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Title: A Defined Hydrogel-Based Method for Generating Three-Dimensional Human Breast Organoids that Recapitulate Mammary Morphogenesis

Authors and Affiliations:

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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? **Yes**
- 3. Filming location:** Will the filming need to take place in multiple locations? **No**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 20

Number of Shots: 43

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Meloryn Seraj:** Our research focuses on human breast development and lactation, tissue organization, environmental exposures, and genetic factors leading to biological changes and cancer.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Meloryn Seraj:** Existing models inadequately capture human breast tissue architecture, developmental timing, and microenvironment; this protocol bridges 2D and animal models in a human system.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Meloryn Seraj:** This hydrogel-based organoid model allows researchers to measure mammary epithelial growth and morphology, with patient-specific tissue responses.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.4. **Meloryn Seraj:** Additional analyses that can be performed following this procedure include reporter transduction, barcoding, perturbation, transformation, and modification of ECM composition and stiffness.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

Human breast tissue samples were obtained under Institutional Review Board-approved protocols at Maine Medical Center and Tufts Medical Center. All samples were anonymized prior to transfer, and written informed consent was obtained from all participants

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Preparation of Single Cells from Cryopreserved Human Breast Tissue

Demonstrator: Meloryn_Seraj

- 2.1. Obtain digested human breast tissue cryopreserved for at least 24 hours [1]. Immediately submerge the cryovial up to the cap in a 37 degrees Celsius water bath and thaw rapidly for 1 minute to minimize cell death [2].
 - 2.1.1. WIDE: Talent taking out the cryopreserved tissue from minus 80 degrees Celsius storage. ***Videographer:** Please obtain CU shot as well for this shot.*
 - 2.1.2. Talent submerging the cryovial up to the cap in a water bath set to 37°C.
- 2.2. Once fully thawed, transfer the contents of the cryovial to a 15-milliliter conical tube containing 10 milliliters of mammary epithelial growth medium or MEGM (M-E-G-M) [1] and centrifuge at 250 g for 5 minutes [2].
 - 2.2.1. Talent transferring the contents of the cryovial into a 15-milliliter conical tube containing MEGM.
 - 2.2.2. Talent placing the conical tube into the centrifuge.
- 2.3. After discarding the supernatant, resuspend the pellet in 500 microliters of dissociation enzyme solution prewarmed to 37 degrees Celsius containing 0.25 percent trypsin [1]. Transfer the suspension to a 1.5-milliliter microcentrifuge tube [2]. Using a P1000 (P-thousand) pipette, triturate the sample 20 times to promote dissociation [3].
 - 2.3.1. Talent resuspending the pellet in the dissociation enzyme solution. **NOTE: Shots 2.3.1 and 2.3.2 were combined into single shot**
 - 2.3.2. Talent transferring the suspension to a 1.5 milliliter microcentrifuge tube.
 - 2.3.3. Talent triturating the sample with a P1000 pipette.
- 2.4. Incubate the tube at 37 degrees Celsius for 10 to 15 minutes [1]. Using a P1000 pipette, mechanically dissociate the sample by triturating 20 to 30 times [2].
 - 2.4.1. Talent placing the microcentrifuge tube into the incubator.
 - 2.4.2. Talent triturating the sample with a P1000 pipette.
- 2.5. Add 1 milliliter of serum-containing wash medium to neutralize the enzymatic reaction [1]. Centrifuge at 500 g for 5 minutes [2].
 - 2.5.1. Talent adding serum-containing wash medium to the suspension.
 - 2.5.2. Talent placing the tube into the centrifuge.

