4°C (short term, until lyophilization) or in -20°C (long-term storage). Decellularized samples were lyophilized in a CRIST LMC2 lyophilizer (IMBB-FORTH). Samples in vials were placed on the lyophilizer shelf set at -10°C. During pre-freezing, shelf temperature was kept at -22°C for 1 hour. During primary drying, the shelf was kept at -18°C for 24 hours and pressure was reduced (via a vacuum pump) to less than 100 Pa. After primary drying, temperature was set at 25°C for 2 hours in order to remove any remaining humidity in the bottle (secondary drying). Finally, lyophilization stopped and shelf pressure was set at 1 atm so that the

lyophilizer door can open. This freeze–drying procedure results in decellularized brain ECM inside bottles in powder form. The outcome of lyophilization is presented in figure 2.3

Figure 2.2: Glass Vial utilized in decellularized brain tissue Lyophilization.

Figure 2.3: Decellularization brain tissue after freeze-dry pocess in glass vial tube.

2.7 Cell culture

All cell manipulations were performed in a laminar air flow hood in order to maintain sterility conditions.

PC12 cell culture

The PC12 cell line was originally derived from a rat pheochromocytoma of adrenal medulla. PC12 have embryonic neural crest origin (a mixture of neuroblastic cells and eosinophilic cells) (Wiatrak, et al. 2020). PC12 cells were grown in full medium [high glucose Dulbecco's Modified Eagles Medium (DMEM) (Thermo 41966029) supplemented with 10% horse serum (HS, Thermo16050122), 5% fetal bovine serum (FBS, Thermo 10270106, 100 units/ml penicillin and 0.1 mg/ml streptomycin (Thermo 15140122)] in a humidified incubator at 37°C and 5% CO_2. Growth medium was changed every 2 days. Upon reaching 95% confluency, PC12 cells were detached by incubation in 0.25% trypsin at 37°C for 5 min. Trypsin was deactivated by full medium, cells were centrifuged at 250×g for 5 min, the supernatant was removed and cells were resuspended in full medium. PC12 were cryo-preserved in freezing medium (70% DMEM, 20% FBS, 10% DMSO) inside

cryo-vials for 24 hours at -80ºC inside in a "Mr Frosty" container. After 24 hours cryo-vials were stored in -80ºC until use.

CellTox Apoptosis Assay

10,000 PC12 cells were seeded per well in a 96-well plate and incubated in 100 μl full medium for three days before treatments. Treatments included 4 conditions: Serum (S), Serum Free (SF), Serum-ECM (S-ECM), Serum Free-ECM (SF-ECM). In each experiment, every condition was performed in 3 wells (technical replicates). Cell counting and viability were performed by trypan blue staining in a Hemocytometer (Neubauer Improved HBG). The day of treatment, full medium was removed, and cells were cultured for 4 hours in 50 μl serum-free medium. Afterwards, SF medium was removed and 50 μl fresh medium (with or without serum, with or without 50 μg/ml decellularized brain ECM) was added. Decellularized brain ECM powder (100mg) was previously diluted in 50 mM acetic acid (100μl, so solution concentration will be 1mg/ml) before supplementing medium to final concentration 50 μg/ml of ECM. After a 24 hour incubation at 37ºC and 5% CO_2, 50 μl CellTox™ Green Cytotoxicity Assay solution (Promega G8741) was added per well (resulting in 1:1 Assay solution: pre-existing medium). CellTox™ Green Cytotoxicity Assay solution had been prepared by diluting CellTox™ Green reagent 1:1000 in cell tox assay buffer and supplementing with 100 ng/ml Hoechst. Cells were incubated in CellTox staining solution for 30 min at 37ºC, and then imaged in a fluorescence microscope (Gravanis Lab, Medical School, University of Crete). After aspirating the medium (contains CellTox staining solution), 100 μl fresh medium (with or without serum, with or without 50 μg/ml decellularized brain ECM) was added and cells were incubated overnight at 37ºC and 5% CO_2. The following day (48h total treatment) cells were imaged again in the fluorescence microscope. In all cases, four images were acquired per well. Acquired image were processed in Image J software. Every image was split into its two channels (blue channel: Hoechst staining; green channel: cell tox staining). For every image and every channel nuclei and celltox (green) were counted using the "Analyze particles" tool of Image J after converting images to binary using the "Threshold" tool, and the "Binary-> Watershed" tool.

NSC34 cell culture

The NSC34 cell line has been derived from a hybridoma of motor neuron enriched, embryonic mouse spinal cord cells and mouse neuroblastoma (Cashman et al. 1992). Each NSC34 culture contains two cell populations: small, undifferentiated cells that have the capacity to undergo cell division and larger, multi-nucleate cells. NSC34 cells transfected with a plasmid containing the human wild Type (WT) SOD1 gene (Menzies et al. 2002) were grown in complete NSC34 medium: high glucose DMEM (Thermo 41966029) supplemented with 10% FBS (Thermo 10270-106), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Thermo 15140122) and 0.25 mg/ml Geneticin (G418) (Sigma). NSC34 cells were grown in a humidified incubator at 37ºC and 5% CO_2. NSC34 were carefully thawed first in G418-free medium and then in complete NSC34 medium. 15,000 NSC34 were seeded in 6-well plates in 2 ml complete NSC34 medium. Growth medium was changed every 2 days. Upon reaching 80% confluency (once per 3 to 4 days) NSC34 were detached by gentle pipetting and sub-cultured in new 6-well plates. NSC34 cells were cryo-preserved in NSC34 freezing medium (90% FBS, 10% DMSO). Cryovials with $500 \cdot 10^3$ NSC34 cells in Mr Frosty were placed in -80ºC overnight. The following day, cryovials were transferred in a liquid nitrogen tank.

NSC34 Live-Dead Viability Assay

One day before cell seeding (Day -1) wells of 48-well plates were coated with either 50 μg/ml decellularized brain ECM powder (diluted in 50 mM acetic) or 50 μg/ml collagen (FibriCol, Advanced BioMatrix, 5133) overnight. Before cell seeding, each well was washed twice with dH_2O. On Day 2, 15,000 NSC34 were seeded per 48-well plate in 350μl NSC34 complete medium. After 2 days incubation (day 4), 200 μl 0.25 mg/ml matrigel (diluted in ice-cold medium) was added per well. After a 90 min incubation at 37°C, a thin matrigel coating (0.16 mg/ml final concentration) formed above cells. 150 μl more medium was added per well and cells were further incubated for 3 more days. On Day 5, cell medium was removed and 150 μl live-dead staining solution (1 μM calcein AM, 1 μM Propidium Iodide (PI), 3 ng/ml Hoechst 33342 in DMEM with 1% P/S but without FBS) was added. Cells were incubated in staining solution at 37°C for 45 min. After one PBS wash, cells were imaged in a Florescent microscope (4 fields per well) using 10x lens. Acquired image were processed in Image J software. Every image was split into its three channels (blue channel: Hoechst staining; green channel: calcein staining and red channel: PI staining). For every image and every channel nuclei and PI (red) positive cells were counted using the "Analyze particles" tool of Image J after converting images to binary using the "Threshold" tool, and the "Binary-> Watershed" tool. For calcein channel (green) positive cells were counted using the "Cell count" tool of Image J.

NSC34 Cell tox Apoptosis Assay

5,000 NSC34 cells were seeded (day 0) per well in a 96-well plate coated and incubated in 100 μl full medium for two days. Before cell seeding, wells had been coated by treatment with 50 μg collagen in 50mM acetic acid overnight at 4°C. On day 2, 70μl 0.25 mg/ml matrigel (diluted in ice-cold medium) was added per well. After a 90 min incubation at 37°C, a thin matrigel coating (0.16 mg/ml final concentration) formed above cells. 110 μl more medium was added per well and cells were further incubated for 3 more days. On Day 5, cell medium was removed, each well was washed with PBS and cells were cultured for 4 hours in 70 μl serum-free medium. Afterwards, SF medium was removed and 50 μl fresh medium (with or without serum, with or without 50 μg/ml decellularized brain ECM) was added. Decellularized brain ECM powder (100mg) was previously diluted in 100 μl 50 mM acetic acid (the resulting concentration of brain ECM will be 1 mg/ml) before supplementing medium to final concentration 50 μg/ml decellularized ECM. After a 24 hour incubation at 37°C and 5% CO_2, 50 μl CellTox™ Green Cytotoxicity Assay solution (Promega G8741) was added per well (resulting in 1:1 Assay solution: pre-existing medium). CellTox™ Green Cytotoxicity Assay solution had been prepared by diluting CellTox™ Green reagent 1:1000 in cell tox assay buffer and supplementing with 100 ng/ml Hoechst. Cells were incubated in CellTox staining solution for 30 min at 37°C, and then imaged in a fluorescence microscope (Gravanis Lab, Medical School, University of Crete). After aspirating the medium (contains CellTox staining solution), 100 μl fresh medium (with or without serum, with or without 50 μg/ml decellularized brain ECM) was added and cells were incubated overnight at 37°C and 5% CO_2. The following day (48h total treatment) cells were imaged again in the fluorescence microscope. In all imaging sessions, four images were acquired per well. . Acquired image were processed in Image J software. Every image was split into its two channels (blue channel: Hoechst staining; green channel: celltox staining). For every image and every channel nuclei and celltox (green) were counted using the "Analyze particles" tool of Image J after converting images to binary using the "Threshold" tool, and the "Binary-> Watershed" tool.

Embryonic Cortical Neural Stem Cell Primary Culture

Embryonic Cortical Neural Stem Cell (NSC) isolation and culture was performed by Constantina Georgelou. All procedures followed the rules and guidelines for animal manipulation of University of Crete as described previously (Kourgiantaki et al., 2020). Briefly, for NSC isolation, pregnant mice (gestational day 13.5) were sacrificed via cervical dislocation, embryos were carefully removed and placed in a petri dish in HBSS supplemented with 5% penicillin/streptomycin. NSC were isolated by mechanically dissociating cortical hemispheres in NSC complete medium [1:1 DMEM/F12 (thermo 11330032) supplemented with 2% B-27 without vitamin A (Thermo scientific 12587010), 0.6 % D-glucose (sigma G8769), 100 μg/mL Primocin, 20 ng/ml FGF2 (R&D), 20 ng/ml EGF]. 1 ml warm (37˚C) complete embryonic medium was added per 4-8 brains. Cells were dissociated using of a 1 ml tip via, by approximately 10 up-downs are until the solution became cloudy. 5 ml warm complete embryonic medium was added, cells were centrifuged at 100xg for 5 min, the supernatant was discarded, and cells were reconstituted in 1-2 ml warm complete embryonic medium using a 1ml tip. Cell number was counted using a hemocytometer and $25{\cdot}10^4$ NSCs in 5 ml complete NSC medium were seeded per T25 flask.1 ml fresh medium was added every other day. Neurospheres formed within 2 days. Neurospheres were dissociated by accutase (Sigma) at day 4 or 5. Dissociated NSCs were either passaged or used for experiments (passage 3 to 8). For splitting, the contents of a T25 flask were poured in a 50 ml tube, centrifuged at 10g for 5 min, the supernatant was discarded and 200μl accutase was added. Cells in falcon were incubated at 37˚C for 5min, meanwhile falcon were shaking every 2 min. NSCs were dissociated by pipetting up/down several times using a 200 μl pipette. 2 ml warm DMEM/F12 medium was added in the tube and the resulting NSC suspension was centrifuged for 5 min at 100g. The supernatant was discarded, 350 μl warm complete NSC medium was added and the pellet was dissociated. Cell concentration was calculated via a hemocytometer. Finally. $25{\cdot}10^4$ NSCs in 5 ml complete medium were seeded in T25 flasks (Kourgiantaki et al. 2020).

NSC Live-Dead Viability Assay

One day before cell seeding (day -1), wells of 48-well plates were coated in three different ways:

i. PDL & laminin coating: wells were treated first with 150μl 1 mg/ml PDL overnight at 4°C and then with 150 μl 15 μg/ml Laminin in dH_2O for 2 hours at 37°C
ii. PDL coating: wells were treated with 150μl 1 mg/ml PDL overnight at 4°C.
iii. PDL & ECM coating: wells were treated with 150 μl PDL overnight at 4°C, and then with 150 μl 50 μg/ml decellularized brain ECM solution overnight at 4°C.

On day 0, $2.5 \cdot 10^4$ NSCs were seeded per well. Three days later (day 3), the medium was removed, NSCs were washed, incubated in 150 μl live-dead staining solution (1 μM calcein AM, 1 μM PI, 3 ng/ml Hoechst 33342 in PBS) at 37°C for 45 min, washed in PBS and imaged in a Florescent microscope (8 fields per well) using a 10x objective lens. Acquired image were processed in Image J software. Every image was split into its three channels (blue channel: Hoechst staining ; green channel: calcein staining and red channel: PI staining). For every image and every channel nuclei and PI (red) positive cells, were counted using the "Analyze particles" tool of Image J after converting images to binary using the "Threshold" tool, and the "Binary-> Watershed" tool. For calcein channel (green) positive cells, were counted using the "Cell count" tool of Image J.

2.8 Statistical Analysis

All results are reported as mean±s.e.m. Comparison of measurement means from two groups was performed using an unpaired t-test. Comparison of measurement means from multiple groups was

performed using t-test, one-way ANOVA or two-way ANOVA followed by Sidak's post-hoc test. Statistical analyses were performed using Prism, version7 (GraphPad Software Inc.). p-values less than 0.05 were considered statistically significant.

3. Results

Sections 3.1 to 3.2 present representative results of mouse brain tissue decellularization as well as various pieces of evidence that the developed decellularization protocol can successfully remove cell content while retaining brain ECM. Sections 3.3 to 3.5 demonstrate the effects of the decellularized brain ECM powder in three kinds of neural cells: the PC12 cell line (section 3.3), NSC34 cell line (section 3.4) and primary embryonic neural stem cells (Section 3.5).

3.1 Brain Decellularization

The progress of a representative decellularization procedure is presented in Figure 3.1. Initially, tissue samples have a pinkish color, possibly due to the presence of blood residues. After the first 8-hour treatment in water, tissue chunks are swollen and are located on the tube bottom. Also, the solution becomes less transparent. After the first 14-hour TritonX-100 treatment, chunks became less pink, less swollen and the color of solution becomes more transparent. After the second water treatment, chunks appear whiter and less soluble. After the second 14-hour tritonX-100 treatment, tissues show no red/pink color and appear white. After the first PBS wash, tissue pieces appear shrunken, whiter and more transparent. After the DNase treatment, tissue chunks appear white and more shrunken. Finally, after the second PBS wash, tissue is totally white and solid. After snap freezing, tissue seems compact and like a brittle.

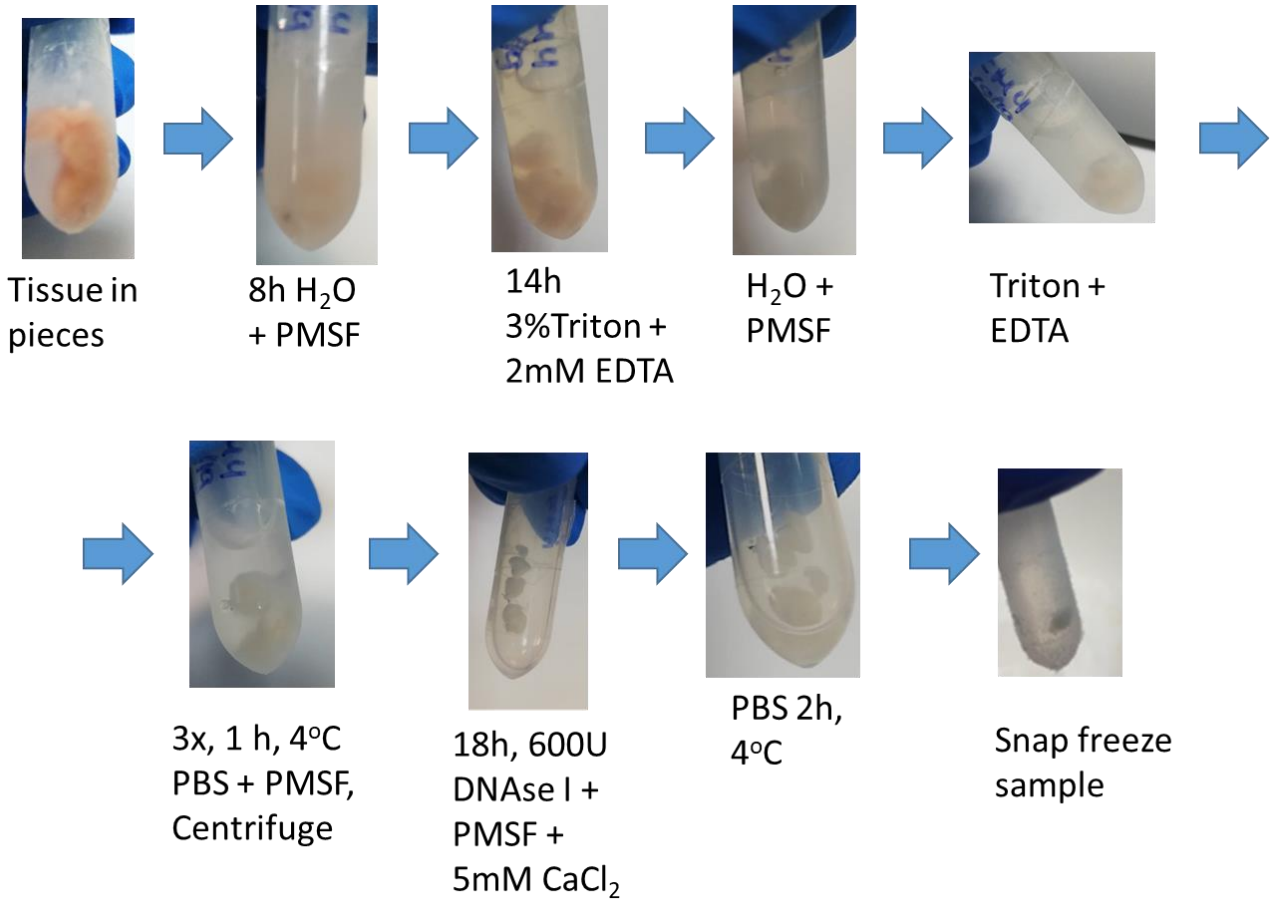


Figure 3.1 Representative images acquired at various steps of the brain decellularization process.

3.2 Decellularization Characterization

3.2.1 Proteomic Analysis

In order to characterize the proteomic content of the tissue obtained in the end of the decellularization procedure shown in Fig 3.1, the decellularized powder was transferred in a tube and was diluted in RIPA buffer in order to obtain a suspension of approximate density 0.5 mg/ml (mg tissue mass per ml RIPA). Tubes that contained control (non-decellularized) brain lysates were also prepared by snap freezing non-decellularized brain tissue in isopentene, homogenizing in a dounce homogenizer, diluting in RIPA buffer and sonicating 3 times for 5 seconds. In all samples, 1% PMSF was added after homogenization and before sonication. All brain samples (decellularized and control) must be well homogenized in order to ensure correct protein quantification via the BCA, a prerequisite for correct immunobloting. Loading the lanes of the gel with equal mass of protein is a prerequisite for the correct interpretation of immunoblotting results, i.e. that the absence of cell housekeeping proteins in decellularized samples is indeed due to successful decellularization.

The protein content of the resulting suspensions was qualified via the BCA assay using established procedures. Since ECM suspensions were in RIPA buffer, BSA standards were also prepared in RIPA buffer.

In order to ensure that protein samples (control and decellularized) are well homogenized, drops of protein samples were imaged in a plain optical microscope in order to confirm that homogenization was successful (figure 3.2).

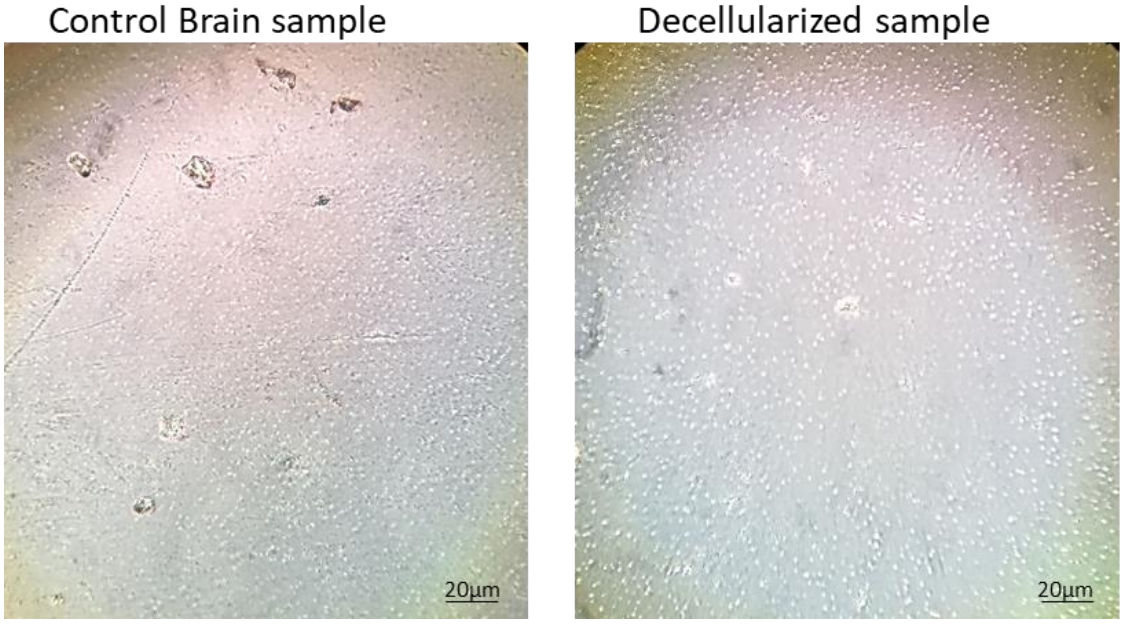


Figure 3.2: Images of decellularized and control brain samples diluted in ripa buffer after rigorous sonication (3 sonication runs per day, 5 sec per sonication). Images suggest that homogenization was successful as the size of tissue chunks is less than approximately 15 μm.

In order to evaluate if decellularized brain samples lack indeed of cells and their components, brain samples (control, decellularized) were analyzed by immunoblotting. 50 μg protein per samples were loaded in 4-20% gradient gels. The utilization of gradient gels was crucial as it enabled smooth electrophoresis in the gel and subsequently efficient protein transfer to the membrane.

