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Title: Direct Bioprinting of 3D Multicellular Breast Spheroids onto Endothelial Networks

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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 similar? **N**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **N**

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 the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, 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 (greater than walking distance)? **N**

Protocol Length
Number of Shots: **31**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Alisa Morss Clyne**: Our protocol facilitates the printing of pre-formed 3D cellular structures, such as breast epithelial spheroids, enabling the rapid formation of biologically relevant models in a variety of bioinks **[1]**.

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

REQUIRED:

1.2. **Swathi Swaminathan**: The main advantage of this technique is that it facilitates the bioprinting of 3D tumor spheroids onto a vascular network that can be used almost immediately for experimental study **[1]**.

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

OPTIONAL:

1.3. **Alisa Morss Clyne**: These 3D bioprinted tissue models provide a rapid in vitro platform that can be expanded for personalized precision medicine **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

OPTIONAL:

1.4. **Swathi Swaminathan**: This highly versatile 3D bioprinting method can be applied to other physiological systems in which 3D interactions with the vasculature are important **[1]**.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

Protocol

2. Breast Epithelial Spheroid Formation

2.1. For breast epithelial spheroid formation, first freeze 200 microliter pipette tips for 30 minutes **[1]** and place growth-factor reduced matrix solution on ice **[2]**.

2.1.1. WIDE: Talent placing tips into freezer

2.1.2. Talent placing solution onto ice

2.2. When the tips have frozen, using one new ice-cold pipette tip per well **[1]**, add 30 microliters of the ice-cold matrix solution onto the side and along the corners of each well of an 8-well chamber slide **[2-TXT]**, adding a final drop to the center to ensure an even coating **[3]**.

2.2.1. Talent attaching tip to pipette NOTE: 2.2.1 – 2.2.3 in one shot

2.2.2. Matrix being added to side and along corners *Videographer: Important step; Videographer: please also capture WIDE shot/Video Editor: shot will be used again* **TEXT: Avoid bubble formation**

2.2.3. Drop being added to center *Videographer: Important step*

2.3. When all of the wells have been coated, incubate the slide at 37 degrees Celsius and 5% carbon dioxide for 15-20 minutes **[1]**.

2.3.1. Talent placing slide into incubator NOTE: use 4.5.2. Videographer: Important step

2.4. While the matrix is polymerizing, resuspend MCF10A (M-C-F-ten-A) cells in MCF10A Assay Medium at a 2 x 10^5 cells/milliliter concentration **[1-TXT]**.

2.4.1. Talent adding medium to cells, with medium container visible in frame **TEXT: See text for all medium preparation details**

2.5. At the end of the incubation, add 50 microliters of cells **[1]** and 450 microliters of MCF10A spheroid growth medium to each well **[2]** and immediately return the slide to the incubator **[3-TXT]**.

2.5.1. Talent adding cells to well(s) Videographer: Important step
2.5.2. Talent adding medium to well(s), with medium container visible in frame Videographer: Important step
2.5.3. Talent placing slide into incubator NOTE: use 4.5.2. **TEXT: See text for MDA-MB-231 cell spheroid formation details**

2.6. Every four days, tilt the chamber slide to a 45-degree angle to allow 200 microliters of supernatant to be replaced with fresh spheroid growth medium per well **[1]**.

2.6.1. Shot of tilted slide, then medium being aspirated and/or replaced from 1 well

3. Human Umbilical Vein Endothelial Cell (HUVEC) Network Formation

3.1. For HUVEC (hue-veck) network formation, prepare a matrix solution-coated 8-well chamber slide as demonstrated **[1]** and pre-stain a 10-centimeter dish of HUVECs with 10 microliters of red cell tracker dye for 30 minutes in the cell culture incubator **[2]**.

3.1.1. Use 2.2.2. WIDE: Talent adding matrix solution to well(s)
3.1.2. Talent adding dye to dish, with dye container visible in frame NOTE: Use take 2

3.2. At the end of the incubation, wash the endothelial cells with 10 milliliters of warm PBS **[1]** before treating them with 2 milliliters of 0.05% Trypsin-EDTA **[2]**.

3.2.1. Talent washing cells, with PBS container visible in frame NOTE: mis-slated 3.1.2 t 1
3.2.2. Talent adding trypsin-EDTA to dish, with trypsin-EDTA container visible in frame

3.3. After 5 minutes at 37 degrees Celsius and 5% carbon dioxide, neutralize the trypsin with 5 milliliters of endothelial growth medium-2 **[1]** and transfer the cells to a 25-milliliter conical tube for centrifugation **[2-TXT]**.

3.3.1. Talent adding medium to plate, with medium container visible in frame NOTE: This and next shot together
3.3.2. Talent adding cells to tube **TEXT: 5 min, 1200 x g, RT**

3.4. Resuspend the pellet in 5 milliliters of serum-free endothelial basal medium-2 for counting **[1]** and dilute the cells to a 1 x 10^6 cells/milliliter concentration **[2]**.

3.4.1. Shot of pellet if visible, then medium being added to tube, with medium container and hemocytometer visible in frame
3.4.2. Talent adding medium to tube NOTE: Use take 2

3.5. Then add 1 x 10^5 HUVECs to each well **[1]** and place the plate in the cell culture incubator for 6 hours **[2]**.

