Fibroblast and keratinocyte routine cell culture

Materials

- DMEM Media (fibroblasts) – Gibco DMEM Media (standard, +4ºC stock room) + 10% Bovine Serum (Watt Lab TC, +4ºC fridge).
- Versene (Gibco, Watt Lab TC, at RT next to centrifuge)
- 0.25% Trypsin-EDTA (Watt Lab TC, -20ºC freezer, bottom shelf)
- PBS (Gibco, stock room)
- Mitomycin C (Watt Lab TC, -20ºC freezer, middle shelf)
- FAD Media (keratinocytes) – (K)FBS (Watt Lab TC, +4ºC fridge), L-Glutamine & Pen/Strep (Watt Lab TC, -20ºC freezer, bottom shelf), HCE (Hydrocortisone, Cholera enterotoxin, and EGF) & Insulin (Watt Lab TC, -20ºC freezer, middle shelf), and $CaCl_2$ (Watt Lab TC, RT top shelf, next to DMSO).

1. Fibroblast (J2 3T3, feeder cells) preparation for keratinocyte seeding (from thaw)

1- Thaw the fibroblasts two weeks before starting the keratinocyte cultures (as described in the "Thaw and freeze cell lines" doc). Fibroblasts cultures are usually passaged weekly, and media is changed every second or third day. Once confluence is attained (that is, after approx. a week), passage as described:
   1.1- Rinse fibroblasts with PBS x1 and trypsinize diluting 1:4 0.25% trypsin-EDTA in versene.
   1.2- Incubate for 5 min (approx., usually less is enough) at 37ºC.
   1.3- Inactivate trypsin with DMEM+BS (*e.g.* if 5 mL of trypsin-EDTA/versene have been used the same volume of media will be used to inactivate it).
   1.4- Harvest cells and transfer to a Falcon for centrifugation. Usually, 1000 rpm for 3 min is enough to have a good pellet.
   1.5- Aspirate the supernatant, resuspend and count cells.
   1.6- J2 3T3s (stock) are passaged at a cell density of $1x10^5$ cells/T75 flask. After approx. a week the fibroblasts will be confluent and therefore ready for splitting (stock) or keratinocyte seeding (feeders).
2- The day before to keratinocyte thaw and seeding, treat confluent flasks of feeder cells (already in the second passage after thaw), with mitomycin C (0.4µg/mL final concentration, that is, 100 µL for a T75 or 230 µL for a T175), for 2-3 hours.
3- Wash x3 with PBS mitomycin C-treated fibroblasts, trypsinize, harvest, resuspend as described before, and count. Seed J2s at a cell density of $2.5x10^6$cells/T75 or $6x10^6$cells/T175 and let settling down over night.

2. Keratinocyte seeding on mitomycin C-treated fibroblasts (from thaw)

1- Wash x1 using DMEM+BS the mitomycin C-treated fibroblasts before keratinocyte seeding.
2- Thaw keratinocytes (as described in the "Thaw and freeze cell lines" doc), and seed onto the fibroblasts using FAD media.
3- Keratinocyte cultures are monitored every day and media is changed every second day. After 7-10 days of seeding, keratinocytes will be ~90-95% confluent and ready for passage/experiment use.

3. Keratinocyte passage

1- When confluence is attained keratinocytes will be passaged using a previously prepared fibroblast/feeder flask the day before.
2- Rinse with versene x3 (5 minutes each time, and incubating at 37ºC), and gently tap to selectively detach the remaining fibroblasts present in the co-culture.
3- Trypsinize the remaining keratinocytes using 0.05% trypsin-EDTA/versene (1:4 dilution of 0.25% of trypsin-EDTA in versene), for 3-5 min at 37ºC. **Beware not to overtrypsinze the keratinocytes!**
4- Tap profusely to detach the keratinocyte colonies from the flask and swiftly inactivate trypsin using FAD media.
5- Harvest, centrifuge, and resuspend keratinocytes as described previously. Count keratinocytes and seed onto previously mitomycin C-treated and washed feeder flasks at a cell density of $1x10^5$cells/T75 flask or $2.5x10^5$cells/T175 flask. Keratinocyte cultures will be ready again to be split/passage in 7-10 days and therefore for the next round of experiments.

