PROTOCOL FOR 3D ORGANOTYPIC CELL CULTURE

(with primary keratinocytes and primary fibroblasts from normal human oral mucosa tissue or with cancer cell lines)

Preparations:

Be sure you have:

- FAD medium
- PBS
- culture medium for isolating fibroblasts: DMEM medium (Sigma) + 10% FBS (InVitrogen) + 1% AB/AM InVitrogen),
- culture medium for keratinocytes: KSFM medium (InVitrogen) +1 ng/ml EGF human recombinant (InVitrogen) + 25 μg/ml bovine pituitary extract (InVitrogen) + 1% AB/AM (InVitrogen)
- Trypsin 1x (Sigma cat. no. T 4049) or TrypLE™ Express Stable Trypsin Replacement Enzyme (from InVitrogen cat. no. 12604-013)

- DMEM 10x (Sigma Cat. No. D 2554) – now they do not prepare it anymore, so I use our routine DMEM with high glucose
- FBS (InVitrogen)
- Collagen type I (from BD Biosciences cat. no. 35 4236)
- sterile RB (reconstitution buffer): 2.2.g $NaHCo_3$ + 0.6g NaOH + 4.766 HEPES in 100 ml dH_2O. Filter after preparation.

- 24 wells plates
- 6 well plates
- For best normal 3D I use Culture dishes: centre-well organotypic culture dish (Falcon, Becton Dickinson, cat. No. 35 3037)
- Lens paper (Special lens-cleaning tissue - Leica)
- Sterile Metal bent spatula for lifting the tissues
- Sterile metal grids
- Sterile tissue forceps (delicate).
- 10 ml plastic pipettes.
- 15 ml centrifuge tubes (Nunc)
- 50 ml centrifuge tubes (Nunc)

- ice

1st day
day 0 of co-culture
Prepare biomatrix *! prepare it on ice !*

Calculate how much of each component you need by using the ratio:
- collagen:DMEM :RB(reconstitution buffer):FBS(+Fibroblasts) =7vol:1vol:1vol:1vol

For 2 ml collagen matrix add and mix in a 50 ml centrifuge tube in the following order:
- 1.4 ml collagen type I
- 0.2 ml DMEM
- 0.2 ml reconstitution buffer
- 0.2 ml FCS with fibroblasts (with 0.25 mil Fibs/ml matrix)

- bring ice
- check if you have all reagents (if RB is prepared!)
- trypsinise the fibroblasts, count and resuspend them in FBS at a conc. of approx. 10 mil cells/ml
- put on ice the collagen, RB and DMEM + some pipettes
- mix in a 50ml centrifuge tube the collagen, with DMEM and RB. *Be careful not to get air bubbles!*
- check the pH of the matrix , to be around 7.2-7.4 (take with a Pasteur pipette a small quantity of the matrix and lay it on a pH meter paper)
- add the FBS + fibroblasts to the matrix at the end so the no of fibs will be 0.25-0.5 mil/ml biomatrix (for human fibs use 0.25 mil cells /ml)
- place 700 µl of the matrix prepared in each well (center well of Falcon organoypic culture dish or 24 well dish) by reverse pipetting in order not to get air bubbles.
- let it 20 min/1h to gellify in the incubator.
- place in the top 1ml DMEM medium with 10% FCS (routine media for fibroblasts).

2nd day
day 1 of co-culture
Seeding of keratinocytes
- trypsinise the primary normal keratinocytes and resuspend them in KSFM at a conc. of 0.25-0.5 mil cells/ml KSFM (cell lines may be resuspended in their routine growth medium and at lower concentration)
- suck off the DMEM medium from top of the gels from the day before (all!).
- place 0.5 mil keratinocytes in 1 ml KSFM medium on the top of each collagen matrix.
- leave it in the incubator overnight for plating.

3rd day
day 2 of co-culture
Release the matrix:
- release the matrix from the walls with a help of a bent spatula

Transfer gently the matrix on the metal grid and lift the tissue (this can be done also at day 4-5):
-deep the greed in OT medium in a 6 cm diameter cell culture dish;
-embeed a square (1-1.5 cm^2) of lens paper in OT medium;
-lay it on the metal greed and place the metal greed in a well of a 6 well plate if you use that.
-lay the OT culture on the top of the lens paper with a help of bent spatula and tweezers;
- drain out the medium from the top of the culture by tilting the metal grid and with a help of a delicate tweezers' tip
-remove ~700 µl of the conditioned media from the centre well dish if you use that
-add ~3 ml of fresh OT medium;

-add with a quick movement the metal grid with the OT culture in top.
-check if there are air bubbles under the grid.
-repeat the procedure till you do not have any air bubbles under the grid.

5th day
day 4 of co-culture
-remove the metal grid and put it on the cover of the dish if you used center well, otherwise just change the medium by tilting the 6 well plate
-change 2-2.5 ml of conditioned medium with new OT medium.
-place the greed back in the position;
-check if there are air bubbles under the greed;
-repeat the procedure till you do not have any air bubbles under the greed.

7th day
day 6 of co-culture
-repeat the procedure of day 4 of co-culture (5th day)

9th day
day 7 of co-culture
-repeat the procedure of day 6 of co-culture (7th day)

11th day
day 10 of co-culture
Harvest the tissue:
-remove with a forceps the OT culture together with the lens paper from the grid;
-place it on the cover of the dish;
-cut the OT culture in 2 pieces with a scalpel;
-place 1 half in 4% buffered formalin overnight for ½h
-place the other half in TissueTek and freeze it down in liquid nitrogen. Store it at –70°C.
-pay much attention to the way you orientate the tissue while freezing. You can first freeze the tissue on a small plastic surface with a drop of TissueTek and then orientate the tissue on the bolk in more Tissue Tek and freeze it down.

Process the fixed half:
- 10min wash in PBS after formalin
- 1h in alcohol 70°, -1h in alcohol 96°, -1h in alcohol 100°
-1h in xylen
-3h in parafin in the oven at 56° C
-embeed in paraffin block: attention to orientate it properly!