# Human Fetal RPE Dissection and Culture Protocol

(Adapted with grateful acknowledgement from Maminishkis A, et al. IOVS, v.47, p.3612 (2006).)

## Materials:

Disposable polypropylene arm guards (UPC code: 643377819396) and face mask to avoid contaminating culture

Sterile HBSS with calcium and magnesium (Thermo catalogue #14025092)

PBS

Antibiotic/antimycotic solution – 1mL of Thermo catalogue #15240096 plus 1mL of gentamycin 10mg/ml (Sigma catalogue #G1272) plus 8 mL HBSS

5-8 27-gauge needles, deep dissecting dish (Living Systems Instrumentation catalogue #DD-100-D), retinal scissors, scalpel, iris scissors, small metal spatula, Dumont tweezers #2, #5B and #5 (World Precision Instruments catalogue #500336, #500234, and #501985). All instruments should be autoclaved prior to use.

1 fetal eye - 16-22 weeks old, shipped in RPMI media from Advanced Bioscience Resources, Inc. (Alameda, CA), dissected ideally within 24 h post-mortem and not more than 48 hours post-mortem. If the eye is closer to 22 weeks old, keep in mind that it may be more difficult to peel the RPE layer off in steps 21-23, and you may have to preempt this with longer incubation in dispase in step 13.

Dispase (Worthington catalogue #LS02109) - reconstitute dispase in the cell culture media base you are using. Prepare in aliquots of 30 units in 500µL, syringe filter, and store at -20°C.

DNase I (Sigma catalogue #10104159001) - prepare in aliquots of 900 units in 300µL of PBS, syringe filter, and store at -20°C.

Trypsin-EDTA solution (0.25%) (Thermo catalogue #25200056)

At least three 15mL conical tubes and tissue culture dishes

Sterile 12-well plate

Sterile 6-well plate (Corning catalogue #353046)

Flame sterilized forceps

Serologic pipettes

Eppendorf repeater pipettes with 2.5mL and 10mL Combitips Advanced, Biopur syringes (Eppendorf catalogue #0030089.650 and #0030089.677)

3mL syringes (BD Biosciences catalogue #309585)

Spinal needles (EXELint Medical Products catalogue #26960)

Ice buckets

Dissecting stereomicroscope

EVOM device with an STX2 electrode for measuring trans-epithelial electrical resistance (TEER) (World Precision Instruments)

Centrifuge that can accommodate 15mL and 50mL conical tubes

Small open plastic dish larger than dissecting dish, with the dissecting dish inside the plastic dish and ice between the two dishes

Ethanol in beaker to sterilize instruments

Primaria 25cm$^2$ flasks (1 flask per fetal eye) (Corning catalogue #353808

24-well Transwells, 0.4µm pore size, polyester (Corning catalogue #3470)

Human placental extracellular matrix (Corning catalogue #354237) or Human fibronectin (Corning

#356008)

MycoAlert Plus mycoplasma detection kit (Lonza catalogue #LT07703)

RPE Complete Media (recipe is for 500mL of media)

| Reagent | Company | Amount | Storage |
|---|---|---|---|
| MEM, alpha modification, with Earle's salts, without ribonucleosides, deoxyribonucleosides, or L-glutamine | Corning 15-012-CV | Fill to 500 mL final media solution (amount of MEM will depend on amount of serum) | +4°C |
| N1 supplement | Sigma N-6530 | 5 mL | +4°C |
| Penicillin-streptomycin | Thermo 15140122 | 5 mL | -20°C |
| GlutaMax | Thermo 35050061 | 5 mL | RT |

| | | | |
|---|---|---|---|
| Non-essential amino acids | Thermo 11140050 | 5 mL | +4°C |
| THT*<br><br>Taurine<br><br>Hydrocortisone-Cyclodextrin<br><br>3,3',5-triiodo-L-thyronine sodium salt | <br><br>Sigma T8691<br><br>Sigma H0396<br><br>Sigma T5516 | <br><br>125 mg<br><br>10 µg<br><br>0.0065 µg | -20°C |
| Heat-inactivated, Premium Select Fetal Bovine Serum** | Atlanta Biologicals S11550H | 5% or 15% | -20°C |

