FINAL SCRIPT: APPROVED FOR FILMING

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Title: Collection of Human Follicular Fluid, Follicle Somatic Cells, and Immature Oocytes from Individuals Undergoing In Vitro Fertilization

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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. **Filming location:** Will the filming need to take place in multiple locations? **Yes, 2 rooms next to each other**

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: 16
Number of Shots: 42

Introduction

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

INTRODUCTION:

1.1. **Elnur Babayev:** We developed reliable methods to collect and analyze IVF byproducts to study human fertility, infertility, and reproductive aging.

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

1.2. **Dilan Gokyer:** Maintaining RNA integrity and cell viability is a challenge that requires rapid, coordinated sample handling between clinical and research teams.

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

CONCLUSION:

1.3. **Dilan Gokyer:** Our protocol yields high-quality RNA, viable granulosa cells, and single-cell datasets revealing molecular underpinnings of human reproductive function.

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

1.4. **Elnur Babayev:** We aim to identify ovarian cell types with age-related gene expression changes to uncover mechanisms of reproductive aging and fertility decline.

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

1.5. **Elnur Babayev:** Integrating multi-omics and longitudinal studies will map dynamic follicular changes across age, environment, and disease states.

1.5.1. 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
This research has been approved by the Institutional Review Board (IRB) at the Northwestern University

Protocol

2. Cryopreservation of Human Follicular Fluid Cell Pellets

Demonstrator: Dilan Gokyer

2.1. To begin, obtain 50 milliliter conical tubes containing follicular fluid **[1].** Centrifuge the tubes at 400 *g* for 10 minutes at 4 degrees Celsius **[2]**. Discard the supernatant from each tube, leaving behind the cell pellet **[3]**.

2.1.1. WIDE: Talent holding 50 milliliter conical tubes with follicular fluid.

2.1.2. Talent placing the 50 milliliter conical tubes with follicular fluid into the centrifuge.

2.1.3. Talent decanting or aspirating the supernatant from each conical tube, showing the pellet remaining at the bottom.

2.2. Add 4 to 5 milliliters of DMEM/F12 *(D-M-E-M-F-Twelve)* to each cell pellet **[1]** and resuspend the pellet by gentle pipetting **[2]**.

2.2.1. Talent adding DMEM/F12 medium to each tube.

Videographer's NOTE: 2.2.1 - 2.2.2 Combined

2.2.2. Talent pipetting gently to resuspend the cell pellet.

2.3. Now, add 100 microliters of dimethyl sulfoxide and 100 microliters of fetal bovine serum into each tube **[1]**. Aliquot 800 microliters of the resuspended cell suspension into cryogenic vials **[2]**, then gently pipette up and down to mix the contents before sealing the cryovials **[3]**. **NOTE:** VO is inverted for the inverted shots

2.3.2 Talent adding 100 microliters of dimethyl sulfoxide and 100 microliters of fetal bovine serum to each vial. **Videographer's NOTE:** 2.3.1 - 2.3.2 need to be swapped

2.3.1 Talent dispensing 800 microliters of the cell suspension into cryogenic vials.

2.3.3 Talent mixing the contents by gentle pipetting and sealing the cryovials.

2.4. Transfer the sealed vials into a freezing container filled with 100 percent isopropyl alcohol **[1].** Place it in a minus 80 degrees Celsius freezer for 24 hours to allow controlled cooling at approximately minus 1 degree Celsius per minute **[2]**. The next day, move the cryovials to a liquid nitrogen tank for long-term storage **[3]**.

2.4.1. Talent placing the cryovials into the freezing container.

2.4.2. Talent loading the cryovials into a minus 80 degrees Celsius freezer.

2.4.3. Talent transferring cryovials from the freezer into a liquid nitrogen tank using

appropriate protective equipment.

3. Thawing, Isolation, and Culture of Human Primary Granulosa Cells from Cryopreserved Follicular Fluid

3.1. Thaw fetal bovine serum, L-glutamine, DMEM-F12 and penicillin-streptomycin in a 37 degrees Celsius bead or water bath **[1]**.

3.1.1. Talent placing vials of fetal bovine serum, L-glutamine, and penicillin-streptomycin into a 37 degrees Celsius bead bath. **Videographer's NOTE:** 3.1.1 Need to also add DMEM / F12

3.2. In a 15-milliliter conical tube, prepare 4 milliliters of thaw media and 8 milliliters of resuspension media **[1]**. In a 50-milliliter conical tube, prepare up to 50 milliliters of culture media **[2]** and place it in a 37 degrees Celsius bead bath **[3]**.

3.2.1. Shot of labeled 15 mL vials with 4 mL Thaw media and 8 mL resuspension media.

3.2.2. Talent preparing culture media in a 50 milliliter conical tube. **Videographer's NOTE:** 3.2.2 We have a pickup shot when when mixing the second tube

3.2.3. Talent placing the culture media into a bead bath.

3.3. Next, thaw the frozen cryovials in a 37 degrees Celsius bead bath for approximately 1 minute or until the pellet begins to loosen **[1]**. Using a P1000 *(P-One-Thousand)* pipette, transfer the entire contents of each cryovial into thaw media and gently resuspend **[2]**.

