Video Article

Engineered 3D Silk-collagen-based Model of Polarized Neural Tissue

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Abstract

Despite huge efforts to decipher the anatomy, composition and function of the brain, it remains the least understood organ of the human body. To gain a deeper comprehension of the neural system scientists aim to simplistically reconstruct the tissue by assembling it *in vitro* from basic building blocks using a tissue engineering approach. Our group developed a tissue-engineered silk and collagen-based 3D brain-like model resembling the white and gray matter of the cortex. The model consists of silk porous sponge, which is pre-seeded with rat brain-derived neurons, immersed in soft collagen matrix. Polarized neuronal outgrowth and network formation is observed with separate axonal and cell body localization. This compartmental architecture allows for the unique development of niches mimicking native neural tissue, thus enabling research on neuronal network assembly, axonal guidance, cell-cell and cell-matrix interactions and electrical functions.

Video Link

The video component of this article can be found at http://www.jove.com/video/52970/

Introduction

The central nervous system (CNS) can be affected by a variety of disorders involving vascular, structural, functional, infectious or degenerative. An estimated 6.8 million people die every year as a result of neurological disorders, which represents a growing socioeconomic burden worldwide[1]. However, only few of the disorders have available treatments. Therefore, there is a critical need for innovative therapeutic strategies for patients suffering from neurological disorders. Unfortunately, many CNS-targeted therapeutics fail during clinical trials, in part due to the utilization of inadequate pre-clinical research models, which do not allow assessment of acute and chronic impacts with physiologically-relevant functional readouts.

Despite significant research efforts over the past decades, there is a vast amount unknown about the structure and function of CNS. In order to gain more knowledge, animal models are frequently used to model pathological states, such as traumatic brain injury (TBI) or dementia, especially in pre-clinical studies. However, animals differ significantly from humans both in anatomy of CNS, as well as in function, gene expression and metabolism[2-4]. On the other hand, 2D *in vitro* cultures are the common method to investigate cell biology and are routinely used for drug discovery. However, 2D cell cultures lack the complexity and physiological relevance as compared to human brain[5-7]. While there is no substitute for the low cost and simplicity of 2D cell culture studies or the complexity provided by animal models, 3D tissue engineering could generate improved research models to close the gap that exists between the 2D *in vitro* and *in vivo* approaches. 3D tissue engineering provides more physiologically-relevant experimental conditions achieved by 3D cell-cell interactions and extracellular cues provided by the biomaterial scaffolds. Despite the significant evidence behind the value of 3D cultures, there are currently only a few 3D CNS tissue models such as stem cell-derived organoid cultures[8-10], neurospheroids[11] and dispersed hydrogel cultures[12,13]. Advanced technical methods including multilayer lithography[14], and 3D printing[15] have been utilized for studying lung, liver, and kidney tissue. However, there is lack of 3D CNS models that allow for compartmentalized neuronal growth, such as mimicking the cortical architecture and biology. Separated growth of neurites from neuronal cell bodies has been previously demonstrated in 2D cultures by using microfabrication[16,17] allowing the study of axon tract tracing, calcium influx, network architecture and activities. This idea inspired us to develop a 3D polarized neural tissue where cell bodies and axonal tracts are located in different compartments mimicking the layered architecture of the brain[18]. Our approach is based on the use of unique silk scaffold design which accommodates high density of cells in a confined volume and allows outgrowth of dense axonal network into a collagen gel. Here we demonstrate the full assembly procedure of brain-like tissue including the scaffold fabrication and neuronal culture.

Protocol

The brain tissue isolation protocol was approved by the Tufts University Institutional Animal Care and Use Committee and complies with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institutional Animal Care and Use Committee B2011-45).

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1. Silk Scaffold Preparation