- 2.6. After discarding the supernatant, resuspend the pellet in 300 microliters of dispase solution [1]. Add 100 microliters of DNase solution [2] and incubate at 37 degrees Celsius for 10 to 15 minutes [3].
 - 2.6.1. Talent resuspending the pellet in dispase solution.
 - 2.6.2. Talent adding DNase solution to the suspension.
 - 2.6.3. Talent placing the tube into the incubator.
- 2.7. Mechanically dissociate the sample by pipetting 15 to 20 times [1]. Add 700 microliters of serum-containing wash medium to the suspension [2].
 - 2.7.1. Talent pipetting the sample repeatedly to mechanically dissociate it.
 - 2.7.2. Talent adding serum-containing wash medium to the suspension.
- 2.8. Filter the cell suspension through a 40-micrometer cell strainer into a clean tube [1].
 - 2.8.1. Talent filtering the cell suspension through a 40-micrometer cell strainer into a clean tube.
- 2.9. Mix the cell suspension with trypan blue in an equal ratio [1]. Load 10 microliters of the mixture into an automated cell counter [2] and determine the cell viability [3]. Centrifuge the suspension at 500 g for 5 minutes at room temperature [4].
 - 2.9.1. Talent mixing the cell suspension with trypan blue.
 - 2.9.2. Talent loading the mixture into an automated cell counter.
 - 2.9.3. SCREEN: Show cells within the field of view of the automated cell counter during cell viability analysis. NOTE: The small screen of the cell counter was filmed, it was displaying results (Footage file C3313)
 - 2.9.4. Talent placing the suspension into the centrifuge.
- 2.10. After discarding the supernatant, resuspend the cell pellet in MEGM at the desired concentration for hydrogel seeding [1].
 - 2.10.1. Talent resuspending the cell pellet in MEGM.

3. Preparation of Hydrogel Master Mix and Cell Suspension

- 3.1. Thaw the frozen laminin, hyaluronic acid, and fibronectin solutions and PBS slowly on ice at 0 to 4 degrees Celsius before use [1]. Prepare extracellular matrix or ECM (E-C-M) supplement stock solution [2]. Mix gently by inverting the tube 3 to 4 times [3]. Store the solution at 4 degrees Celsius for up to 1 month, avoiding repeated freeze-thaw cycles [4].
 - 3.1.1. Talent placing the frozen components on ice.

3.1.2. TEXT ON A PLAIN BACKGROUND:

25× ECM Supplement Stock Solution (1 mL)

Laminin: 425µL

HA: 250µL

FN: 250µL

1× PBS: 75 µL

Keep the tube on ice during preparation NOTE: We filmed the creation of the stock solution just in case. Bonus shot

3.1.3. Talent inverting the tube 3 to 4 times.

3.1.4. Talent placing the stock solution into storage at 4 degrees Celsius.

3.2. Place collagen I (*collagen-one*), ECM supplement, sodium hydroxide, and growth medium on ice at 0 to 4 degrees Celsius to prevent premature polymerization [1].

3.2.1. Talent placing collagen I, extracellular matrix supplement, sodium hydroxide, and growth medium on ice.

3.3. Add the calculated volume of collagen I to a chilled microcentrifuge tube [1], followed by the pre-chilled growth medium [2] and 0.1 normal sodium hydroxide to neutralize the solution [3].

3.3.1. Talent adding collagen I to a chilled microcentrifuge tube. NOTE: 3.3.1, 3.3.2, and 3.3.3 all got combined into a single shot as the process needs to happen back-to-back.

3.3.2. Talent adding pre-chilled growth medium to the collagen solution.

3.3.3. Talent adding 0.1 normal sodium hydroxide to the solution.

3.4. Mix the solution by inverting the tube vigorously at a rate of approximately one inversion per second for at least 10 inversions [1].

3.4.1. Talent vigorously inverting the tube a few times.

3.5. Add the calculated volume of ECM supplement to the mixture [1].

3.5.1. Talent adding the extracellular matrix supplement to the mixture.

3.6. Using a P200 pipette or wide-bore pipette tip, add the calculated volume of single cells or tissue fragments [1]. Mix immediately by inversion as previously demonstrated [2].

