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Title: Cultivating a Three-dimensional Reconstructed Human Epidermis at a Large Scale

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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. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No, filming will be done in 1 laboratory.**

Current Protocol Length

Number of Steps: 15
Number of Shots: 34

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. Marc Eeman: In vitro reconstructed human epidermis models were first developed in the nineties with a primary goal of developing alternative testing methods to animal experimentation. Those 3D epidermal models are now commonly used to test both the safety and efficacy of cosmetic ingredients. Whereas they can be purchased from several tissue suppliers, being able to reconstitute a 3D epidermal model in a laboratory gives more flexibility for the endpoints of interest. There are several research articles describing briefly the reconstitution protocol of 3D epidermal model but there is to date no video available showing the critical steps of the reconstitution process. This is the reason behind the publication of this video paper.

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

1.2. **Irini Dijkhoff:** The preparation of in-house reconstructed human epidermis allows for a lot of freedom in the culturing process, such as changes to the culture medium composition, pro-inflammatory or oxidative stress triggers, silencing of genes of interest, and the inhibition or stimulation of certain biological processes.

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

Protocol

2. Culture and seeding of NHEKs

2.1. To begin, thaw a vial with 1 million cryopreserved normal human epidermal keratinocytes, or NHEKs **[1]**, in a water bath at 37 degrees Celsius by submerging part of the vial in the water **[2]**. Incubate the vial for 1 to 2 minutes in the water bath, until only a small sliver of ice is visible **[3]**. *Videographer: This step is important!*

2.1.1. WIDE: Establishing shot of talent taking a vial of cells out of the liquid nitrogen tank.

2.1.2. Talent submerging the vial in the water.

2.1.3. Properly thawed cells.

2.2. Resuspend the cells very carefully by pipetting up and down 2 to 3 times **[1]**. Transfer the cell suspension into two T75 flasks containing a total of 15 milliliters of pre-warmed thawing medium, resulting in a seeding density of 6.7 x 10^4 cells per centimeter squared **[2]**. *Videographer: This step is important!*

2.2.1. Talent pipetting the cells.

2.2.2. Talent transferring the cells to a flask.

2.3. Pre-fill 24-well plates with 1.5 milliliters of submerged medium, ideally using a dispenser pipette **[1]**. After culturing the cells to 80% confluency, they are ready for seeding **[2a]** in inserts for the cultivation of reconstructed human epidermis, or RhEs **[2b]**. Remove the basal medium from the T75 flasks with the NHEKs **[3]**.

2.3.1. Talent pre-filling the plate.

2.3.2. A. Talent taking the flask out of the incubator.

B. Added shot: Zoom on label

2.3.3. Talent removing the medium from the flask.

2.4. Rinse the cells by adding 5 milliliters of pre-warmed PBS to each T75 flask **[1]**, then remove PBS from the flasks **[2]**. Add 2 to 3 milliliters of pre-warmed 0.05% trypsin-EDTA to each flask, making sure that the trypsin solution is equally distributed on the cell culture area of the flask **[3]**.

2.4.1. Talent adding PBS to the flask.

2.4.2. Talent removing the PBS from the flask.

2.4.3. Talent adding trypsin to a flask.

2.5. Place the flasks in the cell culture incubator for 4 minutes **[1]**, then check whether the cells detach using the microscope at a 10x magnification **[2]**. Rap the flask to help the cells release from the surface of the flask **[3]**.

2.5.1. Talent putting the flask in the incubator and closing the door.

2.5.2. Talent looking at the cells under the microscope.

Author Note: A video recording of the microscope of the cells was made with the microscope camera. However, the video format seems to be corrupted. We are working on preparing a new video with the microscope of trypsinized cells.

2.5.3. Talent rapping the flask.

2.6. Once all the cells are detached, add an equal volume of pre-warmed trypsin inhibitor to each T75 flask **[1]** and transfer the cell suspension from the flasks to a centrifuge tube **[2]**.

2.6.1. Talent adding trypsin inhibitor to the flask.

2.6.2. Talent transferred the cells to a centrifuge tube.

2.7. Rinse the flasks with 5 milliliters of pre-warmed PBS **[1]** and transfer it to the centrifuge tube containing the cell suspension **[2]**. Centrifuge the harvested cells at 400 *x g* for 5 minutes **[3]**. Carefully discard most of the supernatant, leaving approximately 100 to 200 microliters in the tube **[4]**.

2.7.1. Talent rinsing the flask.

2.7.2. Talent adding the PBS to the centrifuge tube.

2.7.3. Talent putting the tube in the centrifuge and closing the lid.

2.7.4. Talent removing the supernatant.

2.8. Gently resuspend the pellet of cells in a low volume of submerged medium, pipetting up and down 5 to 10 times to ensure a uniform cell suspension. Start with a low volume to avoid the formation of cell aggregates and add up to 1 milliliter of submerged medium per initial T75 flask **[1]**.

2.8.1. Talent resuspending the cells.

2.9. Count the cells in the suspension using the trypan blue exclusion method **[1]**. Dilute the cell suspension with additional submerged medium to reach a concentration of 3.525×10^5 cells per milliliter using the first equation provided in the text manuscript **[2-TXT]**.

2.9.1. Talent using the cell counter.

2.9.2. Talent resuspending the cells. **TEXT:** $V_2 = C_1 * \frac{V_1}{C_2} - V_1$ **; C_1 = counted cell concentration ; V_1 = volume used to resuspend the pellet of cells ; C_2 = targeted cell concentration ; V_2 = volume to be added**

2.10. Perform a second cell count of the diluted solution **[1]** and use the second equation in the text manuscript to calculate the cell suspension volume to be seeded into the culture insert **[2-TXT]**.

