Submission ID #: 67786
Scriptwriter Name: Poornima G
Project Page Link: https://review.jove.com/account/file-uploader?src=20674778

Title: Application of a Novel Hyaluronan Hydrogel for Three-Dimensional Follicle Culture and Methodology for Mouse Ovarian Follicle Cryopreservation

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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 **Yes**, can you record movies/images using your own microscope camera?

   No

   Scope SHOTS: 2.3.2, 2.3.3, 2.4.1, 2.4.2, 3.1.1, 3.2.2, 3.2.3, 4.1.4, 4.1.5, 4.2.1, 4.2.2, 4.5.2, 4.5.3, 5.4.2, 5.5.1, 5.5.2, 5.7.2, 5.7.3

Videographer: Please film the above-mentioned shots using the scope kit

Videographer's NOTE: The author did not want to shoot the microscope footage with my iPhone, so instead, per her request, she just screened the scope through her HDMI to a TV screen and I just filmed that. As you can see the TV was crooked, I filmed it with entire TV in frame so that you could adjust/crop with all the detail

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

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

Current Protocol Length
Number of Steps: 20
Number of Shots: 55

Introduction

Videographer: Obtain headshots for all authors available at the filming location.
Videographer's NOTE: You can ignore everything under the Audio Roll on the slate for all shots/takes.

Videographer's NOTE: The headshots are the best I can do. When I got there, most of the team had to leave very quick, so I only had 10 minutes to setup and shoot all their headshots, so not much time to make it look good. Hopefully its okay. The authors also said they wanted to do headshots in the lab itself, which I told them wouldn't look great, but they insisted

Videographer's NOTE: Did the best I could with the interview, the author was extremely camera shy. She would usually do 1 or 2 takes and then just say that she wanted to move on. She also insisted on doing the interview after the protocol, which ended up being at night, so we had no natural light to use and the overhead lights were flickering, so didn't have much light - though i think it still turned out okay

1.1. **Nina Desai:** Our research has been focused on developing a 3-D culture system for in vitro follicle growth and oocyte maturation. We believe that creating such a system using components of a cell's native extracellular matrix will enhance follicle development.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.4.1*

What are the current experimental challenges?

1.2. **Nina Desai:** The biggest challenge is applying the knowledge from animal models to human follicle culture. Access to human ovarian tissue is limited, and human follicles require significantly longer time in culture to achieve oocyte maturation.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 5.2.1*

How will your findings advance research in your field?

1.3. **Nina Desai:** Our 3-D culture model may be especially useful for culturing human ovarian follicles, allowing retention of 3-D architecture during the prolonged time needed in culture for oocyte maturation.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.3.2*

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 Cleveland Clinic

Protocol

2. Follicle and FL-Cluster (FL-C) Isolation

Demonstrators: Maribeth Spangler, Meghan Wirtjes, Arsela Gishto

2.1. To begin, obtain ovaries from 10 to 14-day old B6D2F1 mouse pups for the procedure **[1]**. Pipette 6 milliliters of pre-equilibrated follicle culture medium or FCM into two 60-millimeter dishes **[2]** and overlay with mineral oil **[3]** and incubate **[4].**

2.1.1. WIDE: Talent placing the dish with ovaries on the work bench. **Videographer's NOTE:** 2.1.1 = Got multiple angles of same shot

2.1.2. Talent pipetting FCM into two 60-millimeter dishes.

2.1.3. Shot of overlaying the medium with mineral oil.

Added shot 2.1.4. Close-up of the ovaries

2.2. Pipette 1 milliliter of collagenase into the center well of a dish and 3 milliliters of ovarian tissue or OT medium into the outer well **[1]**. Move the ovaries to the collagenase solution **[2-TXT]**.

2.2.1. Talent pipetting collagenase into the center well and OT medium into the outer well.

2.2.2. Talent transferring the ovaries to the collagenase solution. **TXT: Incubation: Heated surface 37 °C ; Laminar flow hood; 30 - 45 min**

2.3. At the end of the collagenase incubation, move the collagenase-treated ovaries to the outer well to rinse **[1]**. Change micropipettes and transfer the ovaries to a fresh dish filled with OT medium **[2]**. To harvest the follicles, repeatedly aspirate and expel enzyme-treated ovarian tissue through pipette tips cut to different sizes **[3]**. Mechanically tease tissue using 27-Gauge needles to aid release of follicles **[4]**.

