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Controlling Electrospun Polymer Morphology for Tissue Engineering Demonstrated Using hepG2 Cell Line

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Prof David Hay
Guest Editor
JoVE video journal

Dear Prof Hay,

We submit for your consideration our manuscript entitled "*Protocol for controlling electrospun polymer morphology for tissue engineering: assessment with hepG2 cell line*" for publication in your methods collection: "Pluripotent stem cell differentiation, genome editing and tissue engineering." for the JoVE video journal.

In this methods article, we describe a protocol for fabricating electrospun polycaprolactone fibres with differing morphologies. This is offered for researchers to produce an array of scaffold morphologies on which they can conduct a baseline analysis on the influence of electrospun fibres on 3D cell cultures.

In summary, we have engineered six groups of electrospun scaffolds and provided a protocol for their fabrication. We have also included a representative study seeding hepG2 cells on the scaffolds. We look forward to your feedback and thank you for the opportunity to publish in JoVE video journal.

Best regards,

TITLE:

Controlling Electrospun Polymer Morphology for Tissue Engineering Demonstrated Using hepG2 Cell Line

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KEYWORDS:

tissue engineering, electrospinning, scaffold morphology, bioengineering, in vitro models, polycaprolactone scaffolds

SUMMARY:

This method provides the means to test different polycaprolactone fiber morphologies and topographies for the purpose of tissue engineering. Small and large fibers are fabricated with random orientations, aligned orientations, and also porous cryogenically electrospun structures and used as platforms for cell culture.

ABSTRACT:

Electrospinning affords researchers the opportunity to fabricate reproducible micro to nanoscale polymer fibers. The 3D fibrous architecture of electrospun polymers is regarded as a structural imitation of the extracellular matrix (ECM). Hence, electrospun fibers fabricated from biocompatible polymers have been widely investigated by tissue engineering researchers for their potential role as an artificial ECM for guiding tissue growth both in vitro and in vivo. All cells are acutely sensitive to their mechanical environment. This has been demonstrated by the discovery of multiple mechanotransduction pathways intrinsically linked to the cytoskeletal actin filaments. The cytoskeleton acts as a mechanical sensor that can direct the functionality and differentiation of the host cell depending on the stiffness and morphology of its substrate. Electrospun fibers can be tuned both in terms of fiber size and morphology to easily modulate the mechanical environment within a fibrous polymer scaffold. Here, methods for electrospinning polycaprolactone (PCL) for three distinct morphologies at two different fiber diameters are described. The morphological fiber categories consist of randomly oriented fibers, aligned fibers, and porous cryogenically spun fibers, with 1 μm and 5 μm diameters. The methods detailed within this study are proposed as a platform for investigating the effect of electrospun fiber architecture on tissue generation. Understanding these effects will allow researchers to

optimize the mechanical properties of electrospun fibers and demonstrate the potential of this technology more thoroughly.

INTRODUCTION:

Tissue engineering methods have developed over recent decades to provide cells with a biomimetic environment in order to encourage successful tissue regeneration, both for in vitro tissue models and in vivo regenerative techniques. These methods typically encourage cells to form 3D aggregates, either by themselves or with the use of a guiding scaffold¹. Scaffolds can be fabricated as porous or fibrous polymer nano/microstructures, or as highly water absorbent hydrogels²⁻⁴. Polymer electrospinning is a common fabrication technique that yields unwoven nano-microscale polymer fiber matrices that can mimic the fibrous architecture of the native extracellular matrix (ECM)⁵. Multiple biocompatible polymers are applicable to the electrospinning process; hence, electrospun fibers are a popular medium to facilitate tissue regeneration⁶⁻⁸. Electrospun fibers have been utilized in the culture of a range of tissues, demonstrating a promising avenue for inexpensive and reproducible scaffold platforms⁹⁻¹³.

Cells have an acute sensitivity towards their mechanical surroundings. Previous research has highlighted this important relationship, termed mechanotransduction, which can influence cell function, morphology, and differentiation^{14,15}. The basis of the eukaryotic cellular cytoskeleton is constructed from filamentous actin and plays key mechanical roles in cellular division, adhesion, migration, contraction, and morphology. It is manipulated by external mechanical stimuli via connection with extracellular surface proteins¹⁶⁻¹⁸. Morphological influence on stem cell differentiation has been demonstrated in numerous studies by altering the shape of an attachable substrate, which is then able to control cell shape and direct the differentiation process^{19,20}. Moreover, the stiffness of the substrate upon which cells attach also influences stem cell fate and lineage^{21,22}. Apart from stem cells, these effects have also been observed to affect cells of different lineages^{23,24}. Three dimensional tissue engineering methods have shown results akin to those found in 2D mechanotransduction studies with both hydrogels and fibrillar scaffolds²⁵⁻²⁷. Further, substrate mechanics are communicated through 3D cell aggregates via cell-cell signalling²⁸. Material mechanics play a crucial role in directing cellular functionality and hence should be a major factor in the design of scaffolds for engineered tissues.

The objective of this method is to understand the effects of electrospun polycaprolactone (PCL) fiber size and morphology on in vitro cell cultures. The method is adapted from previous research focused on kidney tissue engineering⁹. A more detailed step-by-step protocol is offered here in order to facilitate scaffold reconstruction and develop a standardized method that is applicable to all cell types. Six groups of scaffolds were constructed with this method: three are large 5 μm fibers (LG) and the other three small 1 μm fibers (SM), using different PCL concentrations. Within these LG and SM groups there are three different morphological constructions: random fibers (RA), aligned fibers (AL), and porous cryogenic fibers (CR). These scaffold groups all have inherently different structures, and thus deliver unique local mechanical environments upon which cells attach. To demonstrate the morphological effect this delivers to cells, the hepG2 cell line was seeded onto each group of scaffolds as a representative study. This method is offered as a baseline analysis for electrospun scaffold design for all cell types from stem cells to cell lines

and primary cells.

PROTOCOL:

1. Solution preparation

1.1. Large (random, aligned, and cryogenic) fibers

1.1.1. Prepare a 5:1 ratio of chloroform and methanol by mixing 50 mL of chloroform and 10 mL of methanol in a glass solvent-proof bottle using a glass serological pipette.

CAUTION: Chloroform and methanol are toxic solvents and mixing must be conducted in a fume hood.

1.1.2. To create a 19 w/v% solution of PCL in 5:1 chloroform:methanol, weigh 1.9 g of PCL and add to 10 mL of 5:1 chloroform:methanol in a glass solvent-proof vial. Double seal the vial using paraffin film and leave the vial on a roller overnight to allow the PCL to fully dissolve.

