Cover Page

Title: Engineered tissues made from human iPSC-derived Schwann cells for investigating peripheral nerve regeneration in vitro

Running Head: hiPSC-derived Schwann cell engineered tissue models

Authors: Rebecca Powell and James B. Phillips

Affiliation: UCL Centre for Nerve Engineering, Department of Pharmacology, UCL School of Pharmacy, University College London, 29-39 Brunswick Square, London WC1N 1AX

Corresponding author: jb.phillips@ucl.ac.uk

Engineered tissues made from human iPSC-derived Schwann cells for investigating peripheral nerve regeneration in vitro

Abstract

Peripheral nerves have a limited ability to regenerate and current clinical approaches involving microsurgery give sub-optimal recovery. Engineered tissues using aligned cellular collagen hydrogels can be used as in vitro models through the incorporation of human Schwann cells. However, primary human Schwann cells are difficult to obtain and can be challenging to culture. The ability to generate Schwann cells from human induced pluripotent stem cells (hiPSCs) provides a more reliable cell source for modelling peripheral nerve tissue. Here we describe protocols for generating hiPSC-derived Schwann cells and incorporating them into 3D engineered tissue culture models for peripheral nerve research.

Key words Pluripotent stem cell, Schwann cell, peripheral nerve, hydrogel, regeneration, tissue engineering, 3D culture model

1 Introduction

Nerve regeneration is an important area of research since nerve injuries can be debilitating and current clinical approaches tend to result in limited functional outcomes. However, studying nerve regeneration in vitro is challenging due to the complexity of recreating the cell-cell interactions and tissue architecture that are present in vivo. Nerve tissue is relatively soft and regenerating axons are naturally supported and guided in 3-dimensions by columns of aligned Schwann cells, features which are not recapitulated in monolayer cultures in conventional cell culture plasticware. Furthermore, most research studies published to date have used rodent cells and tissues to study nerve regeneration, which may not be equivalent to the human situation. We have therefore developed an in vitro engineered nerve tissue model containing aligned human Schwann cells in a tethered

collagen hydrogel matrix, which provides a standardised soft 3D environment for studying cellular and molecular features of the nerve regeneration environment.

Human induced pluripotent stem cells (hiPSCs) can be differentiated into Schwann cells [1,2] via a defined earlier precursor stage (see Figures 1 & 2), then used to construct engineered tissues using cellular self-alignment in collagen gels (see Figures 3 & 4) [3]. hiPSCs can be clinical-grade and, by using good manufacturing practice (GMP) approved components, the model has greater translational relevance. Miniaturising the process reduces the cell number and volume of culture media used, improving cost benefit and efficiency [4]. These refinements, which are presented here, build on similar technology which has been used to generate aligned cellular biomaterials for nerve repair [5-11] and drug-screening applications [12]. In the course of those studies various different sources of cells were demonstrated to be compatible with the cellular self-alignment process, including primary rat Schwann cells [11], the F7 rat Schwann cell line [5], differentiated rat adipose-derived Schwann cells [6], human differentiated dental pulp stem cells [8] and human differentiated neural stem cells [9]. Repair Schwann cells are important in the peripheral nerve injury response and repair process [13-15], however the use of primary human Schwann cells for in vitro models is limited by the availability of samples from biopsies [16] or degenerated nerves [17], and the difficulty in culturing pure populations without contaminating fibroblasts [18]. Differentiating hiPSCs into Schwann cell precursors or Schwann cells overcomes these issues, providing a reliable and reproducible supply of human Schwann cells with which to construct engineered tissues that mimic key features of the peripheral nerve microenvironment. These model tissues can be used for a range of applications including studying the molecular and morphological behaviour of denervated human Schwann cells, and exploring the support and guidance provided to regenerating axons by these artificial Bands of Büngner in co-culture studies. Detailed protocols are provided for the differentiation of hiPSCs to Schwann cells and precursors, and the subsequent formation of engineered tissue constructs based on cellular self-alignment in tethered collagen hydrogels.