2.3.1. Talent moving the treated ovaries to the outer well for rinsing.

2.3.2. SCOPE: transferring the ovaries to a fresh dish filled with OT medium.

2.3.3. SCOPE: aspirating and expelling ovaries to release follicles. **Videographer's NOTE:** 2.3.2 and 2.3.3 were combined into 1 shot. The first take of that shot had no microscope-shots and was slated on a piece of paper. The 2nd take of this ALSO adds shot 2.3.4

2.3.4. SCOPE: Shot of mechanically teasing tissue using 2 needles. **NOTE:** Look for the needles to locate this shot

2.4. Examine under a dissecting microscope at 40x magnification **[1]** and pick secondary pre-antral follicles with a centrally located oocyte enclosed within an intact basement

membrane to a fresh OT Dish **[2-TXT]**. Place the FCM2 dish in the incubator for 60 minutes before starting the embedding process **[3]**.

2.4.1. SCOPE: examining follicles under a dissecting microscope. **Author's NOTE**: Need to select best portion of clip where there are the most released follicles, and follicle morphology most easily seen. (and no naked eggs) We took lots of footage . Last bits probably had what we wanted to show. **Please show the footage only there are a lot of follicles**.
Videographer's NOTE: 2.4.1 and 2.4.2 combined to 1 shot

2.4.2. SCOPE: transferring the follicles to dish with FCM**. TXT: Rinse the selected follicles in FCM dish 1 and then transfer to FCM2** **NOTE**: An extra shot 2.4.4 was shot, but it is not required here.

2.4.3. Talent placing the dish inside the incubator.

3. Embedding Follicles and Follicle Clusters

3.1. Move follicles or follicle clusters to be embedded from the FCM dish to a drop of the hyaluronan or HA gel to rinse free of culture medium **[1-TXT]**. Place the FCM dish back under the bubbler to gas **[2]**.

3.1.1. SCOPE: transferring follicles or follicle clusters to a drop of HA gel on lid of 60 mm dish **Videographer's NOTE:** 3.1.1 = just a note, we did 2 attempts of this action in 1 take
NOTE: Extra shot was filmed. 3.0.1 = showing the tubes before talent paces the FCM dish back under the bubbler, but not required to put here

3.1.2. Talent placing the FCM dish under the bubbler. **TXT: Heated surface 37 °C and gassing with 5% CO_2, 5% O_2**

3.2. For embedding follicles **[1]**, place 1 microliter of 0.03% hydrogen peroxide drop onto lid of 60 millimeter dish. Now add 25 microliters of 3 milligrams per milliliter HA gel and mix **[2]**. Pipette approximately 8 to 10 microliters of the HA-hydrogen peroxide mixture into two separate wells of the 8-well culture dish **[3]** and quickly transfer the follicles, follicle clusters or ovarian tissue fragments into the center of each drop **[4].** Ensure there are no bubbles within the drop **[5][6]** **NOTE:** VO added for the extra shots
Added shot: Set up shot with Talent holding labelled tubes of peroxide and HA gel.

3.2.1. Talent adding 1 microliter of 0.03% hydrogen peroxide drop onto 60 mm Petri dish lid and then adding 25 ul of HA gel to this drop. Mix **Videographer's NOTE:** 3.2.1 = do NOT use take 1, per author, actions were modified

3.2.2. SCOPE: pipetting HA-hydrogen peroxide mixture into two wells.
Videographer's NOTE: 3.2.2 and 3.2.3 were combined to 1 shot, do NOT use take 1, per author

3.2.3. SCOPE: transferring individual follicles into HA drops. **TXT: HA: 3 mg/mL in global medium; 37 °C; 60 mm dish with eight 100 µL wells**

Added shot 2.3.4: SCOPE: Image of follicle cluster in HA drop *Video Editor: Use a split screen to show both the added 2.3.4 shots (images) simultaneously*

Added shot 2.3.4: Image of large ovarian tissue fragment in HA drop

3.3. After 3 minutes, add 100 microliters of pre-equilibrated follicle culture medium to each well **[1]**. Overlay with warm pre-equilibrated mineral oil and incubate the dish **[2]**.

3.3.1. Talent adding follicle culture medium to each well. **Videographer's NOTE:** 3.3.1 and 3.3.2 were combined into 1 shot

3.3.2. Talent overlaying with mineral oil on the well. **NOTE**: This may have been slated as 3.3.3

4. Vitrification of Follicles and Follicle Clusters

Demonstrator: Alyssa Brown

4.1. Prepare the equipment and the solutions required for the vitrification process **[1]**. Position the RI carrier into the slot in the cryobox containing liquid nitrogen **[2-TXT].** Place two drops of prewarmed vitrification solution 1 or VS1 side by side on the lid of a 60-millimeter dish near the top **[3]**. Now, place two follicles in the first drop, rinse, **[4-TXT]** and quickly move them to the second drop of VS1 for a 5-minute incubation **[5]**. Then, quickly move the follicles sequentially through the three VS2 drops within 60 seconds **[6]**.