CAUTION: Mixing must be conducted in a fume hood.

1.2. Small (random and aligned) fibers

1.2.1. To prepare a 7 w/v% solution of PCL in hexafluoroisopropanol (HFIP), weigh 0.7 g of PCL and mix with 10 mL of HFIP in a glass solvent-proof vial. Double seal the vial using paraffin film and leave on a roller overnight to allow the PCL to fully dissolve.

CAUTION: HFIP is highly toxic and mixing must be conducted in a fume hood.

1.3. Small cryogenic fibers

CAUTION: Mixing must be conducted in a fume hood.

1.3.1. Prepare a 3:1 ratio of chloroform and methanol by mixing 30 mL of chloroform and 10 mL of methanol in a glass solvent-proof bottle using a glass serological pipette.

1.3.2. To create a 14 w/v% solution of PCL in 3:1 chloroform:methanol, weigh 1.4 g of PCL and add to 10 mL of 3:1 chloroform:methanol in a glass solvent-proof vial. Double seal the vial using paraffin film and leave on a roller overnight to allow the PCL to fully dissolve.

2. Electrospinning setup

NOTE: The parameters must be set before beginning the electrospinning process.

2.1. Needle size and placement

2.1.1. For large fibers (random, aligned, and cryogenic) use a 1 mm OD/0.8 mm ID brass needle and place the needle 230 mm away from the mandrel surface.

2.1.2. For small fibers use a 1 mm OD/0.4 mm ID brass needle and place the needle 15 mm away from the mandrel surface.

2.2. Lateral needle motion

NOTE: The scaffolds detailed in **Figure 1** were fabricated with a 100 mm needle traverse at 50 mm/s, with repeated motion left and right throughout the process.

2.2.1. To set the lateral motion, press **Motion | TNS 1** and set **Start position [mm]** to **-50**, **Distance [mm]** to **100**, **Speed [mm/s]** to **50** and **Turn Delay [ms]** to **750**.

2.3. Syringe pump flow rate

2.3.1. For large fibers (random, aligned, and cryogenic), press the **Material** tab on the user interface and set the syringe pump to 4 mL/h.

2.3.2. For small (random and aligned) fibers, set the syringe pump to 0.8 mL/h.

2.3.3. For small cryogenic fibers, set the syringe pump to 2.5 mL/h.

2.4. Voltage settings

2.4.1. For the large fibers, set the needle voltage at +15 kV and the mandrel voltage at -4 kV by pressing the **Voltage** tab on the user interface and changing the positive and negative voltage values.

2.4.2. For the small (random and aligned) fibers, set the needle voltage at +17 kV and the mandrel voltage at -4 kV.

2.4.3. For the small cryogenic fibers set the needle voltage at +18 kV and the mandrel voltage at -4 kV.

2.5. Mandrel rotation (large and small fibers)

2.5.1. For random fibers, press **Motion | Target** and set the mandrel rotation speed to 250 rpm.

2.5.2. For aligned fibers, set the mandrel rotation speed to 1,800 rpm.

2.5.3. For cryogenic fibers, set the mandrel rotation speed to 180 rpm.

3. Electrospinning of random and aligned fibers (large and small fibers)

3.1. Insert the needle (refer to section 2.1 for information on needle size and placement) into 1 mm bore PTFE tubing and secure within the electrospinner needle holder.

3.2. Under a fume hood, transfer the 10 mL of polymer solution into a polypropylene or glass (solvent-proof) syringe. Make sure to remove any bubbles by letting the syringe stand before priming.

3.3. Connect the syringe to the needle via the PTFE tubing and secure the syringe within the syringe pump. Once connected, prime the PTFE tubing by squeezing the syringe plunger and filling the tube with polymer solution.

3.4. Wrap the mandrel in aluminum foil and secure onto the mandrel rotation device.

3.5. Switch on the mandrel rotation by pressing **Motion | Target | Rotate** and the syringe pump by pressing **Material | Start Pump 1**.

3.6. When a small bead of polymer has formed at the end of the needle, switch on the voltage by pressing **ACTIVATE HIGH VOLTAGE**. A jet of polymer should then be emitted from a conical protrusion of polymer solution, called the Taylor cone. Check the mandrel to confirm fiber deposition on the mandrel surface.

3.7. Run the electrospinning process until the desired amount of PCL solution is electrospun.

NOTE: For the scaffolds presented in the **Figure 1**, 4 mL of solution was electrospun.

3.8. Once the electrospinning has terminated, remove the aluminum foil covered with the fiber from the mandrel and leave in a fume cupboard overnight to allow for excess solvent evaporation.

3.9. After evaporation, store the fibers at room temperature (RT).

4. Electrospinning of cryogenic fibers

4.1. For cryogenic electrospinning, use the cryo-spinning mandrel. Repeat steps 3.1–3.3 then proceed with the following steps.

4.2. Wrap the mandrel in aluminum foil.

4.3. Fill the mandrel with dry ice pellets. Secure the cryo-spinning mandrel cover onto the mandrel to ensure that dry ice cannot escape the mandrel but pressure can escape. Ice crystals should form on the surface of the mandrel almost immediately.

CAUTION: Use protective gloves when handling dry ice to avoid cold burns.

4.4. Secure the mandrel onto the mandrel rotation device and immediately switch on the mandrel rotation by pressing **Motion | Target | Rotate** and the syringe pump by pressing **Material | Start Pump 1**.

4.5. Once a bead of polymer has formed at the end of the needle, switch on the voltage by pressing **ACTIVATE HIGH VOLTAGE**. A jet of polymer should then be emitted from a conical protrusion of polymer solution (i.e., the Taylor cone). Check the mandrel to confirm fiber deposition on the mandrel surface.

4.6. After 1 h of electrospinning, pause the process by pressing **STOP ALL** and top up the mandrel with dry ice. Ensure adequate protection from solvent vapor while refilling the mandrel. Perform this for a total of 3x for 4 h total of electrospinning time.

NOTE: Electrospinning can be resumed by repeating steps 4.4 and 4.5.

4.7. Once the electrospinning ends, carefully remove the mandrel and immediately place it into a freeze drier for 24 h to sublimate the ice crystals on the mandrel surface.