Troubleshooting and additional information about cell lines

- Fibroblasts are used between passages 2 and 12, after passage 12 their proliferation becomes too high and not adequate for fine culture of keratinocytes.
- Keratinocytes are used between cultures 2 and 8, after passage 8 keratinocyte colonies start to display stochastic behaviours that preclude their use for experiments (*e.g.* for clonal growth assays).
- It is advisable to filter supplemented medias (both DMEM and FAD) and aliquot in 50 mL Falcons to reduce the risk of potential widespread contamination of cultures.
- It is advisable to maintain a stock of fibroblasts running being passaged every week, from which the next round of feeder fibroblasts (that is, the ones that will be treated with mitomycin C), will be seeded on the same day of keratinocyte seeding. Using this method confluence of feeder cells will coincide with confluence of keratinocytes cultures helping not to run out of feeders **(which can happen, often when several batches a week of keratinocytes are being used)**. In parallel, the stock flask of fibroblasts will be used for the seeding of feeders for the next week and to perpetuate a pool of stock (non-feeder) fibroblasts.
- Refer to **Mr. Simon Broad** for advice with fibroblast/keratinocyte cell culture and troubleshooting. Culture of such cell lines is often laborious and requires a period of adaptation that other cell lines do not need. It is advisable to invest three to four weeks to solely master the routine culture of both cell lines and its timing and co-culture.

References

Watt, F. M., & Prowse, D. M. (2006). Cultivation and retroviral infection of human epidermal keratinocytes. In *Cell Biology (Third Edition)* (pp. 133-138).

Thaw and freeze cells lines

1. Thaw cells

1- Take the vial from liquid nitrogen storage and warm it up at 37ºC.
2- Thaw the vial up to the point where there is only some frozen liquid (i.e. most of the solution is liquid but some ice remains).
3- **(Inside the hood)** Bring to 10 mL of adequate media specific for the cell line and pipette up and down gently until all the volume of the vial has been collected.
4- Transfer the volume to a 15 mL Falcon tube and centrifuge. Usually, 1000 rpm for 3 min is enough for most cell lines.
5- Aspirate the supernatant with due care to avoid aspirating the pellet.
6- Resuspend the pellet pipetting up and down gently and bring to desired volume. Seed the cells.

2. Freeze cells

1- Harvest cells that will be frozen and centrifuge (as described above).
2- Resuspend cells in 10% DMSO in adequate serum for the specific cell line (e.g. for keratinocytes (K)FBS serum would be used to dilute the DMSO).
3- Take 1 mL of the cell solution, transfer it to a freezing vial, label properly and introduce into a previously thawed (at RT) Mr. Frosty (check level of isopropyl alcohol).
4- Store at -80ºC over night.
5- The next day bring vial to liquid nitrogen storage.

Troubleshooting

Step 1.4. If the cell line is quite sensitive to alterations reducing the centrifugation speed (*e.g.* to 950-900 rpm) is advisable.

Step 1.6. As a rule of thumb, and taking into consideration that a proportion of cells are going to die due to the process of thaw, splitting into 2 or 4 flasks is advisable.

Step 2.3. Usually, a reasonable cell density to freeze is around $1x10^6$ cells/mL (that is, per vial).

Clonal growth assays of keratinocytes

1. Fibroblast preparation

1- The day before to keratinocyte seeding treat fibroblasts (feeder cells) with mitomycin C (final concentration 0.4 µg/mL, standard procedure) for 2 to 3 hours.
2- Wash x3 the flasks with mitomycin C-treated fibroblasts with PBS, trypsinize, and harvest cells (as described in the "Fibroblasts and keratinocyte cell culture" doc).
3- Seed $4x10^5$ cells/well of 6-well plate and let them settle down and spread over night.