3.6.1. Talent adding single cells or tissue fragments to the mixture with a P1000 pipette or wide-bore pipette tip. .

3.6.2. Talent inverting the tube to mix the contents.

4. Hydrogel Deposition and Culture Establishment

- 4.1. Pipette the hydrogel mixture into culture vessels [1] at the desired volume per well [2]. Spread the hydrogel into a thin pad across the surface when using chamber slides [3]. Position the pipette tip at the upper center edge of the chamber well and drag it across the surface while dispensing the gel to create an even pad [4]. For 96-well plates, position the pipette tip at the center of the well, maintain contact with the surface, and dispense the gel centrally to maintain a dome structure [5].
 - 4.1.1. Shot of the hydrogel mixture being dispensed into culture vessels. NOTE: 4.1.1, 4.1.3 and 4.1.4 combined into same shot, filmed a few times. Close up for shot 4.1.3/4.1.4 was unslated in footage file C3341
 - 4.1.2. TEXT ON A PLAIN BACKGROUND:
Hydrogel Volume per Well:
4-well chamber slide: 200 μ L/well
8-well chamber slide: 100 μ L/well
96-well plate: 20 μ L/well
 - 4.1.3. Shot of the hydrogel being spread into a thin pad across the surface in a chamber slide.
 - 4.1.4. Close-up of the pipette tip positioned at the center upper edge of the chamber well and dragged across the surface while dispensing the hydrogel to create an even pad.
 - 4.1.5. Close-up of the pipette tip positioned at the center of the 96-well plate well, maintaining contact with the surface while dispensing the hydrogel centrally to create a dome structure. NOTE: An optimal angle can't be achieved to make the shot work exactly as written. 2 shots were filmed (wide and CU) of the hydrogel being dispensed into some wells of the 96-well plate
- 4.2. Incubate the culture vessels at 37 degrees Celsius in a humidified incubator with 5 percent carbon dioxide for 60 minutes to allow complete polymerization [1].
 - 4.2.1. Talent placing the culture vessels into the incubator.
- 4.3. Add prewarmed MEGM to each well and adjust the volume according to the culture format used [1].
 - 4.3.1. Talent adding prewarmed mammary epithelial growth medium to each well.
- 4.4. Using a pipette tip, gently detach the hydrogel from the culture surface to allow the gel to float [1]. Slide the pipette tip around the perimeter of the gel [2]. Gently lift the polymerized hydrogel from beneath to release it from the surface [3].
 - 4.4.1. Close-up of a pipette tip gently detaching the hydrogel from the culture surface to allow the gel to float. NOTE: 4.4.1 and 4.4.2 combined into single shot
 - 4.4.2. Close-up of the pipette tip sliding around the perimeter of the hydrogel.

- 4.4.3. Close-up of the pipette tip lifting beneath the polymerized hydrogel to release it from the culture surface.

Results

5. Results

- 5.1. Following culture establishment, breast organoids derived from single epithelial cells displayed progressive morphogenesis and formed branching structures by day 21 of culture [1].
 - 5.1.1. LAB MEDIA: Figure 2. *Video editor: Emphasize the organoid structure in the panel labelled 'Normal Breast'.*
- 5.2. Organoids derived from tumor fragments formed denser and more irregular structures with reduced organization and increased radial projections [1].
 - 5.2.1. LAB MEDIA: Figure 2. *Video editor: Emphasize the dense central structure and the numerous projections in the panel labelled 'Breast Tumor'.*
- 5.3. Salivary gland organoids formed compact structures at day 3 [1], whereas kidney organoids developed elongated and asymmetric morphologies by day 17 [2].
 - 5.3.1. LAB MEDIA: Figure 2. *Video editor: Emphasize the structure labelled 'Salivary gland'.*
 - 5.3.2. LAB MEDIA: Figure 2. *Video editor: Emphasize the elongated structure labelled 'Kidney'.*