Added shot: 4.1.0 showing gear, equipment and solutions for the vitrification process

4.1.1. Talent placing RI carrier into slot in cryobox. **TXT: Fill the insulated cryo box with liquid nitrogen** **Videographer's NOTE:** For the 4 shots in the script between 4.1.2 and 4.1.5, the action in the script is labeled differently on the slate, just adding 1 number to the final number in the shot. So, 4.1.2 in the script is labeled 4.1.3 on the slate, 4.1.3 in the script is labeled 4.1.4 on the slate. 4.1.4 in the script is labeled 4.1.5 on the slate. 4.1.5 in the script is labeled 4.1.6 on the slate.

4.1.2. Talent placing two VS1 drops on a Petri dish.

4.1.3. Talent transferring follicles to the first VS1 drop

4.1.4. SCOPE: follicles being transferred to the second VS1 drop.**TXT: Incubation: 5 min**

Added shot 4.1.6 SCOPE: transferring follicles through three VS2 drops.

Videographer's NOTE: For 4.1.6, do NOT use takes 1 or 2, per author, use take 3, which had a tail-slate

4.2. For vitrification, pick up the follicles from VS2. View the RI carrier under the dissecting scope and adjust the focus to visualize the tiny hole in the plastic stick **[1]**. Deposit follicles with minimal fluid into the hole **[2]**. Drop the plastic stick into the pre-cooled outer straw **[3]** and use an ultrasonic sealer to close the straw **[4]**. Place the straw in a goblet attached to the cryocane **[5]**, cover the cane with a plastic protective sleeve and plunge it into liquid nitrogen **[6]**.

4.2.1. SCOPE: Close-up of the carrier with tiny hole

4.2.2. SCOPE: Talent placing follicles into the tiny hole of the plastic stick.

4.2.3. Talent dropping the stick into the pre-cooled outer straw.

4.2.4. Close-up of the ultrasonic sealer sealing the straw. **Videographer's NOTE:** 4.2.4 - punched in for a close up at the end of the shot

4.2.5. Talent placing the sealed straw into the goblet.

4.2.6. Talent covering the cane with a plastic sleeve and placing it in liquid nitrogen.

4.3. For open carrier vitrification, prepare the carrier by inserting the metal CL stem into the magnetized vial cap, ensuring a firm hold **[1]**.

4.3.1. Close-up of CL stem being inserted into a magnetized vial cap. **Author's NOTE**: Show still setup shot of vial and cap for a fraction of a second and then the action in the video

4.4. Place a rack into the cryo box to hold cryovials, ensuring liquid nitrogen is below the top of the vials **1]**. Fill a magnetized and vented cryo vial with liquid nitrogen and place in the rack **[2]**.

4.4.1. Talent placing a rack into the cryo box.

4.4.2. Close-up of the magnetized vial filled with liquid nitrogen.

4.5. Grasp the CL open carrier by its attached magnetic cap using a magnetic wand **[1]**. Dip the CL carrier in a separate drop of VS2 to create a cryoprotectant film **[2]**. Pick up all follicles or follicle clusters and place them on the film with minimal fluid **[3]**.

4.5.1. Talent holding the CL open carrier with a magnetic wand.

4.5.2. SCOPE: CL carrier being dipped in VS2. **Videographer's NOTE:** 4.5.2 and 4.5.3 were combined to 1 shot

4.5.3. SCOPE: placing follicles onto the cryoprotectant film.

4.6. Immediately immerse the CL into the cryovial filled with liquid nitrogen to vitrify the sample **[1]**. Place the cryovial on the cryocane and then cover with a plastic sleeve **[2]**. Plunge the cane into a liquid nitrogen storage tank **[3]**.

4.6.1. Talent immersing the CL into the cryovial.

4.6.2. Talent placing the cryovial onto the cryocane and covering with plastic sleeve.

4.6.3. Talent plunging the cane into the storage tank.

5. Warming of Vitrified Follicles and Follicle Clusters

5.1. Prepare a center well dish with 3 milliliters of pre-equilibrated FCM in the outer well and 1 milliliter in the center well **[1]**. After overlaying the medium with oil, place the dish under the bubbler to gas **[2]**.
   5.1.1. Talent adding FCM to the dish. **Videographer's NOTE:** 5.1.1 and 5.1.2 were combined to 1 shot
   5.1.2. Talent placing the dish under the bubbler

5.2. Place 0.5 milliliters of warming solutions WS1 and WS2 pre-equilibrated to 37 degrees Celsius into two labeled center well dishes **[1-TXT]**. Move the cryo canes with samples from the storage tank into a cryo box filled with liquid nitrogen **[2]**.

