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Title: Stepwise Cell Seeding on Tessellated Scaffolds to Study Sprouting Blood Vessels

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 20
Number of Shots: 33

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Ariel Szklanny:** Our protocol allows researchers to study the vessel network formation process in a clear and easily trackable fashion, opening the doors to new studies and shedding light on blood vessel behavior.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Shulamit Levenberg:** This technique presents the possibility of creating highly organized and repeatable vascular networks which respond to the surrounding environment and cannot be achieved using more traditional techniques.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Ariel Szklanny:** Endothelial cells from patients suffering from a vascular disease can be retrieved and used in this system, making it possible to recreate the vascular disease and find an appropriate treatment.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.4. **Shulamit Levenberg:** The proposed method can be extended for studying vascular network behavior when paired with different cell types, allowing to observe the interaction of developing vessels with a specific tissue type.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Scaffold fibronectin coating

2.1. Begin by submerging the scaffolds in 70% ethanol for a minimum of 15 minutes ~~**[1],**~~ then wash them twice in PBS **[2]**. NOTE: Shots 2.1.1 and 2.1.2 are together

~~2.1.1. WIDE: Establishing shot of talent putting the scaffolds in the ethanol.~~

2.1.2. Talent dipping the scaffolds in ethanol and rinsing the scaffolds in PBS.

2.2. Mix 1.5 microliters of fibronectin stock solution with 28.5 microliters of PBS per scaffold to be seeded. Prepare one fibronectin dilution to be used for all scaffolds to avoid pipetting errors **[1]**.

2.2.1. Talent mixing the fibronectin solution.

2.3. Place the scaffolds sparsely on top of a hydrophobic surface ~~**[1]**~~ and cover each scaffold with 30 microliters of the fibronectin dilution **[2]**. Replace the plate's lid and put the fibronectin-covered scaffolds into an incubator set to 37 degrees Celsius and 100% humidity for a minimum of an hour **[3]**. *Videographer: This step is important!* NOTE: Shots 2.3.1 and 2.3.2 are combined.

~~2.3.1. Talent placing the scaffolds on a hydrophobic surface.~~

2.3.2. Talent placing the scaffolds on a hydrophobic surface and covering a scaffold with fibronectin.

2.3.3. Talent putting the plate in the incubator and closing the door.

2.4. After incubation, lightly rinse the scaffolds in PBS to remove fibronectin remnants **[1]**. The scaffolds can be kept in PBS at 4 degrees Celsius for up to a week **[2]**.

2.4.1. Talent rinsing the scaffolds.

2.4.2. Talent putting the scaffolds in the refrigerator and closing the door.

3. Endothelial cells seeding

3.1. Prepare endothelial cell, or EC, medium by mixing the basal medium with the corresponding medium kit components, including an antibiotic solution, FBS, and endothelial cell growth supplements, as indicated by the manufacturer **[1]**.

3.1.1. Talent preparing EC medium.

3.2. Make a human adipose microvascular endothelial cell suspension in EC medium with a concentration of 4 million cells per milliliter **[1]**.

3.2.1. Talent suspending cells in the medium.

3.3. Using forceps, place one fibronectin-coated scaffold per well in a non-TC 24-well plate ~~[1].~~ Cover each scaffold with 25-microliter droplets of the cell suspension, making sure not to let the suspension flow away from the scaffold **[2]**. *Videographer: This step is important!* NOTE: Shots 3.3.1 and 3.3.2 are combined.

~~3.3.1. Talent placing a scaffold in a well.~~

3.3.2. Talent placing a scaffold in a well and adding cell suspension to a scaffold.

3.4. Put the lid on the plate and place it in an incubator at 37 degrees Celsius, 5% carbon dioxide, and 100% humidity for 60 to 90 minutes **[1]**.

3.4.1. Talent placing the plate in the incubator.

3.5. After the incubation, fill each well with 700 microliters of EC medium **[1]**. Incubate the endothelialized scaffolds until EC confluence can be observed using fluorescent microscopy or for 3 days. Change the medium every other day **[2]**.

3.5.1. Talent adding medium to a few wells.

3.5.2. Talent putting the plate back in the incubator.

4. Support cell seeding and co-culture

4.1. Prepare DPSC medium by mixing 500 milliliters of low-glucose DMEM, 57.5 milliliters of FBS, 5.75 milliliters of non-essential amino acids, 5.75 milliliters of GlutaMAX, and 5.75 milliliters of penicillin-streptomycin-nystatin solution **[1-TXT]**.

4.1.1. Talent preparing DPSC medium. **TEXT: DPSC: dental pulp stem cell**

4.2. Transfer the endothelialized scaffolds into a new non-TC 24-well plate **[1]**. Discard all media from the current plate using a pipette or vacuum suction, taking care to not apply vacuum directly to the scaffold **[2]**.

