FINAL SCRIPT: APPROVED FOR FILMING

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Title: Production and Use of Customizable Agarose Molds for Scaffold-Free Mouse Ovarian Follicle Culture

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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? **Yes**

   If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit.

   If your microscope does not have a camera port, the scope kit will be attached to one of the eyepieces and **you will have to perform the procedure using one eye**.

   SCOPE: 3.4.2, 3.8.2, 3.8.4, 3.11.2, 3.11.4, 4.1.2, 4.2.3

   Videographer: Please use a SCOPE KIT for shots labeled SCOPE

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

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: 27
Number of Shots: 50

Introduction

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

1.1. **Hannes Campo:** Our research aims to develop a customizable, physiomimetic and scaffold-free follicle culture method to determine if it can improve the quality of *in vitro* grown follicles and the oocytes they develop.

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

What technologies are currently used to advance research in your field?

1.2. **Prianka Hashim:** Currently, the most advanced culture method uses a hydrogel-encapsulated in vitro follicle growth system, which allows follicles to maintain their three-dimensional architecture during folliculogenesis.

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

What are the current experimental challenges?

1.3. **Emily Zaniker:** Hydrogel encapsulation is technically challenging, laborious, low-throughput, and is unfortunately not compatible with automatic imaging methods. Additionally, 3D encapsulation provides uniform support which does not mimic in vivo physiology.

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

What significant findings have you established in your field?

1.4. **Prianka Hashim:** We created 3D-printed biocompatible molds that support oocytes, highly sensitive cells. Follicles grown in this scaffold-free environment showed improved growth and ovulation without compromising hormone production compared to established techniques.

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

What advantage does your protocol offer compared to other techniques?

1.5. **Emily Zaniker:** We're addressing the need for a user-friendly and customizable culture system that could better mimic the follicle's natural environment. Timelapse imaging also allows live tracking and analysis of follicle performance.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:5.4*

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

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at Northwestern University

Protocol

2. Custom CAD Design of 24-Well Silicone Micromolds for Follicle Culture Systems

Demonstrator: Hannes Campo

2.1. To begin, launch the CAD *(Cad)* software on a computer system **[1].** Open the **24-well master mold base design .step** *(dot-step)* file on the software **[2]**.

2.1.1. WIDE: Talent sitting at workstation and opening CAD software.

2.1.2. SCREEN: 2.1-2.2.mkv. 00:00-00:09

2.2. Select the internal surface area of the object **[1]**, then navigate to the **Solid** tab in the **Design** workspace and select **Create Sketch** to insert the desired micromold design **[2]**.

2.2.1. SCREEN: 2.1-2.2.mkv. 00:10-00:12

2.2.2. SCREEN: 2.1-2.2.mkv. 00:13-00:19

2.3. Now, introduce the desired x and y dimensions and number of the microwells **[1]**, then select **Finish Sketch** on the toolbar **[2]**.

2.3.1. SCREEN: 2.3B.mkv. 00:00-00:23

2.3.2. SCREEN: 2.3B.mkv. 00:27-00:30

2.4. Create the 800 micrometer deep microwells using the **Extrude** function by sequentially selecting **Solid , Create** and **Extrude [1]**. ~~Choose the **Cut** operation to form the microwell cavity **[2].**~~

2.4.1. SCREEN: 2.4.mkv. 00:00-00:12

~~2.4.2. SCREEN: 2.4.mkv.~~

2.5. Now, choose a 0.1 millimeter radius fillet for the top of the microwells by clicking on **Design, Solid, Modify** and **Fillet [1]**. Add a 0.25 millimeter radius fillet to create a round-bottom microwell **[2]**. Save the new master mold design and export a copy of in .step format by selecting **File** and pressing **Export [3]**.

2.5.1. SCREEN: 2.5.1.mkv. 00:00-00:05, 00:44-00:51

2.5.2. SCREEN: 2.5.2.mkv. 00:30-00:43

2.5.3. SCREEN: 2.5.3.mkv. 00:00-end

2.6. Open the **24-well silicone cast container 1 .step** file **[1]**, then insert the new master

mold design as an external component by right-clicking the saved file and selecting **Insert into Current Design** ~~choosing **Solid , Insert** and **Insert Component**~~ **[2]**.

2.6.1. SCREEN: 2.6.mkv. 00:02-00:14

2.6.2. SCREEN: 2.6.mkv. 00:15-00:24

2.7. Next, right-click on the inserted component and select **Break Link** to remove the reference to the master mold design **[1]**.