NOTE: There might be an added shot here 5.0.2 to show the dishes. Do not include it

   5.2.1. Talent pipetting WS1 and WS2 into labeled center well dishes. **Videographer's NOTE:** 5.2.1 - do NOT use take 1, per author
   5.2.2. Talent transferring cryo canes from storage into the cryo box.

5.3. First, remove the plastic cane cover **[1]**. Then, keeping the straw immersed, remove it from the goblet and slide it into the holding slot in the cryo box **[2]**. Cut the outer straw just above the black dot **[3]**.
   5.3.1. Talent removing the plastic cane cover.
   5.3.2. Talent sliding the immersed straw into the holding slot. **Videographer's NOTE:** 5.3.1 and 5.3.2 were combined to 1 shot
   5.3.3. Close-up of the outer straw being cut above the black dot.

5.4. Lift the inner plastic stick out of the outer straw **[1]** and quickly immerse the stick into WS1, gently swirling to unload the follicles within 10 seconds **[2-TXT]**.
   5.4.1. Talent lifting the inner plastic stick from the straw.
   5.4.2. SCOPE: immersing the stick in WS1 while swirling. **TXT: Confirm complete follicle unloading under the dissecting microscope**

5.5. After 2 minutes in WS1, use a micropipette to transfer follicles or follicle clusters to WS2 for a 3-minute incubation **[1-TXT]**. Then, transfer them to FCM dish and incubate the follicles for 1 to 2 hours in the center well before embedding **[2]**.
   5.5.1. SCOPE: transferring follicles to WS2 using a micropipette. **TXT: Avoid medium tracking**
   5.5.2. SCOPE: transferring follicles to FCM. **TXT: Rinse follicles in the outer well of the FCM dish before transferring to the central well**

5.6. For open carrier CL warming, repeat the FCM and WS dish preparation **[1]**. Transfer the cryo cane into a cryo box and remove the plastic cane cover **[2]**. Use a magnetic wand to lift the cryovial cap until the metal stem of the CL is visible **[3]**.
   5.6.1. Talent placing WS dishes on the work surface and FCM dish under bubbler.
   5.6.2. Talent transferring the cryo cane and removing the plastic cover.

5.6.3. Close-up of the magnetic wand lifting the cryovial cap.

5.7. Grasp the cap with attached CL carrier using the magnetic wand **[1]** and quickly immerse the CL into WS1, swirling to unload follicles within 10 seconds **[2, 3].**

5.7.1. Talent grasping the metal stem with forceps.

5.7.2. SCOPE: immersing the CL into WS1. **Videographer's NOTE:** 5.7.2 - do NOT use take 1, per author

5.7.3. SCOPE: Close up of follicles *Video editor: Use split screen to show both 5.7.2 and 5.7.3*

Results

6. Results

6.1. Preantral follicle growth was maintained in the three-dimensional hyaluronan gel culture, preserving native follicle architecture, with an antrum visible by day 12 **[1]**.
6.1.1. LAB MEDIA: Figure 6. *Video editor: Highlight DAY 12 images*.

6.2. Encapsulated follicles in hyaluronan gel exhibited radial expansion due to granulosa cell proliferation **[1]**, with follicle diameters increasing from around 139.8 micrometers to 385.6 micrometers over the culture period **[2]**.
6.2.1. LAB MEDIA: Figure 7. *Video editor: sequentially highlight each row*
6.2.2. LAB MEDIA: Figure 7. *Video editor: highlight image I*.

6.3. Ovulated metaphase II *(2)* oocytes were found near the follicle after human chorionic gonadotropin trigger **[1]** oocytes measured approximately 84.8 micrometers in diameter **[2]**
6.3.1. LAB MEDIA: Figure 8. *Video editor: highlight A*,B
6.3.2. LAB MEDIA: Figure 8. *Video editor: highlight C,D*.

6.4. With fresh ovaries, both individual follicles and follicle clusters exhibited good maturation rates **[1]**. Follicle clusters from cryopreserved ovaries exhibited lower maturation rates, leading to premature oocyte extrusion **[2]**.
6.4.1. LAB MEDIA: Table 1 *Video editor: Mark the row " MII oocyte formation (%0 for Fresh Ovaries column*
6.4.2. LAB MEDIA: Table 1. *Video editor: Mark the row " MII oocyte formation (%" in " Frozen Ovary" column*.