1. Cryopreserved
Pronunciation link: <https://www.merriam-webster.com/dictionary/cryopreserve>
IPA: /ˌkraɪ.oʊ.priˈzɜːvd/
Phonetic Spelling: kry·oh·prih·zurvd
2. Cryovial
Pronunciation link: <https://www.merriam-webster.com/medical/cryovial>
IPA: /ˌkraɪ.oʊˌvaɪ.əl/
Phonetic Spelling: kry·oh·vye·uhl
3. Mammary
Pronunciation link: <https://www.merriam-webster.com/dictionary/mammary>
IPA: /ˈmæm.ə.ri/
Phonetic Spelling: mam·uh·ree
4. Epithelial
Pronunciation link: <https://www.merriam-webster.com/dictionary/epithelial>
IPA: /ˌep.əˈθiː.li.əl/
Phonetic Spelling: ep·uh·thee·lee·uhl
5. Dissociation
Pronunciation link: <https://www.merriam-webster.com/dictionary/dissociation>
IPA: /dɪˌsoʊ.siˈeɪ.ʃən/
Phonetic Spelling: dih·soh·see·ay·shuhn
6. Trypsin
Pronunciation link: <https://www.merriam-webster.com/dictionary/trypsin>
IPA: /ˈtrip.sɪn/
Phonetic Spelling: trip·sin
7. Triturate
Pronunciation link: <https://www.merriam-webster.com/dictionary/triturate>
IPA: /ˈtrɪtʃ.əˌreɪt/
Phonetic Spelling: trich·uh·rayt
8. Dispase
Pronunciation link: <https://www.howtopronounce.com/dispase>
IPA: /dɪˈspeɪs/
Phonetic Spelling: dih·spays
9. DNase
Pronunciation link: <https://www.howtopronounce.com/dnase>
IPA: /ˈdiː.ɛn.eɪs/
Phonetic Spelling: dee·en·ays
10. Trypan
Pronunciation link: <https://www.merriam-webster.com/medical/trypan%20blue>
IPA: /ˈtraɪ.pæn/
Phonetic Spelling: try·pan
11. Viability
Pronunciation link: <https://www.merriam-webster.com/dictionary/viability>
IPA: /ˌvaɪ.əˈbɪl.ə.ti/
Phonetic Spelling: vy·uh·bil·uh·tee

12. Hyaluronic
Pronunciation link: <https://www.merriam-webster.com/dictionary/hyaluronic>
IPA: /ˌhaɪ.ə.ləˈrɑː.nɪk/
Phonetic Spelling: hy·uh·luh·ron·ik
13. Fibronectin
Pronunciation link: <https://www.merriam-webster.com/medical/fibronectin>
IPA: /ˌfaɪ.brəˈnek.tɪn/
Phonetic Spelling: fy·bru·nek·tin
14. Polymerization
Pronunciation link: <https://www.merriam-webster.com/dictionary/polymerization>
IPA: /pəˌlɪm.əˈzeɪ.ʃən/
Phonetic Spelling: puh·lim·ur·uh·zay·shuhn
15. Hydrogel
Pronunciation link: <https://www.merriam-webster.com/medical/hydrogel>
IPA: /ˈhaɪ.droʊ.dʒel/
Phonetic Spelling: hy·droh·jel
16. Organoid
Pronunciation link: <https://www.merriam-webster.com/medical/organoid>
IPA: /ˈɔːr.gəˌnɔɪd/
Phonetic Spelling: or·guh·noyd
17. Morphogenesis
Pronunciation link: <https://www.merriam-webster.com/dictionary/morphogenesis>
IPA: /ˌmɔːr.foʊˈdʒen.ə.sɪs/
Phonetic Spelling: mor·foh·jen·uh·sis
18. Asymmetric
Pronunciation link: <https://www.merriam-webster.com/dictionary/asymmetric>
IPA: /ˌeɪ.səˈmet.rɪk/
Phonetic Spelling: ay·suh·met·rik
19. Salivary
Pronunciation link: <https://www.merriam-webster.com/dictionary/salivary>
IPA: /ˈsæl.əˌver.i/
Phonetic Spelling: sal·uh·vair·ee
20. Extracellular
Pronunciation link: <https://www.merriam-webster.com/dictionary/extracellular>
IPA: /ˌek.strəˈsel.jə.lə/
Phonetic Spelling: ek·struh·sel·yuh·ler