Figures 3.3 and 3.4 clearly demonstrate that β actin was consistently not detected in five decellularized brain samples. On the contrary, β actin was consistently detected in three control samples. Quantification of β actin from membrane images revealed that β actin was significantly less in decellularized samples compared to control samples (decellularized: mean±s.e.m, control: mean±s.e.m; $p < 0.001$ (t-test)). The presence of β actin in control samples was expected as it is an intracellular protein expressed in brain cells. The absence of β actin band in decellularized samples suggests that the developed decellularization protocol successfully removed cells.

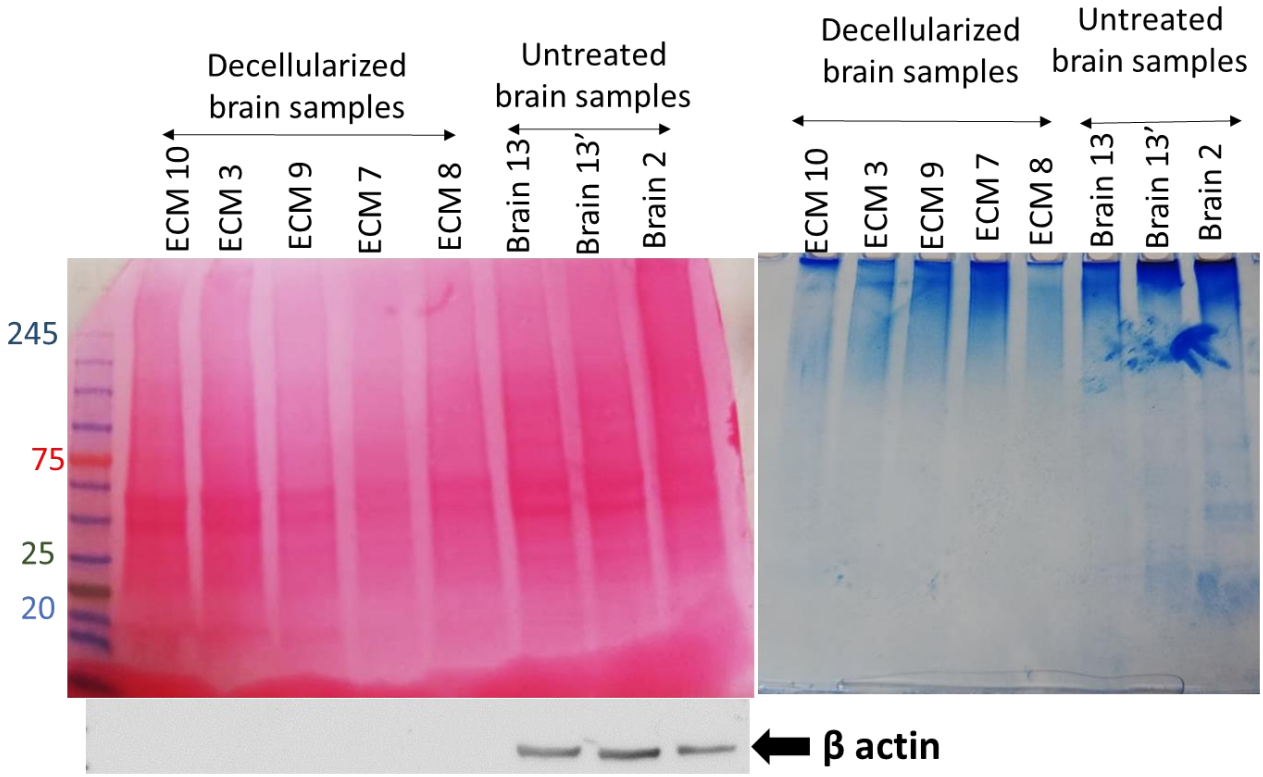


Figure 3.3: Detection of β-actin in control (untreated) and decellularized brain samples via immunoblotting. ***Top right****: coomassie staining G250 of the gel after gel transfer reveals that proteins smaller than 100 kDa are successfully transferred to the membrane. Some proteins bigger than 100kDa are still detected in the gel.* ***Top left****: Ponceau staining of the membrane reveals that most sample proteins were successfully transferred to the membrane.* ***Bottom left****: Immunodetection for β actin demonstrates that β actin is detected only in control samples (n=3 samples) but it is absent from decellularized samples (n=5).*

sample	sample conc	µl for 50 µg protein	Arbitrary Units (AU)
ECM 10	5,74	8,71	0,214
ECM 3'	6,76	7,40	0,571
ECM 9	7,59	6,59	0,208
ECM 7	5,10	9,80	0,394
ECM 8	5,83	8,58	0,277
Brain 13	5,01	9,98	32
Brain 13'	6,00	8,33	43
Brain 2	5,57	8,98	24

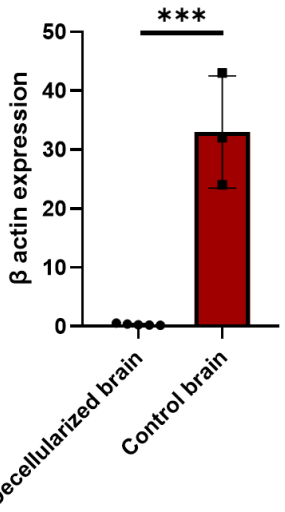


*Figure 3.4: Quantification of β actin expression in the membrane shown in Fig. 3.3 (bottom left) and statistical analysis of β actin expression in decellularized and control (untreated) brain samples. Data are presented as mean±s.e.m. ***: P<0.001 (t-test).*

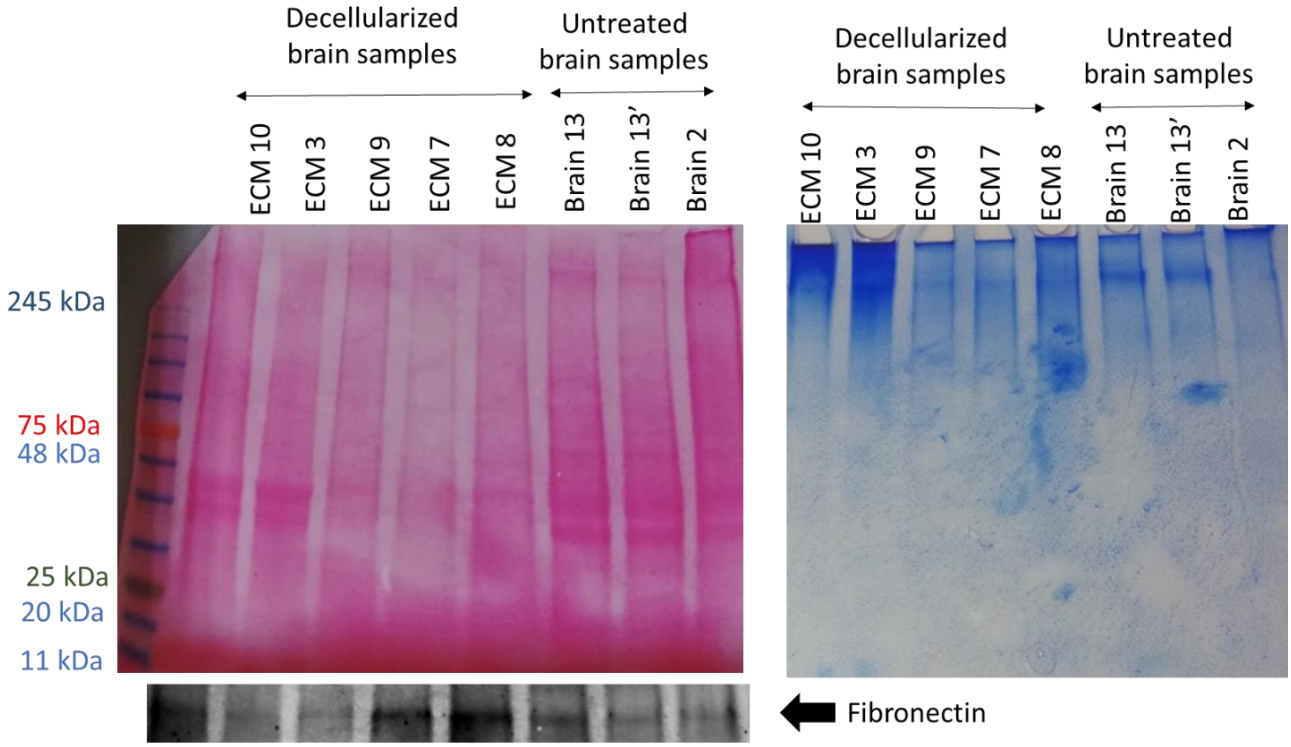


*Figure 3.5: Detection of fibronectin in control (untreated) and decellularized brain samples via immunoblotting. **Top right**: Coomassie G250 gel staining after gel transfer reveals that proteins smaller than 250 kDa have successfully been transferred to the membrane. Some proteins bigger than 250kDa are still detected in the gel. **Top left**: Ponceau staining of the membrane reveals that most sample proteins have been successfully transferred to the membrane. **Bottom left**: Immunodetection for fibronectin demonstrates that fibronectin is detected in all samples consisting of three control (untreated) and five decellularized samples.*

sample	sample conc	µl for 50 µg protein	Arbitrary Units (AU)
ECM 10	5,74	8,71	11,784
ECM 3'	6,76	7,40	16,234
ECM 9	7,59	6,59	15,276
ECM 7	5,10	9,80	19,014
ECM 8	5,83	8,58	8,397
Brain 13	5,01	9,98	6,874
Brain 13'	6,00	8,33	14,468
Brain 2	5,57	8,98	13,598

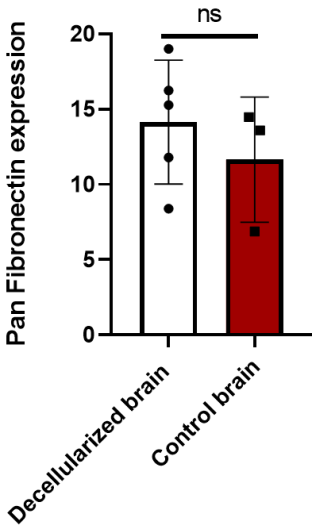


Figure 3.6: Quantification of fibronectin expression in the membrane shown in Fig. 3.5 (bottom left) and statistical analysis of fibronectin expression in decellularized and control (untreated) brain samples. Data are presented as mean±s.e.m. ns: P >0.05 (t-test).

Figs. 3.5, 3.6 clearly demonstrate that fibronectin was consistently detected in five decellularized brain samples and three control samples. Quantification of fibronectin from membrane images revealed that fibronectin was not significantly different in decellularized samples compared to control samples (decellularized: mean±s.e.m, control: mean±s.e.m; p=0.99 (t-test)). The presence of fibronectin in both control and decellularized samples was expected as fibronectin is a component of the brain extracellular matrix.

Combined together, the presence of fibronectin and the absence of β actin in decellularized samples (Fig. 3.3 to 3.6) suggests that the protocol described in Fig. 3.1 successfully removed cells without significantly affecting extracellular matrix proteins.

3.2.2 Immunohistochemical Characterization

In order to further characterize the efficiency of the developed decellularized process, control brain samples and decellularized samples were fixed, frozen in OCT, and cryo-sectioned in a cryotome. Sections were immuno-stained using a pan-fibronectin antibody (Hynes lab, MIT) or an anti-laminin antibody (Sigma L9393) and counter-stained using Hoechst 33342.

Figures 3.7 & 3.8 show that Hoechst$^+$ nuclei were not detected in sections of decellularized brain tissue stained via IHC using an anti-laminin antibody. On the other hand, Hoechst$^+$ nuclei were abundant in sections of control brain samples. The anti-laminin antibody stained well both control brain tissue and decellularized brain. Laminin stains perineurial nets that surrounds cells and vessels. I control brain sections both perineurial nets and vessels were stained for Laminin comparing to decellularized sections that only vessels were stained for laminin. This further suggests that the developed decellularization protocol achieved to efficiently remove the vast majority of cell nuclei (figure 3.10) without affecting the presence and structure of brain extracellular proteins.

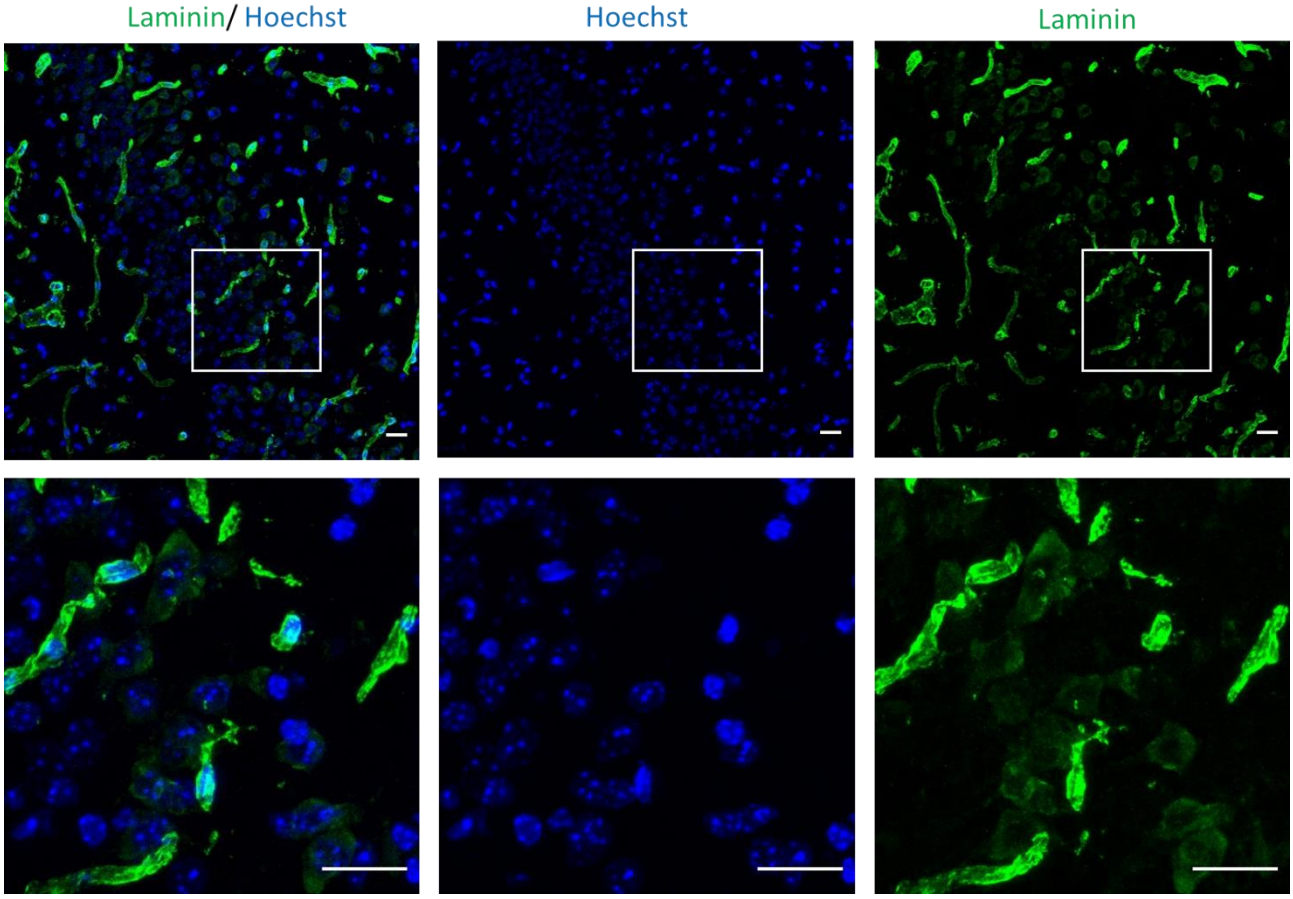


Figure 3.7: Immunohistochemical localization of laminin in cryo-sections from control (untreated) brain tissues. Top row: representative confocal fluorescence microscopy images. Bottom row: higher-magnification images of ROIs in top row images (white boxes). Scale bars: 20 µm.

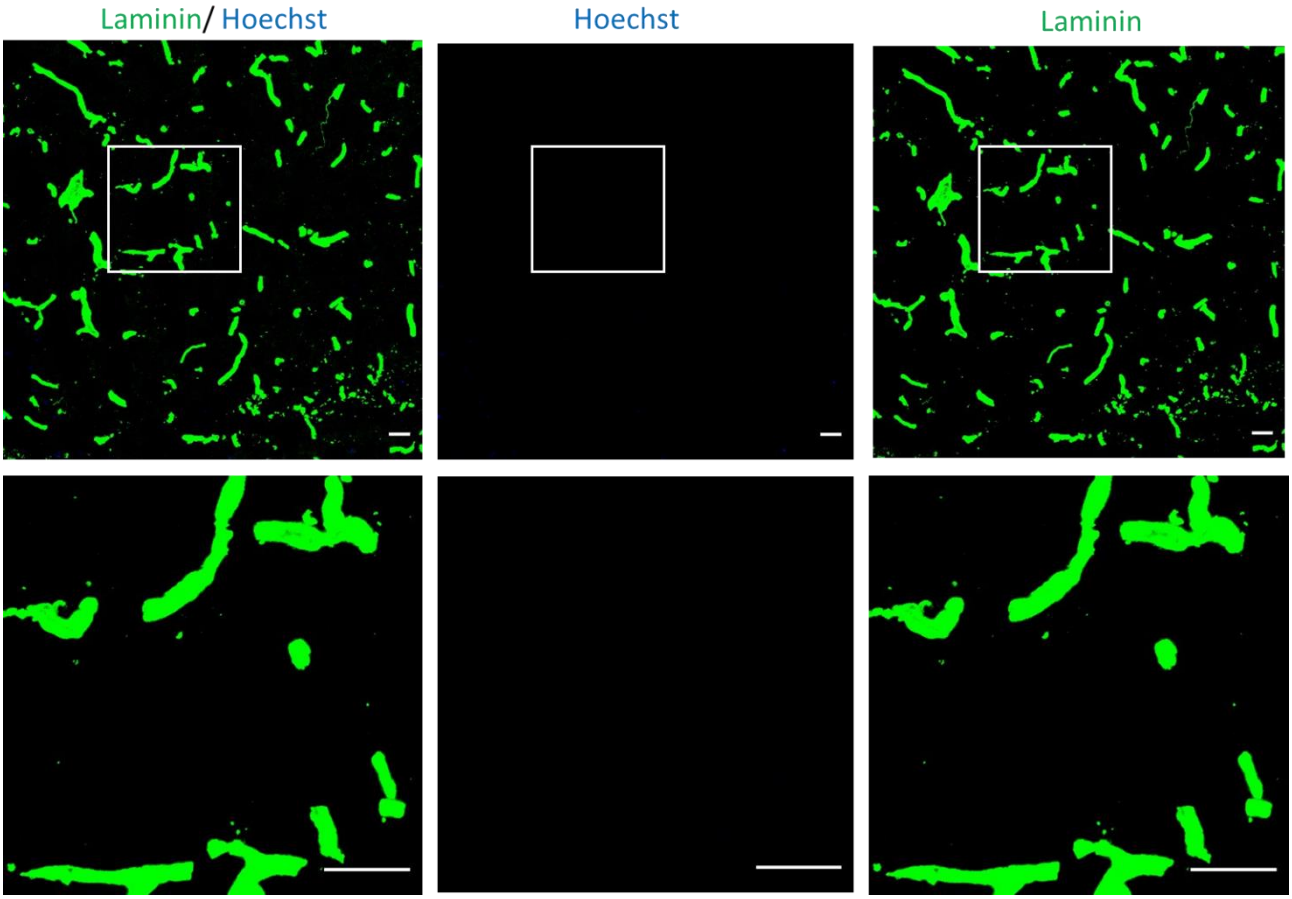


Figure 3.8: Immunohistochemical localization of laminin in cryo-sections from decellularized brain tissues. Top row: representative confocal fluorescence microscopy images. Bottom row: higher-magnification images of ROIs in top row images (white boxes). Scale bars: 20 µm.

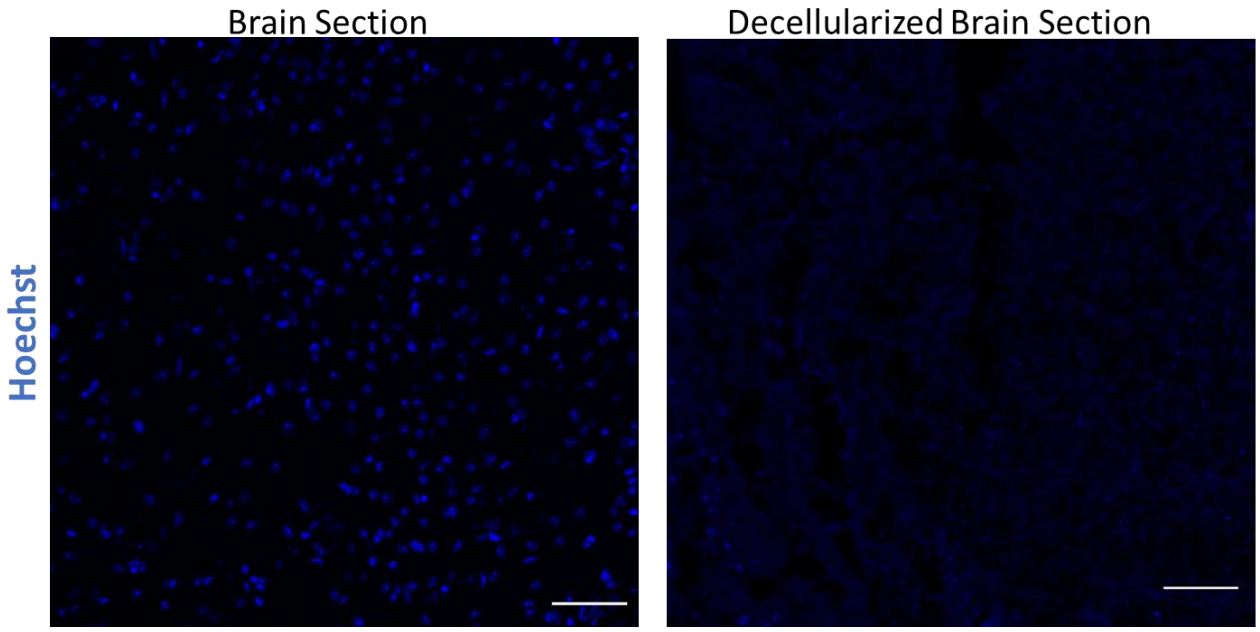


Figure 3.9: Effect of decellularization protocol in brain slices. Hoechst staining in cryo-sections from control (untreated; left) and decellularized (right) brain tissues. Representative confocal fluorescence microscopy images. Scale bars: 50 µm

Figure 3.10 summarizes the results of Hoechst$^+$ nuclei counting in sections from decellularized and control brain samples. Decellularized brain sections contain much fewer Hoechst$^+$ nuclei compared to control brain sections (decellularized: 12.7±3.4 Hoechst$^+$ nuclei per field. Control: 395.8±28.4 Hoechst$^+$ nuclei per field; $P<0.001$). Furthermore, Hoechst$^+$ nuclei detected in decellularized appear smaller and less bright compare to Hoechst$^+$ nuclei in control brain sections (qualitative observations).