* THT is made by dissolving taurine, hydrocortisone-cyclodextrin, and triiodo-thyronine (T3) in PBS before making the medium. Multiple aliquots are made and stored at -20°C to simplify preparation of the culture medium. Note that the hydrocortisone product is actually combined with cyclodextrin (to help with solubility). Each 500mL of media contains 10µg of the powder, which will contain only 0.85µg of hydrocortisone. Thus, final hydrocortisone concentration in the complete media is about 4.7nM. To make 20 doses of THT (enough for 10L of media), dissolve the 1mg T3 with 1mL 1N NaOH; gently swirl to dissolve; add 49 ml PBS. This results in a 20µg/ml stock solution. Add 0.14µg (7µL) of this solution to a 50mL conical tube containing ~48.5mL PBS. Then add 2.5g of taurine to the conical tube. Finally,create a stock solution of hydrocortisone-cyclodextrin by dissolving 20 mg of hydrocortisone-cyclodextrin in 20mL PBS. Add 200µL of this solution to the conical tube containing T3 and taurine. Shake the tube vigorously until completely dissolved (taurine will take a while to dissolve), sterile filter, then aliquot the solution into 2.5-2.6 mL aliquots. Note that taurine is only sparingly soluble at this concentration. Be sure it solubilizes. It will come out of solution after freeze down, so it will have to be re-dissolved once the aliquot is thawed.

** We have used both Atlanta's FBS product above as well as ThermoFischer's certified, heat-inactivated, USA origin FBS (product #10082-147) with success. For 500mL of media, add serum last. Make up 403mL of alpha-MEM with all other ingredients above except for serum. For 15% serum, add 75mL of serum without any additional alpha-MEM. For 5% serum, add 25mL of serum and then top off with 50 additional mL of alpha-MEM.

**Human Fetal RPE Dissection Protocol:**

1) Prepare a sterile 12-well plate with multiple solutions (for each eye, 4 wells are needed); all solutions are ice-cold except for dispase solution. Each well takes between 3 and 4 mL of media:
    a. Antibiotic/antimycotic solution prepared as above - 1 well/eye
    b. HBSS rinse – 2 wells/eye
    c. Dispase solution – 4.5 units/mL in RPE complete media with 5% serum
2) Prepare dissecting dish by unpacking 5 fixation needles and filling it with ice-cold HBSS. Place dish in the larger plastic dish, with ice water between the dissecting dish and the larger dish.
3) Unpack eye and place it into antibiotic-antimycotic solution for 3-5 mins.
4) Rinse eye in two wells (prepared in step 1) with HBSS, then transfer them to dissecting dish. Place the 12-well plate in the incubator so that the dispase well reaches 37°C.
5) Trim excessive muscle and connective tissues around the eye but leave enough to pin posterior portion of eye down to dish.
6) Using 27G needles, pin the eye down to silicon base of the dissecting dish, aligning the eye so the cornea faces up.
7) Using a scalpel, make an incision just below the limbus.
8) Using iris scissors, make a cut around the eye, providing counter-traction with forceps that are holding the cornea. After the corneal button is complete, cut through the anterior vitreous with the same scissors and lift this anterior portion of the eye away.