2. Keratinocyte seeding

1- Wash x1 with DMEM+10%BS (feeder media) the feeders prepared the day before.
2- Harvest confluent keratinocytes (as described in the "Fibroblasts and keratinocyte cell culture" doc), and seed 500 keratinocytes per 6-well plate well.
3- Spread them properly doing the characteristic cell culture "cross". This helps to ensure that the colonies will be evenly spread within the well which will eventually help to count colonies and measure the colony formation efficiency (CFE, %) and area.

3. Keratinocyte treatment

1- After seeding, change the media every second day until the 6^{th} day of growth.
2- The 6^{th} day, change the media as usual adding the correspondent treatments to each well.
3- Keep changing the media, every second day, and treating the cultures until the 14^{th} day of the experiment.

4. Fix and stain keratinocyte colonies

1- Wash out gently, pipetting up and down, the remaining fibroblasts from the wells. Usually a couple of times are enough, however, make sure to remove all the fibroblasts from the well.
2- Fix keratinocytes colonies with 4% PFA for 10 minutes at RT.
3- After incubation time, remove the PFA (collect and dispose PFA appropriately, do not discard into the sink) and wash x3 with PBS the colonies.
4- Stain the colonies using 1% Rhodanile Blue (1:1 Rhodamine B and Nile Blue A in Milli-Q water – Acros Organics) for 30 minutes in the rocker.
5- Remove the excess of Rhodanile Blue using tap water (gently but thoroughly washing the wells) and let dry over night.
6- Take pictures of the whole plates using the Molecular Imager® Gel Doc™ XR+ System (Bio-Rad) in the gel electrophoresis room of the main lab.
7- CFE can be calculated following the procedure of Walko et al. (2017). Colony area can be measured using the software ImageJ/Fiji and the tool "Analyze particles".

Troubleshooting

Step 3. For instance, if keratinocytes are seeded on Monday, change media (only media) on Wednesday, and Friday; on Sunday the treatment will start. Then, media with the correspondent treatment will be added on days; Sunday, Tuesday, Thursday, and Saturday of the next week. The very next Monday (that is, two weeks into the experiment), cells can be fixed and stained.

Step 4.1. The characteristic roundish shape of keratinocyte colonies helps to differentiate them from fibroblast clusters still remaining in the well. Wash gently, but thoroughly, keratinocyte colonies will not detach as they display much stronger adhesion properties than fibroblasts.

Step 4.6. Is handier to take the images with the plates upside down and with the white background that is stored in the drawer below the imager. Refer to **Matteo Vietri** for assistance with the whole procedure.

References

Walko, G. *et al.* A genome-wide screen identifies YAP/WBP2 interplay conferring growth advantage on human epidermal stem cells. *Nat. Commun.* **8**, 14744 (2017).

Micropatterning

Necessary reagents to bring to the 17th Floor **(Contact: Ciro Chiappini) – INDUCTION required**:

PLL-g-PEG, glass slide (specific, refer to **Blaise Louis** or **Alice Vickers** for reference), micropipette and tips and parafilm.

Plasma activation of glass

1- Switch on the plasma oven (with pump-OFF and ventilation-ON). Introduce glass support in the oven and close (hold the glass door, until negative pressure is established in the oven). Turn OFF ventilation and ON the pump. **Wait for the pressure to drop.**
2- **Once the green indicator is visible** introduce 4 minutes (GREEN) in the timer and turn (LEFT) the O_2 flow **(really slow)** until the pressure reaches **0.4**-0.6mbars. Then, switch ON the generator.
3- **Once it has finished to run,** close the O_2 flow (RIGHT), switch OFF the pump and ON the ventilation **(beware with the glass door, it will detach due to positive pressure inside the oven)**. Open the oven and extract the glass support.
4- Place the glass (do not touch the upper side) **upside down** onto a parafilm with several -scattered- drops of PLL-g-PEG.
5- Let it curate at 37°C for **two** days in the incubator.

***In order to close the plasma oven**, make sure that the O_2/Air valves are closed (RIGHT) and that the door is attached to the oven (that is, by negative pressure; so, switch OFF the ventilation and ON the pump and only then switch off the actual oven).