	Nuclei number/ field	Nuclei number / 1mm^2
Brain 2	334.0	2,719.8
Brain 1	387.4	3154.2
Brain 3	463.7	3776.1
Brain 4	458.8	3735.6
Brain 5	335.1	2728.6
ECM 8	9.6	77.9
ECM 3	5.7	46.4
ECM 22	24.3	197.9
ECM 22'	7.7	62.8
ECM 20	16.3	132.3

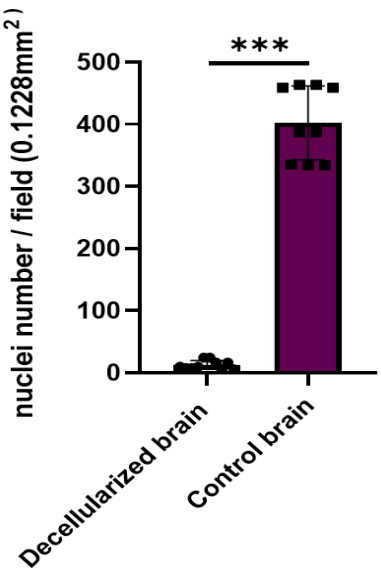


*Figure 3.10: Quantification of the nuclei percentance per image field (0.1228mm^2) in decellularized and control (untreated) brain samples. Data are presented as mean±s.e.m. ***: P< 0.001 (t-test)*

This significant reduction in the density of cell nuclei detected along with the absence of β actin in decellularized samples and the presence of fibronectin and laminin in decellularized samples suggest that the proposed decellularization procedure efficiently managed to remove nuclear cell material, while at the same time did not destroy the proteins of the surrounding extracellular matrix.

3.3 Effects of Soluble Decellularized Brain ECM on PC12 Survival

The pheochromocytoma-derived PC12 cell line, a neoplastic rat cell line arising from neural crest tissue (Wiatrak et al. 2020), has been used as a model to screen *in vitro* for neuroprotective compounds that can reverse apoptosis induced by serum withdrawal. Here, PC12 cells were incubated for 24 and 48 hours in four kinds of PC12 medium (with or without serum, with or without 50 μg/ml decellularized brain ECM). The CellTox assay was utilized to stain cells undergoing apoptosis, while the Hoechst33342 counterstain stained all cell nuclei.

Fig. 3.11 shows representative images from the four cell groups (with or without serum, with or without 50 μg/ml decellularized brain ECM) after 24h culture. Fig. 3.12 provides the corresponding quantification of the fraction of CellTox$^+$ nuclei in the four groups. Serum withdrawal induces apoptosis in PC12 cells, as in the serum$^-$ecm$^-$ group the fraction of CellTox$^+$ nuclei is significantly larger (17.75±2.98 %) compared to the control serum$^+$ecm$^-$ condition(0.67±0.19 %). Importantly, the addition of 50 μg/ml brain ECM in serum-free medium resulted in significantly less apoptotic cells (5.21±1.07 %) compared to the case of serum-free medium without ECM enrichment. In serum$^+$ecm$^+$

group cell apoptosis (0.74±0.22 %) had no significant difference with serum^{+}ecm^{-}. ($P_{2\text{-way anova row factor}}$ = 0.0021, $P_{2\text{-way anova column factor}}$ <0.0001, $P_{\text{sidka's posthock test}}$ <0.0001 for serum-ecm^{-} vs serum free-ecm^{-}, $P_{\text{sidka's posthock test}}$ <0.0001 for serum free-ecm^{-} vs serum -ecm^{+}, $P_{\text{sidka's posthock test}}$ =0.0007 serum free -ecm^{-} vs serum free -ecm^{+})

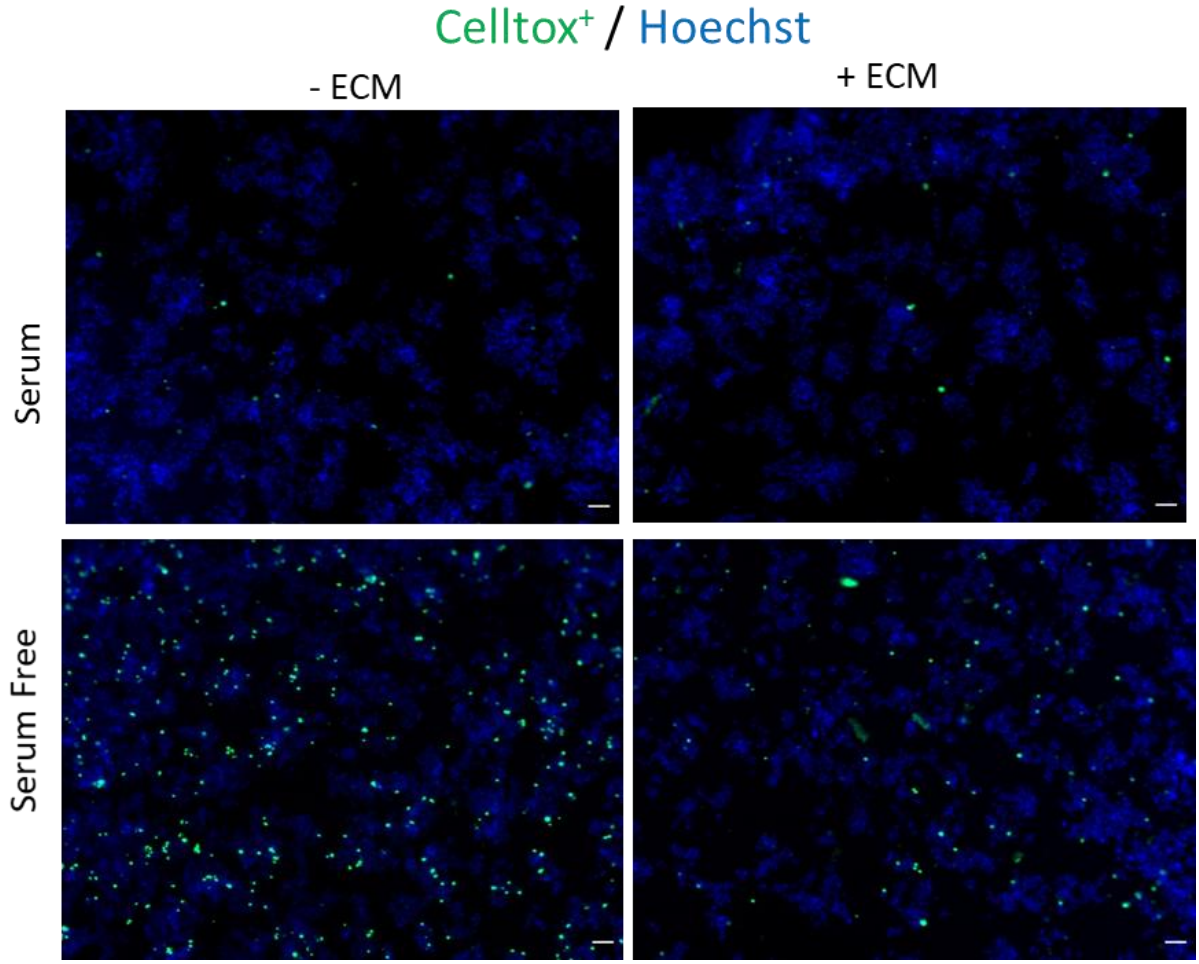


Figure 3.11: Representative fluorescence microscopy images of PC12 cells cultured in four conditions (with or without serum, with or without soluble decellularized brain ECM) for 24h. Green: CellTox apoptosis marker. Blue: Hoechst nuclear counterstain. Scale bars: 50 µm.

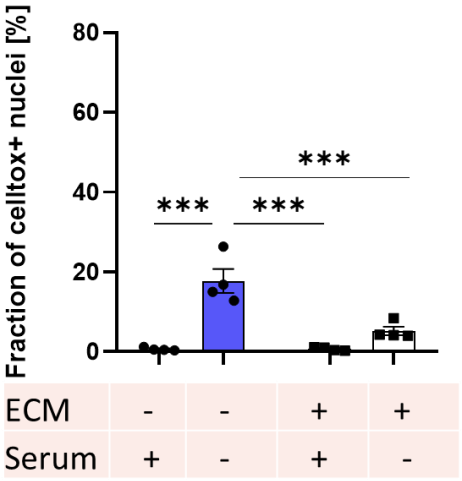


*Figure 3.12: Quantification of apoptosis in PC12 cells grown for 24h in four medium conditions, as shown in Fig. 3.11. Data are presented as mean±s.e.m, n=4 ***: P< 0.001 (two-way Anova followed by Psidka's posthock test).*

Fig. 3.13 shows representative images from the four cell groups after 48h culture. Fig. 3.14 provides the corresponding quantification of the fraction of CellTox$^+$ nuclei in the four cell groups. Results show a similar yet more severe apoptotic pattern, as the one observed after 24h culture. In serum conditions cell apoptosis is limited (2.76±1.26 %) much smaller comparing to the fraction of apoptotic cells in serum-free conditions (59.59± 3.07 %) in the absence of ECM. Again, the addition of 50 μg/ml brain ECM in serum-free medium resulted in reduction of CellTox$^+$ nuclei fraction (17.59±4.98) compared to the case of Serum Free medium without ECM enrichment. In serum$^+$ecm$^+$ cell apotosis (0.02±0.01) had no significant difference with serum$^+$ecm$^-$ ($P_{2\text{-way anova row factor}}$ <0.0001, $P_{2\text{-way anova column factor}}$ <0.0001, $P_{\text{sidka's posthock test}}$ <0.0001 for serum-ecm$^-$ vs serum free-ecm$^-$, $P_{\text{sidka's posthock test}}$ =0.0257 for serum-ecm$^-$ vs serum free-ecm$^+$, $P_{\text{sidka's posthock test}}$ <0.0001 for serum free-ecm$^-$ vs serum -ecm$^+$, $P_{\text{sidka's posthock test}}$ <0.0001 serum free-ecm$^-$ vs serum free-ecm$^+$, $P_{\text{sidka's posthock test}}$ =0.008 serum-ecm$^+$ vs serum free-ecm$^+$.

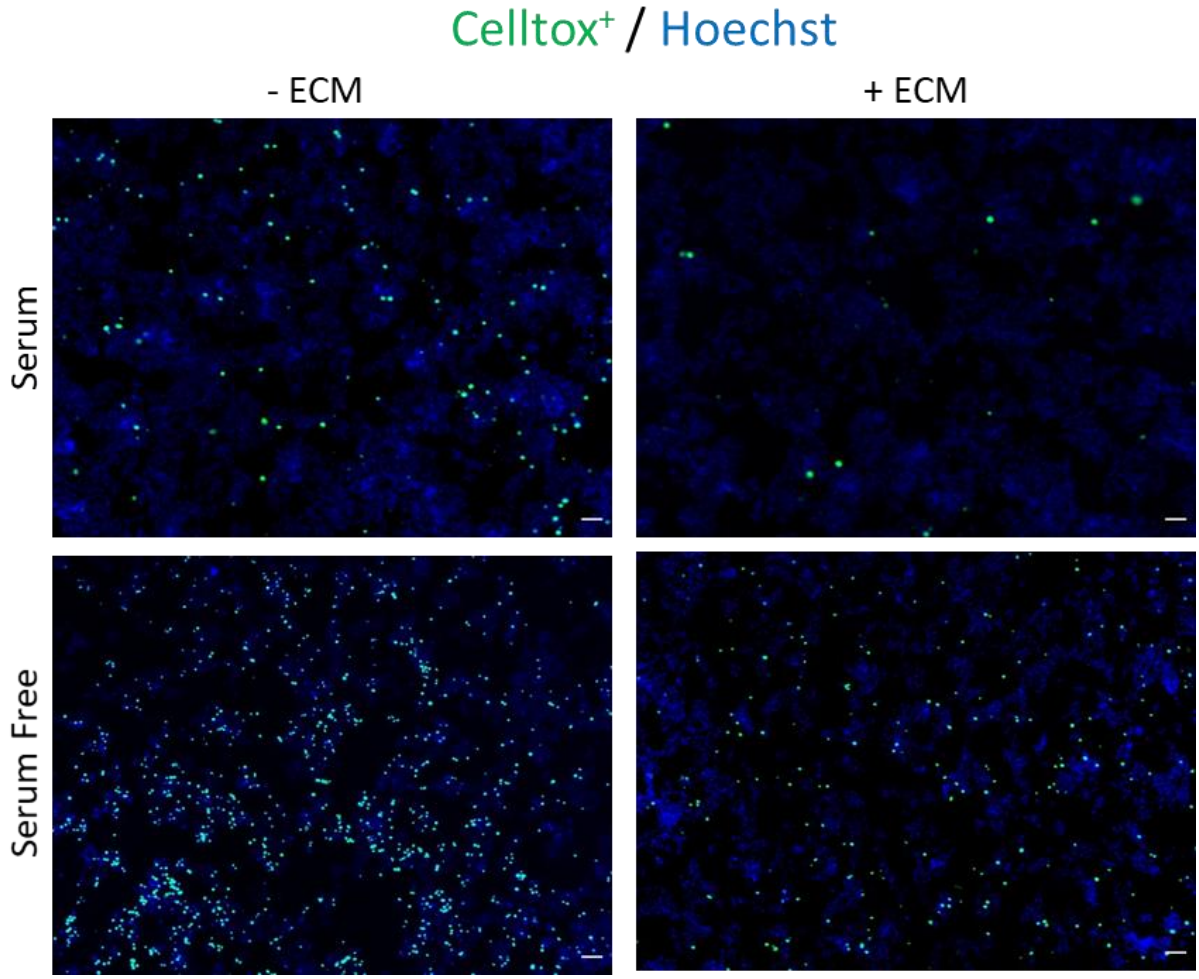


Figure 3.13: Representative fluorescence microscopy images of PC12 cells cultured in four conditions (with or without serum, with or without soluble decellularized brain ECM) for 48h. Green: CellTox apoptosis marker. Blue: Hoechst nuclear counterstain. Scale bars: 50 μm.

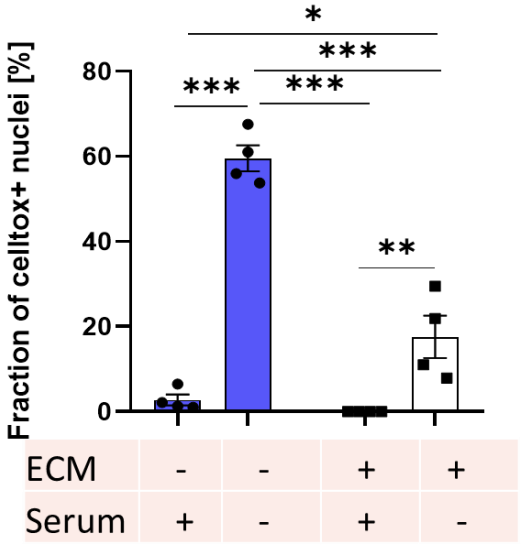


*Figure 3.14: Quantification of apoptosis in PC12 cells grown for 48h in four medium conditions, as shown in Fig. 3.13. Data are presented as mean±s.e.m, n=4 ***: P< 0.001, **: P<0.01, *: P<0.05 (two-way Anova followed by sidka's posthock test).*

In summary, Figs. 3.11 to 3.14 suggest that the addition of 50 μg/ml decellularized brain ECM achieved to significantly reduce PC12 apoptosis induced by serum withdrawal both at 24h and 48h. This finding suggests that the resulting decellularized brain ECM has neuroprotective effects on PC12 cells.

3.4 Decellularized Brain ECM Effects on NSC34 Cells

The NSC34 cell line was derived from a hybridoma of motor-neuron enriched, embryonic mouse spinal cord cells and mouse neuroblastoma (Menzies et al. 2002). Prior *in vitro* studies of NSC34 in the IMBB Neural tissue engineering lab utilize a sandwich culture on collagen I-coated wells in 96-well plates since NSC34 adhere poorly to plastic surfaces and collagen I coating enhanced NSC34 adhesion (Sarlidou O. et al. 2018)

3.4.1 ECM as coating and effects in adhesion and survival on NSC34 cell line.

In order to evaluate the ability of decellularized brain ECM coatings to enhance neural cell adhesion, NSC34 cells were seeded on wells coated with collagen I coating (the established protocol) or on wells coated with decellularized brain ECM. Three days after cell seeding, NSC34 were stained using a fluorescent viability assay that stains live cell cytoplasm (green; calceinAM dye) and dead cell nuclei (red; PI stain). Fig. 3.15 shows representative images of NSC34 grown on the two kinds of coatings. Images revealed no difference in the adhesion pattern of NSC34 on collagen or decellularized brain ECM. Fig. 3.16 shows the corresponding quantification of calcein^{+} live NSC34 cells. Statistical analysis reviled no effect of the coating type on NSC34 survival.

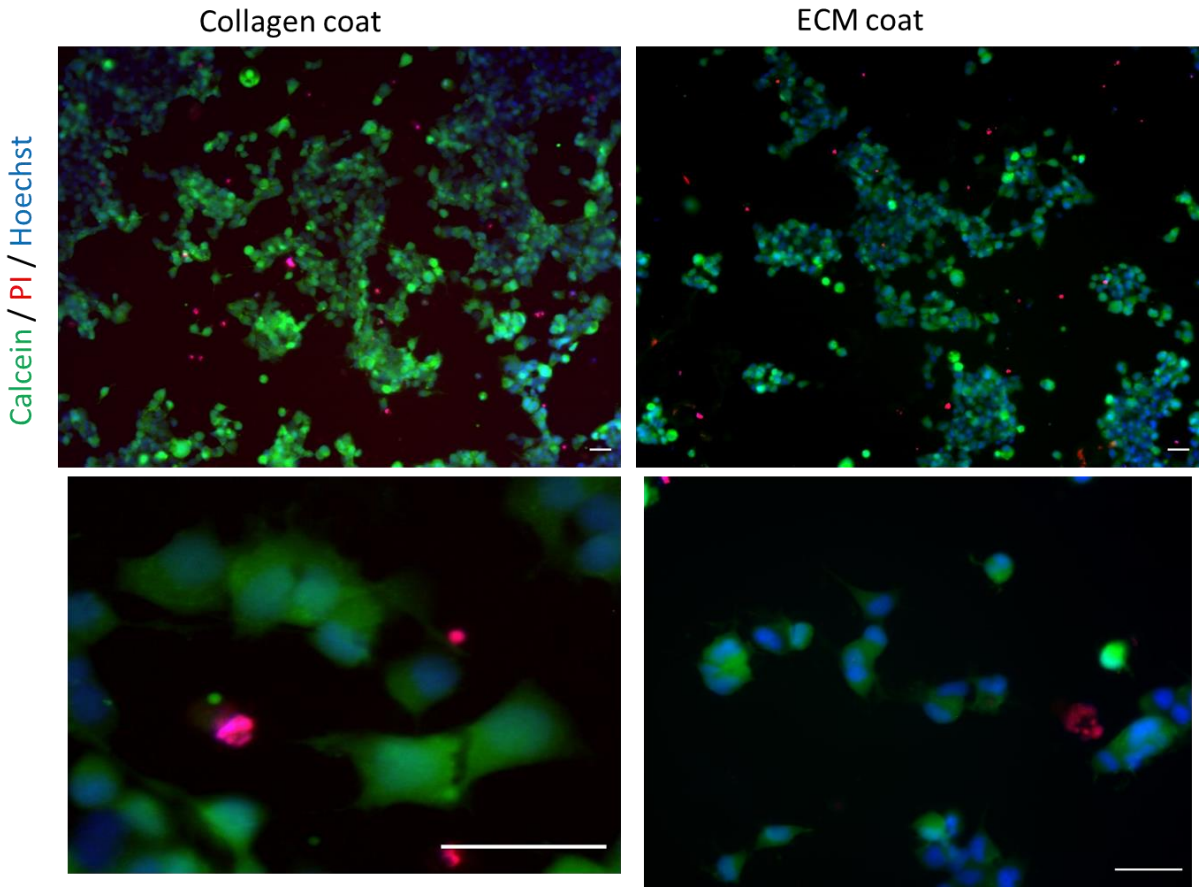
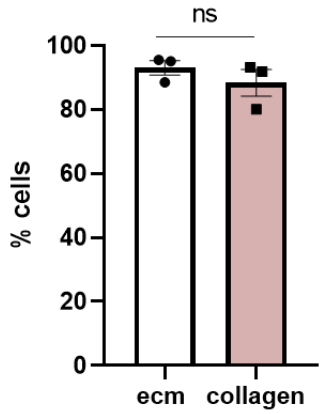


Figure 3.15: Representative fluorescence microscopy images NSC34 culture on well coated with collagen (left) or with decellularized brain ECM (right). Cells were stained with calcein AM (green), propidium iodide (PI; red) and Hoechst33342 (blue). Scale bars: 50 µm.



	Ecm			collagen		
Calcein [%]	88,5	95,1	95,5	80,1	93,2	91,9

Figure 3.16: Quantification of the fraction of calcein⁺ (live) NSC34 grown on wells coated with either collagen or decellularized brain ECM coating at day 3, corresponding to images shown in Fig. 3.22. Data are presented as mean±s.e.m. ns:P=0.4 (t-test).

3.4.2 Effects of Soluble Decellularized Brain ECM on NSC34 Survival

NSC34 cells have been shown to induce apoptosis upon serum withdrawal (D. Tzeranis, unpublished data). In contrast to PC12 cells, NSC34 are reported not to express TrkA. Yet, they are reported to express TrkB, TrkC and p75NTR receptors (Turner et al., 2004; Matusica et al., 2008; F. Baboula unpublished data). It was of interest to evaluate the ability of decellularized brain ECM to rescue NSC34 from serum withdrawal-induced apoptosis. Therefore, NSC34 cells were incubated for 24 and 48 hours in four kinds of NSC34 medium (with or without serum, with or without 50 μg/ml decellularized brain ECM). Again, the CellTox assay was utilized to stain NSC34 cells undergoing apoptosis, while the Hoechst33342 counterstain stained all cell nuclei.