4.2.1. Talent placing a scaffold in a well. *Videographer: Obtain multiple usable takes, this will be reused in 4.3.1.*

4.2.2. Talent removing medium from current plate. *Videographer: Obtain multiple usable takes, this will be reused in 4.3.2.*

4.3. Place one scaffold into the center of each well **[1]**, then dry the surrounding area with light vacuum **[2]**. *Videographer: This step is important!*

4.3.1. *Use 4.2.1.*

4.3.2. *Use 4.2.2.*

4.4. Dilute thrombin and fibrinogen stock solutions with PBS to a final concentration of 5 units per milliliter and 15 milligrams per milliliter, respectively ~~[1].~~ Prepare an 8 million DPSC per milliliter suspension in the thrombin dilution and distribute 12.5

microliters of the suspension into individual microtubes per scaffold to be seeded ~~[3]~~ [2]. NOTE: 4.4.2 and 4.4.3 are combined

~~4.4.1. Prepared thrombin and fibrinogen solutions in labeled containers.~~

4.4.2. Talent diluting cells in the thrombin solution and aliquoting the cell suspension in individual tubes

~~4.4.3. Talent aliquoting the cell suspension in individual tubes.~~

4.5. Set a 5 to 50-microliter pipette to 12.5 microliters and fill it with the fibrinogen solution. Without removing the tip, set the pipette to 25 microliters. The material in the tip should rise and leave an empty volume ~~[2]~~ [1]. *Videographer: This step is important!* NOTE: Shots 4.5.1 and 4.5.2 are combined

4.5.1. Talent filling a pipette with fibrinogen and setting the pipette to 25 microliters.

~~4.5.2. Talent setting the pipette to 25 microliters.~~

4.6. Slowly press the plunger button until the liquid reaches the tip opening but does not leak out **[1]**. Hold the plunger in this position and put the tip into one of the microcentrifuge tubes containing the cells in thrombin suspension, making sure the tip is in contact with the liquid **[2]**. *Videographer: This step is difficult and important!*

4.6.1. Talent pressing the plunger and solution reaching the tip.

4.6.2. Talent putting the tip in a tube.

4.7. Gently release the plunger button and draw the cell suspension into the tip. Thoroughly mix both materials, avoiding bubble formation **[1]**. *Videographer: This step is difficult and important!*

4.7.1. Talent drawing in the cell suspension and mixing it.

4.8. Quickly dispense the mixed materials on top of an endothelialized scaffold. Repeat the previous steps for each scaffold, making sure to change tips between uses to avoid unexpected fibrin gel formation within the tip **[1]**.

4.8.1. Talent dispensing the suspension on a scaffold.

4.9. Replace the plate lid and incubate the scaffolds at 37 degrees Celsius, 5% carbon dioxide, and 100% humidity for 30 minutes **[1]**.

4.9.1. Talent placing the plate with scaffolds in the incubator and closing the door.

4.10. After incubation, fill each well with 1 milliliter of 1 to 1 DPSC and EC medium **[1]**. Culture for 1 week, changing the medium every other day **[2]**.

4.10.1. Talent filling the wells with medium.

4.10.2. Talent putting the plate back in the incubator.

4.11. At different time points during culture, remove the medium from the well and image the constructs using a confocal microscope to study the vascular development or any other parameter of interest **[1]**.

4.11.1. Talent at the microscope, imaging the scaffolds.

Results

5. Results: Analysis of organized vascular networks on tessellated scaffold geometries

5.1. This protocol allows for the fabrication of tessellated scaffolds made of SU-8 photoresist. Scaffolds with distinct compartment geometries and highly accurate and repeatable features were obtained **[1]**.

5.1.1. LAB MEDIA: Figure 1.

5.2. With traditional simultaneous seeding of both endothelial cells and support cells, the cells were homogeneously distributed over the scaffold, resulting in unpredictable and disorganized vascular networks **[1]**.

5.2.1. LAB MEDIA: Figure 2. *Video Editor: Emphasize the top row.*

5.3. Contrarily, stepwise cell seeding resulted in highly organized vascular networks **[1]**.

5.3.1. LAB MEDIA: Figure 2. *Video Editor: Emphasize the bottom row.*

5.4. When using fluorescent endothelial cells, the vessels can be imaged in real time. Red fluorescent protein expressing endothelial cells were cultured on hexagonal scaffolds and imaged **[1]**.

5.4.1. LAB MEDIA: Figure 3 A. *Video Editor: Emphasize Day 0.*

5.5. The support cells were added at Day 1 and the vascular networks were imaged every other day to quantify vessel development **[1]**. For each time point, wide images of the whole scaffold were taken **[2]**. Vessel growth was quantified as total vessel length and area **[3]**.

5.5.1. LAB MEDIA: Figure 3 A. *Video Editor: Emphasize Days 1, 3, 5 and 7.*

5.5.2. LAB MEDIA: Figure 3 B.

5.5.3. LAB MEDIA: Figure 3 C.

5.6. A confocal imaging time lapse was performed to allow single vessel tracking using multicolored endothelial cells. The vessel path was generated from the time lapse, making it possible to observe vessel migration **[1]**.

5.6.1. LAB MEDIA: Supplementary Video 1.mp4.

5.7. Vessel maturation was observed by the presence of smooth muscle actin and support cells. For the circular, hexagonal, and squared compartments, the cells multiply and organize into structures. By day 7, all shapes showed a rich and complex vascular network **[1]**.

5.7.1. LAB MEDIA: Figure 5 A.

5.8. Higher magnification images revealed a denser smooth muscle actin and support cell presence co-localized with formed vessels, evidencing support cell recruitment and differentiation surrounding vascular structures **[1]**.

5.8.1. LAB MEDIA: Figure 5 B.

Conclusion

6. Conclusion Interview Statements

6.1. **Ariel Szklanny:** This technique can be used to study the behavior of three-dimensional vascular networks when exposed to different conditions, such as specific cell inhibitors, or in the presence of additional cell types.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.