6.5. Isolated follicle cryopreservation was more effective than whole ovary preservation. Follicle vitrification on both carriers achieved high maturation rates, regardless of cooling rate differences **[1]**. The CL open carrier system allowed for efficient cryopreservation of multiple follicles, reducing processing time **[2]**.
6.5.1. LAB MEDIA: Table 2. *Video editor: Mark the " RI Closed" column*.
6.5.2. LAB MEDIA: Table 2. *Video editor: Mark the " CL Open" column*.

1. pipette
Pronunciation link:
https://www.merriam-webster.com/dictionary/pipette YouTube+4YouTube+4YouTube+4Merriam-Webster+13Merriam-Webster+13Oxford English Dictionary+13
IPA: /paɪˈpɛt/
Phonetic Spelling: pie-PET

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2. collagenase
Pronunciation link:
https://www.merriam-webster.com/dictionary/collagenase Wikipedia+8Cambridge Dictionary+8Merriam-Webster+8Collins Dictionary+13Merriam-Webster+13Oxford English Dictionary+13
IPA: /kəˈlædʒəˌneɪz/
Phonetic Spelling: kuh-LAJ-uh-naze

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3. hyaluronan (also called hyaluronic acid)
Pronunciation link:
https://www.merriam-webster.com/dictionary/hyaluronate Merriam-Webster+2YouTube+2TheFreeDictionary.com+2Oxford English Dictionary+15Merriam-Webster+15Merriam-Webster+15
IPA: /ˌhaɪəˈlʊrənæn/
Phonetic Spelling: hi-uh-LOOR-uh-nan

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4. vitrification
Pronunciation link:
https://www.merriam-webster.com/dictionary/vitrifaction How To PronounceCambridge Dictionary+5Merriam-Webster+5Wikipedia+5
IPA: /ˌvɪtrəfəˈkeɪʃən/
Phonetic Spelling: vit-ruh-fuh-KAY-shun

1. granulosa
Pronunciation link (YouTube audio): Cambridge Dictionary+15YouTube+15YouTube+15
IPA: /ˌgrænjʊˈloʊsə/
Phonetic Spelling: gran-yoo-LOH-suh

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2. oocyte
Pronunciation link (Merriam-Webster & Cambridge): YouTube+8How To Say Guide+8YouTube+8OpenMD+7Merriam-Webster+7Merriam-Webster+7
IPA: /ˈoʊ.oʊ.saɪt/
Phonetic Spelling: OH-oh-sight

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3. antrum
Pronunciation link (Merriam-Webster & Cambridge): OpenMD+5Cambridge Dictionary+5Cambridge Dictionary+5science101.bizMerriam-Webster+5Merriam-Webster+5Merriam-Webster+5
IPA: /ˈæn.trəm/
Phonetic Spelling: AN-trum

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4. metaphase
Pronunciation link (Merriam-Webster & Cambridge): Word Pronouncer App - How do You Spell+12Cambridge Dictionary+12Cambridge Dictionary+12Merriam-Webster+7YouTube+7How To Pronounce+7
IPA: /ˈmɛt̬.ə.feɪz/
Phonetic Spelling: MET-uh-fayz

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5. cryocane
(No confirmed pronunciation link found)
IPA (constructed): /ˌkraɪ.oʊˈkeɪn/
Phonetic Spelling: kry-oh-KAYN

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6. follicle
Pronunciation link (Merriam-Webster): https://www.merriam-webster.com/dictionary/follicle
IPA: /ˈfɒlɪkəl/ or /ˈfɑləkəl/
Phonetic Spelling: FOL-i-kul

1. laminar

Pronunciation link:

- Merriam-Webster: https://www.merriam-webster.com/dictionary/laminar audioenglish.org+10dictionary.cambridge.org+10youtube.com+10en.wikipedia.org+14merriam-webster.com+14merriam-webster.com+14
  IPA (American): /ˈlæm.ə.nɚ/
  Phonetic Spelling: LAM-uh-ner

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2. cryoprotectant

Pronunciation link:

- Merriam-Webster: https://www.merriam-webster.com/dictionary/cryoprotectant howtopronounce.com+13merriam-webster.com+13howtopronounce.com+13
  IPA (American): /ˌkraɪ.oʊ.prəˈtɛk.tənt/
  Phonetic Spelling: kry-oh-pruh-TEK-tuhnt