Fig. 3.17 shows representative images from the four cell groups (with or without serum, with or without 50 μg/ml decellularized brain ECM) after 24h culture. Fig. 3.18 provides the corresponding quantification of the fraction of CellTox+ nuclei in the four groups. Serum withdrawal induces apoptosis in NSC34 cells, as in the $serum^-ecm^-$ group the fraction of CellTox+ nuclei is significantly larger (33.36±1.14 %) compared to the control $serum^+ecm^-$ condition (3.10 ± 0.43). Importantly, the addition of 50 μg/ml brain ECM in serum-free medium resulted in significantly less apoptotic cells (8.03±0.94 %) compared to the case of serum-free medium without ECM enrichment (33.36±1.14 %). In $serum^+ecm^+$ group cell apoptosis (2.77±0.33 %) had no significant difference with $serum^+ecm^-$. $P_{2\text{-way anova row factor}} < 0.0001$, $P_{2\text{-way anova column factor}} < 0.0001$, $P_{\text{sidka's posthock test}} < 0.0001$ for $serum\text{-}ecm^-$ vs serum free-ecm^-, , $P_{\text{sidka's posthock test}} = 0.0132$ for $serum\text{-}ecm^-$ vs serum free-ecm^+, $P_{\text{sidka's posthock test}} < 0.0001$ for serum free-ecm^- vs $serum\text{-}ecm^+$, $P_{\text{sidka's posthock test}} = 0.0089$ for $serum\text{-}ecm^+$ vs serum free-ecm^+, $P_{\text{sidka's posthock test}} < 0.0001$ serum free-ecm^- vs serum free-ecm^+.

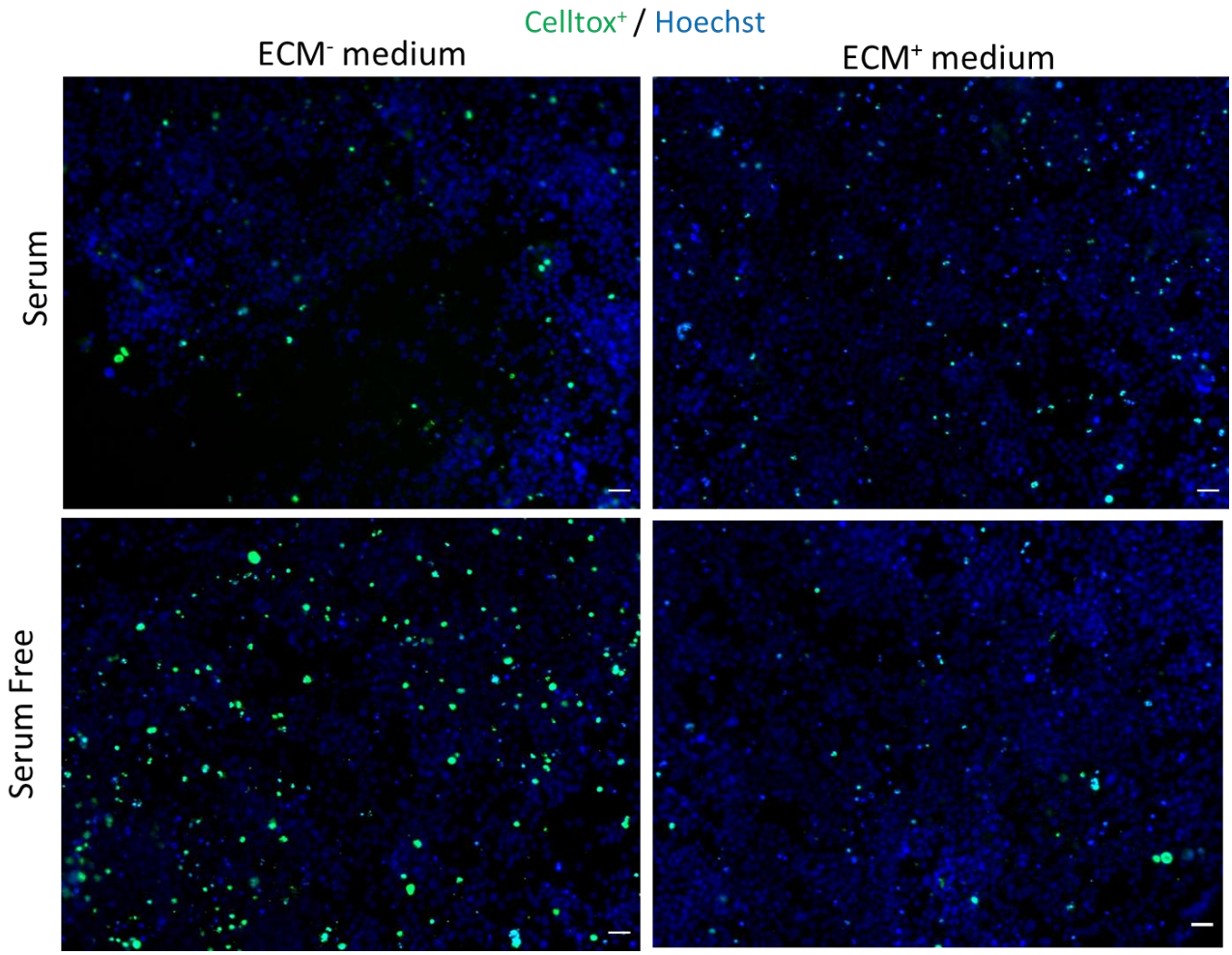


Figure 3.17: Representative fluorescence microscopy images of NSC34 cells cultured in four conditions (with or without serum, with or without soluble decellularized brain ECM) for 24h. Green: CellTox apoptosis marker. Blue: Hoechst nuclear counterstain. *Scale bars: 50 µm.*

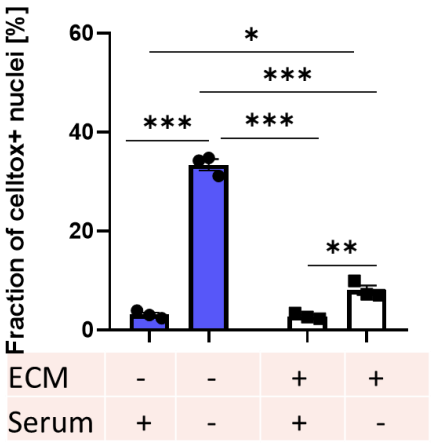


Figure 3.18: Quantification of apoptosis in NSC34 cells grown for 24h in four medium conditions, as shown in Fig. 3.17. *Data are presented as mean±s.e.m, n=3 ***: P< 0.001, **: P<0.01, *: P<0.05 (two-way Anova).*

Fig. 3.19 shows representative images from the four cell groups after 48h culture. Fig. 3.20 provides the corresponding quantification of the fraction of CellTox+ nuclei in the four cell groups. Results show a similar yet more severe apoptotic pattern, as the one observed after 24h culture. In serum conditions cell apoptosis is limited (8.40±0.80 %) much smaller comparing to the fraction of apoptotic cells in serum-free conditions (47.73±1.66 %) in the absence of ECM. Again, the addition of 50 μg/ml brain ECM in serum-free medium resulted in reduction of CellTox+ nuclei fraction (17.3±1.91%) compared to the case of Serum Free medium without ECM enrichment. In serum^{+}ecm^{+} cell apotosis (7.13±1.30%) had no significant difference with serum^{+}ecm^{-} .($P_{2\text{-way anova row factor}}$ <0.0001, $P_{2\text{-way anova column factor}}$ <0.0001, $P_{\text{sidka's posthock test}}$ <0.0001 for serum-ecm^{-} vs serum free-ecm^{-}, $P_{\text{sidka's posthock test}}$ =0.0164 for serum-ecm^{-} vs serum free-ecm^{+}, $P_{\text{sidka's posthock test}}$ <0.0001 for serum free-ecm^{-} vs serum -ecm^{+}, $P_{\text{sidka's posthock test}}$ <0.0001 serum free-ecm^{-} vs serum free-ecm^{+}, $P_{\text{sidka's posthock test}}$ =0.0074 serum-ecm^{+} vs serum free-ecm^{+}.

After 48 hours of ECM treatment in NSC34 cell culture, group with ECM in Serum Free, revealed significant less number of cell apoptosis comparing to Serum free without ECM. ECM solution with or without Serum reviled less cell apoptosis comparing with its control group (for Serum-ECM control group is Serum, and for Serum Free-ECM, control group is Serum Free).

According to total percent of apoptotic number of NSC34 cells in 4 coditions at 24 and 48 hours, ECM supplement reviled significant cell protection form apoptosis in Serum Free condition. This reveals that ECM could prevent cell apoptosis and increase cell survival under stress conditions (Serum Free).

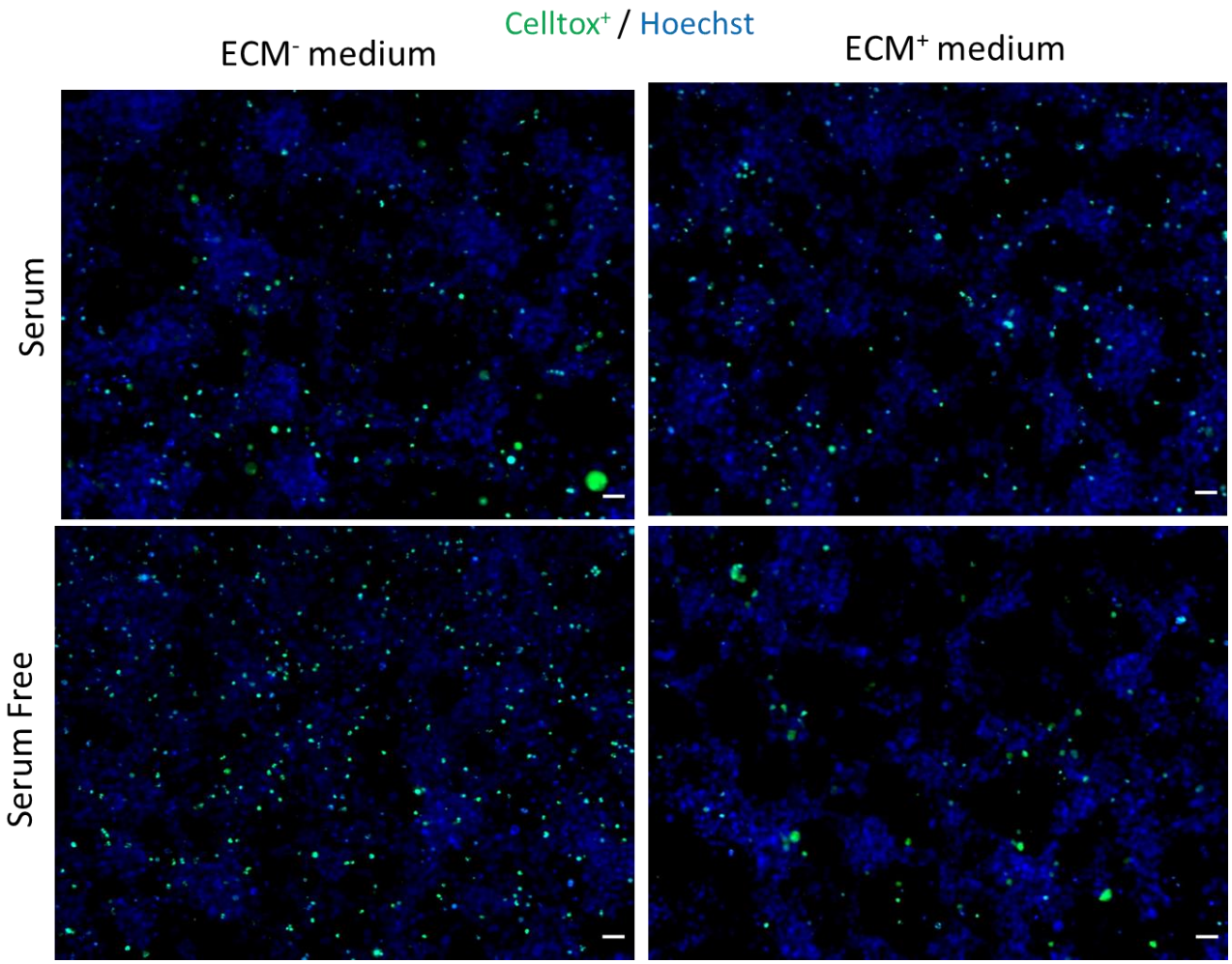


Figure 3.19: Representative fluorescence microscopy images of NSC34 cells cultured in four conditions (with or without serum, with or without soluble decellularized brain ECM) for 48h. Green: CellTox apoptosis marker. Blue: Hoechst nuclear counterstain. *Scale bars: 50 µm.*

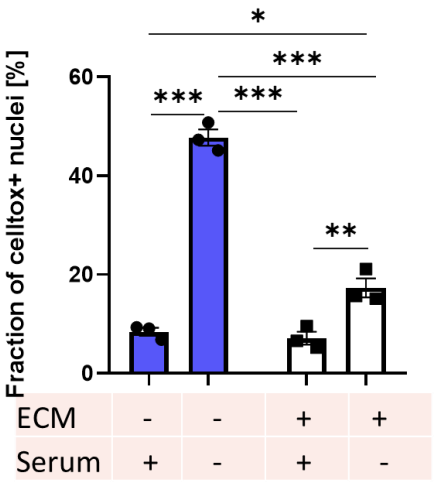


Figure 3.20: Quantification of apoptosis in NSC34 cells grown for 48h in four medium conditions, as shown in Fig. 3.19. *Data. Data are presented as mean±s.e.m, n=3 ***: P< 0.001, **: P<0.01, *: P<0.05 (two-way Anova).*

3.5 Decellularized Brain ECM Effects on Primary Embryonic Cortical Neural Stem Cells (NSCs)

3.5.1 ECM Effects on NSC Adhesion and the Survival

A primary culture of cortical embryonic (E13.5) Neural Stem Cells (NSCs) was used to evaluate the effects of decellularized brain ECM effects on NSC adhesion and survival *in vitro*. NSCs were grown on plastic surfaces coated with four types of coatings: i) PDL & laminin coating (the standard coating utilized for NSC monolayer culture), ii) PDL coating, iii) PDL & decellularized brain ECM coating, iv) decellularized brain ECM coating. NSCs were cultured in full NSC medium. One fifth experimental group was added, where NSCs were seeded on PDL coating and the NSC medium was supplemented with 50 µg/ml decellularized brain ECM.

Fig. 3.23 shows representative images of NSC growth in each one of the five conditions on 0 (1h after seeding), 1, 2 and 3 days after seeding. According to control coating (PDL-Laminin) in NSCs culture, many differences are detected in cultures with ECM as a coating and as enrichment in medium. Even though cells are seeded in all coating groups in single cell morphology, after day 2 cells have start to change their morphology up to the coating material. In PDL laminin coat NSCs present specific attached morphology with laminin, and axons are detected in all cells. Using ECM as a coating leads to cells to have neurosphere morphology and no attached morphology with axons, (axons of cells attached to material). Using ECM in NSCs culture provide an environment that promote cells to specific cell morphologies: cell aggregates, and neurosphere formations.

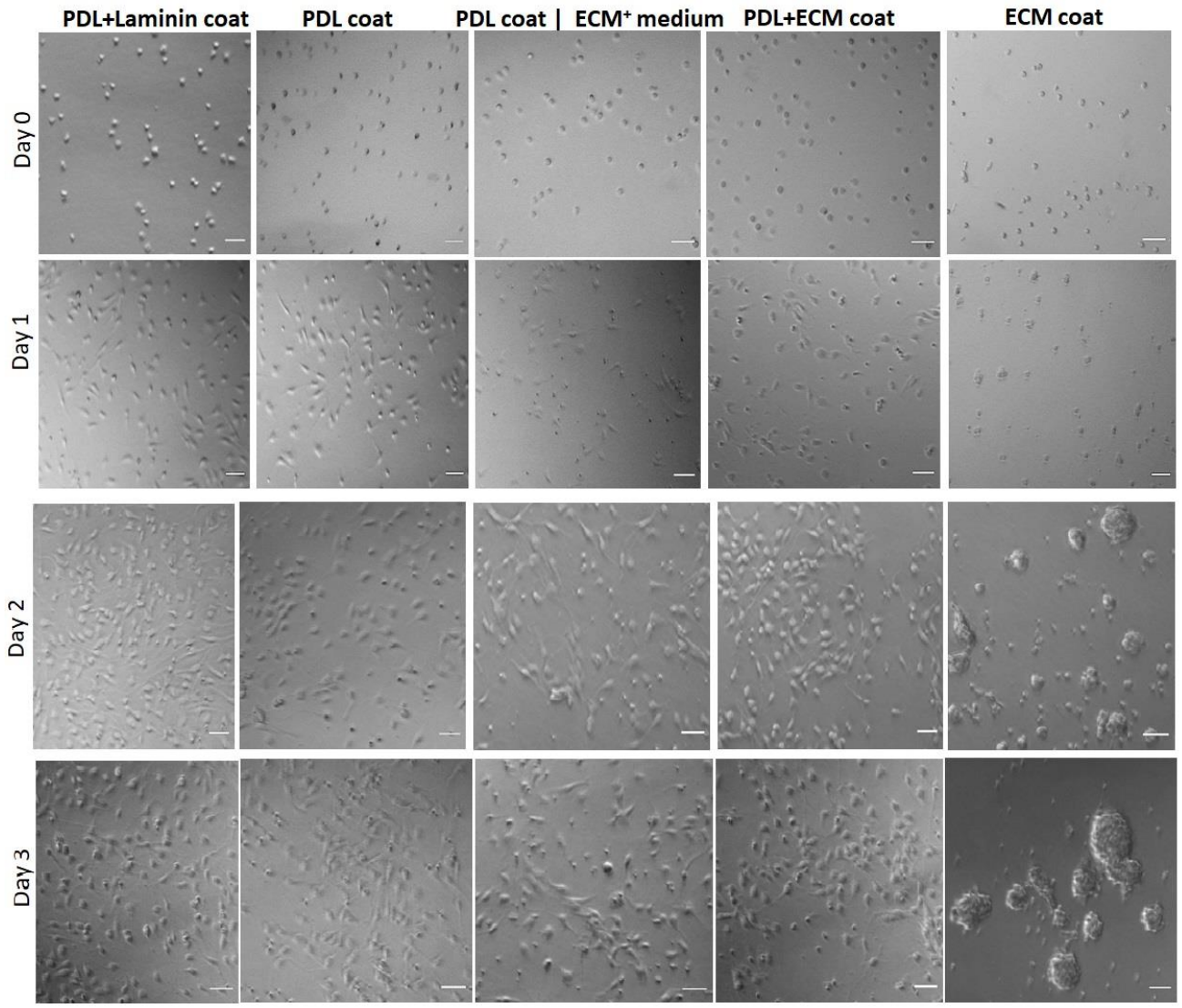


Figure 3.21: Representative microscopy images of NSCs grown on five different conditions (four kinds of coatings, plus one group where medium was supplemented with decellularized brain ECM). Cells were seeded on Day 0. Scale bars: 50 μm.

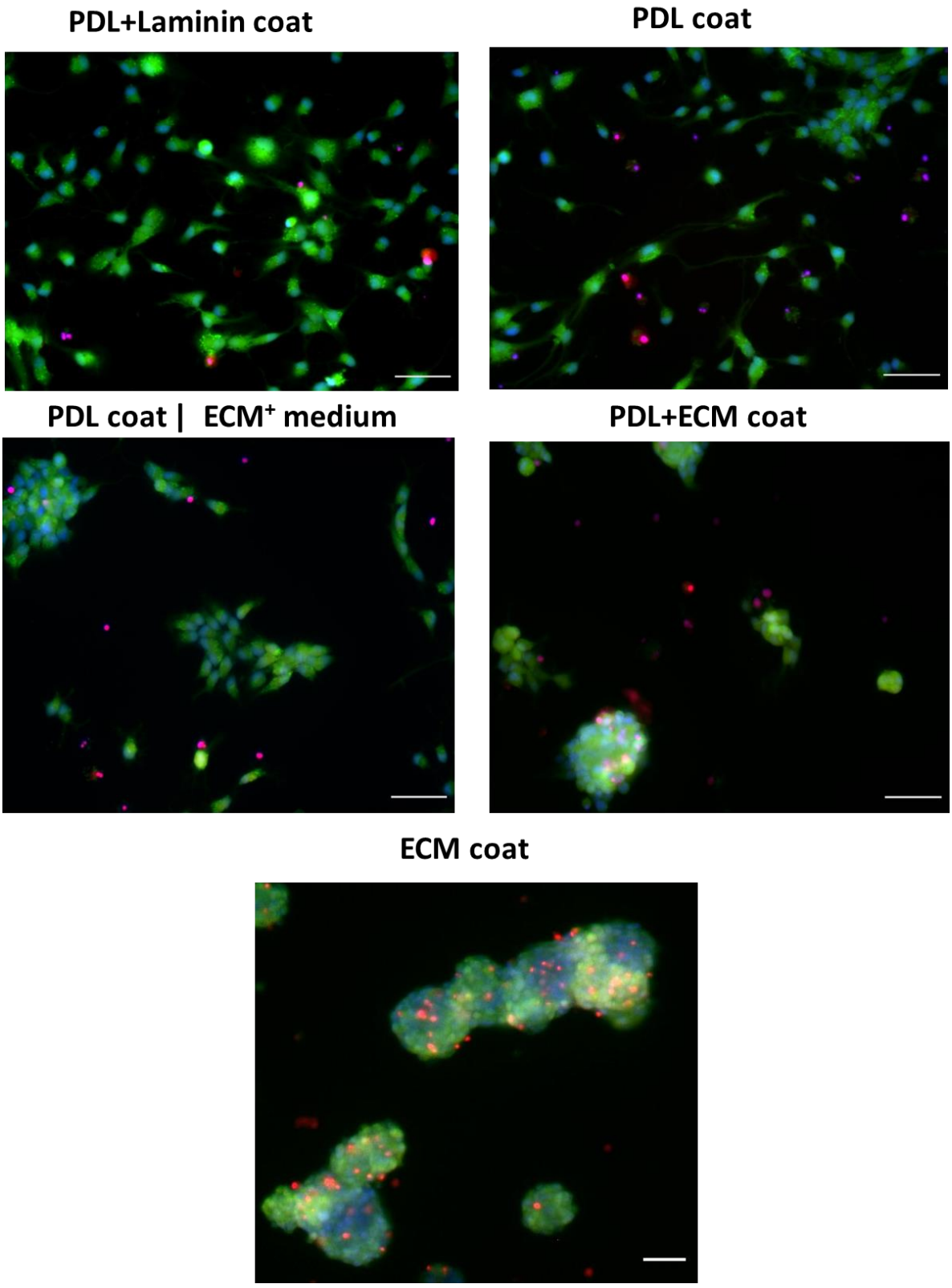


Figure 3.22: Representative fluorescence microscopy zoom images of NSCs grown on PDL&laminin coat, PDL coat, PDL coat and ECM⁺ in medium, PDL and ECM coat and ECM coat. Cells were stained at day 3 with calcein AM (green), propidium iodide (PI; red) and Hoechst33342 (blue). Scale bars: 50 µm.

