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Title: Single Cell Collection of Trophoblast Cells in Peri-implantation Stage Human Embryos

Authors and Affiliations:

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Yes**

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

   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 (through a camera port or one of the oculars). Please list the make and model of your microscope.

   Olympus DP2-SAL system attached to a Nikon SMZ1500 Stereomicroscope

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: 44

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Ye Yuan:** Many pregnancies fail early in human embryo development, around day seven or eight post fertilization. This protocol offers investigators an opportunity to allow conceptuses to develop in vitro during this mysterious implantation window in order to investigate the mechanisms that underpin the success of implantation and early placenta formation.

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

1.2. **Ye Yuan:** Using an extended culture system to grow peri-implantation stage human embryos in vitro is probably the only way to obtain materials to study human implantation and early trophoblast differentiation. This straightforward technique is a powerful tool that can be used to answer many fundamental questions about early human development.

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

OPTIONAL:

1.3. **Ye Yuan:** Research conducted with the use of this protocol will largely contribute to understanding early pregnancy loss, recurrent implantation failure, and placental pathologies.

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

Introduction of Demonstrator on Camera

1.4. **Ye Yuan:** Demonstrating the procedure will be Deirdre Logsdon, a PhD student from my laboratory.

1.4.1. INTERVIEW: Author saying the above.

1.4.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Ethics Title Card

1.5. Procedures involving human embryos have been approved by the Western Institutional Review Board (study no. 1179872) and follow international guidelines.

Protocol

2. Preparation and Zona Removal

2.1. One day prior to embryo warming, prepare media and recovery plates in a sterile laminar flow hood **[1]**. Fill two center-well organ culture wash dishes with 500 microliters of BM with 10% SPS **[2]**, then cover the BM with 500 microliters of embryo culture grade oil **[3]**.

2.1.1. WIDE: Establishing shot of talent at the hood preparing plates and media. **TEXT: Filter and equilibrate all media in advance**

2.1.2. Talent filling the dishes with BM, with the BM container in the shot.

2.1.3. Talent covering the BM with the oil.

2.2. In a 60-millimeter tissue culture dish, layer 8 milliliters of embryo culture oil **[1]** and anchor 20-microliter drops of BM with 10% SPS to the bottom **[2]**. Equilibrate the wash dishes and recovery plate in an incubator at 37 degrees Celsius, 6% carbon dioxide, and 5% oxygen for at least 4 hours **[3]**.

2.2.1. Talent putting culture oil into the culture dish.

2.2.2. Talent anchoring BM drops to the bottom of the dish.

2.2.3. Talent putting the wash dishes and recovery plate in the incubator and closing the door.

2.3. Aliquot approximately 4 milliliters of IVC1 into a 5-milliliter snap cap tube **[2]** and prepare one wash dish with 500 microliters of IVC1 *(pronounce 'eye-V-C-one')* with no oil overlay **[1]**. Equilibrate the dish and tube in the incubator for at least 4 hours **[3]**.

2.3.1. Talent adding IVC1 to a dish, with the IVC1 container in the shot.

2.3.2. Talent aliquoting the IVC1 into a snap tube. NOTE: switch the order of 2.3.1 and 2.3.2

2.3.3. Talent putting the dish and the tube into the incubator.

2.4. Dilute fibronectin from human serum in PBS to 30 micrograms per milliliter **[1]**. Open the 8-well chambered coverslip package, taking care to not touch the wells **[2]**, then gently pipette 250 microliters of the fibronectin into each well **[3]**. Replace the lid on the coverslip and incubate it at 4 degrees Celsius for 20 to 24 hours **[4]**.

2.4.1. Talent diluting the fibronectin with PBS, with the PBS container in the shot.

2.4.2. Talent opening the 8-well chambered coverslip package.

2.4.3. Talent pipetting the fibronectin into a few wells.

2.4.4. Talent putting the covered coverslip into the refrigerator and closing the door.

2.5. Prepare the extended culture plate on the morning of embryo warming. Retrieve the chambered coverslip with fibronectin and place it in the laminar flow hood **[1]**. Then, remove the fibronectin mixture with a 1-milliliter pipette and discard it **[2]**.

2.5.1. Talent taking the coverslip out of the refrigerator.

2.5.2. Talent removing the fibronectin from the coverslip.

2.6. Pipette 300 microliters of the equilibrated IVC1 into each well **[1]** and place the coverslip into the incubator until removal of the zona pellucida **[2-TXT]**.