1. Preparation of silk solution from *Bombyx mori* cocoons as described previously[19,20].
    1. Cut each cocoon into 8 equal pieces using scissors. Use about 11 cocoons for 5 g of fragmented cocoons. (15 min)
    2. Prepare 2 L of 0.02 M Na_2CO_3 solution and bring it to boil using a hot plate. (15 min)
    3. Weigh 5 g of fragmented cocoons and boil them in Na_2CO_3 solution for 30 min. Stir the boiling silk every couple of minutes with a spatula. This step, also called de-gumming, purifies silk fibroin from hydrophilic proteins, sericins. (30 min)
    4. Wring the fibroin out by hand and rinse it in distilled water at least three times to wash out any remaining sericin and chemicals. (5 min)
    5. Place the wet fibroin on a petri dish and dry the fibroin extract in the flow hood O/N.
    6. The next day weigh the dry fibroin mass and place the fibroin in a glass beaker. (15 min)
    7. In order to dissolve the fibroin in 9.3 M LiBr solution, calculate the required volume (in ml) of LiBr by multiplying the mass of dry fibroin by 4. Slowly pour LiBr solution over the silk fibroin and immerse all the fibroin fibers using spatula. Cover the beaker to prevent evaporation and place it at 60 °C for 4 hr to allow the fibers to dissolve. (15 min)
    8. Using the syringe, collect the fibroin solution from the beaker and load it into the MWCO 3,500 dialysis cassettes. Perform dialysis against distilled water for 48 hr. Change the water every couple of hours.
    9. Using the syringe, collect the fibroin solution from the cassettes into 50 ml conical tubes and centrifuge twice at 9,000 rpm (~12,700 x g) at 4 °C for 20 min. Pour the supernatant into a fresh tube after each centrifugation step and discard the pellet. (40 min)
    10. Measure the concentration of the fibroin by estimating the dry weight. Pour 1 ml of silk solution into a weigh boat. Dry the sample in the oven at 60 °C for 2 hr. Weigh the dry silk fibroin and calculate the concentration of silk fibroin solution by multiplying the obtained weight by 100. The expected concentration of silk solution is 6-9% (w/v).
    11. Adjust the silk concentration to 6% (w/v) by diluting it in distilled water.
    Stopping point: The liquid silk fibroin can be stored at 4 °C for up to one month in a closed container.

2. Preparation of porous scaffolds from silk solution.
    1. Sieve the granular NaCl to separate the granules sized 500-600 µm, which will be used in later steps. Discard the granules sized below 500 µm and above 600 µm. (15 min)
    2. Pour 30 ml of 6% silk solution into the polytetrafluoroethylene (PTFE) mold (**Figure 1**). Gently scatter 60 g of sieved NaCl over the silk. Tap the container to obtain a uniform layer of salt. Incubate 48 hr at RT to polymerize the silk.
    3. Place the scaffold-containing PTFE mold in the oven at 60 °C for 1 hr to finalize the polymerization and evaporate any remaining liquid.
    4. Place the content of the PTFE mold in a beaker containing 2 L of distilled water for 48 hr to leach out the salt. Change the water 2-3 times per day. Remove the sponge scaffolds from the molds when the salt is completely leached out. *Stopping point*: The sponges can be stored immersed in water at 4 °C in a closed container to prevent the scaffolds from dehydration.
    5. When ready, cut out the scaffolds with 5 mm diameter biopsy punch. Pre-cut the scaffolds to reach about 2 mm in height. Punch out the center of the scaffold with the 2 mm diameter biopsy punch (**Figure 2A**). (1 hr)
    6. Autoclave the scaffolds immersed in water to sterilize them (wet cycle, 121 °C, 20 min). *Stopping point*: The sponges can be stored immersed in water at 4 °C in a closed container to prevent the scaffolds from dehydration.
    7. Before the planned cell seeding, immerse the scaffolds in sterile 0.1 mg/ml poly-D-lysine (PDL) solution. Incubate for 1 hr at 37 °C.
    8. Wash the scaffolds 3x with phosphate buffered saline (PBS) to remove non-bound PDL. (30 min)

2. Isolation of Rat Cortical Neurons

1. Dissect cortices from embryonic day 18 (E18) Sprague-Dawley rats as previously described by Pacifici and Peruzzi[27]after approved animal protocol is obtained. (2 hr)
2. Incubate 10 cortices in 5 ml of 0.25% trypsin with 0.3% DNase I (from bovine pancreas) for 20 min at 37 °C.
3. Inactivate the trypsin by adding equal volume of 1 mg/ml soybean protein.
4. Triturate the cortices using 10 ml Pasteur pipette by pipetting up and down 20 times until a single cell suspension is generated. Be gentle and avoid air bubble formation. (10 min)
5. Centrifuge the cell suspension at 127 x g for 5 min.
6. Re-suspend the cell pellet in 10 ml of culture medium (Neurobasal medium, 1x B27 supplement, 1x Glutamax, 1% penicillin/streptomycin). Count the cells. The expected cell concentration is about $2x10^7$/ml. (20 min)