2.7.1. SCREEN: 2.7.mkv. 00:00-end

2.8. Center the **new master mold design** within the **24-well silicone cast container 1** surface, ensuring it faces inward **[1]**. Select **OK** to align the objects **[2]**.

2.8.1. SCREEN: 2.8.mkv. 00:00-00:26

2.8.2. SCREEN: 2.8.mkv. 00:27-00:30

2.9. Select the **Cut** operation, using the container as the target body and the master mold as the tool body to create the silicone molding cavity **[1]**.

2.9.1. SCREEN: 2.9.mkv 00:00-end

2.10. Save and export the new **24-well silicone mold 1** as .stl *(dot-S-T-L)* and .step files **[1]**.

2.10.1. SCREEN: 2.10.mkv. 00:00-end

3. Fabrication and Sterilization of Silicone Micromolds for Follicle Culture

Demonstrator: Hannes Campo

3.1. Open the .stl files of the new **24-well silicone mold 1** and **24-well silicone cast container 2** using 3D print preparation software **[1]**. Orient the print with micropillars facing upwards and use the **Drill Hole** function to create a 1-millimeter-wide opening to the side of the print **[2]**.

3.1.1. SCREEN: 3.1.1.mkv. 00:00-00:10

3.1.2. SCREEN: 3.1.2.mkv. 00:00-00:24

3.2. Print both container designs at a 25-micrometre layer thickness **[1]**.

3.2.1. SCREEN: 3.1.2.mkv. 00:15-00:23

3.3. Wash the finished prints according to the manufacturer's instructions **[1].** Thenspray the micropillar section extensively with 95% isopropanol **[2].** Remove any remaining ethanol with compressed air **[3]**.
Added shot: Taking out of the 3D printer

3.3.1. Talent placing print in isopropyl alcohol washer.
Videographer's Note: 3.3.1 and pre 3.3.1 slated wrong as 3.1.1

3.3.2. Talent spraying print with isopropyl alcohol.

3.3.3. Talent drying print with compressed air.

3.4. After drying and curing, inspect each print using a stereomicroscope **[1-TXT]**. Carefully ensure that all micropillars are separated and uniform in size and appearance **[2]**.

3.4.1. Talent placing print under stereomicroscope. **TXT: Discard any 3D prints with imperfections**

3.4.2. SCOPE: Clear view of uniformly separated micropillars under the stereomicroscope.
Videographer: Please capture all shots labeled SCOPE with a SCOPE kit

3.5. After curing,cover the outside of the print with parafilm if the drill channel is not filled and store until further use **[1]**.

3.5.1. Talent wrapping parafilm around the print and placing it in storage box.

3.6. Next, place the prepared silicone in a vacuum desiccator for 5 minutes to remove air bubbles **[1-TXT].** Pour the silicone mixture with a uniform flow into the 3D-printed mold, ensuring the level matches or is slightly below the mold surface for flatness **[2]**. Remove trapped air with a P200 *(P-Two-hundred)* pipette tip and degas again if needed **[3]**.

3.6.1. Talent placing silicone mixture into vacuum desiccator and running degassing cycle. **TXT: Repeat degassing upto 2x**

added shot: Showing bubbles escaping / mixture being degassed.

3.6.2. Talent pouring silicone into mold with steady stream.

3.6.3. Talent using pipette tip to remove bubbles and reinserting into desiccator.

3.7. Cure the silicone at room temperature in a desiccator for at least 5 hours or preferably overnight **[1]**.

3.7.1. Talent placing mold in desiccator and starting a 5 h timer.

3.8. After curing, remove the silicone from the mold **[1]** and inspect the micromolds under a stereomicroscope **[2]**. Remove any excess material from the drill channel **[3]** and discard molds with bridging or damaged wells **[4]**.

3.8.1. Talent demolding silicone carefully.

3.8.2. SCOPE: Shot of the micromolds being seen under a stereomicroscope.

3.8.3. Talent trimming excess material from drill channel.

3.8.4. SCOPE: Micromolds with bridging defects being identified and discarded.

3.9. Next, place the silicone mold into silicone mold container 2 for the second molding step, ensuring the side opening is aligned **[1]**. Spray the silicone mold lightly with embryo-safe mineral oil **[2].** Remove excess oils from the microwells using compressed air **[3]**.

3.9.1. Talent placing first mold into second container and verifying position.

3.9.2. Talent spraying the silicone mold with mineral oil.
Videographer's Note: 3.9.1-3.9.2 combined

3.9.3. Talent uses compressed air to remove oil from the microwells ~~blotting the microwells carefully~~.

3.10. Repeat the silicone mixing procedure and pour the new mixture into the second container, maintaining the level at or below the mold surface **[1-TXT]**.