At Day 3 day, post-seeding, NSCs were stained with a live-dead staining assay consisting of calceinAM (green, stains live cells), Propidium Iodide (PI; red, stains nuclei of dead cells) and Hoechst33342 nuclear counterstain (blue). Acquired images were utilized to study cell morphology and also measure the fraction of live cells in the five experimental groups.

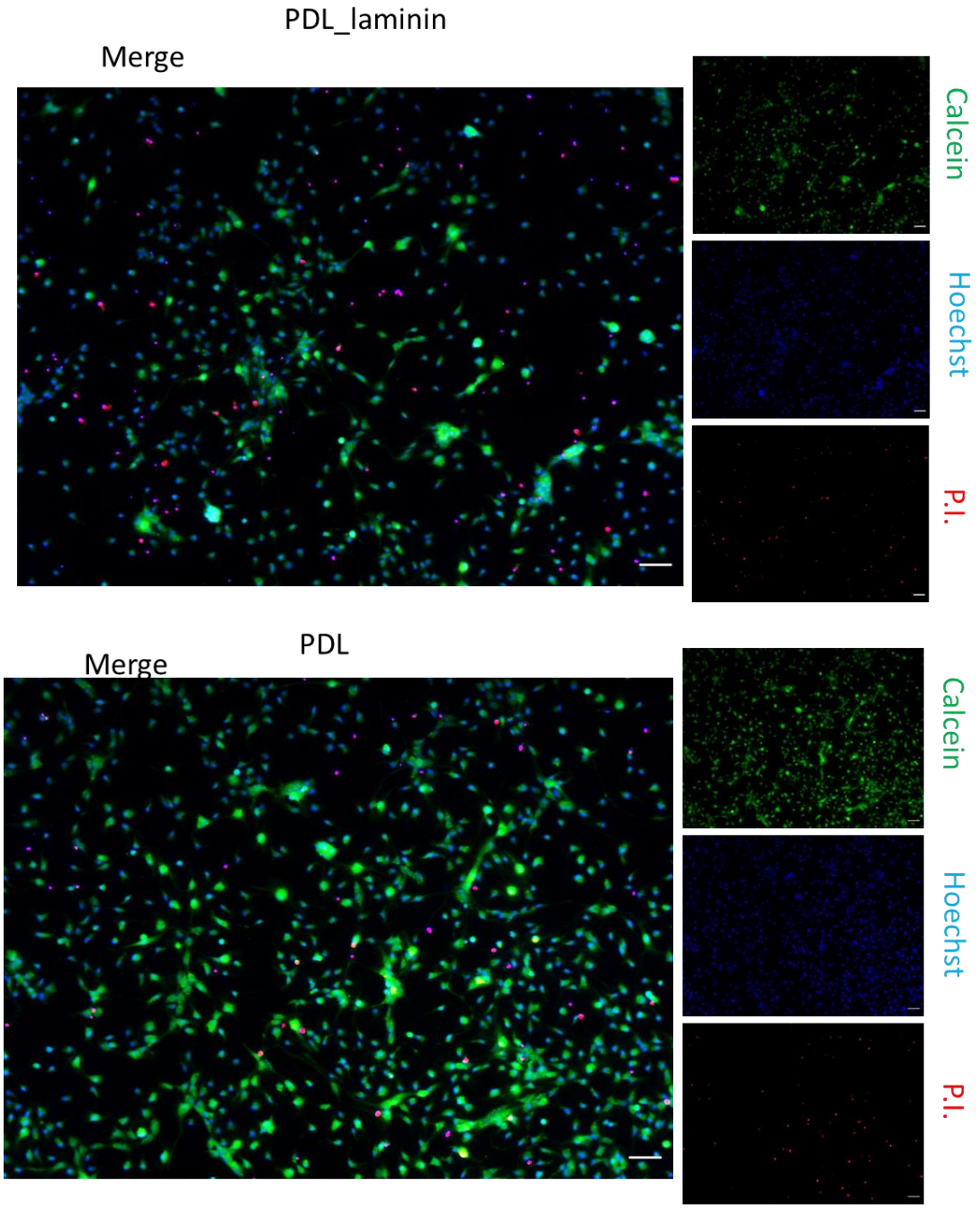


Figure 3.23: Representative fluorescence microscopy images of NSCs grown on PDL&laminin and PDL coated wells for 3 days. Cells were stained with calcein AM (green), propidium iodide (PI; red) and Hoechst33342 (blue). Scale bars: 50 µm.

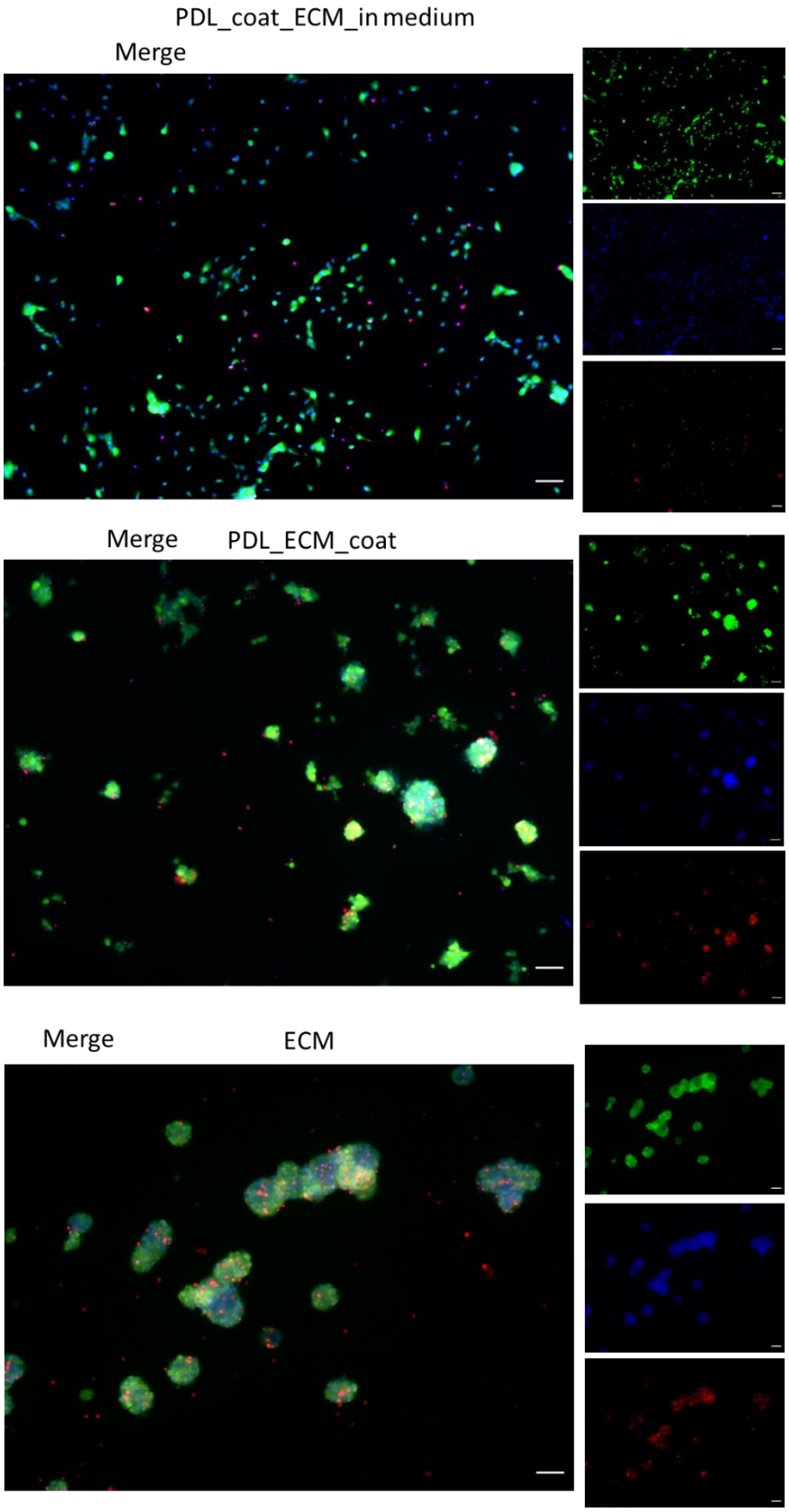


Figure 3.24: Representative fluorescence microscopy images of NSCs grown on wells coated with PDL (medium supplemented with decellularized brain ECM), PDL&ECM (decellularized brain ECM), or ECM-coated wells for 3 days. Cells were stained with calcein AM (green), propidium iodide (PI; red) and Hoechst33342 (blue). *Scale bars: 50 µm.*

Figures 3.21, 3.22, 3.23 & 3.24 reveal that NSC morphology and adhesion pattern depended strongly on the nature of the coating used:

i. PDL + Laminin coating: NSC adhere well, spread well, remain single cells
ii. PDL coating: NSC adhere well, spread good, still single cells, however more dead cells were detected!
iii. PDL coating & medium supplemented with decellularized brain ECM: NSCs remained mostly single cells but NSCs seem not to attach well (less axons are detected) on the PDL coating.
iv. PDL & decellularized brain ECM coating: NSCs form aggregates. More cells with attachment morphology are detected than cells in PDL coat + ECM at medium. The morphology of NSCs here seems to be something inbetween what observed in PDL coat + ECM at medium and ECM coating group.
v. ECM coating: NSCs form only neurospheres. All single cells are dead. No attached morphology is detected.

Finally, live-dead staining (figures 3.22, 3.23 & 3.24) revealed that 3 days post-seeding, ECM coating had no effect on NSC survival in non-stress conditions (NSC grown in complete NSC medium).

3.5.2 ECM Effects on NSC Differentiation

In order to evaluate the effects of decellularized brain ECM coating on NSC differentiation, NSCs were grown for 3 days using the same five groups presented in Figs. 3.21 to 3.23. Subsequently, cells were fixed and immunostained for Nestin (marker of neural precursor cells), Sox2 (marker of multipotent neuronal stem cells) and Glast (marker of radial immature glia cells). Representative imaging results are shown in Figs. 3.25-3.35. Results suggest that NSCs in all coating group express Nestin, Sox2 and Glast.

No difference is reviled in each category and as a result ECM has no effect in cell differentiation as an adhesion molecule or as a coating substrate.

Figures 3.28, 3.29 & 3.30 show that the plate coating did not affect NSC differentiation (more multipotent cells stained with Sox2 and negative for Nestin, or more mature precursor cells positive for Nestin and negative for Sox2). These results agree with figure 3.35 in that the number of $Sox2^{+}$ $Nestin^{-}$ and $Sox2^{-}Nestin^{+}$ cells is limited and the majority of cells are $Sox2^{+}Nestin^{+}$. Similarly, Figures 3.25,3.26, 3.27 & 3.35 show that the vast majority of cells stain for both Glast and Nestin in all coating groups that cells are $Nestin^{+}Glast^{+}$. The number of cells that stain positive for $Nestin^{+}$ but do not express $Glast^{-}$ is limited. Only a small percent of cell population in PDL coat & ECM in medium are positive for $Nestin^{-}Glast^{+}$ but this difference is not statistically significant.

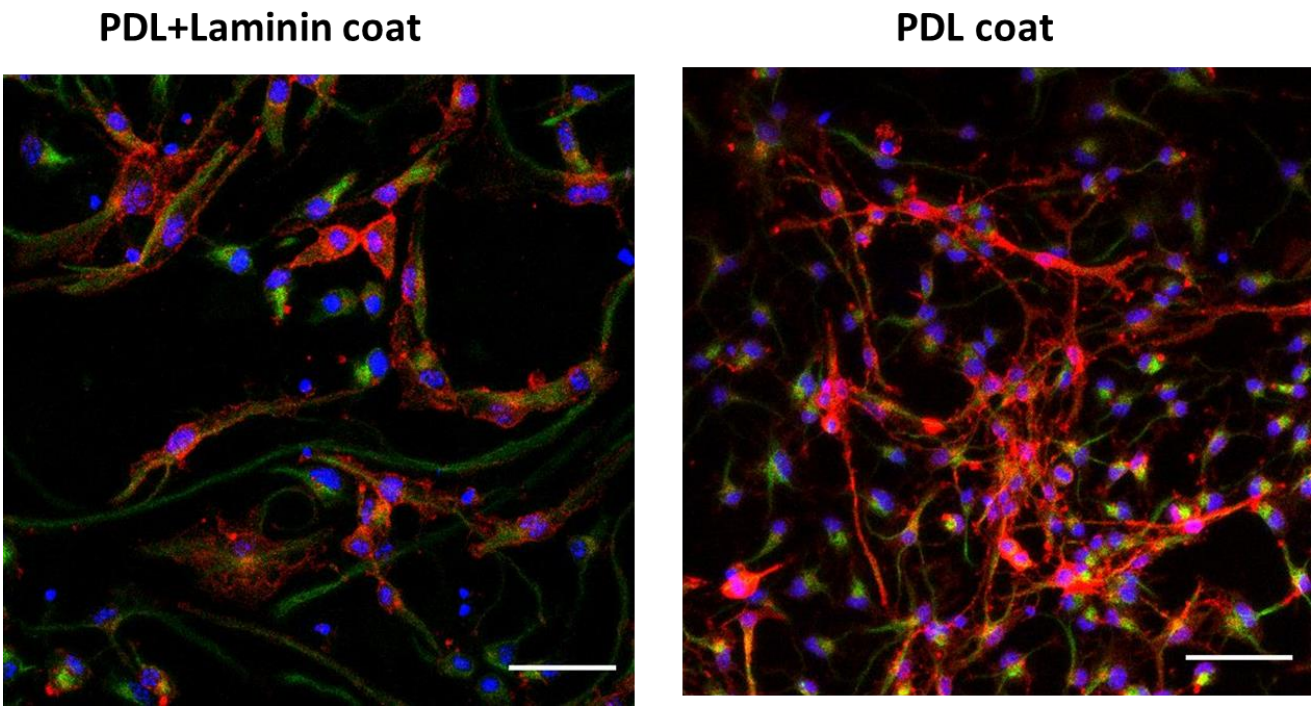


Figure 3.25: Representative confocal microscopy images of 3-day NSC cultures immuno-stained for Nestin (green) and Glast (red), and counterstained with Hoechst (blue). NSCs were grown in wells coated with PDL & Laminin coat, PDL coat. Scale bars: 50 µm.

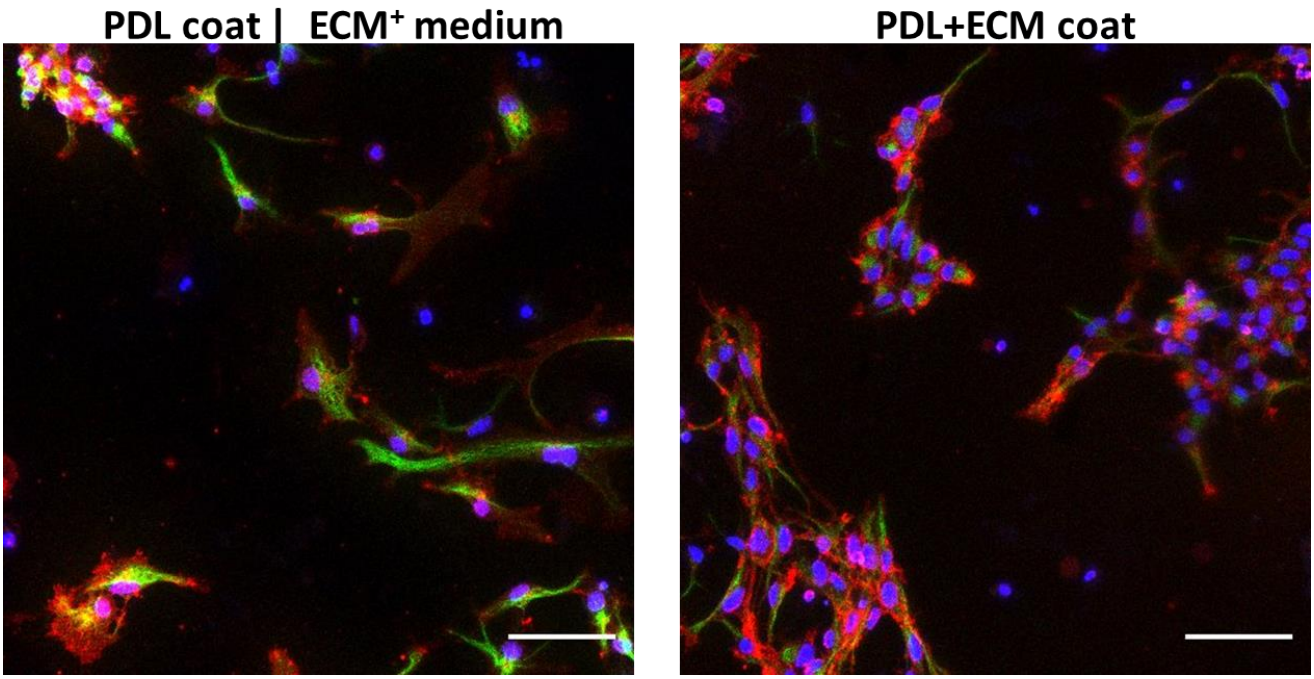


Figure 3.26: Representative confocal microscopy images of 3-day NSC cultures immuno-stained for Nestin (green) and Glast (red), and counterstained with Hoechst (blue). NSCs were grown in wells coated withPDL coat and ECM+ medium, PDL&ECM coat. Scale bars: 50 µm.

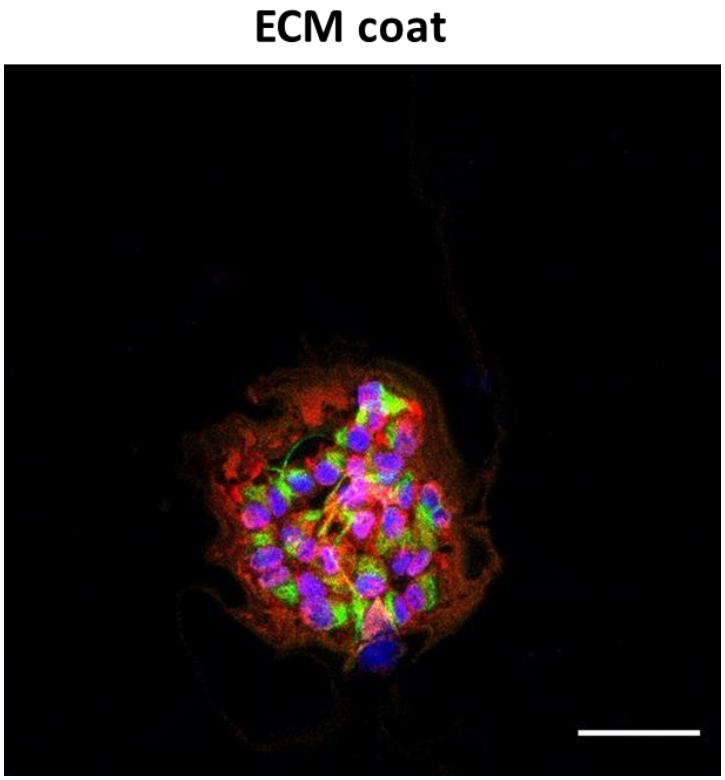


Figure 3.27: Representative confocal microscopy images of 3-day NSC cultures immuno-stained for Nestin (green) and Glast (red), and counterstained with Hoechst (blue). NSCs were grown in wells coated with ECM coat. Scale bars: 50 µm.

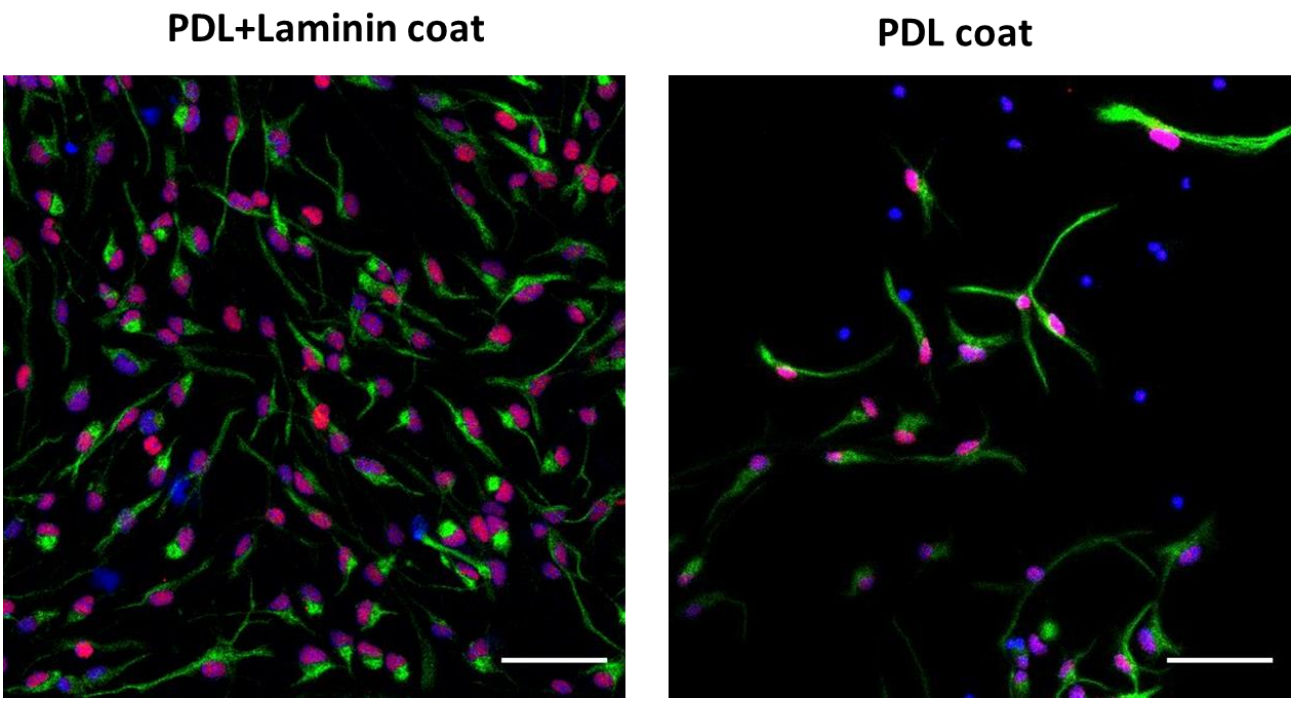


Figure 3.28: Representative confocal microscopy images of 3-day NSC cultures immuno-stained for Nestin (green) and Sox2 (red), and counterstained with Hoechst (blue). NSCs were grown in wells coated with PDL & Laminin , PDL coat. Scale bars: 50 µm.