3. Construct Assembly and Culture

1. Scaffold seeding with cells.
    1. Move the sterile scaffolds and all the required utensils inside a cell culture hood. Using sterile forceps place the scaffolds in a 96-well cell culture plate allocating one scaffold per well. (10 min)
    2. Immerse the scaffolds in cell culture medium to equilibrate them prior to cell seeding. Incubate for 1 hr at 37 °C. (10 min)
    3. Aspirate the excess of the medium from the scaffolds.
    4. Apply 100 µl of cell suspension/scaffold. (10 min)
    5. Incubate the cells at 37 °C O/N to allow for cell attachment to the scaffold.
    6. The following morning aspirate the non-attached cells and apply 200 µl/well of fresh culture medium. (10 min)

2. Scaffold embedding with collagen matrix. (2 hr)
    1. Place 10x PBS, water, 1 N NaOH and rat tail I collagen on ice. Prepare a working solution of collagen as per the manufacturer instructions. Maintain on ice until the cell-seeded constructs are ready (up to 1 hr).
    2. Remove the cell-seeded silk constructs from the incubator and aspirate the excess of medium.

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3. Using sterile forceps transfer the scaffolds to the empty wells on the well plate and immerse each scaffold in 100 µl of 3 mg/ml collagen solution. Place the tissue culture plate back in the incubator for 30 min to allow polymerization of collagen.
4. Apply 100 µl of pre-warmed cell culture medium/well. Culture the constructs for one week changing the medium every day by replacing only half the volume of the medium.

4. Microscopy Analysis

1. Live/dead staining (1 hr)
    1. Prepare stock solution of propidium iodide (PI) 1 mg/ml (1.5 mM) in distilled water.
    2. Prepare stock solution of fluorescein diacetate (FDA) 2 mg/ml in acetone.
    3. Prepare working solution containing 10 µM PI and 0.15 µM FDA diluted in PBS. Pre-warm the solution to 37 °C in a water bath.
    4. Wash the cells with pre-warmed PBS to remove the serum esterases. Aspirate the PBS.
    5. Apply the pre-warmed working solution on the cells for 2 min and wash it with PBS.
    6. Use epifluorescent microscope to image the cells (PI ex λ=490 nm, em λ=570 nm; FDA ex λ=490 nm, em λ=514 nm). The stain remains stable over 40 min. Prolonged staining may result in unspecific staining resulting from PI uptake by the cells and co-localization of PI/FDA in cells.

2. Immunostaining
    1. At the desired time point of culture fix the cells with 4% paraformaldehyde (PFA) for 30 min at RT. Due to the toxicity of the PFA fumes the fixing step should be performed in the chemical fume hood.
    2. Wash scaffolds with 3x PBS. *Stopping point*: The PFA-fixed constructs can be stored in PBS at 4 °C for several days before proceeding with further steps.
    3. Incubate the scaffolds in 0.2% Triton, 0.25% bovine serum albumin (BSA) in PBS containing primary antibody O/N at 4 °C.
    4. The following day discard the staining solution and wash the scaffolds 3 x 30 min with PBS to remove the unbound primary antibody.
    5. Incubate the samples with secondary antibody diluted in 0.2% Triton, 0.25% BSA in PBS for 2 hr at RT.
    6. Remove the staining solution and wash the scaffolds 3 x 30 min with PBS to remove the unbound secondary antibody.
    7. Image the scaffolds using a confocal microscope with 20X objective by taking 100 µm z-sections of the sample with 1 µm step. To visualize the thin neurites the image resolution should be of minimum 1,024 x 1,024 pixels.

3. RNA, DNA and protein isolation
   Note: RNA, DNA and protein isolation is performed using a commercial AllPrep DNA/RNA/Protein Mini Kit according to the manufacturer instructions.
    1. Using sterile forceps transfer the scaffolds out of culture plate to a 2 ml sterile tube. Place one scaffold per tube.
    2. Add 200 µl of RLT buffer to each tube.
    3. Disrupt the construct by fragmenting it with sterile microscissors.
    4. To homogenize the disrupted tissue, transfer the content of the tube to the spin column. Spin for 2 min at full speed in a microcentrifuge. The lysate is homogenized as it passes through the spin column.
    5. Collect the flow through and use it immediately to isolate RNA, DNA and protein following the manufacturer protocol.
    6. Quantify the yield of the nucleic acids any other compatible method (*e.g.*, NanoDrop).
    7. Quantify the protein yield using BCA Protein Assay according to the manufacturer instructions. Measure the outcome of assay using microplate reader at wavelength 562 nm.
       Note: The expected yield of nucleic acids and protein per construct in relation to the cell density is shown in **Figure 4**.