2.10.1. Talent counting the cells in the suspension.

2.10.2. Talent calculating the seeding volume. **TEXT:** $V_4 = C_3 * \frac{V_3}{C_4}$ **; C_3 = targeted cell concentration ; V_3 = targeted volume of the cell suspension to be seeded ; C_4 = counted cell concentration ; V_4 = actual volume of the cell suspension to be seeded**

2.11. Hang the 24 well culture inserts in the highest position of the carrier plate **[1]** and transfer the carrier plate to the 24-well plate pre-filled with submerged medium **[2]**. Add the determined volume of the cell suspension to each insert, taking care to not touch the insert **[3]**. *Videographer: This step is difficult and important!*

2.11.1. Talent hanging the culture inserts in the carrier plate.

2.11.2. Talent transferring the carrier plate into the plate with the medium.

2.11.3. Talent adding the cell suspension to a few inserts.

2.12. After seeding, incubate the 24-well plates for 10 to 15 minutes at room temperature, to overcome an edge effect. Do not move the plates during this time **[1]**. Transfer the plates to the cell culture incubator and leave them in submerged conditions for three days **[2-TXT]**.

2.12.1. Talent leaving the plate at RT.

2.12.2. Talent putting the plate in the incubator and closing the door. **TEXT: 37 °C, 5% CO_2, and 95% RH**

3. Cultivation at ALI

3.1. After a three-day incubation in the cell culture incubator, expose the cells that have adhered to the membrane surface to the air-liquid-interface, or ALI, by removing the submerged medium from the apical compartment with an aspiration system and a glass Pasteur pipette **[1-TXT]**. *Videographer: This step is difficult and important!*

3.1.1. Talent removing the submerged medium. **TEXT: Avoid touching the inserts**

3.2. Fill new 24-well plates with 1.5 milliliters of fresh pre-warmed ALI medium **[1]** and transfer the carrier plates with the culture inserts to the new multi-well plates **[2]**.

3.2.1. Talent filling a plate with ALI medium.

3.2.2. Talent transferring the carrier plates.

3.3. Transfer the multi-well plates back to the cell culture incubator. Refresh the ALI medium every 2 to 3 days for 14 days **[1]**.

3.3.1. Talent putting the plate in the incubator.

Results

4. Results: Characterization of RhEs cultivated from neonatal NHEKs

4.1. Keratinocytes in 2D culture display a traditional morphology with a consistent polygonal shape **[1]**. After 15 days at ALI, the reconstructed human epidermis forms a fully stratified tissue, which is indicated by its four main epidermal layers **[2]**.

4.1.1. LAB MEDIA: Figure 2 A.

4.1.2. LAB MEDIA: Figure 2 B.

4.2. Ultrastructural analysis of the reconstructed human epidermis at different time points in the reconstitution protocol reveals the cornification process with an increased number of corneocyte layers over time **[1]**.

4.2.1. LAB MEDIA: Figure 2 E.

4.3. Keratinocytes in the epidermis show different protein expression profiles according to their differentiation stage **[1]**. Involucrin expression appears more predominantly in the SG layer **[2]**, whereas the expression of filaggrin and loricrin is located in the upper layers **[3]**. Keratin 10 expression was found in all the viable layers, except for the SB layer **[4]**.

4.3.1. LAB MEDIA: Figure 3.

4.3.2. LAB MEDIA: Figure 3 D.

4.3.3. LAB MEDIA: Figure 3 B – C.

4.3.4. LAB MEDIA: Figure 3 E.

4.4. The reconstructed human epidermis displays functional desmosomal junctions, as indicated by the expression of desmoglein 1 in the intercellular space of the viable epidermal layers **[1]**.

4.4.1. LAB MEDIA: Figure 3 F.

4.5. The barrier properties of the reconstructed epidermis model were investigated by assessing the tissue viability upon topical treatment with a known barrier disruptor and by assessing the tissue integrity **[1]**. The tissue integrity was determined after 15 days by measuring the TEER with a voltohmmeter **[2]**.

4.5.1. LAB MEDIA: Figure 4 B.

4.5.2. LAB MEDIA: Figure 4 A.

4.6. Responsiveness of the epidermis to lipopolysaccharide and tumor necrosis factor alpha was investigated. Both LPS and TNF-alpha treatments were non-toxic relative to the Triton X-100 control **[1]**.

4.6.1. LAB MEDIA: Figure 5.

4.7. The release of interleukin 1 alpha and interleukin 8 in the RhE medium was quantified using ELISAs **[1]**. LPS treatment resulted in a statistically significant upregulation in the release of interleukin 1 alpha and interleukin 8 **[2]**. TNF-alpha only upregulated interleukin 1 alpha **[3]**.

4.7.1. LAB MEDIA: Figure 6.

4.7.2. LAB MEDIA: Figure 6 A, D, C, F.

4.7.3. LAB MEDIA: Figure 6 B, E, C, F.

Conclusion

5. Conclusion Interview Statements

5.1. **Irini M. Dijkhoff:** Following this protocol, TEER can be measured as a value for barrier integrity, the viability or cytotoxicity can be measured by a MTT or LDH assay, respectively, and the supernatant can be used to measure the secretion of cytokines. Furthermore, the tissue can be used to study the expression of proteins of genes of interest.

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