2 Materials

2.1 Differentiation of hiPSCs to Schwann cells

1. **hiPSC medium**: for example, Essential 8, Essential 8 Flex, MTesR or StemPro.
2. **Neural differentiation medium (NDM)**: 1:1 mix of Advanced DMEM/F12 and Neurobasal medium add 1x N2 supplement, 1x B27 supplement, 0.005 % BSA , 2 mM GlutaMAX, 0.11 mM β-mercaptoethanol, 3 μM CT99021 (Tocris Biosciences), and 20 μM SB431542 (Tocris Biosciences).
3. **Schwann cell precursor differentiation medium (SCPDM)**: 1:1 mix of Advanced DMEM/F12 and Neurobasal medium add 1x N2, 1x B27, 0.005% BSA, 2 mM GlutaMAX, 0.11 mM β-mercaptoethanol, 3 μM CT99021 (Tocris Biosciences), 20 μM SB431542 (Tocris Biosciences) and 50 ng/mL neuregulin-1/heregulin-1 (NRG1) (PeproTech).
4. **SCPDM for maintenance of SCPs**: SCPDM add 100 ng/mL NRG1.
5. **Schwann cell differentiation medium (SCDM):** DMEM/low glucose with GlutaMAX add 1 % FBS, 200 ng/mL NRG1, 4 μM forskolin, 100 nM all-trans RA and 10 ng/mL PDGF-BB.
6. **SCDM** without forskolin and retinoic acid.
7. **SCDM** without forskolin, retinoic acid and PDGF-BB.
8. **SCDM** with only 1 % FBS and 200 ng/mL NRG-1.
9. hiPSC coating solution will depend on hiPSC line e.g. vitronectin or Geltrex: Geltrex™ hESC-Qualified, Ready-To-Use, Reduced Growth Factor Basement Membrane Matrix OR Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix at 1:100 dilution.
10. 50 mM EDTA.
11. Culture vessels.
12. Phosphate buffered saline (PBS).
13. Accutase.

14. Syringes.
15. 0.22 µM syringe filters may be required if laboratory not routinely antibiotic-free.
16. Incubator.
17. Sterile hood.
18. Spatula.
19. Tweezers.

2.2 Tethered mini collagen hydrogels

1. Type I rat tail collagen solution: 2 mg/mL in 0.6 % acetic acid.
2. 10x minimum essential medium (MEM).
3. 5M and 1M dilutions aqueous solution sodium hydroxide.
4. Tethering moulds [3,4,19].
5. 70 % ethanol.

2.3 Immunocytochemistry & mRNA extraction

1. 4 % paraformaldehyde.
2. 0.5 % Triton-X.
3. PBS.
4. Antibodies can be used to detect Oct4 in pluripotent hiPSCs, Sox10 and P75 in Schwann cell precursors and Schwann cells, and S100 in mature Schwann cells, as well as β-III tubulin which will detect neurites in co-cultures with neurons. DAPI or Hoechst can be used to stain nuclei.
5. 5 % serum from the species the secondary antibody was produced in can be used to block non specific binding.
6. Qiagen RNEasy® Mini Kit
7. Vortex.
8. Syringe needle with gauge 27G or lower.