4.8. After sublimation remove the fiber-covered foil from the mandrel and store at RT, taking care to avoid crushing.

5. Scaffold punching

5.1. Punch scaffolds from the sheet of fibers using a 10–12 mm biopsy punch. If the fibers remain adhered to the aluminum foil, place the scaffolds in 70% ethanol to facilitate detachment.

6. Sterilization and plasma coating

6.1. Place the scaffolds (1 scaffold per well) into a 24 or 48 well plate and wash them 3x in 70% ethanol. Leave submerged in the final wash, place into a -80 °C freezer, and allow to freeze.

6.2. Once frozen, place the plate into a freeze drier and lyophilize for 24 h. The dried scaffolds can now be plasma coated.

NOTE: If there is no access to a plasma coater, submerge the scaffolds in 1% antibiotic-antimycotic (Anti-Anti) in phosphate buffered saline (PBS) and leave to incubate at 37 °C overnight.

6.3. Place the plate into the plasma chamber and remove the lid. Run the plasma coater at 500 mTorr and medium radiofrequency (RF) power for 30 s.

6.4. After plasma coating, submerge the scaffolds in 1 mL of PBS and 1% Anti-Anti. The scaffolds

should readily absorb the PBS solution.

NOTE: The scaffolds are now ready to be seeded with cells.

7. Preparation and seeding of cells

NOTE: The basic cell culture technique provided below is for the hepg2 cells used for demonstration purposes in the representative results. These principles can be applied to any adherent cell type; however, different cells may require different materials and different processes. Cell handling must be conducted in a biological safety cabinet, following aseptic technique.

7.1. Take 1×10^6 cryopreserved hepg2 cells and seed into a T75 cell culture flask with 12 mL of complete media consisting of Eagle's minimum essential media (EMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin-streptomycin, 5 mM L-glutamine, and 1% nonessential amino acids.

7.2. Once the cells are at 80% confluence detach the cells from the culture flask by washing 3x in PBS and incubating in 2 mL of trypsin at 37 °C for 4 min. After tapping the flask, the cells should detach.

7.3. Deactivate the trypsin by adding 8 mL of complete media. Transfer the cell suspension into a 15 mL tube and spin down the cells in a centrifuge at $120 \times g$ for 5 min.

7.4. Remove the supernatant above the cell pellet with an aspirating pipette and replace with 5 mL of complete media. Resuspend the cells by pipetting up and down with a 5 mL serological pipette.

7.5. Count the resulting cell suspension via the trypan blue exclusion method. Take 100 μ L of cell suspension and add to 100 μ L of trypan blue in a 1.5 mL vial. Introduce the mixture onto a hemocytometer under a glass coverslip and count the cells under a microscope.

7.6. Adjust the cell concentration to 2.6×10^6 cells/mL by adding or removing complete media. To remove media, centrifuge cells at $220 \times g$ for 5 min, remove the required media, and resuspend the cells.

7.7. Aspirate the 1% Anti-Anti in PBS from the well plate and seed 50 μ L of 2.6×10^6 cells/mL cell suspension onto the scaffold surface using a 200 μ L pipette.

7.8. Allow cells to attach under incubation at 37 °C and 5% CO₂ for 1–3 h, making sure they do not dry out. Once attached, add 300 μ L (for a 48 well plate) or 500 μ L (for a 24 well plate) of culture media and incubate at 37 °C and 5% CO₂.

NOTE: Standard cell culture techniques can be used from here until further functional analysis.

REPRESENTATIVE RESULTS:

Figure 1 shows scanning electron microscope (SEM) images of both small ($\sim 1\ \mu\text{m}$) and large fibers ($\sim 5\ \mu\text{m}$) fabricated from PCL solutions. The fibers were sputter-coated with gold-palladium (60:40) for 30 s using a sputter coater prior to SEM imaging. Large fibers were produced using a high 19 w/v% concentration of PCL in a 5:1 solution of chloroform and methanol to create a high viscosity PCL solution. This high viscosity solution was then electrospun at a high flow rate to emit a large thread of polymer solution within the electric field. Therefore, a large 230 mm distance between the needle and the mandrel was necessary to ensure effective evaporation of the solvent before deposition on the mandrel. Conversely, smaller fibers were produced using a low 7 w/v% concentration of PCL in HFIP to create a low viscosity PCL solution. Electrospinning the low viscosity PCL solution at a low flow rate ensured that a thinner thread of PCL solution was emitted from the needle within the electric field. The thinner thread facilitated a quicker evaporation of solvent and the needle needed to be closer to the mandrel at 70 mm to ensure a stable deposition of fibers. For electrospinning small cryogenic fibers, altering the PCL solution was necessary to achieve a reliable protocol. PCL in HFIP electrospun onto the cryogenic mandrel repeatedly resulted in a disintegrated fiber structure. Electrospinning a 14 w/v% PCL concentration in 3:1 chloroform methanol solved this problem; however, this created a larger variation in fiber size throughout the scaffold. The extent of fiber alignment (fiber radius and orientation) for each scaffold was analyzed through the ImageJ DiameterJ plugin²⁹ and shown in **Figure 2** and **Figure 3**.

[Figure 1]

[Figure 2]

[Figure 3]

This method offered us a set of scaffolds with six distinctly different tensile properties as determined using a tensile testing machine according to previously described methods⁹. As shown in **Table 1** the macroscale Youngs' Moduli ranges from $46.94 \pm 3.48\ \text{MPa}$ in the large aligned fibers to $0.20 \pm 0.01\ \text{MPa}$ in the large cryogenic fibers. Physiological tissue stiffness values range from 100 Pa (neural tissue) to 2–4 GPa (bone tissue)³⁰; all of the scaffold stiffness values reported here were within this range.

[Table 1]

Figure 4 shows the DAPI and phalloidin staining used to visualize the cellular morphology. The staining method used is established in the literature^{11,12}. Images were obtained using a microscope (**Table of Materials**) with a 40x magnification objective. The images show that the aligned fibers produced by this method elongated the hepG2 cell morphology and directed proliferating cells along the length of the fibers, creating directionality within the cell culture. In contrast, the hepG2 cells on random fibers did not display the directional proliferation and elongation observed in the aligned structures. On the random structures the hepG2 cells

displayed a more regular morphology with extended protrusions attaching to the surrounding PCL fibers.

[Figure 4]

FIGURE AND TABLE LEGENDS:

Figure 1: SEM images showing the morphological qualities of each scaffold group. The top row shows the small fibers and the bottom row the large fibers. Morphological groups are listed left to right as random fibers, aligned fibers, and cryogenic fibers, respectively.