3.10.1. Talent mixing and pouring silicone into the container holding mold 1. **TXT: Cure at RT for 5 h or overnight**

3.11. The next day, separate the silicone from the 3D printed mold and the silicone molds 1 and 2 from each other **[1].** After inspecting them microscopically, discard the molds with bubbles or excess oil in the wells **[2]**.

3.11.1. Talent gently separating two silicone molds.

3.11.2. SCOPE: Shot of the micromolds being seen under a stereomicroscope.

3.11.3. Talent separating out defective molds and discarding them.

3.11.4. SCOPE: Micromolds with bridging defects being identified and discarded.

3.12. Now, wash the silicone mold with 70 percent ethanol **[1].** Let it air dry for 30 minutes inside a laminar flow hood **[2]**.

3.12.1. Talent spraying mold with 70% ethanol.

3.12.2. Talent placing the sterilized molds on a sterile surface in hood.

3.13. Once dry, place the mold into a sterilization pouch before autoclaving **[1]**.

3.13.1. Talent sealing pouch and placing it in autoclave. **TXT: Jacket pressure: 20 psi, Chamber temperature: 250 °F, Sterilizing time: 15 min**

4. Scaffold-Free Follicle Culture and Timelapse Imaging Using Agarose Micromolds
Demonstrator: Prianka Hashim & Emily Zaniker-Gomez

4.1. ~~Seed 10 multilayer secondary follicles from mice ovaries into pre-equilibrated agarose molds submerged in growth medium **[1].**~~ Under a microscope, use a 200-micrometer stripper tip to transfer 10 multilayer secondary follicles per micromold, placing them in adjacent microwells **[2]**.

~~4.1.1. Talent preparing agarose mold setup.~~

4.1.2. SCOPE: Stripper tip removing follicles.
Videographer: Please capture SCOPE shots with a SCOPE kit

4.2. Bend the stripper tip slightly to enhance precision during follicle transfer **[1]**. Transfer the follicles quickly to prevent pH or temperature fluctuations **[2]**. Confirm that all 10 follicles are similarly sized before finalizing the setup **[3]**.

4.2.1. Talent bending stripper tip with sterile tweezers.

4.2.2. Talent working efficiently under microscope.
AUTHOR'S NOTE: Move 4.2.1-4.2.2 before 4.1.2

4.2.3. SCOPE: Stripper tip seeds follicles in microwells and end with final view of evenly spaced and sized follicles.
AUTHOR'S NOTE: Move after 4.1.2

4.3. Next, place a light focuser cap on a handheld microscope **[1]**. Insert the microscope with mount into incubator and connect to laptop with software **[2]**.

4.3.1. Talent placing light cap on microscope.

4.3.2. Talent positioning microscope and connecting laptop.

4.4. Position and align the culture well under microscope and adjust height and focus so all follicles are visible **[1]**. Use **Auto White Balance (AWB)** *(Auto-White-Balance-A-W-B)* and **LED** *(L-E-D)* **Control**, turn off **Auto Exposure (AE)** *(Auto-Exposure-A-E)*, and select optimal exposure time **[2-TXT]**.

4.4.1. Talent adjusting microscope focus.

4.4.2. SCREEN: 4.4.2.mkv. 00:00-00:13
TXT: Ensure mold alignment is the same before and after each media change

4.5. Start timelapse imaging with a **duration** of **8 days** and an interval of **30 min** *(minutes)*. Select **Photo** and turn off **LED** when not capturing images **[1]**.

4.5.1. SCREEN: 4.5.1.mkv. 00:00-00:20

Results

5. Results

5.1. Follicles cultured in agarose micromolds showed continuous growth and antral cavity formation **[1]**.

5.1.1. LAB MEDIA: Figure 6A. *Video editor: Sequentially show images for D0 to D8*

5.2. After induction of ovulation, follicles cultured in agarose micromolds yielded more ovulated eggs compared to those in alginate **[1]**. Further demonstrating biocompatibility, the spindle morphology of MII *(M-Two)* eggs did not differ significantly between groups **[2]**.

5.2.1. LAB MEDIA: Figure 6B. *Video editor: Highlight the post-hCG image in the agarose row*

5.2.2. LAB MEDIA: Figure 6C. *Video editor: Show the images side-by-side for both alginate and agarose conditions*

5.3. Alginate-encapsulated follicles did not maintain their position during culture and are incompatible with timelapse imaging **[1].**

5.3.1. LAB MEDIA: Figure 6F *Video Editor: Please sequentially highlight images labeled 0 h, 8 h, 16 h and 24 h*

5.4. During timelapse imaging, individual follicle morphology, including Feret's diameter, circularity, and aspect ratio could be tracked and measured instantly throughout the culture period **[2]**.