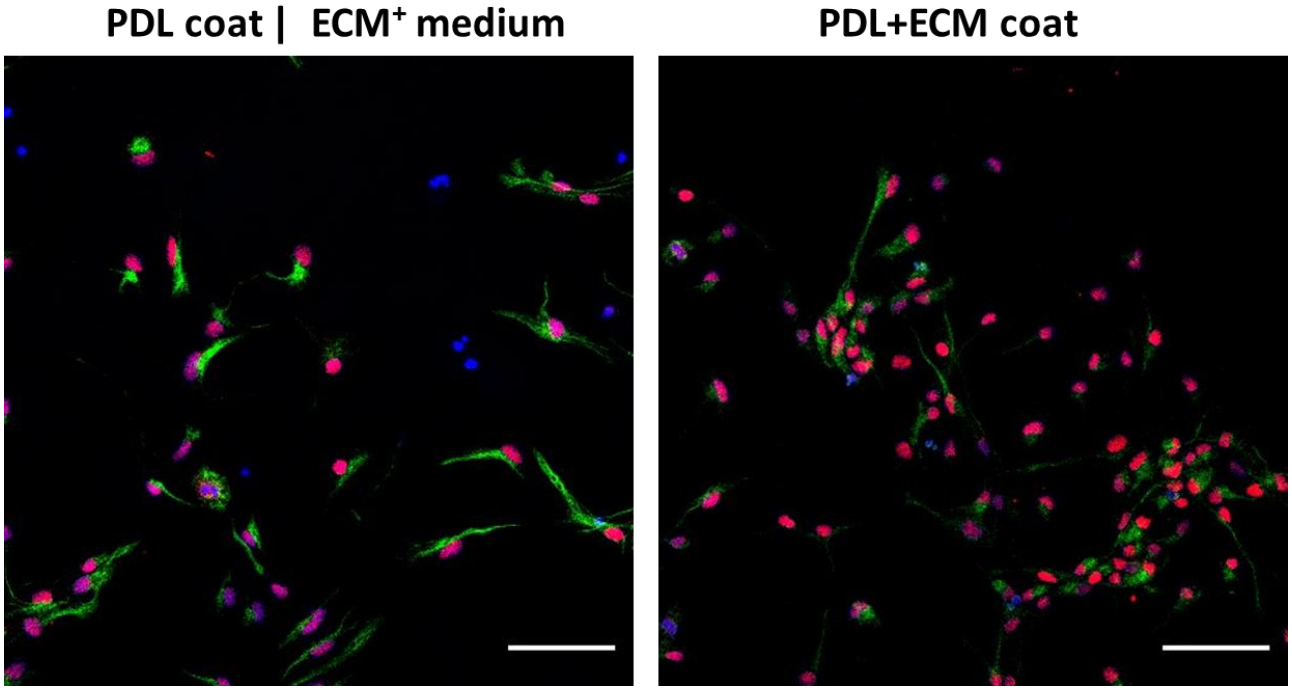


Figure 3.29: Representative confocal microscopy images of 3-day NSC cultures immuno-stained for Nestin (green) and Sox2 (red), and counterstained with Hoechst (blue). NSCs were grown in wells coated with PDL coat& ECM+ in medium, PDL&ECM coat. Scale bars: 50 µm.

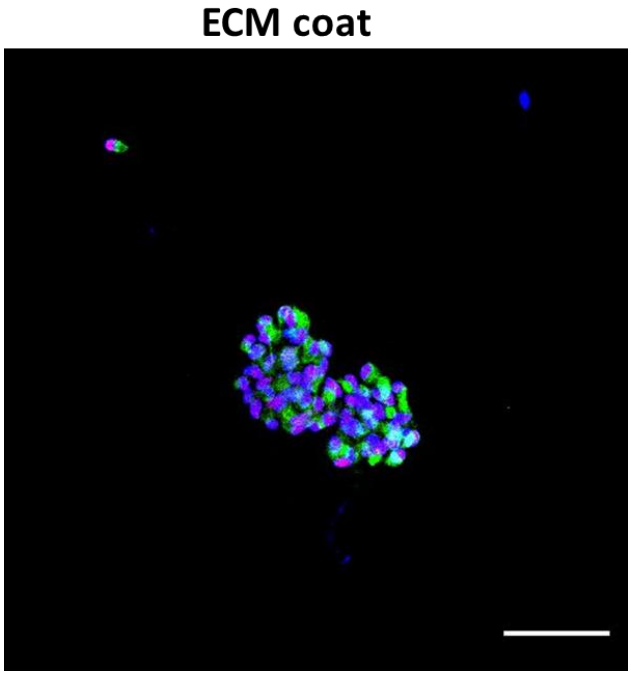


Figure 3.30: Representative confocal microscopy images of 3-day NSC cultures immuno-stained for Nestin (green) and Sox2 (red), and counterstained with Hoechst (blue). NSCs were grown in wells coated with ECM coat. Scale bars: 50 µm.

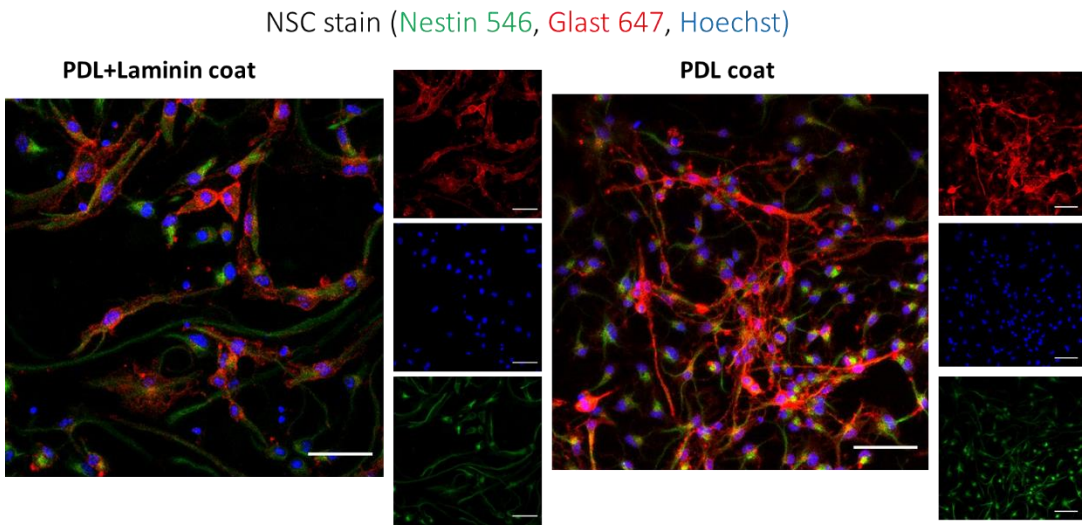


Figure 3.31: Representative confocal microscopy images of 3-day NSC cultures immuno-stained for Nestin (green) and Glast (red), and counterstained with Hoechst (blue). NSCs were grown in wells coated with either PDL & Laminin or with plain PDL. Scale bars: 50 µm.

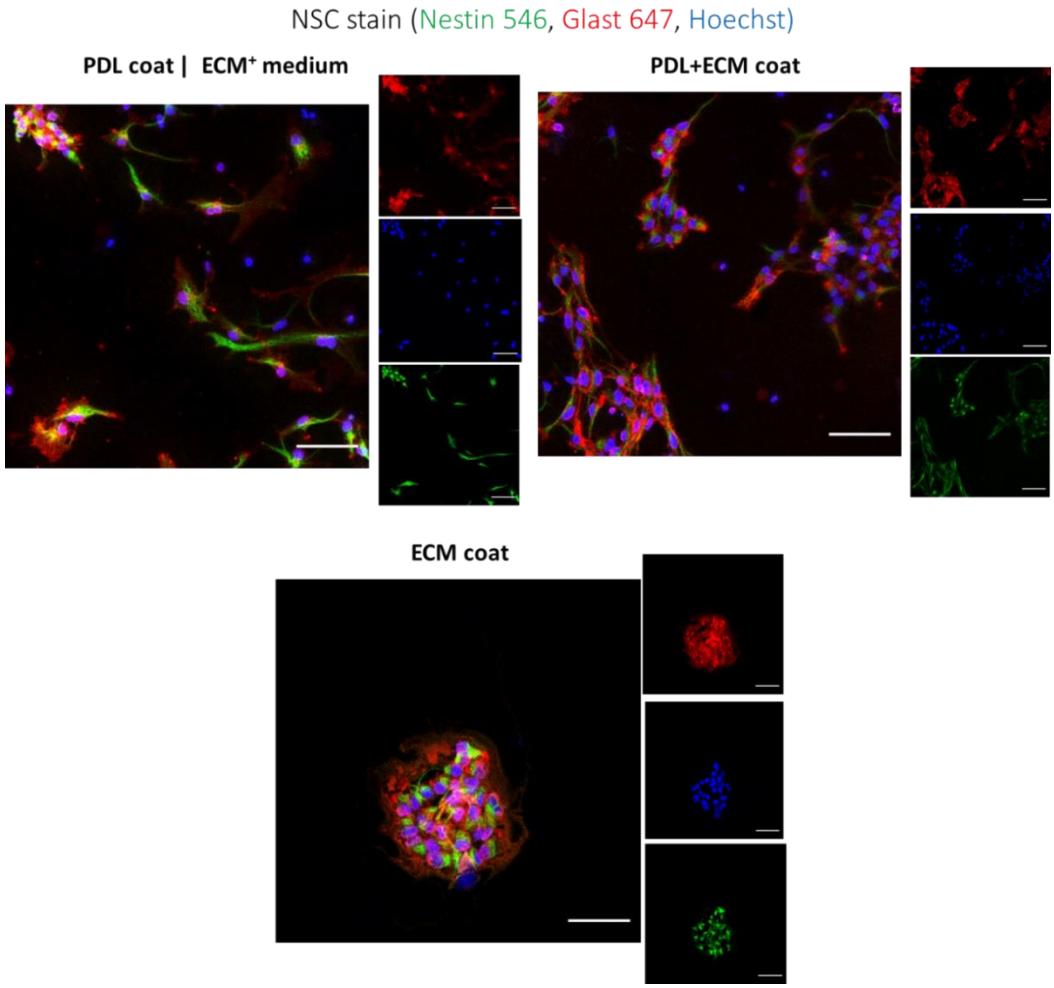


Figure 3.32: Representative confocal microscopy images of 3-day NSC cultures immuno-stained for Nestin (green) and Glast (red), and counterstained with Hoechst (blue). NSCs were grown in wells coated with PDL & ECM, coated with PDL coat and in medium supplemented with decellularized brain ECM, or on wells coated with decellularized brain ECM. Scale bars: 50 µm.

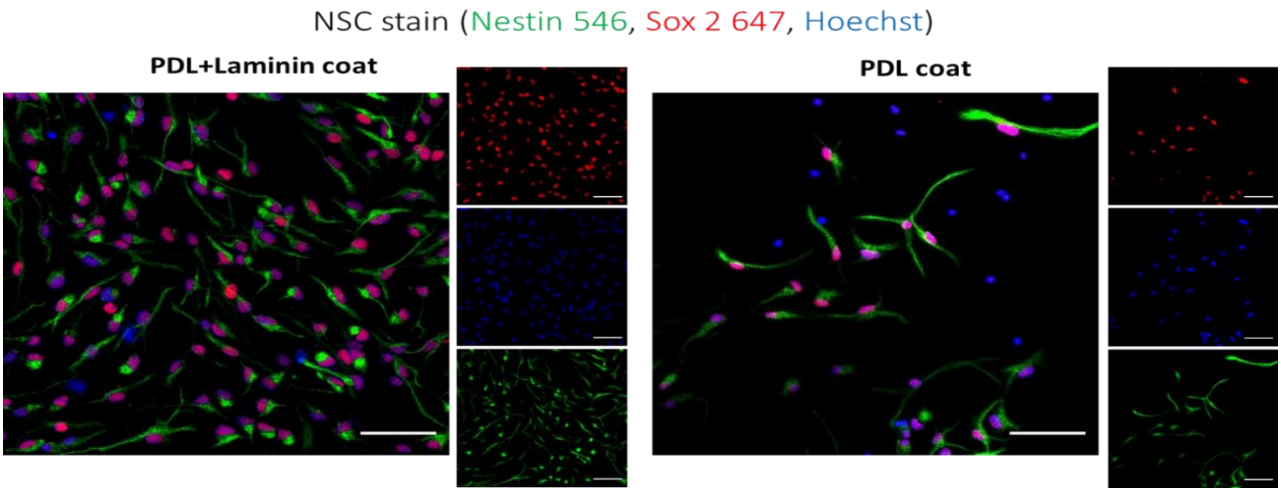


Figure 3.33: Representative confocal microscopy images of 3-day NSC cultures immuno-stained for Nestin (green) and Sox2 (red), and counterstained with Hoechst (blue). NSCs were grown in wells coated with either PDL & Laminin or with plain PDL.

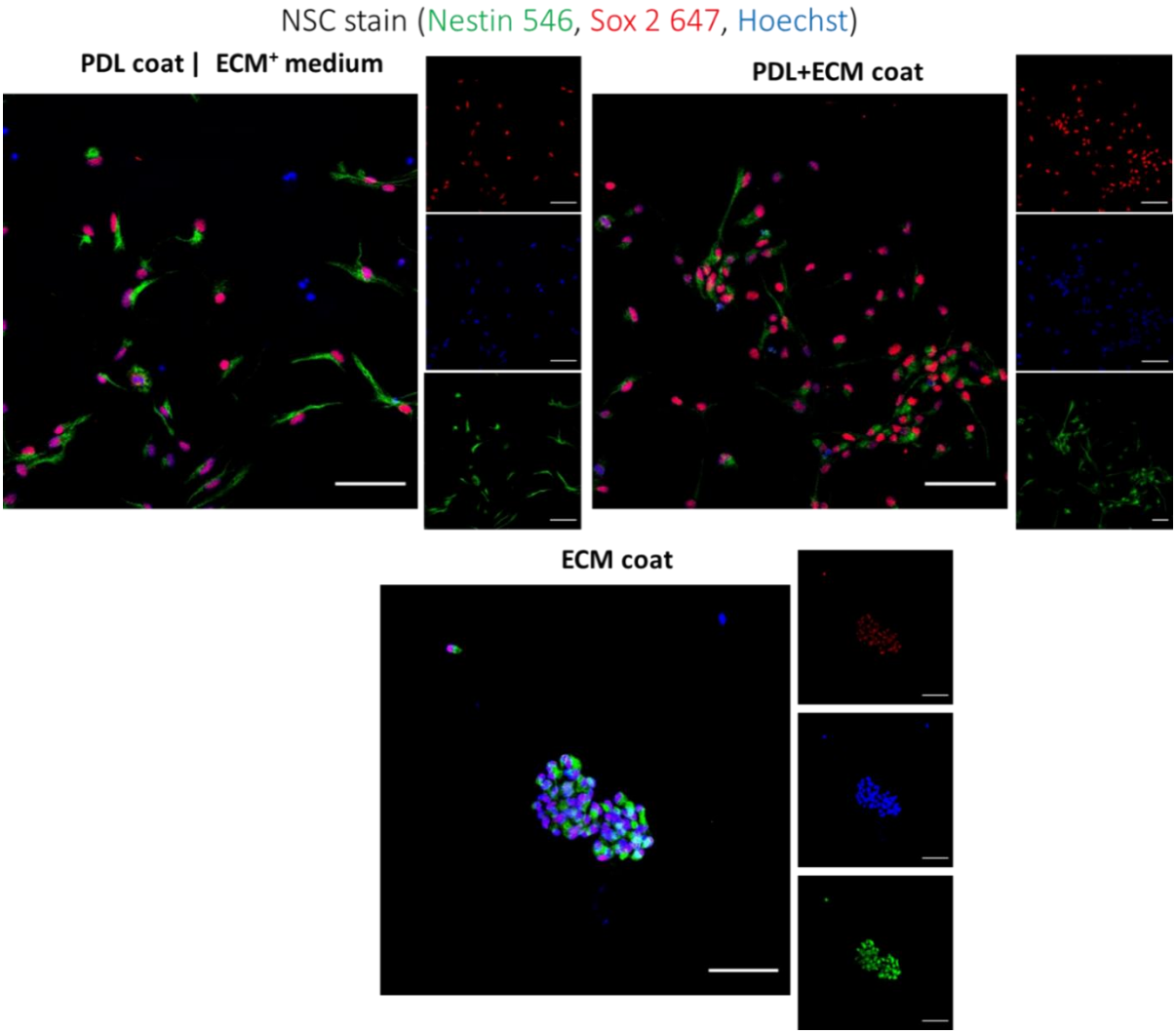
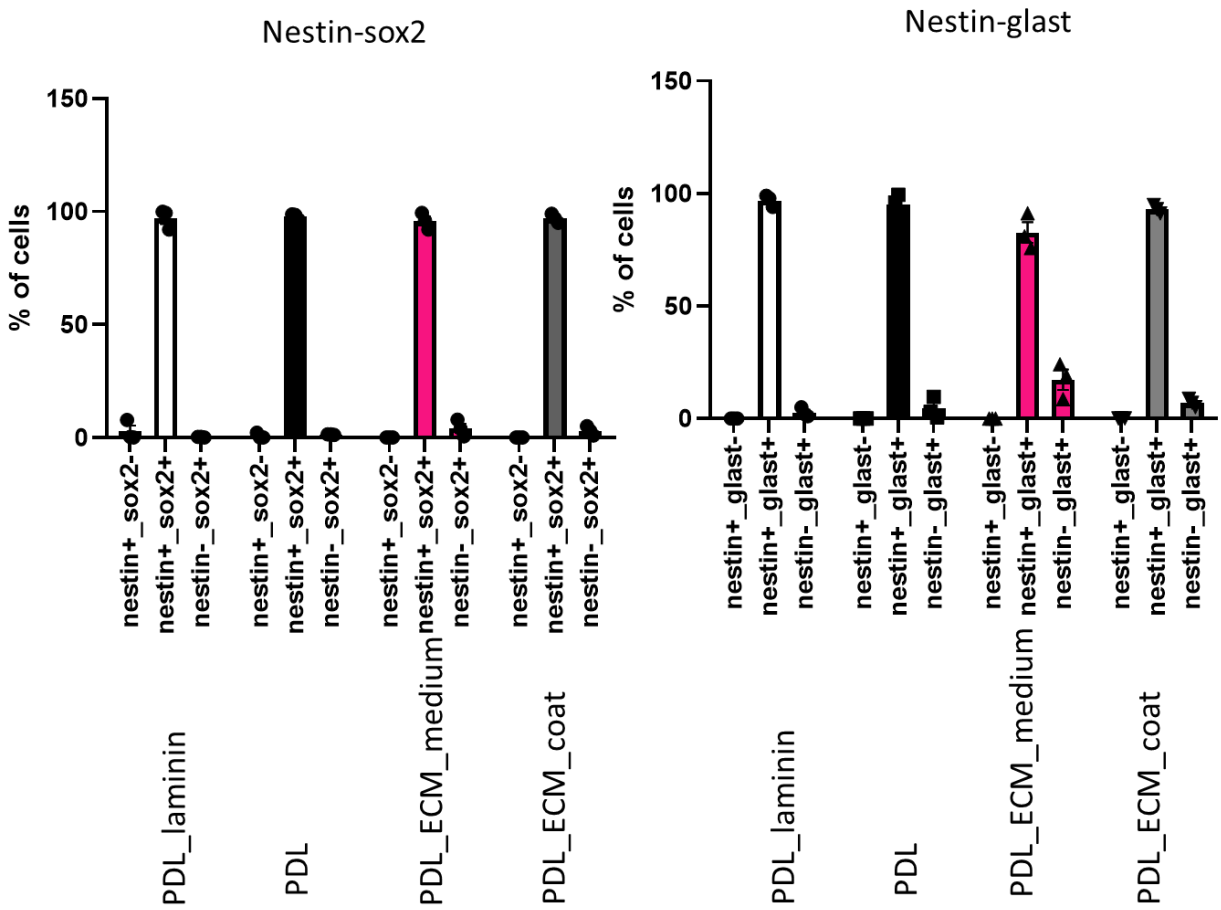


Figure 3.34: Representative confocal microscopy images of 3-day NSC cultures immuno-stained for Nestin (green) and Sox2 (red), and counterstained with Hoechst (blue). NSCs were grown in wells coated with PDL & ECM, coated with PDL coat and in medium supplemented with decellularized brain ECM, or on wells coated with decellularized brain ECM. Scale bars: 50 µm.



Group	Nestin⁺ Sox2⁺	Nestin⁺ Sox2⁻	Nestin⁻ Sox2⁺
PDL&laminin coating	97.1 ±2.6 %	2.7 ±2.6 %	0.2 ±0.1 %
PDL coating	97.8 ±0.8 %	0.7 ±0.7 %	1.3 ±0.3 %
PDL&ECM coating	97.0 ±1.2 %	0 .0 ± 0.0%	3.1±1.2 %
PDL coating + ECM in medium	95.9 ±2.1 %	0. 0 ± 0.0 %	4.1±2.1 %

Group	Nestin⁺ Glast⁺	Nestin⁺ Glast⁻	Nestin⁻ Glast⁺
PDL&laminin coating	97.0 ± 1.5%	0. 0 ±0.0 %	2.7±1.2 %
PDL coating	95.2± 2.7 %	0.0 ±0.0 %	4.4 ±2.7 %
PDL&ECM coating	93.0 ± 1.1 %	0.0 ±0.0 %	7.0 ±1.1 %
PDL coating + ECM in medium	82.7± 4.6 %	0.0 ±0.0 %	17.3±4.6 %

Figure 3.35: Quantification of the fraction of cells that stains positive for nestin, sox2 and glast after growing NSCs for 3 days in five specific conditions shown in Figs. 3.24 to 3.27. Data are presented as mean±s.e.m, n=3. The mean among groups are not statistically different P>0.99 (one-way Anova). Due to difficulties in quantifying cells in neurosphere, this quantification do not include the ECM only coating.

4. Discussion

This study presents a novel procedure for the decellularization of adult mouse brain tissue pieces. The developed procedure provides decellularized brain ECM in powder form, appropriate for use as a medium supplement or as a raw material for biomaterial fabrication.to support long-term growth of multiple cell types.