3.3.1. Talent retrieving cryovials from liquid nitrogen and placing them in the bead bath. **Videographer's NOTE:** 3.3.1 is in two parts

3.3.2. Talent transferring cryovial contents to thaw media and gently pipetting to mix.

3.4. Centrifuge the thawed cell suspension in thaw media at 400 *g* for 10 minutes at 4 degrees Celsius **[1]**. Then aspirate the supernatant, leaving only the cell pellet in the 15-milliliter conical tube **[2]**. Now add 4 milliliters of resuspension media to the pellet and vigorously pipette to ensure complete resuspension without clumps **[3]**.

3.4.1. Talent loading the tubes into the centrifuge and starting the spin.

3.4.2. Talent carefully aspirating the supernatant, leaving the pellet at the bottom.

3.4.3. Talent adding resuspension media and pipetting vigorously.

3.5. Next, gently pipette 1 part density gradient medium at the bottom of a tube containing the cell suspension, releasing slowly to form a clear layer **[1-TXT]**. Then, add DPBS gently to maintain layering **[2]**. Centrifuge the tube at 850 *g* for 10 minutes at 4 degrees Celsius to activate the density gradient medium **[3]**.

3.5.1. Talent adding density gradient medium into a tube containing 4 mL suspension. **TXT: Use 4 mL cell suspension**

3.5.2. Talent gently adding Dulbecco's phosphate-buffered saline to the tube without disturbing the layers.

3.5.3. Talent placing the prepared tube into the centrifuge and initiating the spin.

3.6. After centrifugation, identify the top layer as the granulosa cell layer and the bottom layer as primarily red blood cells **[1]**. Using a P1000 pipette, gently remove the top layer of cells **[2]** and resuspend them in the remaining 4 milliliters of resuspension media **[3]**.

3.6.1. Shot of the separated layers post centrifugation.

3.6.2. Talent carefully removing the top granulosa cell layer with a P1000 pipette.

3.6.3. Talent resuspending the granulosa cells in resuspension media.

3.7. Now, combine 10 microliters of the resuspended cells with 10 microliters of Trypan Blue solution in a 0.5 microliter microcentrifuge tube **[1]**. Pipette 10 microliters of this mixture onto a hemocytometer for cell counting **[2-TXT]**. Then centrifuge the cell suspension at 380 *g* for 5 minutes at room temperature **[3]**.

3.7.1. Talent mixing cell suspension and Trypan Blue solution in a microcentrifuge tube.

3.7.2. Talent loading the hemocytometer with the stained sample. **TXT: Count cells to determine total cell numbers and calculate required media volume**

3.7.3. Talent placing the tube with cell suspension into the centrifuge.

3.8. Aspirate the supernatant from the centrifuged cell suspension, leaving behind the cell pellet **[1]**. Then add the required volume of warmed culture media **[2]** and resuspend the pellet thoroughly **[3]**.

3.8.1. Talent aspirating supernatant from the 15-milliliter conical tube.

3.8.2. Talent adding warmed culture media. **Videographer's NOTE:** 3.8.2 - 3.8.3 Combined

3.8.3. Talent pipetting the suspension up and down to fully resuspend cells.

3.9. Plate the cells at the desired seeding density **[1].** View the cells under a microscope **[2]** and then incubate the cells **[3-TXT].** Rinse the cells with warmed DPBS and replace the media every 2 days **[4]**.

3.9.1. Talent plating cells into culture wells.

3.9.2. Talent placing the cells under a microscope.

3.9.3. Talent placing the cells in an incubator. **TXT: Incubation: 37 °C**

3.9.4. Talent rinsing culture wells with warmed Dulbecco's phosphate-buffered saline and replacing the medium.

3.10. To process human follicular fluid, first centrifuge the samples at 380 *g* for 10 minutes at 4 degrees Celsius **[1]**. Then transfer the supernatant from each tube into fresh 15 milliliter conical tubes **[2]**.

3.10.1. Talent placing tubes into a centrifuge and starting the run.

3.10.2. Talent transferring supernatant into new conical tubes.

3.11. Add protease inhibitor cocktail and phosphatase inhibitor to each tube **[1]** and mix thoroughly by pipetting **[2]**.

3.11.1. Talent adding inhibitors to the tube.

3.11.2. Talent pipetting the suspension to mix well.

3.12. Distribute 1 milliliter of the prepared follicular fluid into sterile 1.5 milliliter microcentrifuge tubes **[1]**. Store the tubes at minus 80 degrees Celsius until further analysis **[2]**.

3.12.1. Talent aliquoting follicular fluid into sterile microcentrifuge tubes.

3.12.2. Talent placing labeled microcentrifuge tubes into a minus 80 degrees Celsius freezer.

Results

4. Results

4.1. Immunocytochemistry performed on day 4 of culture showed that approximately 90% of cells stained positive for AMHR2 *(A-M-H-R-Two)* , confirming strong expression of this granulosa cell marker **[1]**. Quantification of AMHR2 staining confirmed that approximately 90% of the cells were AMHR2-positive **[2]**.