UV oven (chemistry room, 28th Floor)

After incubation:

1- Rinse the glass slide and mask with water. Dry well (with soft tissues) the mask and place the glass slide upside down (PLL-g-PEG facing down) onto the mask and make sure that you remove all the bubbles between the mask and the glass. ***The glass is placed upside down in the gold face of the mask.**
2- Place the mask (upside down, that is, silver face facing up) and switch on the UV oven. Incubate for 35 to 40 min. ***Make sure that the hood is closed (window) and the ventilation running -<u>the UV oven produces O_3</u>-.**
3- Take the glass from the UV oven and cover it with tissues in a box.
4- Use glue to paste the glass to the bottom of a 96-well plate (***mix well the glue – Loctite**).
5- Paste and incubate for 48h (minimum) at 45°C in one of the ovens.

Collagen-coating and seeding of cells (96-well plate format)

1- Add ~150 µl of 20µg/ml rat tail collagen type I (calculate appropriate concentration each time from specific stock) and incubate at 4°C o/n.
2- After the incubation period, remove the solution and rinse x3 the well using PBS.
3- Remove all the volume and do a final washing step with PBS (again, with 150µl).
4- **Keratinocytes.** Trypsinize, harvest, centrifuge (as described in the "Fibroblast and keratinocyte cell culture" doc) and resuspend **in FAD media.**
5- **Alice's pattern (1mm).** Around 60.000 **(OPTIMIZE)** cells in 100-200 µl are used per 96-well plate well (use 40µm strainer if necessary to make sure that we are plating single cells).
6- Incubate for 2-3h at 37°C and once the cells are attached to the substrate change FAD media to remove non-attached cells.

7- Incubate for **24** to 48h.
8- Fix the cells using 70% EtOH and stain as desired.

Troubleshooting

- Please, refer to Alice Vickers (1 mm micropatterns) or to Blaise Louis (20-50 µm micropatterns) for troubleshooting about this assay.

PDMS substrates

1. Polymerization

1- Mix the PDMS precursor with the curing agent (SYLGARD™ 184 Silicone Elastomer Kit, Dow Corning; chemistry room, drawer below the left hand side oven). Usually for 4-5 sets of 9 different small micropatterned moulds 5 mL of the precursor and 500 µL of the curing agent are enough. **Mix appropriately both reactives.**
2- Centrifuge the Falcon for 1 minute at 3000 rpm to get rid of bubbles.
3- Evenly pour onto the moulds avoiding bubbles.
4- Leave over night at room temperature to polymerize and dry out.
5- Finally, the next morning incubate them at 80ºC in the chemistry room ovens for 3 hours.
6- Store covered at room temperature upside down (to avoid dust accumulating on the substrates) and use within 2 to 3 weeks.

2. Seeding

1- Introduce PDMS already polymerized into a 12-well plate well. As long as they are packed in a big unit of the 9 subunits they will stick to the bottom. If single subunits want to be used it is advisable to use a drop of silicone (without the curing agent) to stick them to the bottom of the well.
2- Sterilize with 70% ethanol for 2-5 min.
3- Wash with PBS (x3).
4- Coat with collagen. Measure appropriate volume of rat tail type I collagen (Watt Lab, +4ºC fridge, usually top shelf; depending on the specific density of the batch). Usually, around 20 µg/mL in PBS will be enough.
5- Incubate for 2h at 37^{o}C.
6- Wash with PBS (x3).
7- Plate cells with a clonal density of around 50.000cells/well for 12-well plates, usually around 1.5ml of cell suspension are needed to cover the whole substrate (the same volume is used for sterilizing and washing).
8- Incubate as desired (from 1h for single cells to over weekend for full coverage of the substrate) and fix.

3. Cell fixation

1- Remove the media from the wells.
2- Using 4% PFA cover completely the sample and incubate at RT for 10 minutes.
3- Wash with PBS (x3).
4- Store at RT with PBS.

Troubleshooting

Step 1. For more information about the moulds and the process of polymerization refer to **Dr. Atefeh Mobasseri** or the original article from Priya *et al.* (2016).

Step 2.7. These cell densities are indicative and further optimization of cell density will be required depending on the cell line, the incubation period, and the plate and number of substrates used.