    **Note:** In steps 3 through 8, avoid any excessive mechanical pressure to eyeball.

9) When dissecting, avoid iris contamination by removing as much iris tissue as possible.
10) Transfer open eye (which includes vitreous) into dispase solution (prepared in step 1 and warmed up in step 4). The eye will be very floppy, so use a spatula to help secure bottom of eye while picking up the lip of the eyecup with forceps.
11) Incubate the eyecup in 4.5 units/mL of dispase in RPE complete media with 5% serum for 90 min (total time) at 37°C with 5 % $CO_2$ (30 units of dispase into 7mL of media). Adjust dispase incubation time or concentration depending on how easily the choroid peels off the RPE.
12) To pre-treat the Primaria flask that will be used to plate the final dissociated RPE solution, pipette 5ml of RPE complete media with 5% serum into the flask and allow incubation in the incubator.
13) After 30-45 min of dispase incubation, remove eye and cut down sclera from the edge of the eyecup towards the optic nerve such that the eye is nearly divided into halves. Perform these cuts by finding a cutting plane between the choroid and the sclera at the edge of the eyecup and then using scissors to cut down towards the optic nerve. This will cut the sclera but leave the choroid and RPE intact. Repeat this 180° from the first cut. The result will be two halves that are still connected to each other by the optic nerve, along with a preserved whole sheet of RPE and choroid. This method will improve exposure of the RPE/choroid interface to dispase but prevent crush injury to the RPE from scissors. After these cuts, return the eyecup to the dispase well and return it to the incubator.

14) Replace HBSS solution in dissecting dish with fresh room temperature HBSS and remove from ice water trough, since the eye no longer needs to be kept cold once dispase treatment has begun.
15) Prepare a 15mL conical tube with 10mL of RPE complete media with 5% serum and keep at room temperature. In addition, place 25mL of RPE complete media with 15% serum at room temperature. Finally, thaw 5mL of trypsin solution, and place the trypsin in the incubator 15 minutes before anticipated use in step (25). The trypsin should be in a dish with a high exposed surface area. This will help equilibrate the pH of the trypsin solution.
16) After 90 min of total incubation time, transfer the cut eyecup from dispase to the dissecting dish. Again, use a spatula and forceps to help transfer.
17) Gently lift the partially separated neural retina and, using retinal scissors, cut retina away from its attachment at the optic nerve.
18) Using all five 27G needles, flatten the eye and stretch out the RPE layer.
19) Extend the cuts made in step (13) to include a cut around the nerve to remove the optic nerve and remaining retina attached to the nerve.

**Note:** Steps 13-18 can be done with low magnification or without a stereo microscope

20) Adjust stereomicroscope to 250x magnification or more.
21) Using two 45° angled forceps, separate RPE-Bruch's membrane from the choroidal tissue layer. Start the peeling around the scleral cuts. Peel the RPE away from the choroid at the edges of the eyecup. Try to minimize the number of new places where forceps are applied to help decrease the extent of crush injury. Readjust forcep position as the RPE pulls up from choroid by moving the forceps close to the point of adherence between the choroid and the RPE. Make sure to scrape off bits of choroid from the forcep tips to avoid transferring them to the RPE sheet. When possible, “push” RPE up from choroid rather than pull it away from choroid since this avoids crushing the RPE with the forceps. Pushing the RPE involves approaching the RPE from the backside – meaning the choroid is closer to the researcher than the RPE. In this orientation, firmly grip the choroid and then, with a second pair of 90° forceps, close the forceps and slide the forceps between the choroid and RPE layers, pushing the forceps away and trying to create a tissue dissection plane between the choroid and RPE.
22) When removing the RPE from the choroid, a very fine white layer stuck to the RPE may be noticeable – this is Bruch’s membrane. It is different than choroid (which is also clear to white but has blood vessels). This fine white layer of Bruch’s does not need to be removed from the RPE.
23) Transfer RPE pieces (ideally, the entire RPE sheet will come up as 4-6 pieces) to the 15mL conical tube containing 10mL of RPE complete media with 5% serum. The RPE is extremely sticky; RPE will only fall into the conical tube with vigorous shaking of the forceps. Do not use a pipette in the conical tube as RPE will stick to plastic of the pipette and not release. Shake the tube occasionally to prevent RPE from adhering to the sides of the tube.
24) After the RPE is collected, gently shake the conical tube up and down for 30 sec – if cells separate into fine suspension, no trypsin is needed. If they don’t fully separate into a suspension, then trypsinization will be required. To prepare for trypsinization, spin the conical tube containing RPE sheet fragments at 255xg for 3.5 min.

**Note:** Much of the cell death in this protocol probably happens in the next steps. The key to preventing cell death is to use enough trypsin to break apart connections, but not so much to kill cells. The other key is to use gentle pipetting rather than vigorous shaking to keep cells alive.