Figure 2: Fiber radius (left) and fiber orientation (right) data from the DiameterJ plugin for the small set of fibers. Morphology listed from top to bottom as random fibers, aligned fibers, and cryogenic fibers, respectively.

Figure 3: Fiber radius (left) and fiber orientation (right) data from the DiameterJ plugin for the large set of fibers. Morphology listed from top to bottom as random fibers, aligned fibers, and cryogenic fibers, respectively.

Figure 4: DAPI (blue) and phalloidin (green) stained hepG2 cells on the different scaffold morphologies: random (left), aligned (middle), and cryogenic (right). The top row shows small fibers and the bottom row shows large fibers.

Table 1: Tensile testing data for each scaffold group at intervals of 2% strain. SM.RA = Small random, SM.AL = Small aligned, SM.CR = Small cryogenic, LG.RA = Large random, LG.AL = Large aligned, and LG.CR = Large cryogenic. Data presented as mean \pm SD, n = 5.

DISCUSSION:

The creation of different fiber sizes was achieved using a combination of different solvents, PCL concentrations, and electrospinning parameters. The type of polymer, its molecular weight, and the solvent strongly affect the viscosity and charge properties of the resulting solution and therefore bear a strong influence on the electrospinning properties³¹. The voltages displayed in this method are subject to change based upon the conditions in which the electrospinning is conducted. Temperature and humidity affect the behavior of the electrospinning process³². Therefore, the reader is advised to be prepared to change the voltage to counter any hindrances and achieve a stable Taylor cone. It is recommended to house the electrospinner within an environmental control enclosure to reduce batch-to-batch variability in the electrospinning process.

Achieving the different morphologies presented in this method is possible due to the capabilities of the IME electrospinning device. A variable speed mandrel is essential for controlling fiber alignment. Mandrel rotation at a low rpm (<250 rpm) yields randomly oriented fibers due to the behavior of the polymer jet. When the charged polymer solution is emitted from the needle, the internal charges and aerodynamic forces induce a chaotic whipping effect that is utilized to lay down a chaotic arrangement of fibers on the mandrel^{33,34}. This whipping effect can be overcome

by increasing the mandrel rotation speed above 1,800 rpm. Fibers deposited on such a high rpm mandrel exhibit an aligned structure because the surface speed is enough to effectively counter any transverse movement exhibited by the whipping fiber. It is important to note that when high mandrel speeds are used, it is likely to disrupt the surrounding air, which can have an adverse effect on the production of a stable Taylor cone.

Highly porous cryogenic scaffolds are produced by filling the mandrel with dry ice at -78.5 °C. This reduces the temperature of the mandrel and promotes condensation and freezing of water droplets on the surface. The crystals formed exhibit peaks that emerge from the mandrel surface and the fibers are deposited throughout the peaks. Once the ice crystals are sublimated out, a porous structure of fibers remains on the mandrel^{35,36}. The weight and fragility of the crystals limit the ability to create porous aligned fibers, as too high mandrel speeds (>250 rpm) result in the crystals and fibers detaching from the mandrel due to excessive centripetal and aerodynamic forces. It is possible to face difficulties with this method due to the fragility of the structures formed. Care must be taken when handling the mandrel and also when handling the scaffolds afterwards, because the structures are prone to being flattened when squeezed and folded when removing from liquids. There is also the possibility that solutions that electrospin successfully without the cryogenic mandrel can be fatally disrupted by the temperature change and the crystal structures. When cryo-spinning, the amount of ice formed on the mandrel surface is highly dependent on the humidity, producing variability in the final result. It is therefore highly recommended to contain the electrospinning process within an environmental control enclosure. To assess cellular performance on these scaffolds, it is necessary to sterilize the material beforehand. There are many options available for sterilizing polymers, including chemical methods, radiation methods, and heat-based methods^{37,38}. It is important to assess the effectiveness of each method and the suitability for both the use of the material and for the material itself. This protocol uses sterilization in 70% ethanol before lyophilizing, plasma treating, and submerging in 1% Anti-Anti solution. The use of ethanol allows proteins and microorganisms to be denatured and dehydrated while not disrupting the PCL material³⁷. The use of stronger solvents may result in the dissolution of the PCL. Plasma coating was incorporated in this method primarily to increase the hydrophilicity of PCL, which is notoriously hydrophobic, and improve cell attachment^{39,40}. Conveniently, this also works as a secondary sterilization step, but care must be taken to keep the scaffolds in a sterile environment pre and post plasma treatment. This method has proven suitable for in vitro studies with antibiotic treated media. However, for in vivo applications, more robust options such as ethylene oxide (EtO) and gamma exposure must be considered. Once sterilization has been performed, cells can be seeded onto the scaffold and standard cell culture techniques can be applied to form 3D cell cultures in vitro.

It is important to note that the stiffness values should be attributed to the different microstructures formed within the scaffolds. The bulk material (PCL) remains constant between scaffolds. Therefore, the bulk material stiffness does not change between groups. The cryogenic scaffolds display a much lower stiffness than the other groups due to the reduced connectivity within the fiber matrix, which can be seen in **Table 1**. The reduced connectivity is thought to induce a more bending-dominant deformation as opposed to stretching-dominant deformation within the fibrous structure⁴¹. Interestingly, the scaffold stiffness is not highly dependent on fiber

size in the random and cryogenic groups. However, the longitudinal stiffness of the aligned fibers shows a significantly higher dependence on fiber size with the Young's Modulus at 0–2% strain being 27.94 ± 8.63 MPa for small fibers and 46.94 ± 3.48 MPa for large fibers. Due to the stretching nature of the tensile test, this implies a higher bulk PCL density within the longitudinal cross section. It is necessary to reiterate that the mechanical data presented here is a macroscale mechanical snapshot of the fibrous architectures. Further micromechanical characterization would be beneficial to fully understand the mechanical influences on the cell scale.

Both random and aligned architectures were included in this method to provide a comparison between isotropic and anisotropic morphologies. The extent of fiber alignment can be observed in the SEM images in **Figure 2** and the fiber analyses presented in **Figure 2** and **Figure 3**. Anisotropic properties are observed throughout many of the tissues in the body. In particular, this is commonly observed in aligned cellular structures such as those found in muscle and nerve tissues. Aligned polymeric fiber structures offer the capability of recapitulating these aligned structures in vitro^{42,43}. As described before, cell function is sensitive to morphological and mechanical changes, so further functional analysis should be conducted on scaffold-bound cells to determine the biological influence of each scaffold type.