5.4.1. LAB MEDIA: Figure 6G *Video Editor: Please sequentially show i to iii*

5.5. Optical coherence tomography enabled 3D visualization of internal follicle structures **[1]**, and also captured dynamic follicle rupture events during ovulation **[2]**.

5.5.1. LAB MEDIA: Figure 7A. *Video editor: Highlight the two large round follicles imaged by OCT.*

5.5.2. LAB MEDIA: Figure 7B.

5.6. Agarose micromolds enabled histological analysis of multiple follicles in the same plane after paraffin embedding and sectioning **[1]**, allowing identification of tissue presence or absence **[2]** and supporting H&E staining **[3]**.

5.6.1. LAB MEDIA: Figure 7C(i-ii).

5.6.2. LAB MEDIA: Figure 7C(iii). *Video editor: Please show the white arrow when VO says "presence" and green arrow when VO says "absence".*

5.6.3. LAB MEDIA: Figure 7C(iv).

5.7. To demonstrate the customizability of this method, a high-throughput, 96-well version was created, featuring 10 microwells per mold **[1]**, and was successfully fabricated **[2]**.

5.7.1. LAB MEDIA: Figure 8A(i–iii). *Video editor: Sequentially show i to iii*

5.7.2. LAB MEDIA: Figure 8C and 8D. *Video editor: Sequentially show 8C and 8D*

5.8. As with the 24-well design, it was compatible with timelapse imaging of follicles **[1]**.

5.8.1. LAB MEDIA: Figure 8E

Pronunciation Guide:
Agarose
Pronunciation link: https://www.merriam-webster.com/dictionary/agarose
IPA: /əˈgɛroʊs/
Phonetic Spelling: uh-GAIR-ohs
Micromold
Pronunciation link: No confirmed link found
IPA: /ˈmaɪkroʊˌmoʊld/
Phonetic Spelling: MY-kroh-mohld
Scaffold-Free
Pronunciation link (scaffold): https://www.merriam-webster.com/dictionary/scaffold
IPA: /ˈskæf.əld/ → so "scaffold-free" = /ˈskæf.əld-friː/
Phonetic Spelling: SKAF-uhld-free
Folliculogenesis
Pronunciation link: https://www.howtopronounce.com/folliculogenesis
IPA: /ˌfɒlɪˌkjʊləˈdʒɛnəsɪs/
Phonetic Spelling: fol-ih-kyuh-LOH-jen-uhsis
Timelapse
Pronunciation link: https://www.merriam-webster.com/dictionary/time-lapse
IPA: /ˈtaɪmˌlæps/
Phonetic Spelling: TIME-laps
Microwell
Pronunciation link: No confirmed link found
IPA: /ˈmaɪkroʊˌwɛl/
Phonetic Spelling: MY-kroh-wel
Autoclaving
Pronunciation link: https://www.merriam-webster.com/dictionary/autoclave
IPA: /ˈɔːtoʊˌkleɪv/ → so "autoclaving" = /ˈɔːtoʊˌkleɪvɪŋ/
Phonetic Spelling: AW-toh-klay-ving
Cumulus-oocyte
Pronunciation link (cumulus): https://www.merriam-webster.com/dictionary/cumulus
Pronunciation link (oocyte): https://www.merriam-webster.com/dictionary/oocyte
IPA: /ˈkjuːmjʊləs-ˈoʊəsaɪt/
Phonetic Spelling: KYOO-myuh-lus-OH-uh-sait
Fillet (as in fillet radius)
Pronunciation link: https://www.merriam-webster.com/dictionary/fillet
IPA: /ˈfɪlɪt/
Phonetic Spelling: FIL-it
Extrude
Pronunciation link: https://www.merriam-webster.com/dictionary/extrude
IPA: /ɪkˈstruːd/
Phonetic Spelling: ik-STROOD

Polyurethane
Pronunciation link: https://www.merriam-webster.com/dictionary/polyurethane
IPA: /ˌpɒliʊˈrɛθən/
Phonetic Spelling: POL-ee-yuh-RETH-uhn
Micropipette
Pronunciation link: https://www.merriam-webster.com/dictionary/micropipette
IPA: /ˌmaɪkroʊpɪˈpɛt/
Phonetic Spelling: MY-kroh-pi-PET