9. Ice bucket.
10. 1.5 mL tubes.

3 Methods

3.1 Differentiation of hiPSCs to Schwann cells via an expandable Schwann cell precursor stage

1. Coat culture flasks with Geltrex™ Ready-to-Use or Geltrex™ LDEV-free hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix at 1:100 dilution (see **Note 1**).
2. Incubate for at least 1 hour in a 37°C, 5 % CO_2 incubator.
3. Seed hiPSCs onto Geltrex™-coated flasks (see **Note 2**). If hiPSCs are not routinely plated on Geltrex™, incubate in usual hiPSC medium for 24 hours at 37°C before changing to neural differentiation medium.
4. Replace medium on hiPSCs with neural differentiation medium (NDM) and incubate cells for 6 days at 37°C until neural rosettes are seen (see **Note 3**).
5. Replace media daily for 18 days with Schwann cell precursor differentiation medium (SCPDM) (see **Note 4**).
6. Passage cells once 80 % confluency is reached using Accutase for dissociation (see **Notes 5 & 6**).
7. After 18 days, Schwann cell precursors (SCPs) can be maintained in SCPDM for maintenance of SCPs, passaging once 90 % confluency is reached (see **Notes 5-7**).
8. For Schwann cell (SC) differentiation, SCPs must be plated at $1.8x10^3/cm^2$ on Geltrex coated culture vessels and medium changed to Schwann cell differentiation medium (SCDM).
9. After 3 days incubation, replace medium with SCDM without forskolin and retinoic acid.
10. After 2 days incubation, replace medium with SCDM without forskolin, retinoic acid and PDGF-BB.
11. After 2-7 days in SCDM with only 1 % FBS and NRG-1, SCs are produced.

12. Media exchanges should occur every 2 days.

[Fig, 1 & 2 here]

3.2 Tethered collagen hydrogels

Collagen hydrogels will contain aligned cells if tethered but can be made in wells without tethering for 3D neural culture, adjusting volumes as necessary. The following produces 1.2 mL collagen gel. (see **Note 8**).

1. Sterilise tethering moulds by soaking in 70 % ethanol (see **Note 9**).
2. Dissociate cells from culture flasks and resuspend the number required for $4x10^6$/mL density in the final 30-50 µL collagen gels.
3. Add 10% total gel volume of 10x MEM to 80 % of total gel volume collagen solution and mix by swirling (see **Notes 10 & 11**).
4. Add sodium hydroxide dropwise to the collagen and MEM solution to neutralise (see **Note 12**).
5. Add the required gel volume to the cell suspension, mixing by swirling and transfer to the mould.
6. Transfer the plate to a 37°C, 5 % CO_2 incubator for 10 minutes to allow gel to set.
7. In a sterile hood, add appropriate cell media to cover the gel and mould before transferring back to the incubator. Contraction is seen after 24-48 hours.

   [Fig, 3 here]

3.3 *Immunocytochemistry and RNA extraction*

1. Aspirate medium and wash gently with PBS twice.
2. For RNA extraction, lift gels into a 1.5 mL tube on ice using a spatula or tweezers.
3. Cover with 350 µL buffer RLT from Qiagen RNEasy Kit.

4. Vortex and pass through a syringe needle until gel has dissolved and buffer is no longer viscous to produce homogenised lysate.
5. Qiagen RNEasy Kit protocol can be continued from step 4.
6. For immunocytochemistry, fix hydrogels by covering with 4 % paraformaldehyde overnight at 4°C or at least 3 hours at room temperature.
7. Lift hydrogels out of moulds into wells of a 24 well plate (see **Note 13**) using a spatula to avoid damage.
8. Wash gently with PBS by filling well and soaking hydrogels for 5 minutes, three times.
9. Cover hydrogel with 0.5 % triton-X if permeabilization is required, incubating for 30 minutes at room temperature and washing off as before.
10. Incubate hydrogel with 5 % blocking serum for 30 minutes at room temperature, washing as previously described.
11. Incubate hydrogels with primary antibody at appropriate dilution overnight at 4°C.
12. After washing unbound primary antibody off, incubate with secondary antibodies for 90 minutes at room temperature before washing and imaging.

[Fig. 4 here]

3.4 Neuronal co-culture for peripheral nerve regeneration

Cell-seeded collagen gels can be co-cultured with neuronal cells lines such as the human SH-SY5Y (neuroblastoma cell line), the rat cell line PC12 or primary dorsal root ganglions. Neurite extension can be quantified by measuring neurite length or area populated by neurites as described previously [4,5,20].