Compared to other existing scaffold fabrication techniques, this method provides a simple way to produce microscale scaffold structures with comparatively high control over the mechanical properties and morphology. Alternative PCL scaffold fabrication such as phase separation, salt leaching, and gas foaming allow for morphological control in terms of void space and pore size. However, the pore and structural geometry remains largely the same^{44–47}. Therefore, qualities such as the level of isotropy cannot be altered as easily in comparison to electrospinning. Hydrogel materials, popular for scaffold production, provide the means to alter the stiffness of the polymer substrate through alteration of the level of cross-linked polymer chains⁴⁸. It is also possible to 3D print via a variety of methods, providing excellent control over morphology⁴⁹. However, achieving electrospinning scale resolution in biocompatible hydrogel materials remains a challenge to be practically implemented^{50,51}. Electrospun scaffolds have featured commonly in tissue engineering research throughout the last decade, and the introduction of new materials and applications with different cell types is always being explored. While new materials are constantly under development for the purpose of electrospinning scaffolds, opportunities for further biological characterization of existing electromaterials and methods remain. The method described is proposed as a method for facilitating in vitro biological study, as it is directly applicable to basic cell culture techniques.

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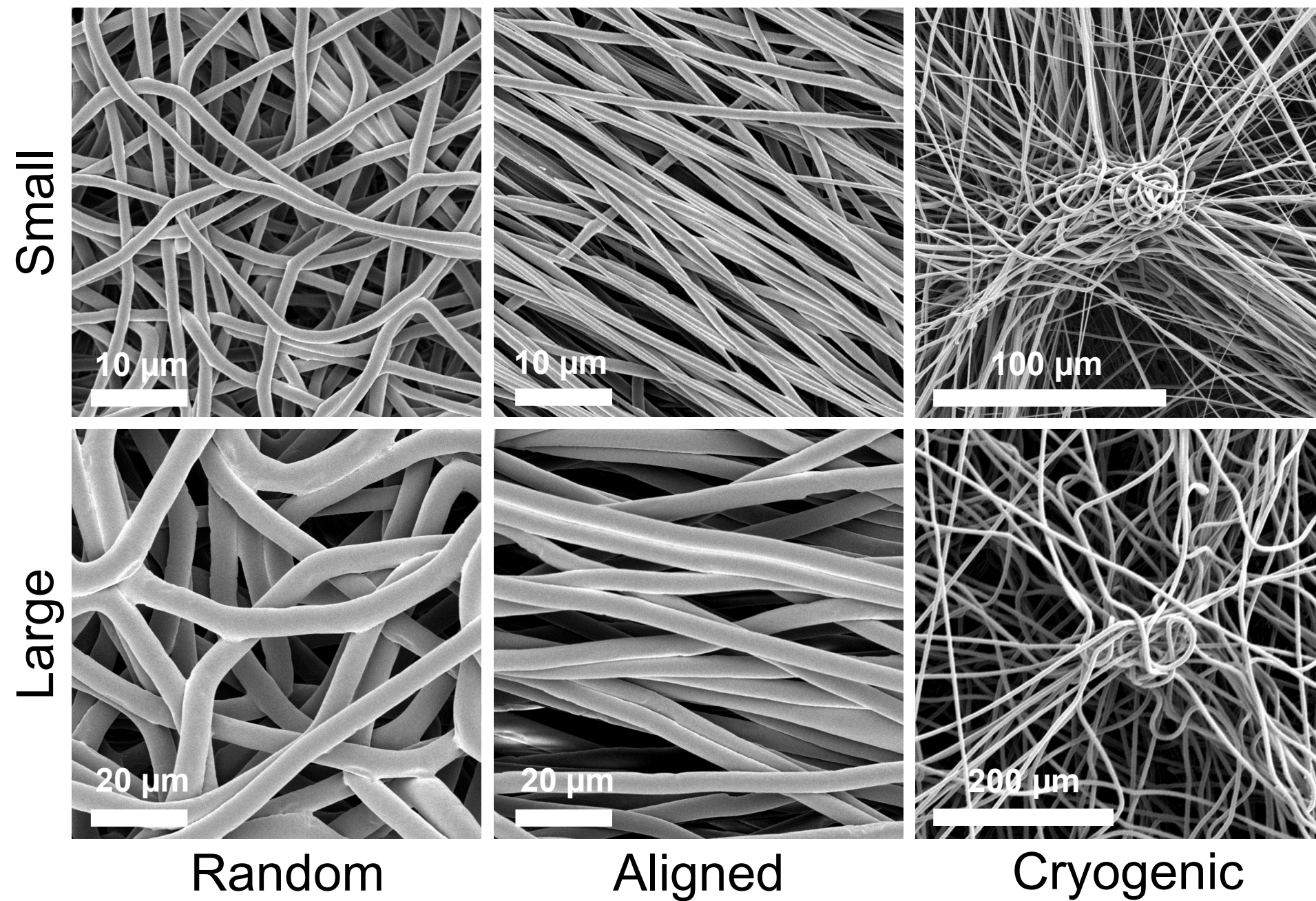


Figure 2

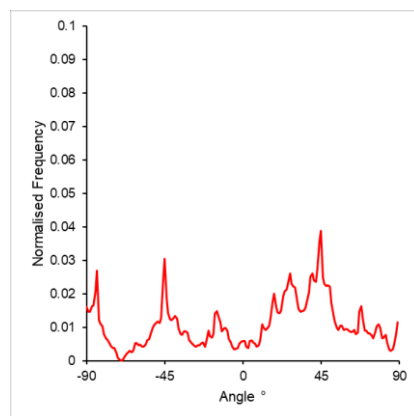
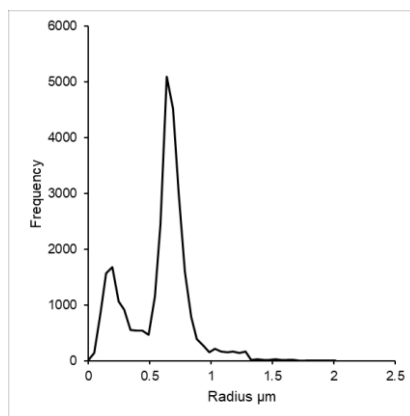
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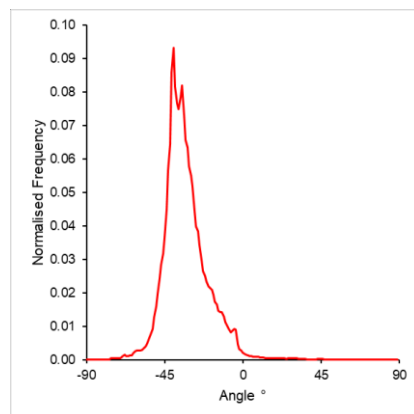
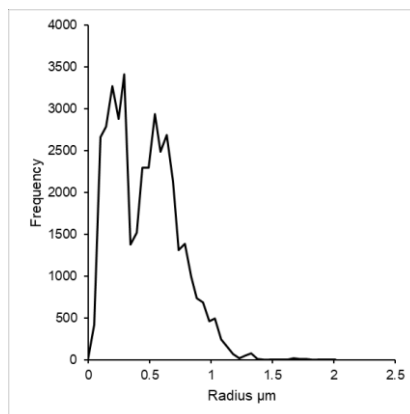
Fibre radius