25) Remove media using a manual pipette instead of a pipette attached to the vacuum trap to avoid accidentally aspirating the cells. Add 5mL warm trypsin-EDTA, incubate for 5-10 min in the incubator, checking every 2-3 min and shaking the tube to ensure maximal exposure to trypsin, then use a serological pipette to pipette cells up and down (relatively gently) until they appear as a fine suspension. A few larger clusters of cells may remain. Do not attempt to pipette these into a suspension. Instead, remove them with a pipette and perform a more vigorous pipetting of these cells in a separate microcentrifuge tube.
26) When a fine suspension has been achieved, add 10mL of RPE complete media with 15% serum. Warning – too long a trypsin treatment will kill RPE, so be judicious with trypsinization. If any small clusters of cells were removed to a microcentrifuge tube for more vigorous pipetting, first add RPE complete media with 15% serum to the main conical tube to stop the trypsin, then turn to the microcentrifuge tube (where the trypsin is still active) and pipette that tube. After the microcentrifuge tube is in fine suspension, quench the reaction with RPE complete media with 15% serum and add the suspension back to the main conical tube.
27) Remove any remaining undissolved fragments (which float to the top) that cannot be broken apart, and discard. Use a pipette tip to do this.
28) Spin down the hfRPE suspension at 255xg for 3.5-4 min, remove supernatant, and *gently* resuspend cells in RPE complete media with 15% serum (5mL per eye dissected); it is important to avoid excessive pipetting since the cells are fragile at this stage. Again, remove any floating cell clusters.
29) Aspirate the pre-treated Primaria flask from step (12) and place 5mL of the cell suspension in the flask.
30) Before placing cells into 5% $CO_2$, 37°C incubator, check to be sure the cells are evenly distributed across the flask. Movements of the flask may cause waves that trigger aggregation of the cells towards the center. Consider swirling the flask to ensure the cells are not aggregating towards the center, taking care to avoid having the cell suspension run into the filter cap of the flask during the swirl. After the cells are evenly distributed, carefully place the flask in the incubator, trying not to introduce any other rocking motions during the transfer. Place the flask on top of another plate to help dampen transmission of vibrations from the incubator fan through the incubator shelf and onto the plate; these vibrations cause clumping in the cells as they bind to the flask.
31) During plating, perhaps 30-70% of cells will be individual and 30-70% will be in small clusters, with the largest clusters comprised of ~15-20 cells.
32) After 24-72 h, check to see if the cells are well attached and beginning to flatten out. If so, replace the media with RPE complete media containing 5% serum. If not enough cells have attached after 24 h, media change can be delayed another 1-3 days to allow cells to recuperate in the RPE complete media with 15% serum.
33) Change media in the flask 2-3x weekly (3x for first several weeks, then 2x weekly after). When changing media, turn on the tissue culture hood UV light at least 20 min prior to use, and

thoroughly wipe down cell culture hood surfaces with ethanol. Additionally, use different serologic pipettes and a different glass pipette aspirator for each hfRPE donor (so that cross-contamination with something like mycoplasma is minimized).

34) No earlier than 1 week after splitting, check for mycoplasma contamination using the MycoAlert Plus kit from Lonza, following manufacturer's directions. Collect supernatant for this test only after media has been present on cells for at least 3 days (just prior to media change). If cells are not contaminated, then the flask can be moved from a quarantine incubator to a non-quarantine incubator.

**Human Fetal RPE P0 to P1 Splitting onto Transwells Protocol:**

Transwell Permeable Supports

| Transwell® Insert Format | Transwell Insert diameter | Approximate Growth Area ($cm^2$) | Average Cell Yield | Recommended Volume (mL) | |
|---|---|---|---|---|---|
| | | | | Well | Insert |
| 96 well | 4.26mm | 0.143$cm^2$ | $1.4 \times 10^4$ | 0.235 | 0.075 |
| 24 well | 6.5mm | 0.33$cm^2$ | $3.3 \times 10^4$ | 0.6 | 0.1 |
| 12 well | 12mm | 1.12$cm^2$ | $1.12 \times 10^5$ | 1.5 | 0.5 |
| 6 well | 24mm | 4.67$cm^2$ | $4.67 \times 10^5$ | 2.6 | 1.5 |
| 100mm dish | 75mm | 44$cm^2$ | $4.4 \times 10^6$ | 13.0 | 9.0 |