Representative Results

Solid silk sponges precut to the donut shape represent a unique and simple idea to achieve a compartmentalized architecture resembling neural tissue (**Figure 2A**). The highly porous structure of the scaffold with the pore size of about 500 µm results in exceptionally high surface area allowing the seeding and growth of high cell density within a small volume ($2x10^7$/ml) (**Figure 2B**). Additionally, the high porosity of the scaffold allows for unrestricted diffusion of nutrients and wastes resulting in superior viability of dense cell cultures over extended time frames. Upon seeding the neurons rapidly and uniformly attach to the surface of the scaffold pores.

After cell attachment is complete and cells are equilibrated on the silk substrate, the sponge scaffold is filled with the soft collagen matrix in order to provide a 3D environment for axonal network formation (**Figure 3**). In the absence of the collagen matrix the compartmentalization of axons and cell bodies is not possible as neurons grow extensions only on the surface of the pores resulting in mixed networks as found with 2D surfaces (**Figure 3B**). In contrast, the collagen gel provides a meshwork which supports extensive axonal outgrowth in 3D, while neuronal cell bodies remain attached to the silk scaffold (**Figure 3C**). This growth pattern leads to compartmentalization of cell bodies and pure axonal networks (**Figure 3D**), which resembles the gray and white matter of the cortex in the brain.

Apart from microscopy, the constructs can be evaluated with a variety of other methods[18], depending on the question behind the experiment. For example, the isolation of DNA, RNA and protein from the constructs can be easily performed using commercial kits (**Figure 4**). The yield of nucleic acids and protein per construct depends on the number of cells initially seeded on the silk scaffolds. The more cells seeded the higher the yield of DNA, RNA and protein.

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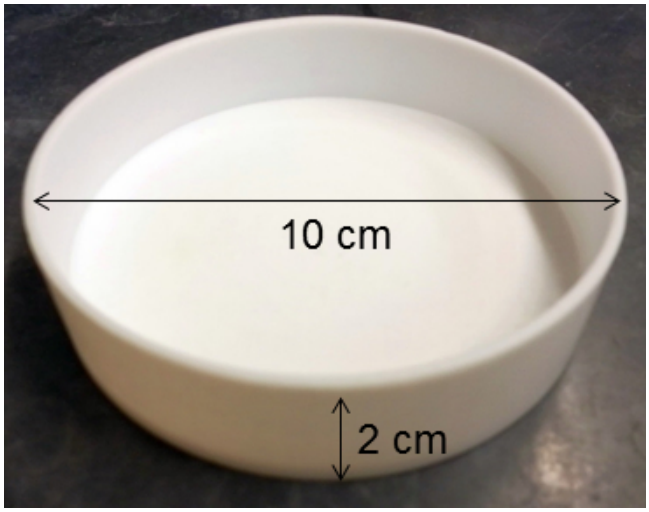

Figure 1. PTFE mold used for silk sponge scaffold preparation. The dimensions: 10 cm diameter, 2 cm height. Please click here to view a larger version of this figure.