Fibre orientation

Random



Aligned



Cryogenic

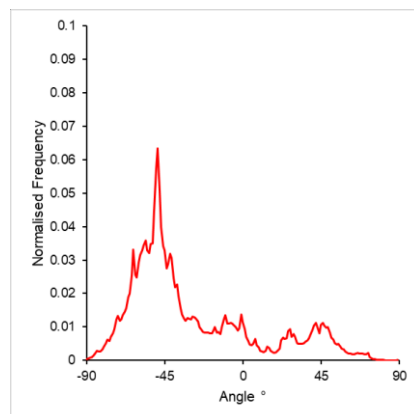
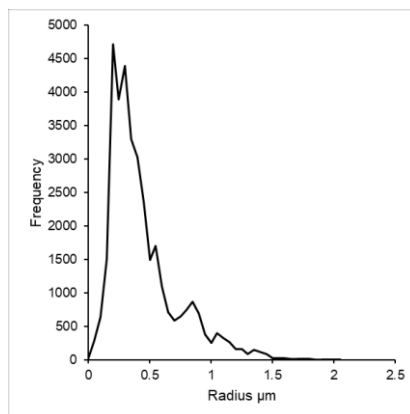


Figure 3

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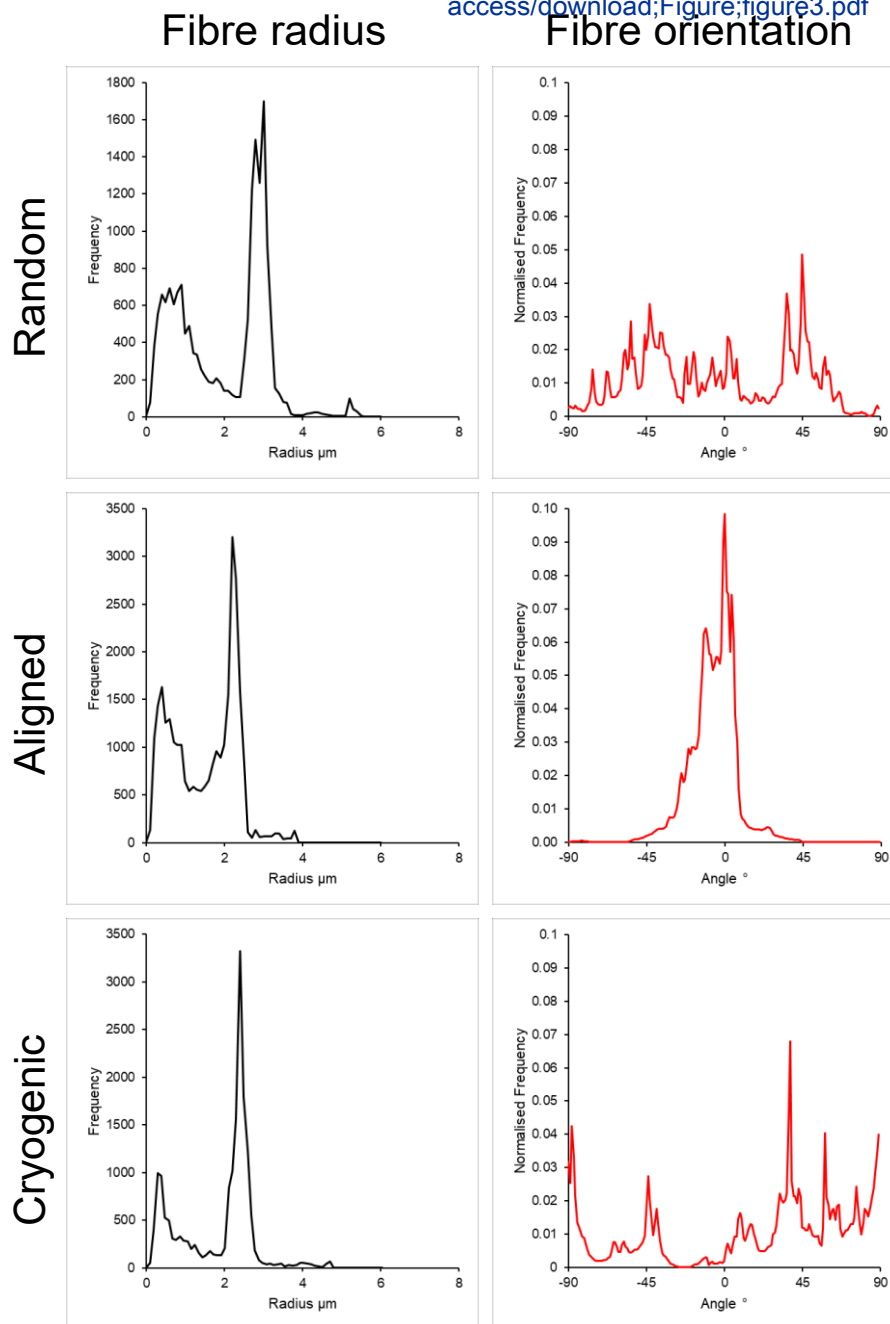
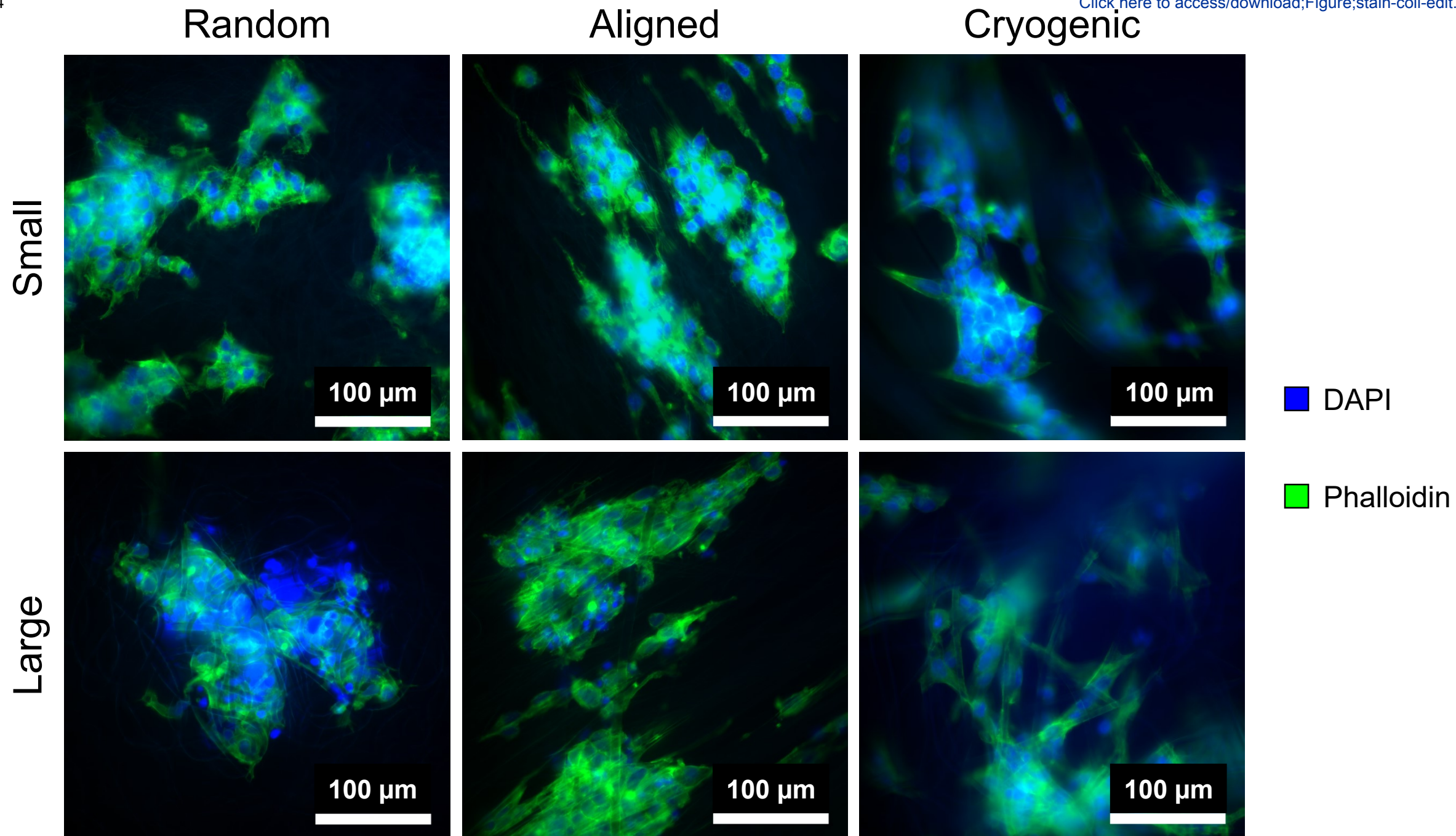