References

Viswanathan, P. e*t al.* Mimicking the topography of the epidermal–dermal interface with elastomer substrates. *Int. Biol.* **8**, 21-29 (2016).

Spin-coated PDMS substrates of different stiffness

Source of the spin-coated substrates: Laboratory of Dr. Thomas Iskratsch, Queen Mary University University of London – School of Engineering and Materials Science.

Contact: Pragati Pandey & Matthew Ward.

For assistance with the protocol refer to Miss Blaise Louis (sulfo-SANPAH) and Chris Aldrich (buffers and other reactives).

1- Place the coverslips in a 6-well plate and cover with 0.2 mg/mL of sulfo-SANPAH (Blaise's freezer in the main lab) solution (sulfo-SANPAH can be diluted in MilliQ water).
2- Place the coverslips and the 6-well plate inside the UV oven of Rashid's laboratory (Refer to Soon Seng for help with the oven), and irradiate for 5 minutes.
3- Remove the sulfo-SANPAH solution and wash x1 with 50 mM of HEPES in PBS (Chris's buffer), and repeat the coating with the sulfo-SANPAH solution a second time in the oven.
4- Wash x2 with 50 mM HEPES in PBS and coat with 20 µg/mL of rat tail collagen type I (Watt Lab TC, +4ºC fridge) in PBS for ~3 hours at RT.
5- Wash x3 with PBS and seed around 200.000 cells (*e.g.* keratinocytes) onto the substrates in each well.
6- Fix the cells after 24 hours:
   6.1 Using warm PBS wash the cultures 2-3 times.
   6.2 Fix the cells using 70% EtOH for 10 min at RT.
   6.3 Wash again using PBS x3 and store in the fridge for subsequent staining.

*__For further assistance__ with the protocol, please, refer to Chris Aldrich. This experiment was only performed one time by me and several points (for example, the cell density) may be subject to further optimization.

Immunofluorescence (IF)

Materials

- 0.5% Tritton x100 (Main lab, chemical section, to shelf); usually, 250 µl of Tritton and 50 mL of PBS. Shake firmly and put in rocker for 15-30 min. Store at +4ºC.
- 3% BSA (main lab, common stock in -80ºC room, +4ºC fridge); dilute 0.3 gr of BSA in 10 mL of PBS and mix properly. Aliquot and store at -20ºC.
- Primary antibodies are diluted in 3% BSA, however, concentrations for each antibody should be optimized. As a rule of thumb, 1:100 dilution is a good starting point.
- For secondary antibodies, although there is no standard rule, a good starting point is 1:200 dilution.

Protocol

1- Vortex and spin down (~1 min/max rpm) the primary antibodies and pipette the adequate volume from the supernatant.
2- Remove the PBS from the sample and wash x1 with PBS.
3- Add tritton x100 to permeabilize the fixed cells for 5 minutes **(sharp)**.
4- Wash x5 with PBS the samples to remove the remaining tritton x100.
5- Put the 1ª antibody solution (in 3% BSA) on the sample and incubate for 30 minutes at RT. **Avoid bubbles!**
6- After incubating remove the excess of 1ª antibody solution and rinse the sample with PBS x3 (fast). Additionally, rinse x3 more with PBS incubating at RT 5 minutes each time the sample.
7- Put the 2ª antibody solution (2ª antibodies are usually found as a common stock in the -80ºC room at +4ºC), DAPI or any other chemical dyes (*e.g.* phalloidin). Incubate for 30 min at RT.
8- Finally, wash x3 with PBS (fast), and x3 more times incubating 5 min at RT each time.
9- Image directly from the well or mount the coverslips onto slides using mounting media (main lab, top shelf at RT).

Troubleshooting

- Refer to **Sebastiaan Zijl** (Watt Lab) for information about antibodies, and antibodies themselves.
- If coverslips are being used for IF, usually 100 µl volumes in each step are enough to stain them. The procedure involves incubating the cells upside-down in a drop of the 1ª or 2ª antibodies (is handier to use some parafilm to help the drop to stand still).