4 Notes

1. We have found 80-100 µL/cm^2 of Geltrex™ solution to be sufficient for coating. Coated plates or flasks can be sealed (e.g. with parafilm) and kept in a 37°C incubator for up to 2 weeks prior to use if no evaporation occurs.
2. Optimal density of hiPSC plating for differentiation is around $2.1x10^4/cm^2$.
3. Cell culture media should be added at room temperature as with hiPSC medium.
4. CT99021 (Tocris Biosciences), SB431542 (Tocris Biosciences) and NRG1 should be added fresh to each flask during each media exchange. Ensure all components used for resuspension of lyophilised powders are filtered through a 0.22 µm syringe filter prior to use, or filter media during each exchange, to ensure sterility.
5. 80-100 µL/cm^2 of room temperature Accutase can be used to dissociate cells. When cells are dissociated, resuspended cells should be added to an equal volume of PBS before centrifugation to reduce the Accutase concentration.
6. Cells in SCPDM and SCPs should be plated at a density of at least $1x10^5/cm^2$, or up to a 1:3 split when passaging from an 80-90 % confluent culture flask or plate.
7. Media exchanges can occur every 2-3 days to allow weekend-free feeding.
8. 1mL gel is the minimum recommended volume – volumes below this are difficult to neutralise accurately.
9. Ensure moulds are air-dried completely in cell culture hood before use.
10. Keep all gel components on ice or on a flat freezer block.
11. Collagen should only be mixed by swirling rather than trituration through a pipette to avoid shearing.
12. Neutralised gel is a light orange/pink colour similar to cell culture media. Total volume of neutraliser added should be less than 5 % total gel volume.
13. Gels can also be kept after fixing in PBS in a sealed sample tube or 24 well plate containing PBS in all wells, sealed with parafilm.

