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Title: Generation of self-assembled vascularized human skin equivalents

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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?

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**

Current Protocol Length

Number of Steps: 23

Number of Shots: 52

Introduction

1. Introductory Interview Statements

REQUIRED:

- 1.1. **Martina M. Sanchez:** The detail provided in this protocol addresses skin equivalent generation which is often technically challenging. Novel human skin equivalent protocol elements such as vasculature and volumetric imaging are included.

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

- 1.2. **Joshua T. Morgan:** The technique enables straightforward construction of skin models that more closely match *in vivo* tissue for study of diseases, aging, and personalized medicine.

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

OPTIONAL:

- 1.3. **Martina M. Sanchez:** The most challenging part of this protocol is the transition between submersion and air liquid interface. It will likely take a few trials to optimize the timing and media levels for consistent results.

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

Protocol

2. Generation of 3D collagen dermal component

- 2.1. To begin, add 516 microliters of media to a cold capped tube and immediately add 375 microliters of cold collagen using the 1000-microliter positive displacement pipette [1-TXT]. Remove the empty pipette tip [2] and switch to the prepared 250-microliter positive displacement pipette to mix [3].
 - 2.1.1. Talent adding media and collagen to tube **TEXT: Avoid dispensing collagen to side of the tube** *Videographer: This step is difficult and important!* **Note: take 2; incorrectly slated as 2.1.2 tk2 (P1330724.MOV)**
 - 2.1.2. Talent removing the empty pipette tip
 - 2.1.3. Talent mixing the solution with the 250-microliter pipette
- 2.2. Mix quickly but gently to prevent excess bubble formation, keeping the tip in the solution [1]. Mix uniformly from different positions of the tube until the solution is of homogenous color, which typically takes about 5 pipette cycles or 10 seconds [2].
 - 2.2.1. Talent mixing the solution *Videographer: This step is important!*
 - 2.2.2. Homogeneous solution.
- 2.3. Immediately disperse 125 microliters of acellular collagen onto the membrane of the 12-well culture insert [1]. To ensure uniform coverage, **tilt the plate and** use the pipette tip to paint the membrane by gently spreading collagen around [2-TXT].
 - 2.3.1. Talent dispersing the collagen onto the membrane
 - 2.3.2. Talent working to uniformly spread the collagen **TEXT: Avoid applying pressure to the membrane.** *Videographer: This step is important!*
- 2.4. Immediately move the 12-well plate to a 37-degree Celsius cell culture incubator to let it gel for at least 20 minutes [1].
 - 2.4.1. Talent incubating the plate

3. Cell laden collagen seeding of dermal compartment

- 3.1. After the gelation period, take the 12-well plate of acellular collagen out of the incubator [1-TXT]. Remove the 1.7-milliliter capped tube from wet ice [2], then remove the stock collagen from refrigeration [3-TXT] and place it on wet ice with the lid open [4].
 - 3.1.1. Talent removing the plate from the incubator **TEXT: If collagen is not gelled, do not continue the procedure**
 - 3.1.2. Talent removing the tube from wet ice

- 3.1.3. Talent removing the stock from refrigerator **TEXT: Stock Collagen: 8 mg/mL**
- 3.1.4. Talent placing the tube on ice and opening the lid
- 3.2. Add the 516 microliters of cooled cell suspension to the cold capped tube [1]. Use the 1000-microliter pipette to immediately pipette 375 microliters of cold collagen solution directly into the solution inside the capped tube [2].
 - 3.2.1. Talent adding the cell suspension to the tube
 - 3.2.2. Talent pipetting the collagen solution to the tube
- 3.3. Expel all collagen from the pipette into the tube and discard the positive displacement pipette tip [1]. Immediately switch to the 250-microliter pipette and mix the collagen solution [2].
 - 3.3.1. Talent expelling collagen from the pipette and discarding the tip
 - 3.3.2. Talent mixing the solution
- 3.4. Once mixed, transfer 250 microliters of cellular collagen solution onto the acellular collagen supports in the 12-well culture insert [1], then move the 12-well plate to a 37-degree Celsius cell culture incubator [2].
 - 3.4.1. Talent transferring collagen to the plate
 - 3.4.2. Talent putting the plate in the incubator **Note: Did not shoot; use 2.4.1**
- 3.5. After the 30-minute gel time, gently tilt the plate to assess the gelation and make sure that the collagen has solidified [1]. Add 500 microliters and 1000 microliters of blend media to the upper chamber and lower chamber of the insert, respectively [2-TXT].
 - 3.5.1. Talent tilting the plate to assess the gelation
 - 3.5.2. Talent adding the media to upper chamber and then to the lower chamber.
TEXT: Blend media: Half endothelial and half fibroblast maintenance media
- 3.6. Ensure that the collagen gel is submerged, adding more media if necessary [1]. Place the well plate in the cell incubator for overnight incubation [2].
 - 3.6.1. Talent adding the media to submerge the collagen gel
 - 3.6.2. Talent incubating the well plate **Note: Did not shoot; use 2.4.1**

4. Seeding of epidermal component and stratification induction

- 4.1. On Submersion Day 7, use a manual pipette to collect and discard media from the bottom and top chamber of each construct well [1]. To collect media stuck directly under the permeable membrane, place the pipette tip under the membrane, knocking the insert out of place temporarily [2].
 - 4.1.1. Talent collecting and discarding the media

- 4.1.2. Talent placing the tip under the membrane and displacing the membrane
- 4.2. Add 1 milliliter of Human Skin Equivalent, or HSE, media supplemented with 5 percent FBS to the lower chamber of each well [1] and 200 microliters of cell suspension to the top chamber of each well. Seed the keratinocytes directly onto the dermal construct surface [2] and allow them to settle for 2 hours in the incubator [3].
 - 4.2.1. Talent adding media to lower chamber of the well
 - 4.2.2. Talent adding cell suspension to the top chamber of the surface
 - 4.2.3. Talent incubating the well plate
- 4.3. Two hours after seeding the keratinocytes, carefully add 300 microliters of HSE media supplemented with 5 % FBS to the top chamber of each construct well [1]. After loading the media, place the construct back into the incubator [2].
 - 4.3.1. Talent adding the media to the top chamber
 - 4.3.2. Talent putting the plate in incubator **Note: Did not shoot; use 2.4.1 or 4.2.3**
- 4.4. Using a manual pipette, lift each construct to the air-liquid interface, or ALI, by removing media waste from the upper chamber only. Try to get as close to the epidermal layer as possible without touching or damaging it [1].
 - 4.4.1. Talent removing media waste from the chamber. *Videographer: This step is important!*
- 4.5. Tilt the plate slightly at different angles to collect the media [1], then add approximately 2 milliliters of sterile water to the surrounding wells in the plate to maintain consistent humidity [2].
 - 4.5.1. Talent tilting the plate and collecting the media.
 - 4.5.2. Talent adding the water to the plate
- 4.6. Check the plate a few hours later to make sure the keratinocytes are still at the ALI. Remove any media in the upper chamber, keeping tracking of how much media is removed from each well. The epidermal layer should look hydrated, not dry, but there should not be media pooled on top of the construct. [2-TXT].
 - ~~4.6.1. Plate with keratinocytes at ALI *Videographer: This step is difficult!*~~
 - 4.6.2. Talent removing the media from the upper chamber **TEXT: Ensure that the VHSEs do not dry out because of low media levels** *Videographer: This step is difficult!*

5. Immunofluorescent staining of 3D constructs

- 5.1. To prepare the construct for immunofluorescent staining, turn an insert upside down and place it over its well on the well plate [1]. Stabilize the insert with one hand while

using fine tip forceps or a precision knife to cut about half of the circumference of the membrane. Cut as close to the plastic housing as possible [2].

5.1.1. Talent turning the insert upside down and placing it on the well

5.1.2. Talent holding the insert and making an incision using forceps/knife
Videographer: This step is important!

5.2. Using the fine tip forceps, grab the edge of the cut membrane flap and gently peel the porous membrane off the insert as well as the VHSE (*Pronounce as V-H-S-E*) construct [1]. If the construct gets stuck on the side of the chamber, use the fine tip forceps or a small scoopula to move it to the well [2].

5.2.1. Talent peeling the membrane of the insert *Videographer: This step is important!*

5.2.2. Talent moving the construct from the wall to the well *Videographer: This step is important!*

5.3. Once the VHSE is in the well, discard any remaining pieces of the insert membrane [1] and keep the culture insert housing in each well to hold the VHSEs in a submerged position during staining [2].

5.3.1. Talent discarding the remaining pieces of the insert membrane

5.3.2. Talent placing insert housing in each well to submerge VHSEs

6. Confocal Imaging of 3D constructs

6.1. Two days before imaging, prepare polydimethylsiloxane or PDMS. Place any clean mixing vessel on a weighing balance and tare the scale [1]. Add the base and then add the crosslinker [2-TXT].

6.1.1. Talent putting the vessel on weighing balance and opting to tare the scale

6.1.2. Talent adding the base and crosslinker to the vessel **TEXT: BASE (27 g):
CROSSLINKER (3 g); 9:1 ratio; total 30 g PDMS**

6.2. Stir the solution vigorously for at least 4 minutes, creating small bubbles [1]. After sufficient mixing, pour the PDMS into a 90 or 100-millimeter Petri dish [2].

6.2.1. Talent vigorously stirring the solution

6.2.2. Talent pouring the PDMS into the Petri dish

6.3. De-gas the PDMS in a vacuum chamber until all bubbles disappear and the PDMS is clear [1]. Release the vacuum slowly [2] and remove the PDMS [3]. Place the dish into an oven to cure overnight at 50 to 60 degrees Celsius, making sure that the dish is sitting flat for PDMS to cure evenly [4].

6.3.1. Talent de-gassing the PDMS in a vacuum chamber

- 6.3.2. Talent releasing the vacuum
- 6.3.3. Talent removing the PDMS
- 6.3.4. Talent putting the PDMS in oven
- 6.4. One day before imaging, use a steel punch or handheld precision knife to punch or cut out a circular well from the PDMS sheet, around the same size as the VHSE construct **[1]**. Cut a square patch around the circular well to create a single PDMS well **[2]**.
 - 6.4.1. Talent cutting the circular well from PDMS sheet
 - 6.4.2. Talent cutting the square patch around circular well
- 6.5. Using a glass coverslip of a similar size as the PDMS well, add cyanoacrylate glue to the bottom surface of the PDMS **[1]** and smear evenly with a disposable pipette tip **[2]**. Press the PDMS well onto the center of the glass while leaving a clear glass window within the punched circle. Let the glue dry overnight before using. **[3]**.
 - 6.5.1. Talent adding glue to PDMS
 - 6.5.2. Talent smearing glue evenly with pipette
 - 6.5.3. Talent pressing the PDMS to the glass

Results

7. Results: Characterization of in vitro vascularized human skin equivalents (VHSE)

- 7.1. Characterization of the epidermis and dermis show appropriate immunofluorescent markers for human skin in the VHSE constructs [1]. Cytokeratin 10 is an early differentiation keratinocyte marker that usually marks all suprabasal layers in skin equivalents [2].
 - 7.1.1. LAB MEDIA: Figure 2
 - 7.1.2. LAB MEDIA: Figure 2 *Video editor: Please highlight the Cytokeratin 10 associated row in the figure, the last two rows.*
- 7.2. Involucrin and filaggrin are late differentiation markers in keratinocytes and mark the uppermost suprabasal layers in skin equivalents [1].
 - 7.2.1. LAB MEDIA: Figure 2 *Video editor: Please highlight the Involucrin and filaggrin associated rows in the figure, the first four rows*
- 7.3. Clearing the VHSE allowed for straightforward imaging in one setting and eliminated the need to reorient the construct to image the dermis and epidermis separately [1]. Volumetric images make it possible to generate 3D rendering to map vasculature throughout each construct [2].
 - 7.3.1. LAB MEDIA: Figure 3
 - 7.3.2. LAB MEDIA: Figure 3 *Video editor: Please highlight the right most part of the image labelled as "Cleared 3D rendering"*
- 7.4. Image stacks were loaded into computational software and a custom algorithm was used for 3D rendering and quantification [1]. Skeletonization determined the definitive center of each Collagen IV (*pronounce: "four"*) marked vessel [2] and the resulting data was used to calculate vessel diameter as well as vascular fraction [3].
 - 7.4.1. LAB MEDIA: Figure 4
 - 7.4.2. LAB MEDIA: Figure 4 *Video editor: Please highlight the upper row labelled as "4-week" and "8-week" in the figure*
 - 7.4.3. LAB MEDIA: Figure 4 *Video editor: Please highlight the lower row (graphs) labelled as "quantification" in the figure*

Conclusion

8. Conclusion Interview Statements

- 8.1. **Martina M. Sanchez:** Following this procedure, characterization methods such as barrier analysis, scanning electron microscopy, lipid profiles, and others could be performed to understand how vascularity specifically effects epidermal maturation and stability.

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

- 8.2. **Joshua T. Morgan:** This protocol enables tissue relevant and cell specific studies in a vascularized skin model. It is particularly applicable for long term experiments in the field of aging and personalized medicine.

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