2.6.1. Talent pipetting IVC1 into a few wells.

2.6.2. Talent placing the coverslip in the incubator and closing the door. **TEXT: 37 °C ; 6% CO_2 ; Atmospheric O_2** *Videographer: Obtain multiple usable takes, this will be reused in 3.2.1, 3.5.3 and 4.1.3.*

2.7. After warming and recovery of the D5 human embryos, assess them for re-expansion and take pictures of each embryo **[1]**. Move each embryo to 500 microliters of a MOPS *(pronounced "mops", rhymes with "hops")* -buffered handling medium with 5% FCS **[2]**, then treat it with acidic Tyrode's solution as described in the text manuscript **[3]**. *Videographer: This step is important!*

NOTE: Authors recorded all scope shots and numbered them according to the script. All files are uploaded to project page but are also available at https://1drv.ms/u/s!Agyx8pNizCnV-EckgLHPaQ9Ir1da?e=2b6ELo

2.7.1. SCOPE: Talent looking at the embryos and imaging them. *Videographer: Please film the display of the benchtop monitor for all SCOPE shots.*

2.7.2. SCOPE: Talent moving an embryo into the MOPS FCS.

2.7.3. SCOPE: Talent moving the embryo to Tyrode's solution.

2.8. Immediately move the embryo with the dissolving zona into 300 microliters of warmed MOPS buffered medium to quench the Tyrode's solution **[1]**. Then, move the embryo into a center-well organ tissue dish containing the equilibrated BM with 10% SPS under the embryo culture grade oil **[2]**.

2.8.1. SCOPE: Talent moving the embryo to MOPS.

2.8.2. SCOPE: Talent moving the embryo to the center-well organ tissue dish.

2.9. Then, return the embryo to the 20-microliter recovery drop **[1]**.

2.9.1. SCOPE: Talent putting the embryo into the recovery drop.

3. Blastocyst Extended Culture

3.1. Individually move the embryos to the wash dish with the equilibrated IVC1 media **[1]**, then carefully move each embryo to a well of the chambered coverslip, making sure to keep track of embryo identification **[2-TXT]**.

3.1.1. SCOPE: Talent moving an embryo to the wash dish.

3.1.2. SCOPE: Talent moving an embryo to the coverslip. **TEXT: Work quickly to minimize evaporation!**

3.2. Return the chambered coverslip to an incubator set to 37 degrees Celsius, 6% carbon dioxide, and atmospheric oxygen for 2 days **[1]**.

3.2.1. *Use 2.6.2.*

3.3. At outgrowth day 2, carefully examine the attachment of the embryos under the microscope and exchange the media **[1]**. Note which embryo is attached to the dish by gently tapping the plate **[2]**.

3.3.1. Talent looking at the embryos under the microscope.

3.3.2. Talent gently tapping the plate.

3.4. To change the media, remove the lid and carefully aspirate 150 microliters of IVC1, taking care to not disturb the attached embryo. If an embryo has not yet attached to the plate, do not exchange the media because the serum in IVC1 will aid in attachment **[1]**. *Videographer: This step is important!*

3.4.1. Talent aspirating the media from a well.

3.5. Slowly pipette 150 microliters of equilibrated extended culture media, IVC2, into each well **[1]** and replace the lid on the coverslip **[2]**. Carefully return the chambered coverslip to the incubator. Repeat media exchange and attachment check every day until embryos are ready for fixation or single cell digestion **[3]**.

3.5.1. Talent adding IVC2 to a few wells.

3.5.2. Talent putting the lid on the coverslip.

3.5.3. *Use 2.6.2.*

3.6. If collection of spent media is necessary for further analysis, snap-freeze the 150 microliters of removed IVC1 into a sterile, low-bind 0.5-milliliter tube **[1]**.

3.6.1. Talent putting the spent medium into a tube, closing it, and plunging in liquid nitrogen.

4. Single Cell Selection and Sample Collection

4.1. Wash each embryo once with 200 microliters of PBS, then add 200 microliters of trypsin solution to each well **[2]**. Return the chambered coverslip to the incubator for 5 minutes **[3]**.