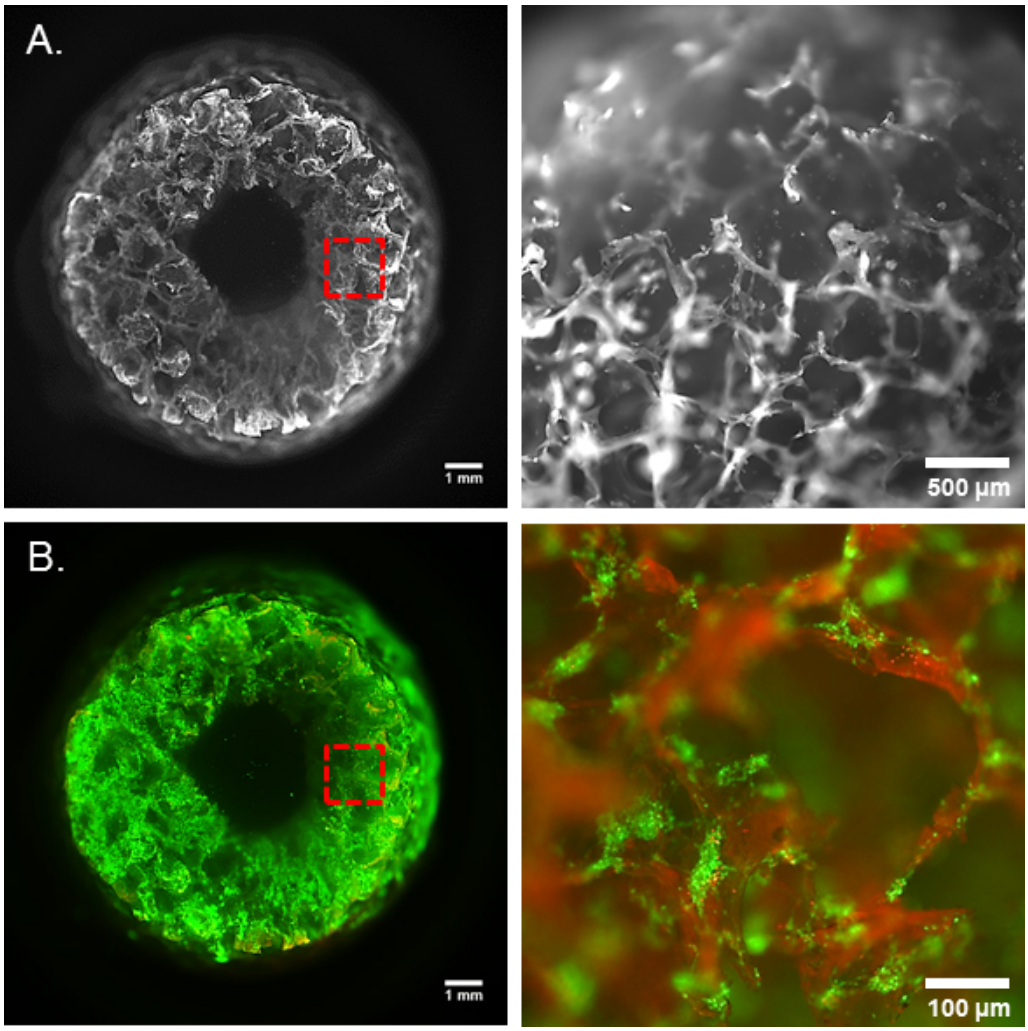

Figure 2. 3D brain-like tissue model. (**A**) Porous silk sponge scaffold. (**B**) Live/dead staining of neurons at day 1 upon seeding on silk scaffold before collagen embedding (green-live cells, red-dead cells). Panels on right side show the magnification of area captured in red frames. Please click here to view a larger version of this figure.

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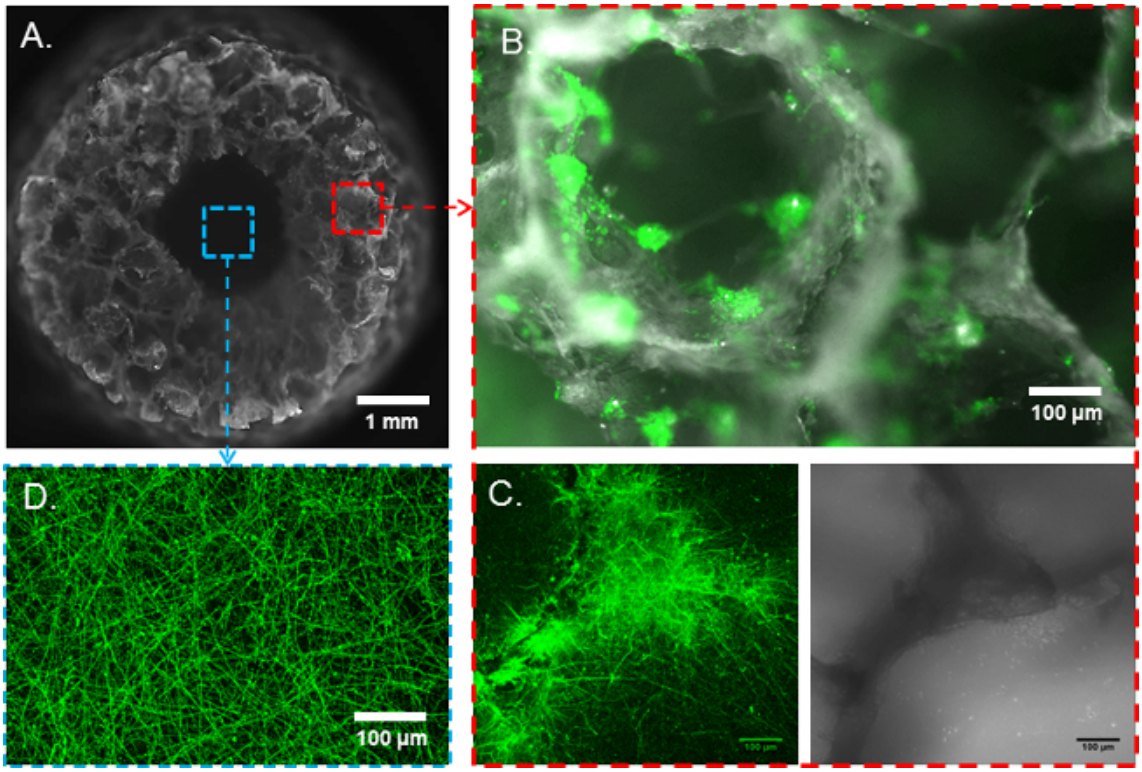