Figure 4

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Scaffold	Youngs' modulus (MPa) for strain%				
	0–2%	2–4%	4–6%	6–8%	8–10%
SM.RA	11.14 ± 2.13	9.31 ± 0.43	6.75 ± 0.64	4.75 ± 0.59	3.32 ± 0.40
SM.AL	27.94 ± 8.63	28.27 ± 7.36	16.34 ± 4.87	9.10 ± 3.75	5.85 ± 3.11
SM.CR	0.27 ± 0.11	0.31 ± 0.44	0.31 ± 0.65	0.29 ± 0.59	0.26 ± 0.40
LG.RA	9.23 ± 0.78	8.22 ± 0.87	5.96 ± 0.68	3.96 ± 0.59	2.63 ± 0.45
LG.AL	46.94 ± 3.48	38.03 ± 2.46	27.69 ± 1.22	16.10 ± 1.61	7.87 ± 1.32
LG.CR	0.20 ± 0.01	0.16 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.09 ± 0.01

Name of Material/Equipment	Company	Catalog Number
10 mm biopsy punch	Acuderm	P1025
15 mL Falcon tube	Thermo-scientific	339650
20 mL glass vial	Fisherbrand	11513542
48 well cell culture plate	Greiner Bio-one	677180
Anti-Anti 100X	Gibco	15240062
Chloroform	Acros Organics	13029236
DAPI	Sigma-aldrich	D9542
Eagles Minimum Essential Media	Gibco	11090081
Electrospinner	IME Technologies	EC-DIG
Fetal Bovine Serum	GE Healthcare	SH30071.03
Gold-Palladium Sputter coater	Emscope	SC500A
HepG2 cell line	Sigma-aldrich	85011430
Hexafluoroisopropanol (HFIP)	Manchester Organics	G26383
L-Glutamine 200 mM	Gibco	25030081
MEM Non-essential Amino Acids	Gibco	11140050
Methanol	Acros Organics	11984591
Penicillin-Streptomycin 10,000 U/mL	Gibco	15140122
Phalloidin	Abcam	ab176754
Phosphate Buffered Saline (PBS)	Sigma-aldrich	P4417-100TAB
Plasma coater	Harrick Plasma	PDC-002
Polycaprolactone (Avg. MW 80,000)	Sigma-aldrich	440744
PTFE Syringe	Fisherbrand	12941031
Scanning Electron Microscope	Hitachi	S4700
T75 Culture Flask	Corning	CLS430641
Trypan blue (0.4%)	Gibco	15250061

Comments/Description



THE UNIVERSITY
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Dr Anthony Callanan
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28th January 2020

Prof David Hay
Guest Editor
JoVE video journal

Dear Prof Hay,

We submit for your consideration our manuscript entitled "*Protocol for controlling electrospun polymer morphology for tissue engineering: demonstration using hepG2 cell line*" for publication in your methods collection: "Pluripotent stem cell differentiation, genome editing and tissue engineering." for the JoVE video journal.

We thank the reviewers for spending the time reviewing the submitted manuscript and offering their invaluable feedback upon which we could improve the manuscript. We have taken on board the advice and criticism offered by the reviewers and we now offer a revised manuscript.