References

1. Huang CW, Huang WC, Qiu X et al (2017) The Differentiation Stage of Transplanted Stem Cells Modulates Nerve Regeneration. Sci Rep 7(1): 17401. doi:10.1038/s41598-017-17043-4
2. Kim HS, Lee J, Lee DY et al (2017) Schwann Cell Precursors from Human Pluripotent Stem Cells as a Potential Therapeutic Target for Myelin Repair. Stem Cell Reports 8(6): 1714-1726. doi:10.1016/j.stemcr.2017.04.011
3. Phillips JB, Brown R (2011) Micro-structured materials and mechanical cues in 3D collagen gels. Methods Mol Biol 695: 183-196. doi:10.1007/978-1-60761-984-0_12
4. O'Rourke C, Lee-Reeves C, Drake RA et al (2017) Adapting tissue-engineered in vitro CNS models for high-throughput study of neurodegeneration. J Tissue Eng 8: 2041731417697920. doi:10.1177/2041731417697920
5. Georgiou M, Bunting SC, Davies HA et al (2013) Engineered neural tissue for peripheral nerve repair. Biomaterials 34(30): 7335-7343. doi:10.1016/j.biomaterials.2013.06.025
6. Georgiou M, Golding JP, Loughlin AJ et al (2015) Engineered neural tissue with aligned, differentiated adipose-derived stem cells promotes peripheral nerve regeneration across a critical sized defect in rat sciatic nerve. Biomaterials 37: 242-251. doi:10.1016/j.biomaterials.2014.10.009
7. Gonzalez-Perez F, Hernandez J, Heimann C et al (2018) Schwann cells and mesenchymal stem cells in laminin- or fibronectin-aligned matrices and regeneration across a critical size defect of 15 mm in the rat sciatic nerve. J Neurosurg Spine 28(1): 109-118. doi:10.3171/2017.5.SPINE161100
8. Martens W, Sanen K, Georgiou M et al (2014) Human dental pulp stem cells can differentiate into Schwann cells and promote and guide neurite outgrowth in an aligned tissue-engineered collagen construct in vitro. FASEB J 28(4): 1634-1643. doi:10.1096/fj.13-243980
9. O'Rourke C, Day AGE, Murray-Dunning C et al (2018) An allogeneic 'off the shelf' therapeutic strategy for peripheral nerve tissue engineering using clinical grade human neural stem cells. Sci Rep 8(1): 2951. doi:10.1038/s41598-018-20927-8
10. Sanen K, Martens W, Georgiou M et al (2017) Engineered neural tissue with Schwann cell differentiated human dental pulp stem cells: potential for peripheral nerve repair? J Tissue Eng Regen Med. doi:10.1002/term.2249
11. Phillips JB, Bunting SC, Hall SM et al (2005) Neural tissue engineering: a self-organizing collagen guidance conduit. Tissue Eng 11(9-10): 1611-1617. doi:10.1089/ten.2005.11.1611
12. Rayner MLD, Laranjeira S, Evans RE et al (2018) Developing an In Vitro Model to Screen Drugs for Nerve Regeneration. Anat Rec (Hoboken) 301(10): 1628-1637. doi:10.1002/ar.23918
13. Navarro X, Vivo M, Valero-Cabre A (2007) Neural plasticity after peripheral nerve injury and regeneration. Prog Neurobiol 82(4): 163-201. doi:10.1016/j.pneurobio.2007.06.005
14. Deumens R, Bozkurt A, Meek MF et al (2010) Repairing injured peripheral nerves: Bridging the gap. Prog Neurobiol 92(3): 245-276. doi:10.1016/j.pneurobio.2010.10.002
15. Jessen KR, Mirsky R (2016) The repair Schwann cell and its function in regenerating nerves. J Physiol 594(13): 3521-3531. doi:10.1113/JP270874
16. Van den Berg LH, Bar PR, Sodaar P et al (1995) Selective expansion and long-term culture of human Schwann cells from sural nerve biopsies. Ann Neurol 38(4): 674-678. doi:10.1002/ana.410380419
17. Haastert-Talini K (2012) Culture and proliferation of highly purified adult Schwann cells from rat, dog, and man. Methods Mol Biol 846: 189-200. doi:10.1007/978-1-61779-536-7_17
18. Rutkowski JL, Kirk CJ, Lerner MA et al (1995) Purification and expansion of human Schwann cells in vitro. Nat Med 1(1): 80-83. doi:10.1038/nm0195-80
19. O'Rourke C, Drake RA, Cameron GW et al (2015) Optimising contraction and alignment of cellular collagen hydrogels to achieve reliable and consistent engineered anisotropic tissue. J Biomater Appl 30(5): 599-607. doi:10.1177/0885328215597818
20. Phillips JB (2014) Monitoring neuron and astrocyte interactions with a 3D cell culture system. Methods Mol Biol 1162: 113-124. doi:10.1007/978-1-4939-0777-9_9

Acknowledgements

Rebecca Powell is supported by Medical Research Council funding MR/N013867/1.

Figure Captions

Figure 1: Brightfield micrographs representative of morphological features in (a) colonies of human induced pluripotent stem cells (hiPSCs), (b) differentiated Schwann cell precursors (SCPs) at the expandable stage after 18 days in Schwann cell precursor differentiation medium and (c) differentiated Schwann cells (SCs) after 8 days in Schwann cell differentiation medium with an elongated, bipolar shape. Scale bars 100 µm.

Figure 2: Immunocytochemistry of Schwann cell precursors (SCPs, left), and Schwann cells (SCs, right). (a) and (b) Both cell types express Sox10 (red) in the nucleus. (c) and (d) SCPs are negative for S100 expression in the cytoplasm, while SCs are positive for the mature SC marker S100 (red). (e) and (f) Both cell types express the extracellular protein P75 (red). DAPI (blue) used for nuclei stain. Scale bars 20 µm.

Figure 3: Tethering mini-moulds (left) fit into the well of a 24-well cell culture plate. Tethering bars are located at either end. Example of a contracted aligned cellular gel (right) in PBS after 24 hours incubation.

Figure 4: Confocal micrographs showing Schwann cells (S100, green) in contracted collagen gels aligned along the longitudinal axis. Cell density $4x10^6$/mL. Scale bar 20 µm.