Figure 3. Neuronal compartmentalized outgrowth in the 3D brain-like tissue model. (**A**) Scaffold compartments: Red-cell body compartment, Blue-axonal compartment. (**B**) Neuronal growth pattern at day 1 upon seeding before collagen embedding. (**C**). Neuronal outgrowth at day 7 upon seeding in the cell body compartment. (**D**) Neuronal outgrowth at day 7 upon seeding in the axonal compartment. Green — βIIITubulin. Please click here to view a larger version of this figure.

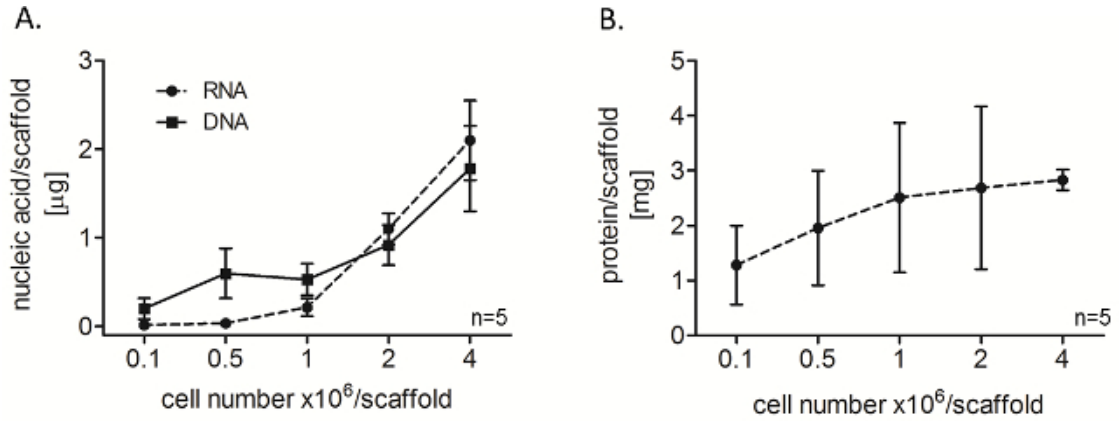

Figure 4. Expected yield of (A) RNA and DNA, and (B) protein per construct in relation to the amount of cells seeded per scaffold (±SD, n=5). Please click here to view a larger version of this figure.

Discussion

Here we described the guidelines to assemble a compartmentalized 3D brain-like tissue model. The model is characterized by dense polarized culture of neurons resulting in the development of two morphologically different compartments: one containing densely packed cell bodies, second containing pure axonal networks. Overall, the scaffold architecture and growth pattern mimic brain cortical tissue including the six laminar layers and white matter tracts[21].

The donut-shaped silk protein-based tissue model allows for modifications and tuning of mechanical properties, versatile structural forms, hydrogel fillers and cellular components. Thus, this tissue model establishes a base platform for a wide range of studies. Silk is a favorable candidate for biomaterial platforms due to its biocompatibility, aqueous-based processing, and robust mechanical and degradation properties[22]. The silk matrix also serves as a stable anchor to reduce collagen gel contraction over time in culture. The properties such as silk concentration and porosity of the scaffold used in this model have been previously adjusted to achieve optimal cell growth and mechanical properties resulting

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in brain-like tissue elasticity[18,23,24]. We suggest to keep these parameters constant to ensure the successful outcome and reproducibility of the experiments.

The 3D neuronal network formation was achieved by combining two types of biomaterials with different mechanical properties: a stiff scaffold to provide neuronal anchoring and a softer gel matrix to permit axon penetration and connectivity in 3D. Selective material preferences of the silk scaffold and soft hydrogel provide the underlying principle for compartmentalizing the neurons from the axonal connections. Due to the inert nature of the silk scaffold, functionalization with poly-D-lysine is required for neuronal attachment. However, other cell adhesion promoting factors can be applied such as RGD[25] or fibronectin[26]. To achieve the 3D network formation the silk scaffold needs to be filled with hydrogel in a timely manner as soon as neurons are attached to the scaffold. Likewise, the collagen matrix filler can be replaced by other hydrogels, thus allowing the study of the influence of 3D extracellular environments on axonal network formation to serve as a platform to evaluate novel hydrogels in terms of neuronal compatibility. Additionally, apart from rat cortical neurons other neuronal sources such as hippocampal neurons or induced pluripotent cell (iPSc)-derived neurons can be utilized. Moreover, the tissue model can be used to study heterocellular interactions by including glial cells along with neurons and to build more complex brain-like tissue.