1) After 4-6 weeks in culture on Primaria flasks, cells should be ready to split and grown on Transwells. Optimal splitting ratio is approximately 1:2.5 - 1:3. Since the Primaria flask has a surface area of 25$cm^2$ and each 24-well Transwell has a surface area of 0.33$cm^2$, a flask can be split into 18 plates of Transwells (there are 12 Transwells per plate) with a small excess of cells, assuming a split ratio of 1:3. In general, if the split ratio is too low, RPE will dedifferentiate into fibroblastic cells. If the split ratio is too high, cell clumping and death will result. Since Transwells are optically semi-opaque, one empty well in the receiver plate (without a Transwell) is also seeded with split RPE to allow for monitoring of the health of the entire group of plates. Since one well in the 24-well receiver plate has a surface area of 1.9$cm^2$, it will require ~6x the number of cells as a Transwell to ensure similar plating density.
2) Coat transwells with human placental extracellular matrix (2.5µg in 50µL HBSS per Transwell of a 24-well plate). Avoid placing Transwells in the 4 corners of the 24-well plate, as evaporation is worst at these locations. To repeatedly add a fixed volume of solution to each of numerous Transwells, utilize the Eppendorf repeater plus pipette with a 2.5mL Combitip for the apical side of the Transwell and a 10mL Combitip for the basolateral side of the Transwell. To ensure the solutions are added without creating backsplash from the force of the stream, a p10 or p20 non-filtered pipette tip should be fitted snuggly onto the end of the Combitip syringe. This smaller pore pipette tip will moderate the flow rate out of the syringe and help prevent backsplash.

   **Note:** Should ECM be unavailable or too expensive, we have tested human fibronectin and found it to be an equivalent alternative (0.5µg in 100µL HBSS or 1ug in 200uL HBSS per Transwell of a 24-well plate). Coat the transwells one day before splitting, then leave plates in the tissue culture hood

at room temperature overnight *without* the UV light on. The next day remove remaining fibronectin solution and pre-incubate with RPE complete media with 5% serum as in step 5 below.

3) Add autoclaved water in between the receiver wells (~0.5mL-0.75mL). This water should NOT be in the receiver wells, but rather between them. The water helps keep the local humidity in the plate high and minimizes evaporation.
4) Place the Transwell plates coated with ECM in a tissue culture hood and cure WITHOUT UV light with the plate lids off for 2 h. To prevent drying out of ECM during this time, turn the hood's blower off. If curing for more than 2 hours, leave a shallow dish of autoclaved water in the hood as well.
5) After 2 h, aspirate the extracellular matrix solution off each Transwell with a glass pipette and then pre-incubate the Transwells for at least 1 h with RPE complete media with 5% serum (both apical and basolateral side). Place 125µL on the apical side and 400µL on the basolateral side. Place plates in incubator so that the basolateral media (which will not be replaced when cells are plated) stays at the appropriate pH.
6) Thaw out 12mL of trypsin per flask. Add 60 units of DNase per mL of trypsin solution. The DNase will help prevent the RPE from clumping together during trypsin digest, since DNA is released from dying cells and creates a viscous web that prevents RPE cells from settling down on the Transwell membrane once they are plated. Place trypsin in a tissue culture dish in the cell culture incubator to allow for pH equilibration.
7) Rinse cells with PBS or another $Ca^{2+}$ and $Mg^{2+}$ free buffer twice by filling flask (~15mL) with buffer, gently swirling on all sides of the flask (without allowing solution to touch the filter top) and removing fluid.
8) Add 4mL warm (37°C) 0.25% trypsin-EDTA with DNase and incubate flask in the incubator, monitoring progress every 10 minutes.
9) Cells should begin to appear more rounded, with about 10% of them curling up and either lifting off or still attached. Only at this point (regardless of how much actual time has passed), use a spinal needle attached to a 3ml syringe to stream-wash attached cells – only irrigate each area once, but be forceful. Do not touch cells with the tip of the needle.
10) Collect cell suspension into a conical tube and add RPE complete media with 15% serum right away (~7mL).
11) If there are still attached cells that cannot be dislodged with the initial stream-wash, add more trypsin to the flask and incubate further, checking cells every 10 min. It can take up to 75 min of trypsin treatment to completely detach the vast majority of cells from the flask bottom. After adding additional trypsin and placing flask into the incubator, proceed to step (12) immediately for the first collected aliquot of cells in the conical tube. For the remaining cells on the flask undergoing a second trypsinization, the spinal needle should be used to stream-wash cells well before all the cells lift off, but not before most of the cells appear rounded up. If not all the cells come off, remove the trypsin solution with the spinal needle, quench the trypsin solution in serum-containing media (as in step (10)), and add a third round of trypsin to the flask. Proceed to step (12) for the second aliquot of collected cells and then stream-wash the flask at the appropriate time to collect the third aliquot of RPE cells.
12) Spin down cells (255xg) in a centrifuge for 3.5-4 min and resuspend in RPE complete media with 15% serum. The final resuspension volume should be 28mL if the intended split ratio is 1:3, 25.7mL if the intended split ratio is 1:2.75, and 23.4mL if the intended split ratio is 1:2.5.
13) Gently pipette the resuspended pellet(s) to achieve a fine suspension. If some larger pieces persist,