4.1.1. LAB MEDIA: Figure 7A. *Video editor: Highlight the red fluorescence panel in the lower row labeled "AMHR2 Primary".*

4.1.2. LAB MEDIA: Figure 7B. *Video editor: Highlight the box plot showing a high percentage of AMHR2-positive cells under "Anti-AMHR2 Primary Ab".*

4.2. Further immunocytochemistry confirmed granulosa cell identity, with approximately 80% of cells staining positive for FOXL2 *(fox-L-Two)* **[1]**. Quantification of FOXL2 staining showed approximately 80% FOXL2-positive cells **[2]**.

4.2.1. LAB MEDIA: Figure 7C. *Video editor: Highlight the green fluorescence panel in the lower row labeled "FOXL2 Primary".*

4.2.2. LAB MEDIA: Figure 7D. *Video editor: Highlight the box plot showing a high percentage of FOXL2-positive cells under "Anti-FOXL2 Primary Ab".*

- follicular

Pronunciation link: https://www.merriam-webster.com/dictionary/follicular Merriam-Webster+1
IPA: /fəˈlɪkjələr/
Phonetic spelling: fuh-LIK-yuh-ler

- conical

Pronunciation link: https://www.merriam-webster.com/dictionary/conical
IPA: /ˈkɒnɪkəl/ or American /ˈkɑnɪkəl/
Phonetic spelling: KON-i-kul

- centrifuge

Pronunciation link: https://www.merriam-webster.com/dictionary/centrifuge
IPA: /ˈsɛntrəˌfjuːʤ/
Phonetic spelling: SEN-truh-fyoog

- resuspension

For “resuspension”: base word “resuspend” —
Pronunciation link: https://www.merriam-webster.com/dictionary/resuspend
IPA: /ˌriːsəˈspɛnd/ → resuspension: /ˌriːsəˈspɛnʃən/
Phonetic spelling: ree-suh-SPEHN-shun

- isopropyl

Pronunciation link: https://www.merriam-webster.com/dictionary/isopropyl
IPA: /ˌaɪsoʊˈproʊpɪl/
Phonetic spelling: eye-soh-PROH-pil

- density

Pronunciation link: https://www.merriam-webster.com/dictionary/density
IPA: /ˈdɛnsɪti/
Phonetic spelling: DEN-sih-tee

- gradient

Pronunciation link: https://www.merriam-webster.com/dictionary/gradient
IPA: /ˈgreɪdiənt/
Phonetic spelling: GRAY-dee-uhnt

- hemocytometer

Pronunciation link: https://www.merriam-webster.com/dictionary/hemocytometer Merriam-Webster+1
IPA: /ˌhiːməˈsaɪtɑmətər/
Phonetic spelling: hee-muh-SY-tuh-mah-ter

- protease

Pronunciation link: https://www.merriam-webster.com/dictionary/protease
IPA: /ˈproʊtiːˌeɪs/
Phonetic spelling: PROH-tee-ays

- phosphatase

Pronunciation link: https://www.merriam-webster.com/dictionary/phosphatase
IPA: /ˈfɑsˌfeɪtɛɪs/ or /ˌfɒsˈfeɪˌteɪs/ depending on vowel
Phonetic spelling: FOS-fay-tays

- inhibitor

Pronunciation link: https://www.merriam-webster.com/dictionary/inhibitor
IPA: /ɪnˈhɪbɪtər/
Phonetic spelling: in-HIB-i-ter

- immunocytochemistry

Pronunciation link: https://www.merriam-webster.com/dictionary/immunocytochemistry

Merriam-Webster+1
IPA: /ˌɪm.jə.noʊˌsaɪtəˈkɛmɪstri/
Phonetic spelling: im-yoo-no-sai-toe-KEM-ih-stree

- microcentrifuge

(from “micro-centrifuge tube”) — base words “micro” + “centrifuge”:
Pronunciation link for centrifuge above; “micro” is common /ˈmaɪkroʊ/ → full:
/ˈmaɪkroʊˌsɛntrəfjuːʤ/
Phonetic spelling: MY-kroh-SEN-truh-fyoog

- aliquot

Pronunciation link: https://www.merriam-webster.com/dictionary/aliquot
IPA: /ˈælɪkwət/ or /ˈeɪlɪkwət/
Phonetic spelling: AL-i-kwut or AY-li-kwut

- DPBS

(acronym for Dulbecco’s Phosphate-Buffered Saline) — pronounce letter-by-letter:
Dee-Pee-Bee-Ess.
Pronunciation link: No confirmed link found.
Phonetic spelling: dee-pee-bee-ess

- DMEM

(acronym commonly pronounced Dee-Em-Ee-Em) — letter-by-letter.
Pronunciation link: No confirmed link found.
Phonetic spelling: dee-em-ee-em

- Trypan (as in “Trypan Blue”) — the word “Trypan”:

Pronunciation link: https://www.merriam-webster.com/dictionary/trypan
IPA: /ˈtraɪpæn/
Phonetic spelling: TRY-pan