~~4.1.1. SCOPE: Talent washing an embryo with PBS.~~

4.1.2. Talent adding trypsin to a few wells.

4.1.3. *Use 2.6.2.*

4.2. Use a small pipette or finely pulled mouth pipette to pick up an individual MTB **[1]**, then use a larger pipette to gently dissociate the embryo by aspirating up and down **[2]**. *Videographer: This step is difficult and important!*

4.2.1. SCOPE: Talent picking up an MTB. *Videographer: Please film the display of the benchtop monitor for all SCOPE shots.*

4.2.2. SCOPE: Talent pipetting the embryo up and down.

4.3. Continue aspirating the embryo gently and repeatedly using a smaller diameter pipette tip until the embryo has been incubated for a total of 10 minutes in trypsin **[1]**. *Videographer: This step is important!*

4.3.1. Talent switching to a smaller pipette tip and continuing to pipette the embryo up and down.

4.4. To perform single-cell selection, move the dissociated cells through three 20-microliter wash drops of PBS and 0.1% PVP under embryo culture oil, taking care to not lose any cells **[1]**. *Videographer: This step is important!*

4.4.1. Talent putting the dissociated cells in a wash drop of PBS.

4.5. After washing the cells, use a finely pulled glass pipette to select one cell **[1]**. Carefully pipette the single cell into a sterile 0.2-milliliter low-bind tube with a minimal volume of PBS and PVP **[2]**. Snap freeze single cells in liquid nitrogen **[3]** and store them at -80 degrees Celsius for future use **[4]**. *Videographer: This step is important!*

4.5.1. SCOPE: Talent picking up one cell with the pipette tip.

4.5.2. Talent putting the cell in the low-bind tube.

4.5.3. Talent snap freezing the tube.

4.5.4. Talent putting the tube in the freezer and closing the door.

Results

5. Results: Morphologies and Trophoblast Marker Expression during Extended Culture

5.1. Healthy embryos exhibited continued proliferation over the course of extended culture **[1]**, while abnormal embryos began to retract from their outer edges and disintegrate **[2]**.

5.1.1. LAB MEDIA: Figure 2 B and C. *Video Editors: Emphasize B.*

5.1.2. LAB MEDIA: Figure 2 B and C. *Video Editors: Emphasize C.*

5.2. At Day 8 post fertilization, most cells in the embryos were cytotrophoblasts, or CTBs, that were positive for trophoblast marker GATA3 *(pronounce 'gah-tah-three')* **[1]**. On the periphery of the embryo, the CTBs were already differentiating into multinucleated syncytiotrophoblasts, which had a sheet-like appearance and stained positive for human CGB **[2]**.

5.2.1. LAB MEDIA: Figure 3 A. *Video Editor: Emphasize the GATA3 image.*

5.2.2. LAB MEDIA: Figure 3 A. *Video Editor: Emphasize the CGB image.*

5.3. At Day 10, the formation of CGB positive migratory trophoblast cells was at a maximum, which was confirmed by the upsurge of hCG production at this time **[1]**. Migratory trophoblasts, which stained positive for HLA-G *(pronounce each letter; H-L-A-G)*, also began to emerge and migrate away from the embryo body **[2]**.

5.3.1. LAB MEDIA: Figure 3 B. *Video Editor: Emphasize the D10, D11, and D12 bars.*

5.3.2. LAB MEDIA: Figure 3 A. *Video Editor: Emphasize the HLA-G image.*

5.4. By Day 12, STB differentiation was in decline and MTB production became more prominent, suggesting a shift of emphasis from hormone production on Day 10 to cell migration on Day 12. These changes can be observed in a time-lapse video of the peri-implantation period **[1]**.

5.4.1. LAB MEDIA: Movie_JoVE.mp4. *Video Editor: Please use the version without the circles for the Jove video and this one for reference.*

5.5. The video demonstrates the collapse of the blastocoel, the formation of the STB **[1]**, and the eventual differentiation and migration of MTB **[2]**.

5.5.1. LAB MEDIA: Movie_JoVE.mp4. *Video Editor: Please use the version without the circles for the Jove video and this one for reference. Emphasize the parts of the video circled in green at 0:18.*

5.5.2. LAB MEDIA: Movie_JoVE.mp4. *Video Editor: Please use the version without the circles for the Jove video and this one for reference. Emphasize the parts of the video circled in orange at 0:30.*

Conclusion

6. Conclusion Interview Statements

6.1. **Deirdre M. Logsdon:** The most important thing to keep in mind when completing this procedure is that you are working with live embryos that are sensitive to changes in temperature, osmolality, and pH. Minimizing the amount of time that the dishes are out of the incubator is critical to maintaining embryo health.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.7.1 – 2.7.3.*

6.2. **Deirdre M. Logsdon:** After completing this procedure, investigators can use isolated single cells for downstream single cell omics assays such as single-cell RNA sequencing and whole genome bisulfite sequencing to understand transcriptomic and epigenetic changes that occur during human implantation and early placenta formation.

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