The outcome of the developed procedure was characterized extensively using proteomics and immunohistochemistry. Indeed, IHC revealed that the resulting decellularized tissue contains very few nuclei, much less compared to control brain tissue (Figs. 3.9, 3.10). Immublotting analysis revealed that extracts from decellularized brain samples lacked detectable intracellular protein β actin (figure 3.3), yet they contained components of CNS extracellular matrix including fibronectin (Figure 3.5) and laminin (figure 3.8).

The extracellular matrix of the brain contains relatively small amounts of fibrous proteins such as fibronectin, laminin and collagen (Crapo et al. 2012, Crapo et al. 2014, Lau et al.2013, Volpato et al. 2013). Yet, these components have been shown able to affect cell survival, proliferation and differentiation, regulate synapses formation, and stabilize neural cell connections. Such proteins (like laminin and fibronectin) were isolated using the developed decellularized protocol from brain tissue samples. During decellularization process ECM proteins (like fibronectin and laminin) have been isolated and the protein outcome of decellularization was evaluated and it was detected in ECM powder could maintain its native role after decellularization.

The characteristics of a brain-derived ECM scaffold, which is very weak and degrades quickly, led to difficulties in rinsing and recovering the ECM matrix for hydrogel scaffold, for use as a coating or as a solution for enrichment cell environment. (Crapo et al. 2012, De Quach et al. 2011, Volpato et al. 2013). At the same time residual of cellular material within the ECM scaffold, particularly DNA, may cause incompatibility *in vitro* and adverse inflammatory responses *in vivo* (Ott et al.2008, Yuan et al. 2011, Among et al. 2016).

Multiple publications have reported tissue decellularization protocols, which have been utilized to provide decellularized tissue in solution, scaffold or coatings and mimic the *in vivo* micro-environment in a variety of cell cultures. Such ECM enrichment methods have shown tissue-specific effects on cellular behavior, and in some instances increased maturation when compared with conventional substrates (De Quach et al, 2011, Among et al. 2016). Biocompatibility is one of the general issues that must be considered when a biomaterials or scaffold is designed (Guo et al. 2010, DeQuach et al. 2011, Arenas-Herrera et al. 2013, Zhu et al. 2015).

In order to characterize possible toxic effects of the derived decellularized brain ECM powder in neural cell, ECM powder was solubilized in 50 mM acetic acid and used as an enrichment in medium. Biocompability of ECM powder as an enrichment for NSC34 and PC12 under control conditions (medium with serum) and stress conditions (serum-free medium) was evaluated using the Celltox Green Assasy. Results revealed that decellularized brain ECM was not toxic to both PC12 (Figs.3.12, 3.14) and NSC34 (Figs. 3.18 & 3.20) cells in serum condition. Furthemore, supplementing serum-free medium with 50 µg/ml decellularized brain ECM significantly reduced cells apoptosis induced by serum withdrawal. These results suggest that decellularized brain ECM powder after solubilization in acetic acid is not toxic to cells. They also suggest that decellularized brain ECM can provide neuroprotective clues to cells under stress conditions and protect cells from apoptosis. Using different concentrations of ECM powder in survival assay of PC12 and NSC34 cell lines will help in identifying the proper concentration of decellularized brain ECM that can protect cells from apoptosis. It would be also important to detect if ECM powder can have an effect in cell apoptosis as

a coating substrate. ECM in solutions reviled significant cell apoptosis reduction in stress conditions, so ECM coating can provide useful ingredients and protect cells even before starvation and as a result to prevent also from apoptosis. Follow-up experiments could focus on the effects of decellularized brain ECM (as solution and coating) in the differentiation of PC12 and NSC34 cells. Both cell lines can differentiate (Wiatrak et al. 2020, Menzies et al. 2002) and it would be interesting to identify if components in the brain ECM can provide signals for survival and differentiation *in vitro*.

Decellularized brain ECM was also evaluated for its effects as coating and in medium on NSC adhesion, survival and differentiation. Fig. 3.23 presents how the morphology of seeded NSCs were altered within 3 days of culture in five different conditions, including four different coatings. The unexpected finding was that NSCs cultured in ECM coating, stayed in neurosphere morphology and did not adhere as single-cells on plastic surfaces coated with decellularized brain ECM. These neurospheres were weakly attached on the ECM coating, in contrast to free-floating neurospheres, which form when NSCs are grown in flasks without coating. When NSCs were seeded in wells coated with decellularized brain ECM plus PDL, they formed aggregates and many NSC with attachment morphology (axons of NSCs are detected to be attached with coating material) are detected. Finally, adding decellularized brain ECM in the medium also affected NSC morphology: when the medium of NSCs seeded on PDL coated wells was supplemented with soluble decellularized brain ECM, NSCs remained mostly single cells although some cell aggregates were still detected.

Follow-up experiments should investigate the effect of multiple concentrations of decellularized brain ECM on neuropheres formation and probe more in detail the interactions between NSCs and decellularized brain ECM coatings. Important observations would be detected also if ECM was double coated with Laminin, a key coating protein utilized in monolayer NSC culture.

Although NSCs do not attached to decellularized brain ECM coatings as well as on PDL-Laminin coatings, live-dead staining (Figures 3.23 & 3.24) revealed no difference regarding the percent of live cells after three days in culture. These results suggest that the derived decellularized ECM powder is biocompable with NSCs. Overall, experiments in PC12, NSC34 and primary NSCs revealed no toxic effects of decellularized brain ECM to cell cultures.

The effects of decellularized brain ECM, when used as a coating (with or without PDL coat) or when used as an enrichment in medium (Figure 3.21), on NSC differentiation revealed that NSC in all coating groups expressed Nestin, Sox2 and Glast at similar amounts. No difference was detected in NSC differentiation as a result of utilizing decellularized brain ECM as a coating substrate or as a medium supplement. Future work could probe the effects of supplementing the medium with larger concentrations of decellularized brain ECM powder.

This master thesis provides a raw source of decellularized brain ECM, which can be utilized to fabricated porous scaffolds. Utilizing porous scaffolds for 3D cell culture or for implant development offers several advantages and already NSC-seeded porous collagen scaffolds have demonstrated effects in spinal cord injury (Kourgiantaki et al. 2020). The fabrication of porous scaffolds made of decellularized brain ECM could enhance key cell phenotypes after CNS injury, including cell survival and axonal regeneration. In future implantantions of collagen-based scaffold enriched with ECM, inflammatory response markers should be detected after injection in mouse spinal cord. It is expected that due to natural ingredients that decellularized brain scaffold contain should not provide inflammatory response in mouse spinal cord injury.

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Appendix A: Decellularization Troubleshooting

A1. Incomplete Decellularization

Various versions of the decellularization protocol were tested before reaching the final decellularization procotol derived in this work (Decellularization protocol in Materials and Methods). Not all decellularization protocols were efficient in terms of cell removal. Figs. A.1 and A.2 shows that cell Hoechst$^+$ nuclei are visible in cryosections of tissue not decellularized completely. Figs. A1.1 and A1.2 show that in sections of poorly decellularized brain tissue there is just a small reduction in Hoechst$^+$ compare to control brain sections.
In this work, the crucial steps for enhancing decellularization efficiency were H_2O treatment and TritonX-100.

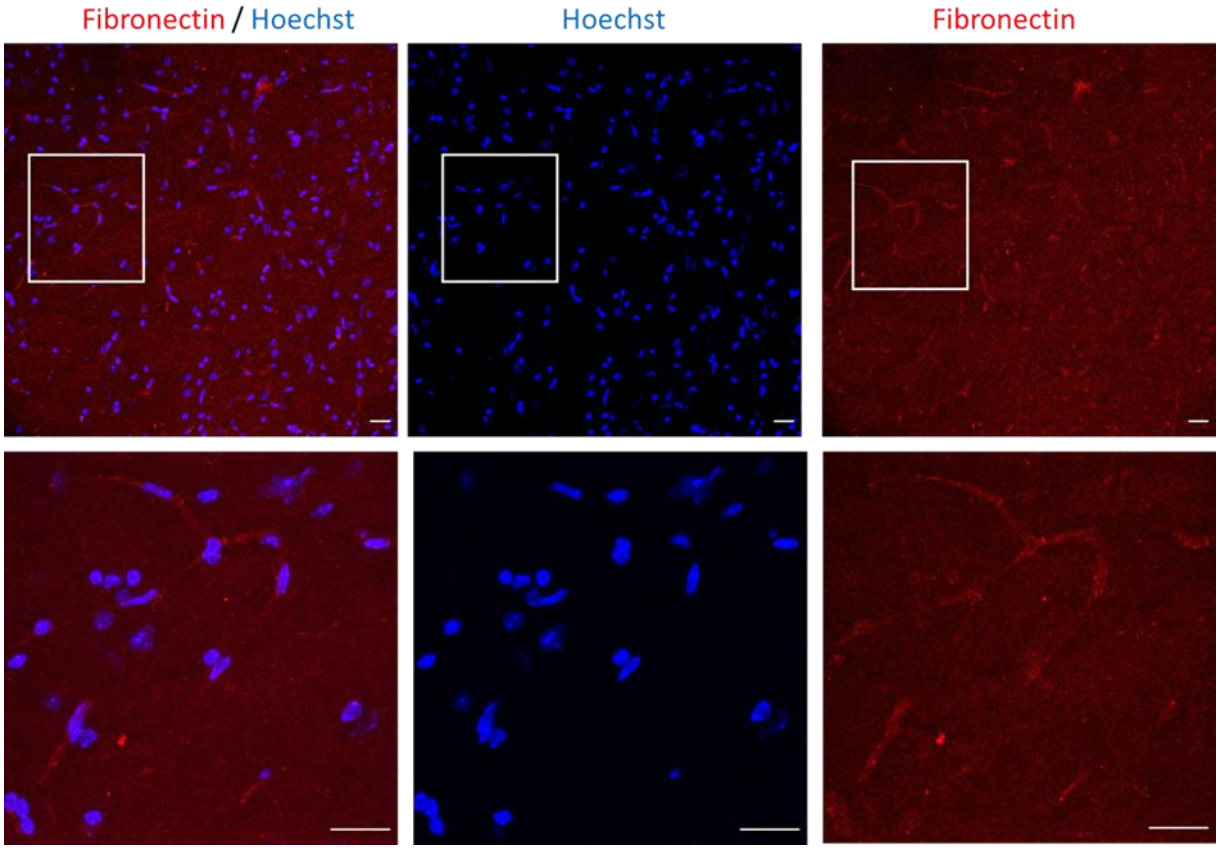

Figure A1.1 Immunohistochemical localization of fibronectin (using a pan-fibronectin antibody) in cryo-sections from control (untreated) brain tissues. Top row: representative confocal fluorescence microscopy images. Bottom row: higher-magnification images of ROIs in top row images (white boxes). Scale bars: 20 µm.

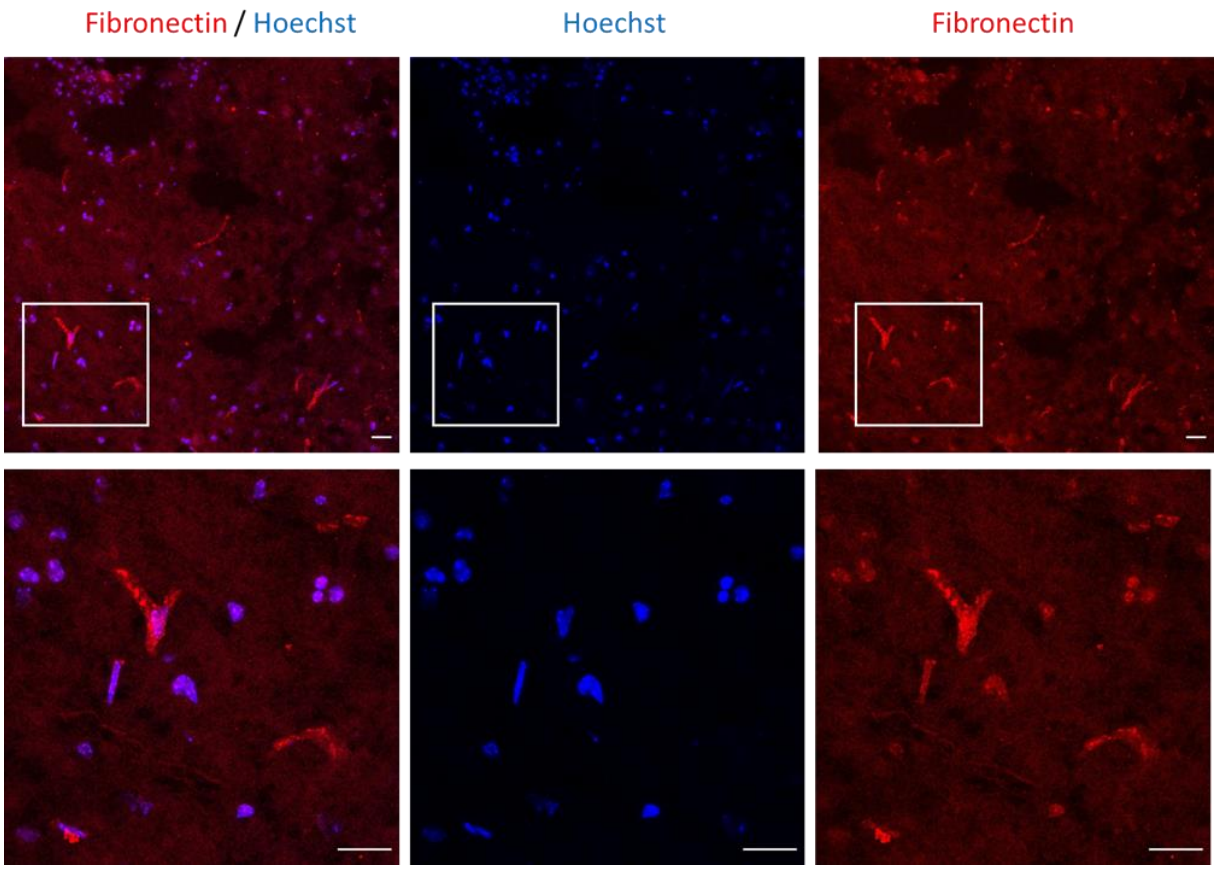

Figure A1.2 Immunohistochemical localization of fibronectin (via a pan-fibronectin antibody) in cryo-sections from decellularized brain tissues. Top row: representative confocal fluorescence microscopy images. Bottom row: higher-magnification images of ROIs in top row images (white boxes). Scale bars: 20 μm.

A2. Challenges in Reliably Measuring Protein Density of Brain Tissue Extracts

For Proteomic characterization of Decellularized tissue after obtained in the end of the procedure, the decellularized powder was diluted in 50mM acetic acid in ½ w/v tissue mass per acetic acid volume. Similar tubes with control (non-decellularized) brain lysates were snap freeze in isopentane, homogenizing in a dounce homogenizer, diluting in acetic acid and sonicating 3 times for 5 seconds. In all samples, 1% PMSF was added after homogenization and before sonication. The protein content of the resulting suspensions was qualified via the BCA assay using established procedures. Based on the protein concentration standard curve derived from BSA dilutions, samples (control & decellularized) in acetic acid had low amount of proteins concentration (about 0.2mg/ml).

In western blot using 12% acrylamide gel, ponceau staining revealed that there no proteins in the nitrocellulose membrane and only some proteins with high molecular weight exists in one of the duplicates of decellularized sample and in control sample. Using acetic acid as lyssis reagent was not the right choice. The result in Figure A2.1 (ponceau staining) showed that the transfer of the proteins was succeed (according to the bands from the lysate NIH-3T3). As a result the other two samples (brain 1 and ECM 5 diluted in acetic acid) had less proteins and probably acetic acid may destroy small molecular weight proteins. For that reason acetic acid was no longer used as solution buffer for protein characterization.

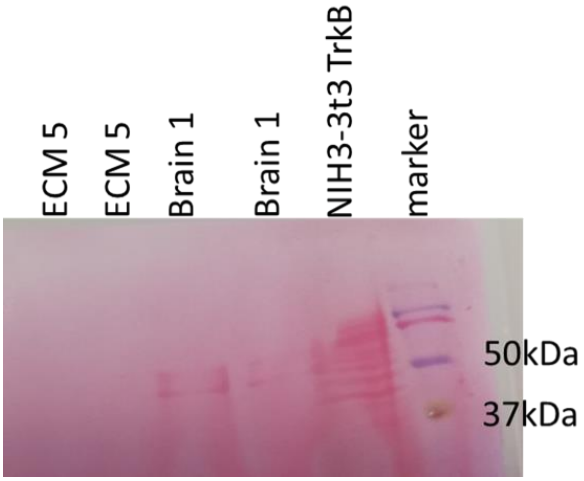

Figure A2.1: Ponceua staining in nitrocellulose membrane after transfer before blocking, of decellularized brain samples (ECM5) and control non-treated brain sample (Brain 1), diluted in 50mM acetic acid, including lysates from NIH-3T3 cell line. All samples were quantify for 20μg of protein. In conrol samples (Brain 1 & NIH3T3) some proteins are detected in membrane. In ECM doublicate sample proteins didn't pass to nitrocellulose membrane, or 20μg of protein were not right quantified.

RIPA buffer was used instead of acetic acid for samples dilutions for protein characterization of control and decellularization samples. For qualification of the tissue samples in RIPA buffer, BCA assay was used and in the western blot wells were loaded with 50 μg of protein. Figures A2.2, A2.3, are presented the first results of characterization the decellularized samples. Using RIPA buffer as a solution for protein characterization reveals more protein bands in nitrocellulose membrane with Ponceau staining but still less proteins are detected in decellularized samples. Additionally, the control brain 4, diluted in RIPA buffer, expresses GAPDH.

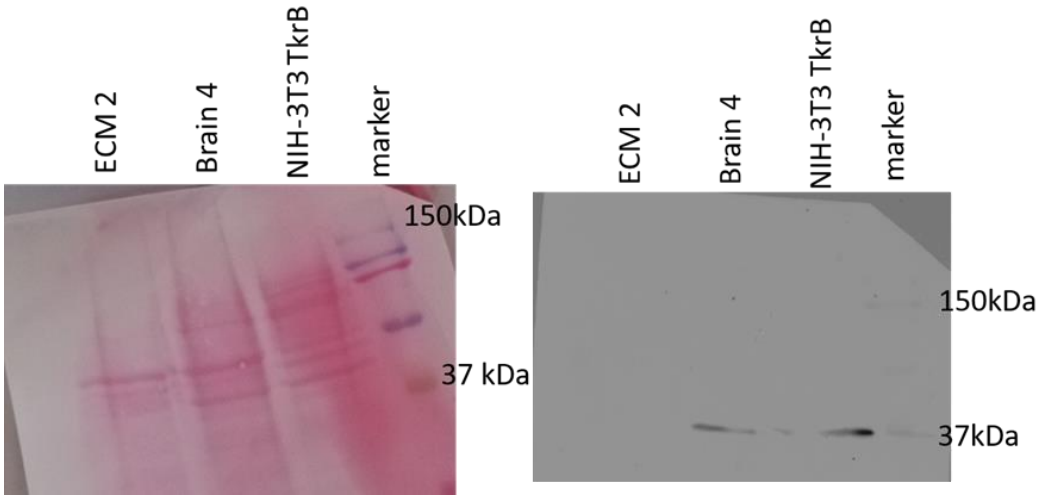

Figure A2.2: Left: Ponceau staining in nitrocellulose membrane before transfer, here ECM2 is the decellularized brain diluted in RIPA buffer and brain 4 is the control brain directly diluted in RIPA buffer. The transfer was succeed and the proteins in each sample have been transferred to membrane. Right: Expression of GAPDH is detected in brain sample (brain 4 loaded 50μg proteins) but do not detected in ECM2 (decellularized brain tissue loaded for 50μg protein). Sample brain 4 and ECM 2 are diluted in RIPA buffer. As a control the expression of GAPDH in lysates NIH-3T3.

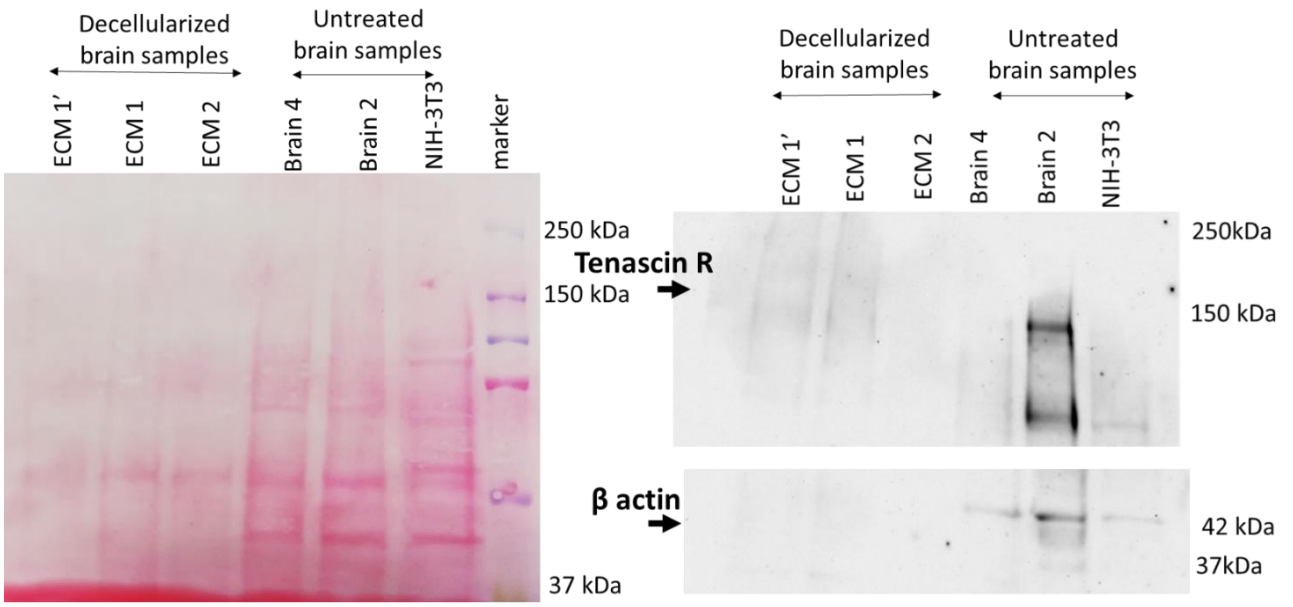

Figure A2.3:Left: Ponceau staining in nitrocellulose membrane after transfer. . The protein transfer was succeed only in control samples but in decellularized samples (ECM 2,1,1') less proteins were transferred to membrane. Right: Expression of Tenascin R is presented at 160kDa and is detected clearly only in Brain 2. GAPDH expression detected only in control samples (Brain 4, 2 & NIH3T3).