As shown previously, our model can be utilized to evaluate neuronal functionality in 3D microenvironment with a variety of assays such as cell viability, gene expression, LC-MS/MS and electrophysiology, thus demonstrating physiological relevance of the model[18]. Other methods, which are frequently utilized to evaluate 3D tissue-engineered constructs, such as immunostaining and microscopy[8,9,11] can also be used to assess cell distribution and extent of axonal network formation. However, it has to be noted, that due to the high density of the collagen matrix, the penetration of antibodies and depth of the imaging is limited to few hundred micrometers. Moreover, the signal to noise ratio may be affected by nonspecific background fluorescence. This can be overcome by using lipophilic cell tracers and genetically expressed fluorescent proteins which diminish the need for immunostaining[11]. Alternatively, the imaging can be performed with 2-photon microscopy instead of usual one-photon technique, which may reduce the signal loss, photobleaching, and can be extended to several hundred micrometers of depth.

Summarizing, the silk and collagen-based brain-like tissue offers a robust platform to study neuronal networks in 3D and is a starting point for the development of more advanced models of neurological disorders in the future. Independent from the mode of evaluation, the relative simplicity of this tissue model supports its utility, success and reproducibility.

Disclosures

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References

1. Dua, T., Janca, A., Kale, R., Montero, F., Muscetta, A., & Peden, M., Chapter 1, Public health principles and neurological disorders, In *Neurological Disorders.*, World Health Organization, Geneva (2005).
2. Ohtsuki, S., Hirayama, M., Ito, S., Uchida, Y., Tachikawa, M., & Teresaki, T. Quantitative targeted proteomics for understanding the blood-brain barrier: towards pharmacoproteomics. *Expert. Rev. Proteomic.* **11** (3), 303-313, doi: 10.1586/14789450.2014.893830 (2014).
3. Hyder, F., Rothman, D.L., & Bennett, M.R. Cortical energy demands of signaling and nonsignaling components in brain are conserved across mammalian species and activity levels. *Proc. Natl. Acad. Sci. U.S.A.***110** (9), 3549-3554, doi: 10.1073/pnas.1214912110 (2013).
4. Wesseling, H., *et al.* A combined metabonomic and proteomic approach identifies frontal cortex changes in a chronic phencyclidine rat model in relation to human schizophrenia brain pathology. *Neuropsychopharmacology.* **38** (12), 2532-2544, doi: 10.1038/npp.2013.160 (2013).
5. Fawcett, J.W., Housden, E., Smith-Thomas, L., & Meyer, R.L. The growth of axons in three-dimensional astrocyte cultures. *Dev. Biol.* **135** (2), 449-458, doi:10.1016/0012-1606(89)90193-0 (1989).
6. Cullen, D.K., Simon, C.M., & LaPlaca, M.C. Strain rate-dependent induction of reactive astrogliosis and cell death in three-dimensional neuronal–astrocytic co-cultures. *Brain Res.* **1158**, 103-115, doi:10.1016/j.brainres.2007.04.070 (2007).
7. East, E., de Oliveira, D.B., Golding, J.P., & Phillips, J.B. Alignment of astrocytes increases neuronal growth in three-dimensional collagen gels and is maintained following plastic compression to form a spinal cord repair conduit. *Tissue Eng. Part A.* **16** (10), 3173-3184, doi: 10.1089/ten.tea.2010.0017 (2010).
8. Dubois-Dauphin, M.L., Toni, N., Julien, S.D., Charvet, I., Sundstrom, L.E., & Stoppini, L. The long-term survival of *in vitro* engineered nervous tissue derived from the specific neural differentiation of mouse embryonic stem cells. *Biomaterials.* **31** (27), 7032-7042, doi: 10.1016/j.biomaterials.2010.06.017 (2010).
9. Lancaster, M.A., *et al.* Cerebral organoids model human brain development and microcephaly. *Nature.* **501** (7467), 373-379, doi: 10.1038/nature12517 (2013).
10. Hogberg, H.T., *et al.* Toward a 3D model of human brain development for studying gene/environment interactions. *Stem Cell Res. Ther.* **4** (Suppl 1), S4, doi: 10.1186/scrt365 (2013).
11. Kato-Negishi, M., Morimoto, Y., Onoe, H., & Takeuchi, S. Millimeter-sized neural building blocks for 3D heterogeneous neural network assembly. *Adv. Healthc. Mater.* **2** (12), 1564-1570, doi: 10.1002/adhm.201300052 (2013).
12. Watanabe, K., Nakamura, M., Okano, H., & Toyama, Y. Establishment of three-dimensional culture of neural stem/progenitor cells in collagen type-1 gel. *Restor. Neurol. Neurosci.* **25** (2), 109-117 (2007).
13. Aurand, E.R., Wagner, J.L., Shandas, R., & Bjugstad, K.B. Hydrogel formulation determines cell fate of fetal and adult neural progenitor cells. *Stem Cell Res.* **12** (1), 11-23, doi: 10.1016/j.scr.2013.09.013 (2014).