The documents attached describes the changes we have made and offers a rebuttal to each of the comments made by the reviewers. We look forward to your feedback and thank you for the opportunity to publish in JoVE video journal.

Best regards,

A handwritten signature in black ink, appearing to read 'Anthony Callanan'.

Editorial Comments

Thank-you for your diligent comments regarding the manuscript content, we have addressed the points as per below:

Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have made some edits to the protocol which highlight button presses and software usage.

- **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

- 1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.

- 2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

- 3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

- 4) Notes cannot be filmed and should be excluded from highlighting.

We have highlighted the appropriate parts of the text that identify the important visual steps that are to be recorded.

- **Results:** Please add at least one paragraph of results text that explains your representative results in the context of the technique you describe; i.e. how do these results show the technique, suggestions about how to analyze the outcome etc. This text should be written in paragraph form under a "Representative Results" heading and should refer to all of the results figures. You may include the figure captions under this heading but the captions and figure text must be separate entities.

We have added paragraph form text within the representative results section that describe the results in the context of the technique.

- **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We have edited the discussion information to fulfil these requirements to the best possible effect. The main edits were made in the addition of the final paragraph, and the movement of some discussion elements to the representative results section where they were more relevant.

- **Figures:** Define the pseudocolors in Fig 4.

Fig 4 caption has been appropriately updated to indicate the colours in the figure.

• **Table of Materials:** Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as dyes and stain used, cell lines, software etc.

The table of materials has been appropriately updated.

Reviewer #1:

Major Concerns:

The assessment of the HepG cell line is not conducted in detail.

Thank you for your comment regarding the assessment of the HepG2 culture and we understand your concerns regarding the lack of detail included about it. It is true that the article currently includes little detail on the cell culture aspect which was a decision made because it is provided as a demonstration only. It is expected that readers of this article have at least been exposed to standard cell culture methods before attempting to carry out studies with these scaffolds. The method describes that it requires only standard cell culture techniques, the sole difference being the inclusion of the scaffold. The results shown are representative results so it was not thought necessary to include a detailed account of how to carry out the cell culture. Also, the application of multiple cell types is encouraged within the introduction and discussion which implies slightly different materials and methods would be applied in each case. To address your comment we have expanded the methods section to include some more detail on the cell culture aspect (Section 7 of the method). We have also changed the title of the article to reflect our intended purpose of demonstrating an example of a potential cell study using the hepg2 cell line.

Reviewer #2:

Thank-you for your comments, these have been addressed as per below:

Minor Concerns:

1- **Proof-read the paper after complete revision and consider the grammatical points and typos.**

The manuscript has been reviewed and we have made edits to the grammar and typos where appropriate.

2- **Please use the recent literature reports in discussion section and compare your work with them**

Comparisons of the described method with others recently published in the literature have been included. The final paragraph in the discussion includes these comparisons and 45% of citations within the discussion are dated between 2018 and 2019.

3- **Please update the references in Introduction and cite very recently published works, if exist, especially publications from 2018 and 2019.**

We appreciate your requirement for more up-to-date articles cited within the introduction. Having updated some citations, we have 39% of citations dated between 2018 and 2019 within the introduction.

Reviewer #3:

Major Concerns:

manuscript does not communicate any new information. science is poor and and just repeat what many other papers have shown already in last decade

Thank-you for your comments regarding the manuscript content. We do acknowledge that the information is not necessarily new and in the introduction we had mentioned that it is adapted from previous work¹. However we must highlight the fact that this method has not been published with the high level of step-by-step detail provided within this manuscript and for a methods based or visual experiment journal. Whilst electrospun polymer technology has been thoroughly explored within the last decade, there is an enduring lack of consideration for morphological consistency and seemingly arbitrary choices of architecture and fibre size²⁻⁴. This method is intended to address these issues and provide researchers with access to a detailed study design through which mechano-biological optimisation of electrospun *in-vitro* systems can be achieved. The level of method detail included is for the purpose of introducing standardised methods into the research field that other researchers are able to follow and reproduce. The representative results described are in accordance with current standards in literature⁵⁻⁷. We have supplied results which show a suitable level of data to legitimise claims made in the justification for the method. The statement 'science is poor' is rather vague and as with any scientific writing a basic requirement is the justification of your argument in order to facilitate understanding.

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12th February 2020

Prof David Hay
Guest Editor
JoVE video journal

Dear Prof Hay,

We submit for your consideration our manuscript entitled "*Protocol for controlling electrospun polymer morphology for tissue engineering: demonstration using hepG2 cell line*" for publication in your methods collection: "Pluripotent stem cell differentiation, genome editing and tissue engineering." for the JoVE video journal.

We thank the editors for spending the time inspecting the revised manuscript and offering their diligent advice. We have edited the revised manuscript according to the changes required by the editors.

The documents attached describes the changes we have made and offers a response to each of the comments made by the editor. We look forward to your feedback and thank you again for the opportunity to publish in JoVE video journal.

Best regards,

A handwritten signature in black ink, appearing to read 'Anthony Callanan'.

Editorial Comments – Revised changes

The diligent comments provided for this revision were greatly appreciated and below are provided our line-by-line responses to each comment.

1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank-you for the formatted text with useful comments included, the formatted manuscript 61043_R1_RE has been edited. Spelling and grammar have been checked and corrected where appropriate.

2. Figure 1: Please include a space between the number and the units of the scale bar (e.g., 100 μm).

This has been corrected, thank-you for noticing.

3. Figure 4: Please use the micro symbol μ instead of u and include a space between the number and the units of the scale bar (e.g., 100 μm).

This has been corrected, thank-you for noticing.

4. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.

The table of materials has been updated according to any new materials described and ordered correctly.

5. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See specific comments marked in the attached manuscript.

Thank-you for the very useful formatting and comments. The edited manuscript has addressed these problems, and described how to do certain tasks such as cell culture in more detail. Where appropriate we have described consecutive button presses in the form '**Button 1 | Button 2...**' as this was deemed a clear and concise way of doing so. Single button presses are emboldened and in quotation marks. References have been provided where a more detailed description is required.

6. In the protocol, please add a section describing how to obtain the data presented in figures and tables. Please note that this section does not have to be filmed and that references can be included to explain how to perform SEM, obtain fibre radius and orientation, and perform staining and morphological data.

A brief seventh section has been added as requested, detailing the methods used to obtain the representative results. Where appropriate references were used as these methods are commonplace in literature.