3.5.1. Talent adding cells NOTE: Mis-slated 3.4.2 take 1 *Videographer: Difficult step*
3.5.2. Talent placing plate into incubator *Videographer: Difficult step*

4. Breast Epithelial Spheroid Bioprinting onto Pre-Formed HUVEC Networks

4.1. After 5-8 days of culture, count the number of spheroids in each well in representative phase contrast microscopy images **[1]** and use a 1000-microliter pipette tip with the last 0.5-centimeter of the tip removed to carefully collect all of the spheroids from each well of the 8-well chamber slide into a 50-microliter tube **[2-TXT]**.

4.1.1. WIDE: Talent at computer, counting spheroids
4.1.2. Spheroids being collected and/or added to tube **TEXT: Collect spheroids without medium**

4.2. Resuspend spheroids in an appropriate bioink at a 100 spheroids/100 microliters of ink concentration **[1]** and load the pooled spheroids into a 10-milliliter sterile syringe **[2]**.

4.2.1. Ink being added to tube, with ink container visible in frame *Videographer: Important/difficult step*
4.2.2. Talent loading spheroids into syringe NOTE: 2 parts *Videographer: Important/difficult step*

4.3. Equip the syringe with a 25-gauge sterile needle **[1]** and attach the syringe to the bio-deposition system **[2]**.

4.3.1. Needle being attached *Videographer: Important step*
4.3.2. Talent attaching syringe to system *Videographer: Important step*

4.4. Remove the medium from each well of HUVEC network **[1]** and extrude 100 microliters of breast epithelial spheroids into each of 6 wells of the HUVEC network chamber slide at a flow rate of 1 milliliter of spheroids/minute **[2-TXT]**.

4.4.1. Medium being aspirated *Videographer: Important step*
4.4.2. Spheroids being printed into well *Videographer: Important step* **TEXT: Last 2 wells spheroid-free controls**

4.5. Then add 400 microliters of MCF10A Spheroid Growth Medium to each well **[1]** and place the co-cultures in the cell culture incubator for 24-96 hours **[2]**.

4.5.1. Talent adding medium to well(s), with medium container visible in frame
4.5.2. Talent placing slide into incubator NOTE: multiple takes

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
2.2., 2.3., 2.5., 4.2.-4.4.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
3.5., 4.2.

Results

5. Results: Representative Breast Epithelial Spheroid and HUVEC Network Imaging

5.1. After 5-8 days of culture as demonstrated **[1]**, non-tumorigenic MCF10A breast epithelial spheroids **[2]** should appear round with a hollow center **[3]**, with integrin alpha-6 polarized to the outer edge of the spheroid **[4]**.

5.1.1. LAB MEDIA: Figure 1
5.1.2. LAB MEDIA: Figure 1 *Video Editor: please emphasize left image*
5.1.3. LAB MEDIA: Figure 1 *Video Editor: please emphasize spheroid in inset*
5.1.4. LAB MEDIA: Figure 1 *Video Editor: please emphasize green outline for at least one spheroid in left image*

5.2. Highly invasive MDA-MB-231 (M-D-A-M-D-two-thirty-one) breast cancer epithelial cells form irregular spheroids **[1]** that may show cells migrating out of the spheroids if maintained in the Matrigel culture for too long **[2]**.

5.2.1. LAB MEDIA: Figure 1 *Video Editor: please emphasize right image*
5.2.2. LAB MEDIA: Figure 1 *Video Editor: please emphasize long arms coming out of at least one spheroid*

5.3. After 6-8 hours of sparse, serum-free culture as demonstrated **[1]**, HUVEC networks can be imaged by phase contrast **[2]** or confocal microscopy **[3]**.

5.3.1. LAB MEDIA: Figure 2
5.3.2. LAB MEDIA: Figure 2 *Video Editor: please emphasize left image*
5.3.3. LAB MEDIA: Figure 2 *Video Editor: please emphasize right image*

5.4. After breast epithelial spheroid bioprinting onto the HUVEC networks **[1]**, both the spheroids **[2]** and networks should maintain their original morphology for at least 24 hours **[3]**.

5.4.1. LAB MEDIA: Figure 3
5.4.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize spheroids in left and right images*
5.4.3. LAB MEDIA: Figure 2 *Video Editor: please emphasize HUVEC network in both images*

Conclusion

6. Conclusion Interview Statements

6.1. **Swathi Swaminathan**: The main thing to remember while attempting this protocol is to handle the spheroids carefully before printing to avoid clustering **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (4.1., 4.2.)

6.2. **Alisa Morss Clyne**: Following bioprinting and co-culture, tumor cell viability, proliferation, migration, and other biochemical interactions with the endothelial network can be measured with and without a pharmacological treatment **[1]**.

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

6.3. **Swathi Swaminathan**: We have demonstrated that drug testing can be initiated as early as 2 hours after co-culture bioprinting and have tested spheroid adhesion to endothelial networks with the anti-cancer drug Paclitaxel **[1]**.

6.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*