According to figures A2.2-A2.3 decellularized samples didn't detected any GAPDH or β actin expression. But according to ponceau staining in membranes decellularized samples had less proteins transferred to membrane comparing to control samples even thought all samples were quantified and loaded for 50μg of protein.
The observations after these experiments was that only control brains express housekeeping proteins, yet sample loading was not constant. Protein loading problem was solved after a lot of sonication in samples and well homogenization. After well homogenization of samples (decellularized and control brain) protein quantification was valid and the same amount of protein in every sample was loaded for 50μg of protein in wells of acrylamide gel.

A3: Difficulties in Analyzing Brain Tissue Samples via Immunoblotting

Trypsin Treatment in Brain tissue and Decellularized tissue for Break ECM Protein Aggregates:

In order to characterize the expression of housekeeping proteins in decellularized samples and brake protein aggregates trypsin treatment was used. After decellularization of ECM samples and dilution in RIPA buffer, samples were further diluted 1:1 v/v with 2,5% trypsin and incubated for 18h, at 37°C. Then samples were centrifuged for 10 min at 4°C in 14.500g. Pellet and supernatant of samples were separated and pellet was re-suspended in 50μl RIPA.

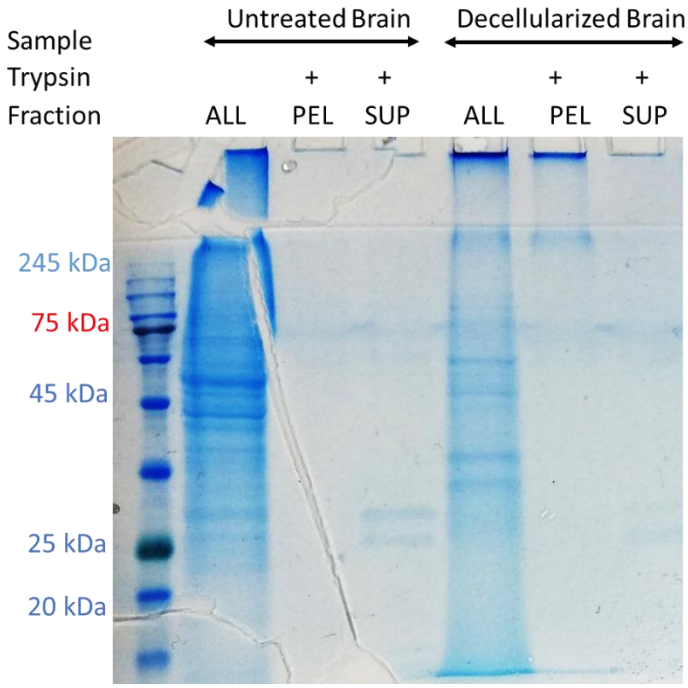

Figure A3.1Coomassie G250 staining in 12% acrylamide SDS-PAGE. Samples were loaded for 50µg of protein. On top of SDS-PAGE is presented the treatment of each sample, the fraction and the time of treatment.

In SDS_page stained for commassie, Figure A3.1, is detected that samples with all fractions, which are not treaded with trypsin, more than 2 bands are detected. Although important amount of protein stayed in wells. In pellets from control sample no band was detected in the gel. Similarly, in pellet of decellularized sample no bands are detected in the gel but some proteins were stuck in the well of stacking gel and one band is presented above 245kDa in separating gel. As a result ECM aggregates didn't break. In supernatants from control and decellularized samples were detected 2 bands between 20 to 25 kDa. Probably one of those bands was trypsin (23,3 kDa molecular weight).
Most of the ECM proteins stayed in the pellet after centrifuge of the treated sample with trypsin.

Different time points of Trypsin Treatment:

Decellularized brain samples (ECM2' & ECM1) and Control brain sample (Brain 2) were diluted 1:2 w/v with RIPA. Then brain 2 & ECM2' were further diluted with 2,5% trypsin 1:1 v/v and incubated for different time points (8h, 22h, 30h) at 37°C. Sample concentration was calculated via A280 (in nanodrop) from the 22h treated samples with trypsin. The untreated samples concentration was calculated via A280 nm (Brain 2 &ECM2'). Run samples in a 12% acrylamide gel (50µg protein /well, 2 hour). Stained via Coomassie Brilliant Blue G-250.

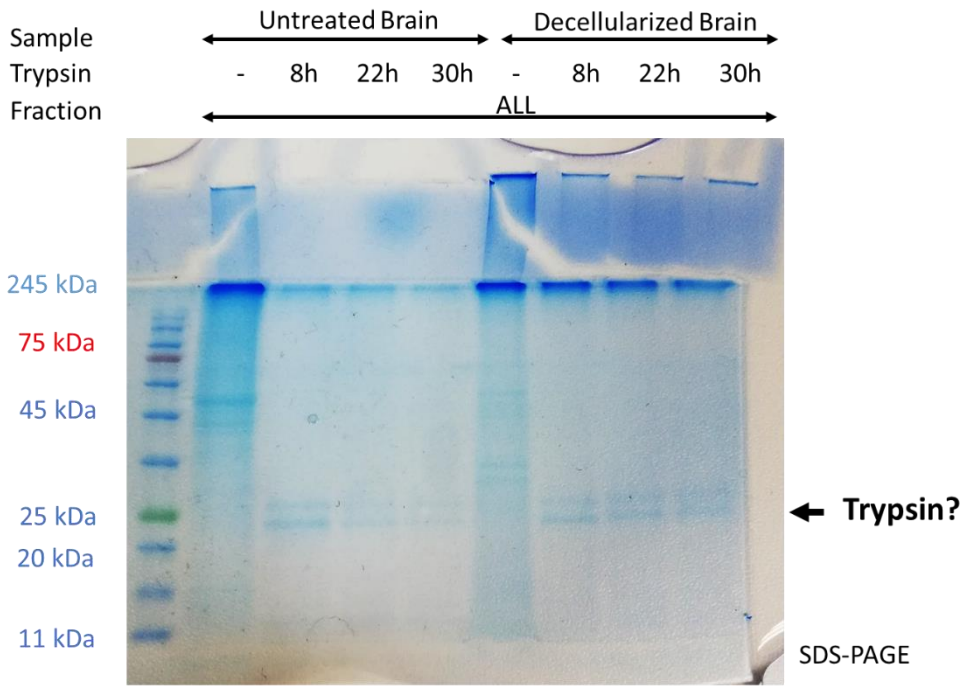

Figure A3.2 Commassie staining G250 in 12% acrylamide gel. Samples were loaded for 50µg of protein. On top of SDS-PAGE is presented the treatment of each sample and the time point of each of treatment.

Duration of trypsin treatment 8-30h doesn't affect protein aggregates (Figure A3.2) and as a result proteins remain at interface between stacking (4%) - separating (12%) gel. The problem of protein aggregates that doesn't let proteins to pass to stacking gel and some of those proteins were stuck in the interface between separating and stacking gel was not solved with trypsin treatment.

Using gradient 4-20% acrylamide gels from Bio-rad let proteins run at the right molecular weight and be detected.

Appendix B: Experimental Protocols

B1: Brain Tissue Decellularization

Materials

- Scalpel for cutting the brain
- 2ml eppendorf
- DNAse 600U/tube (Sigma, catalog number: 10104159001
- Homogenizer dounce
- Centrifuge
- 50mM EDTA (Sigma, 101903086)
- Protease (Calbiochem, DO0170007) and Phosphatases (Millipore, 524629-1SET P) Inhibitors
- PBS 1% p/s
- Triton-X100 (Sigma, 101425569)
- dH_2O
- isopenatene
- dry ice
- 50 mM acetic acid (MERCK, K25686663)
- 1x RIPA buffer

Procedure

Decellularization of mouse brain tissue

1. Cut brain tissue in small pieces and put them in 2ml epppendorf
2. Weight tissue in each eppendorf (0,130gr-0,3gr tissue)
3. Add 1ml sterile dH_2O supplemented with Penicillin/Streptomycin (P/S) in the eppendorf and 1% PMSF. Incubate 7-8h at 4°C while rotating (cold room, IMBB)
4. Centrifuge for 2 min in 6.000g at 4°C
5. Discard supernatant
6. Add 1ml 3.0% Triton X-100 supplemented with 2mM EDTA and 1,1% PMSF. Incubate 12-14h at 4°C while rotating (cold room, IMBB)
7. Centrifuge for 2 min in 6.000g at 4°C
8. Discard supernatant
9. Repeat steps 3 to 8
10. Add 1ml PBS supplemented with P/S and 1% PMSF. Incubate 2h at 4oC while rotating (cold room, IMBB).
11. Centrifuge for 2 min in 6.000g at 4°C.
12. Discard supernatant
13. Add 400μl 600 U/tube DNase (1500 Units/ml) in 37oC for 18h.
14. Centrifuge for 2 min in 6.000g at 4°C.
15. Discard supernatant.
16. Add 1ml PBS supplemented with P/S and 1% PMSF. Incubate 1.30h at 4°C while rotating (cold room, IMBB).
17. Centrifuge for 2 min in 6.000g

18. Repeat step 16
19. Centrifuge for 3min in 8.000g at 4°C
20. Weight the decellularized brain tissue in the tube (using accuracy weight scale weight the tube with the tissue and then subtract the weight of a tube without tissue inside, the number that you take from the subtraction is the weight of the tissue.

Solubilize Decellularized mouse brain

21. Put a beaker in dry ice and leave it for 2 minutes (until it cools down)
22. Put in the frozen beaker (on dry ice) already cold isopentane (it is stored in -20oC) and leave it for 2 min.
23. Snap freeze brain tissue by quickly immersing eppendorf into isopentane located on top of dry ice. Leave inside isopentane for 1 min.
24. In the meantime place a dounce homogenizer in dry ice
25. Put the frozen tissue sample in the dounce homogenizer (on dry ice) and brake the sample via with 50 up and down motions.
26. After the brake of the tissue, leave pestle (the area of the pestle that touches the tissue) in dry ice and leave it until cools down
27. Take the resulting powder tissue with the cold pestle and place it in a 2ml tube in dry ice.
28. Weight the powder tissue in a scale
29. Keep powder tissue at -20°C until use

B1.2: Western blot characterization (Protocol 1)

Protocol starts after step 29 in "Solubilize Decellularized mouse brain"

30. Add 1x RIPA buffer in the tube with the powder tissue 1:2 w/v dilution. For example decellularized brain 2 after freezing with isopentane was weighting 0,109gr, so I put 0,200ml 1x RIPA buffer in the tube with the powder tissue. In every sample add 1% Protease and Phospatase Inhibitors after RIPA homogenization.
31. Store tube in -20°C until use.

Protein Quantification

Before Protein quantification samples should be well homogenized in ripa buffer.

1. Homogenize samples via a dounce homogenizer; 3 rounds x 2 min up and down)
2. Sonicate samples using SONICS Virba cell Ultrasonic Processor 72434 for 3 times x 30 seconds at pulsed 40 amplitude.

Repeat process (homogenization and sonification) for 3-4 more times per day, two days in row until samples were fully homogenized (store in between overnight at 4oC).

The protein concentration of control and decellularized brain lysate solutions was measured via the bicinchoninic acid (BCA) assay.
Protein quantification were calibrated using a standard curve of BSA solutions known concentration (0, 0.1, 0.5, 0.75, 1 mg/ml) in 1:10 ripa: PBS buffer.

1. Vortex samples for 1 min
2. In 96 well plate add 10 μl of each BSA solution and sample, all loaded in duplicates
3. Add 200 μ l BCA working reagent (1:50 reagent B solution: reagent A solution) per well.
4. Incubate plate at a 37oC in oven for 30 min while covered with aluminum foil.

5. Measure the optical absorbance of each sample at 545 nm using a platereader (ChroMate Awareness Technology; Garinis lab, IMBB).
6. Calculate sample concentration (μg/μl) based on absorbance measurements of BSA standards, which were utilized to derive a linear calibration curve.
7. Store samples in -20oC until use.

B1.3: Lyophilization (Protocol 2)

Protocol starts after step 29 in "Solubilize Decellularized mouse brain"

1. Dilute Decellularized tissue in 50mM acetic acid and homogenized it in ice.

For example: 100 mg decellularized tissue was reconstituted in 250 μl 50 mM acetic acid.

2. Gentle pipet decellularized tissue in acetic acid.
3. Transfer decellularized tissue in glass vials for freeze-drying, see Fig. 2.2.
4. Keep reconstituted samples at 4^{o}C (short term, until lyophilization) or in -20^{o}C (long-term storage).
5. Lyophilize decellularized samples in a CRIST LMC2 lyophilizer (IMBB-FORTH). Place samples in lyophilizer shelf when the shelf temperature is set at -10^{o}C.
6. Set in pre-freezing condition, shelf temperature at -22^{o}C for 1 hour.
7. At primary drying, set shelf temperature at -18^{o}C for 24h and reduce pressure to less than 100 Pa.
8. After primary drying, set temperature at 25^{o}C for 2 hours.
9. Stop lyophilization and set shelf pressure set at 1 atm so that the lyophilizer door can open. This freeze–drying procedure results in decellularized brain ECM inside bottles in powder form. The outcome of lyophilization is presented in figure 2.3

For characterization using staining (protocol 3) look B2 Appedix

B2: Immunohistochemistry in Brain Tissue Cryosections (Protocol 3)

Protocol by Kanelina Karali, November 2014

This protocol starts after step 23 in "Solubilize Decellularized mouse brain"

This method is used for brain and decellularized *sections* (10 μm), brains are fixed with phosphate buffered saline (PBS) and then 4% paraformaldehyde in phosphate buffer ph7.4, post-fixed for 24hr. The fixed brain is cut along the midline and sagitally mounted on to vibratome stage using superglue and sections cut and stored in cryoprotective medium at -20^{o}C.
*Cryoprotectant medium: 30% glycerol/30% ethylene glycol in PB, store at -20^{o}C.

Materials

- 1x PBS

- 0.1% PBStx (Triton X-100, 0.1% in PBS)
- 0.3% PBStx (Triton X-100, 0.3% in PBS)
- Block (10% Normal Serum, 0.1% BSA in PBStx)
- HOECHST (10mg/ml, Invitrogen, 1249542)
- Vectashield mounting medium (Vector, cataloge number H-100)

Procedure

DAY 1:

1. Wash 3 x 15min, PBS RT on shaker
2. Wash 1 x 15min, PBStx (Triton X-100, 0.1% in PBS) RT on shaker
3. Wash 1 x 30min, PBStx (Triton X-100, 0.3% in PBS) RT on shaker
4. Block (10% Normal Serum, 0.1% BSA in PBStx) 1hr –use serum of the species the secondary is raised in
5. Primary Ab: overnight at 4o C (made in blocking solution)

Anti- Laminin (rabbit) 1:100 dilution (L9393, Sigma)

Anti-Fibronectin (rabbit) 1:200 dilution (Hynes Lab, MIT)

DAY 2:

1. Wash 3 x 15min, PBStx (Triton X-100, 0.1% in PBS) RT on shaker
2. Secondary antibody (1:500) made up in PBStx for 1hour RT on shaker cover with aluminum foil (anti rabbit 488 1:1000 dilution)
3. Wash 1 x 15min, PBStx (Triton X-100, 0.1% in PBS) RT on shaker
4. Wash 1 x 15min, PBS RT on shaker
5. Apply nucleus stain (1:10000 for HOESCHT) in PBS for 15 min at RT on Shaker
6. Wash 1 x 15min, PBS RT on shaker
7. Wash 1x 15min PB and leave for mounting
8. Mount onto superfrost slides and dry for a few minutes
9. Coverslip with Vectashield mounting medium, and fix coverslip with nail polish

B3: Immunocytochemistry

Protocol by Kanelina Karali, November 2014

Materials

- 4% PFA in PBS
- 1x PBS
- 0.1% PBStx (Triton X-100, 0.1% in PBS)
- 0.3% PBStx (Triton X-100, 0.3% in PBS)
- Block (10% Normal Serum, 0.1% BSA in PBStx)
- HOECHST (10mg/ml, Invitrogen, 1249542)

Procedure

DAY 1:

1 Wash cells with PBS at RT for 1 min
2 Fix cells with 4% PFA for 10 min at RT on shaker
3 Wash cells with PBS at RT for 2x5 min
4 Wash cells with 0.1% PBSTx at RT for 5 min
5 Block for 1 hr at RT on shaker

Total volume required for blocking: 100μl in 48 well plate

A. 10% Normal Serum (specific to the species the 2ary Ab(s) is/are raised) 80μl in 48 well plate
B. 0.1% BSA (from 10% stock)
C. 0.3% PBSTx to make total volume:

6 Primary antibodies at 4ºC overnight on shaker

Primary Ab dilution:

A. anti-nestin Ab dilution 1:1000
B. anti-sox2 Ab dilution 1:100
C. anti-glast Ab dilution 1:200

DAY 2:

(STEPS 2 ONWARDS PROTECT FROM LIGHT WITH FOIL)

1 Wash in 0.1% PBSTx 2x5 min at RT on shaker
2 Secondary antibodies (1:1000) made in 0.1% PBSTx at RT for 1h

Total volume required: 80μl per 48 well plate

A. anti-chicken (nestin) 546, 1:1000 dilution
B. anti-rabbit (sox2) 647, 1:1000 dilution
C. anti-rabbit (glast) 647, 1:1000dilution

3 Wash in 0.1% PBSTx 2x5 min at RT on shaker
4 Wash in PBS for 5 min at RT on shaker
5 Apply nucleus stain (1:10000 for HOESCHT) in PBS for 10 min at RT on SHaker
6 Wash in PBS for 2x5 min at RT on shaker

B4. Immunoblotting

Protocol by Kanelina Karali, November 2014,
Edited by Erasmia Ioanna Maravgaki, January 2020

Materials

1. TBS (10x) 1L (store at RT)
24.2 g Tris-Base (20mM)
80 g NaCl (150mM)
Up to 1 L in dH2O (pH=7.6)

2. TBS-0.1% Tween20 (TBST) (store at RT)
100 mL TBS x10
1 mL Tween-20 (very viscous, cut pipette tip)
Up to 1 L in dH2O

3. Ponceau S (store at RT)
Ponceau S (Sigma P3504), 0.1 g
Acetic Acid, 5 mL
Distilled Water, 95 mL
*Note: Ponceau S is light sensitive

4. Electrophoresis Buffer x10 1L (store at 4^{o}C)
30.3 g Tris-Base (0.25M) and measure pH=8.3
Add next:
144.1 g Glycine (1.92M)
10g SDS (1%)
Up to 1L in dH2O

5. Transfer Buffer x10 1L (store at 4^{o}C)
30.3 g Tris-Base (0.25M) and measure pH=8.3
Add next 144.2 g Glycine (1.92M)

6. 1xTransfer Buffer 1L (store at 4^{o}C)
100 mL Transfer Buffer x10
50mL Methanol
850 mL ddH2O

7. 5x Laemmli Buffer 10 mL (store at -20^{o}C)
1 g SDS (10% w/v)
2.5 mL mercaptoethanol (2.5% v/v)
5 mL glycerol (50% v/v)
1.25 mL 2M Tris-HCl pH=6.8 (0.25M)
Some bromophenol blue particles

8. Coomassie Colloidal Staining Solution
0.8g Coomassie Brilliant Blue G-250
80g ammonium sulfate
120g citric acid
20ml methanol

9. 4-20% gradient acrylamide gels (Bio-rad 4561094)

Procedure

SDS-PAGE (SDS-Polyacrylamide Gel Electrophoresis)

Sample preparation

- Take samples from -20oC keep samples in ice until they are fully defrost.
- Brain and Decellularized samples in RIPA buffer, after BCA quantification were calculated and diluted for 50µg of protein and 1x and diluted Laemmli Buffer (from stock 5x Laemmli Buffer)
- Prepare samples by heating them to 95°C for 5 minutes

Running the gel

- Place Biorad gradient gels in the electrophoresis apparatus.
- Fill in the tank and the space between the two gels with 1x Electrophoresis Buffer
- Load 3µl of protein ladder (New England) and each of the samples in a predetermined order. The maximum sample loading volume for each casting set up is 35µl in 1mm gel in 4-20% Biorad gels
- Attach the electrophoresis apparatus to an electric power supply. Apply a voltage of 50mA to the gel for 60 min

Protein transfer

- Cut an appropriate size of nitrocellulose membrane (8x6 cm) and filter papers (3 pairs, 9x7 cm).
- Soak them in 1xTrasfer buffer
- Remove the plastic plates from the electrophoresis apparatus and place them on a paper towel. Using a spatula, pry the plates apart.
- Trim off the wells and place a pre-soaked filter paper over the gel. Remove all trapped air bubbles by gently rolling over the surface with a roller (15 mL tube)
- Turn the plate over so that the gel and filter are facing downwards over a piece of glad wrap
- Carefully release the gel
- Wet the surface of the gel with transfer buffer and position the pre-soaked nitrocellulose membrane on the gel, ensuring all air bubbles have been removed.
- Place another pre-soaked filter paper on top of the membrane. Remove any trapped air bubbles.
- Carefully pick up the gel membrane assembly and place on transfer cassette as shown below.
- Place the cassette in the transfer tank and fill it with 1x Transfer buffer, place an ice block in the tank and the tank itself on ice
- Transfer at 300mA for 120min

Blocking and Primary Antibody Incubation

- Briefly rinse the blot with dH2O and stain in with Ponceau S solution to check transfer quality Stain Gel with Coomassie stain for 3h in Shaker and destain overnight in shaker with dH_20

- Wash 2x10min in TBST to wash off Ponceau S stain
- Block in 5%BSA for β actin antibody, 7% BSA blocking for fibronectin antibody (in order to reduce backgroung in membrane imaging) in TBST for 1h RT
- Incubate blot in primary antibody made up in blocking solution overnight at 4°C at 4oC in shaker (in 1:5000 dilution β actin antidody (anti-mouse) in 5% BSA in TBST, 1:1000 dilution for fibronectin (anti-rabbit) antibody in 5% BSA in TBST
- Rinse blot 3x10min in TBST
- Incubate blot in secondary Ab anti mouse and anti-rabbit 1:5000 in TBST 1h RT.
- Rinse blot 3x15min in TBST

Imaging

- Prepare Working Solution by mixing equal parts of the Stable Peroxide Solution and the Luminol/Enhancer Solution (SuperSignal™ West Pico PLUS Chemiluminescent Substrate)
- Use 2 mL Working Solution per membrane. The Working Solution is stable for 8 hours at room temperature.
- Incubate the blot in Working Solution for 5 minutes.
- Remove the blot from Working Solution and drain excess reagent.
- Place the blot in clear plastic wrap or sheet protector and remove bubbles.
- Expose the blot to the imaging system, Bio-rad Chemi Doc imager (IMBB)