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14. Gurkan, U.A., *et al.*.Simple precision creation of digitally specified, spatially heterogeneous, engineered tissue architectures. *Adv. Mater.* **25** (8), 1192-1198, doi: 10.1002/adma.201203261 (2013).
15. Mironov, V., Boland, T., Trusk, T., Forgacs, G., & Markwald, R.R. Organ printing: computer-aided jet-based 3D tissue engineering. *Trends. Biotechnol.* **21** (4), 157-161, doi: 10.1016/S0167-7799(03)00033-7 (2003).
16. Taylor, A.M., Blurton-Jones, M., Rhee, S.W., Cribbs, D.H., Cotman, C.W., & Jeon, N.L A microfluidic culture platform for CNS axonal injury, regeneration and transport. *Nature.* **2** (8), 599-605, doi:10.1038/nmeth777 (2005).
17. Tang-Schomer, M.D., Davies, P., Graziano, D., Thurber, A.E., & Kaplan, D.L. Neural circuits with long-distance axon tracts for determining functional connectivity. *J Neurosci. Methods.* **222**, 82-90, doi: 10.1016/j.jneumeth.2013.10.014 (2014).
18. Tang-Schomer, M.D., *et al.* Bioengineered functional brain-like cortical tissue. *Proc. Natl. Acad. Sci. U.S.A.* **111** (38), 13811-13816, doi: 10.1073/pnas.1324214111 (2014).
19. Rockwood, D.N., Preda, R.C., Yücel, T., Wang, X., Lovett, M.L., & Kaplan, D.L. Materials fabrication from Bombyx mori silk fibroin. *Nat. Protoc.* **6**, 1612-1631, doi: 10.1038/nprot.2011.379 (2011).
20. Lawrence, B.D., Pan, Z., Weber, M.D., Kaplan, D.L., & Rosenblatt, M.I. Silk film culture system for *in vitro* analysis and biomaterial design. *J. Vis. Exp.* (62), e3646, doi: 10.3791/3646 (2012).
21. Maddah, M., Grimson, W.E., Warfield, S.K., & Wells, W.M. A unified framework for clustering and quantitative analysis of white matter fiber tracts. *Med. Image Anal.* **12** (2), 191-202, doi: 10.1016/j.media.2007.10.003 (2008).
22. Vepari, C., & Kaplan, D.L. Silk as a biomaterial. *Prog Polym Sci.* **32** (8-9), 991-1007, doi: 10.1016/j.progpolymsci.2007.05.013 (2007).
23. Yao, D., *et al.* Salt-leached silk scaffolds with tunable mechanical properties. *Biomacromolecules.* **13** (11), 3723-3729, doi: 10.1021/bm301197h (2012).
24. Kim, U.J., Park, J., Kim, H.J., Wada, M., & Kaplan, D.L. Three-dimensional aqueous-derived biomaterial scaffolds from silk fibroin. *Biomaterials.* **26** (15), 2775-2785, doi:10.1016/j.biomaterials.2004.07.044 (2005).
25. Gil, E.S, Mandal, B.B., Park, S.H., Marchant, J.K., Omentetto, F.G., & Kaplan, D.L. Helicoidal multi-lamellar features of RGD-functionalized silk biomaterials for corneal tissue engineering. *Biomaterials.* **31** (34), 8953-8963, doi: 10.1016/j.biomaterials.2010.08.017 (2010).
26. Hopkins, A.M., *et al.* Silk hydrogels as soft substrates for neural tissue engineering. *Adv. Funct. Mater.***23** (41), 5140-5149, doi: 10.1002/adfm.201300435 (2013).
27. Pacifici, M., & Peruzzi, F., Isolation and culture of rat embryonic neural cells: A quick protocol. *J Vis Exp.* (63), e3965, doi: 10.3791/3965 (2012).

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