carefully remove those into another tube or Eppendorf and pipette the clump of cells aggressively, thereby avoiding damage to the already suspended cells in the main conical tube. After aggressive pipetting of cell clumps, return the suspension to the main tube.

14) Remove the apical “pre-incubation” medium from the Transwell plates but keep the basolateral “pre-incubation” medium. Plate 125µL of cell suspension per Transwell. Media volumes on the apical and basolateral side are calculated to ensure the apical column is always higher than the basolateral column, thereby facilitating attachment of the monolayer to the Transwell through a positive hydrostatic pressure gradient. Finally, plate 700µL onto a single plastic well in the receiver plate that does not contain a Transwell. Avoid plating into one of the four corners of the plate, since these have a higher evaporation rate than other wells.

    **Note:** When plating the cell suspension, cells may clump in the barrel of the repeater pipette if left to sit. To ensure the suspension stays uniform, mix the conical tube frequently.

15) After plating cells, ensure that the cell suspension is not stuck preferentially on one side of the well. If this happens, it will create surface tension that will cause the cells to aggregate away from that side of the well.
16) After plating cells, do not tap or shake the plates. This will cause the cells to aggregate into the center of the plate. If they do happen to aggregate into the center of the plate, gently repipetting the suspension will more evenly redistribute the cells.
17) After plated Transwells are placed in the incubator, any plate touching the incubator shelf will suffer from an uneven redistribution of the cell suspension on the Transwell bottom in a circular wave-like (interference) pattern. This may be caused by the vibration of the incubator fan, which sets up a "standing wave" in the bottom plate and "jiggles" the RPE cells on the bottom plate into concentric clumps. This also happens if newly plated Transwells are left in the hood with the blower on (again, likely due to the vibrations of the blower). To avoid these heterogeneities in plating, place plates outside the incubator on a table immediately after plating. The plates sit on this non-vibrating table for 20 minutes before being placed into the incubator. In the incubator, plates with new cell suspensions are placed on top of old plates, such that the new plates are not directly touching the incubator shelves. This dampens vibrations sufficiently to avoid inhomogeneous plating densities.
18) After 24 – 72 h (depending on cell health), media is replaced with RPE complete media with 5% serum.
19) For all media changes after initial plating, basolateral media is removed first, then apical media is removed, then apical media is replaced (125µL), and finally basolateral media is replaced (575-600µL, NOT the 400µL used during initial plating). This order of media changes is important for preventing hydrostatic pressure from the basolateral compartment triggering a detachment of the monolayer. For all media changes, use the Eppendorf repeater plus pipette against the side of the Transwell rather than the bottom of the Transwell, and with a small-bore pipette tip attached to the end of the repeater pipette syringe. The repeater pipette ejects a forceful fluid stream, and if the pipette is aimed at the bottom instead of the side of the Transwell, the stream will knock cells off the microporous support.
20) Change media 2x per week initially, and after 3-6 weeks (once TEER values have stabilized), media changes can move to 1x per week or remain at 2x per week. When changing media, turn on the UV light in culture hood at least 20 min prior to use, and thoroughly wipe down hood surfaces with

ethanol. Additionally, use different serologic pipettes and a different glass pipette aspirator for each group of hfRPE (so that cross-contamination with something like mycoplasma is minimized).

21) Grow cells for at least two weeks and ideally 4-6 weeks before experiments. The stabilization of TEER is a good indication that the cells are fully mature.
22) Cells are ready to use when their pigment and morphology are native-like and their TEER is > 150 ohms*$cm^2$ [calculated such that TEER of cells only = (experimental well ohms – empty or blank well ohms)*(area of transwell membrane in $cm^2$)].

## Plating on the Basolateral Side of the Transwell Protocol:

For better imaging of cells on the Transwell through an inverted microscope, it can be helpful to plate the cells on the basolateral side of the Transwell. To do so, apply the following modifications to the P0 to P1 splitting protocol above:

1) Flame sterilize forceps and transfer each Transwell upside-down into a well of a 6-well plate (so that the lip of the Transwell sits at the bottom of the 6-well plate). Add sterile water in between the receiver wells of the 6-well plate since small volumes of cell culture suspension are used and evaporation is a significant issue.
2) Coat the basolateral surface rather than the apical surface of the Transwell – use 50µL of human placental extracellular matrix in HBSS and cure with the 6-well receiver plate lid off under UV light in the tissue culture hood with the blower off and sash down.
3) After a 2 h cure time, aspirate the extracellular matrix solution off each membrane, flip the Transwell right side up with forceps and while holding it, place 75µL into the apical chamber. Flip the Transwell upside-down again and place 100µL of media onto the basolateral surface. After this is done for all Transwells on the 6-well plate, place the plate lid on, which will attract the basolateral media to the underside of the lid by capillary action. It is critical that the lid of the 6-well plate remain sterile at all times. Place the Transwells in the incubator and proceed with trypsinizing RPE from the Primaria flask.
4) When ready to plate the RPE cell suspension, each Transwell's basolateral surface should be aspirated and then 100µL of the suspension placed on the surface using a regular pipette rather than an Eppendorf repeater pipette.
5) After dispensing 100µL of cell suspension onto each well, immediately place the 6-well plate lid on the plate. The surface tension of the cell suspension droplet without the plate cover will cause the cells to aggregate into the center of the Transwell and placing the cover on helps evenly distribute this surface tension.
6) Incubate for somewhere between 12 and 24 h. After 12-15 h, check to see if the cell culture media has evaporated. If the incubator has an inner clear door and an outer jacket, try to observe the media's evaporation status without opening the inner door of the incubator; this prevents a drop in incubator humidity. If there appears to be enough media still on top of the well, continue incubating until 24 h. If there appears to be significant evaporation, more media

can be added to each Transwell basolateral side. After 24 h, place 600µL of media into a receiver 24-well plate and then turn each Transwell over into the 24-well plate without aspirating the media on the Transwell's basolateral side. If media is removed, cells may be aspirated off the basolateral Transwell surface, and air bubbles will stick to the bottom of the Transwell when it is placed in the 24-well receiver plate.

7) After the Transwell is placed in the 24-well receiver plate, aspirate the apical side of the Transwell and replace with 100µL of media.
8) For all media changes of the "upside-down" RPE cultures, use 100µL in the apical chamber and 600µL in the basolateral chamber to ensure proper hydrostatic pressure.

If cultures aren't working, think about a bad batch of serum or mycoplasma contamination. The following is a picture of a mixed culture in which fibroblasts have overtaken the culture due to poor culture conditions or to contamination during dissection: