

Organotypic 3D culture (OTC) protocol

Fibroblasts: FEF3, fetal human esophageal fibroblasts (a standard fibroblast line) and other fibroblast lines (adult/dermal/CAF, etc.)

Making a master frozen stock

1. For the first time, grow FEF3 (p5-p6) in two 150-mm² dishes until they reach 100% confluent status and undergo contact inhibition in DMEM-10% FBS-P/S.
2. Rinse twice with DPBS and then trypsinize at 37°C for a few minutes.
3. Suspend cells in 10-12 ml DMEM-10% FBS-P/S.
4. Pellet cells by centrifugation at 1,000 rpm for 3 min at 4°C (or room temperature).
5. Make 30-50 frozen vials (1x10⁶ cells/vial) for the first time and store in liquid nitrogen. They will serve personal-lab frozen stocks for FEF3.

Fibroblast Culture Media: DMEM-10% FBS-1% P/S

1. DMEM: Dulbecco's Modified Eagle Medium (DMEM)(1X) liquid (high glucose) without Pyruvate: **DMEM (-)**(Gibco #11965-084)(4°C)
2. FBS: fetal bovine serum (Hyclone from Cell Center)(-20°C)
3. P/S: 10,000 µg/ml of streptomycin sulfate)(Gibco-BRL: Cat. #15140-122)(-20°C)

Add 100 ml of FBS and 10 ml of P/S into 1000 ml of DMEM (-).
Store at 4°C.

Epithelial cells: EPC1/EPC2-hTERT and derivatives, ESCC cell lines

Grow cells according to a regular cell culture protocol for the cell lines to be used.

For EPC1/2-hTERT derivatives, see [Protocol for human esophageal epithelial cell culture](http://www.med.upenn.edu/molecular/core_culture.shtml). (This is available at http://www.med.upenn.edu/molecular/core_culture.shtml)

For ESCC cell lines, use DMEM-10% FBS-1% P/S.

Note: Epithelial cells will be seeded 4-7 days after matrix formation.

Matrix and EP media components

1. **DMEM (-)**(Gibco #11965-084)(4°C)
2. Ham's F12 (Gibco #11765-054 or Mediatech 10-080-CV)(4°C)
3. 10x EMEM (BioWhittaker #12-684F)(room temperature)
4. 7.5% Na-Bicarbonate (BioWhittaker #17-613E)(room temperature)
5. FBS (Hyclone from Cell Center)(stored as an ~10 ml aliquot, -20°C)
6. L-Glutamine (Cellgro #25-005CI)(-20°C)
7. Collagen (Organogenesis #200/50)(Stored as ~50 ml aliquots at 4°C)
8. Matrigel (BD Biosciences #354234)(-20°C)
9. Hydrocortisone (Sigma #H0888): MW = 362.46
 - Dissolve 0.0269 g in 2.5 ml EtOH
 - Add into 97.5 ml DMEM (-): 0.74 mM
 - Filter-sterilize, dispense into aliquots and store at -20°C.
10. ITES (BioWhittaker #17839Z)(-20°C)
11. O-phosphorylethanolamine (Sigma #P0503) MW = 141.06
 - Dissolve 0.705 g in 100 ml DMEM (-): 50 mM
 - Filter-sterilize, dispense into aliquots and store at -20°C.
12. Adenine (Sigma #A9795) MW = 171.59
 - Dissolve 1.55 g in 100 ml warm (37°C) ddH₂O: 0.09 M
 - Filter-sterilize, dispense into aliquots and store at -20°C.
13. Progesterone (Sigma #P8783) MW = 314.46
 - Dissolve 1 mg in 1 ml ETOH
 - Add 14.7 ml ddH₂O.
 - Dilute 1ml of that in 100ml DMEM (-): 2.0 µM
 - Filter-sterilize, dispense into aliquots and store at -20°C.
14. Triiodothyronine (Sigma #T5516) MW = 672.96
 - Dissolve 1 mg in 1 ml 1N NaOH.
 - Add 19 ml of DMEM (-).
 - Dilute 4 µl of that in 31 ml plain DMEM (-): 1 nM
 - Filter-sterilize, dispense into aliquots and store at -20°C.
15. Newborn Calf Serum (Hyclone #SH 3011802)
16. Gentamycin, 50 µg/ml (Cellgro #MT30-00050CR)

Plates/Inserts

1. Plates: 6-well Transwell Carrier (Organogenesis, TS01-001)
Organogenesis, Inc. 150 Dan Road, Canton, MA 02021. Telephone: +1 (781) 575-0775
2. Corning Costar Transwell 3414 (24 mm diameter inserts, 3.0 μm pore size)(6 per plate)

OTC matrix (stromal compartment) preparation

Start growing FEF3 cells (**a week ahead**)

1. Thaw a frozen vial (passage # p6-p7), seed directly into a 150-mm² dish.
2. Grow until they reach 80% confluent in DMEM-10% FBS-P/S.
3. Use for OTC matrix preparation by p8-p9. Avoid using cells at p10 or after.

The day before matrix preparation: Thaw Matrigel overnight (one vial for two OTC plates) at 4°C. Avoid placing the vial too close to the door of a refrigerator as temperature may rise beyond 4°C when people open the door.

Day 1

To do 15-30 min prior to making matrix:

1. Thaw FBS (Hyclone) and L-Glutamine, clean the tube with 70% EtOH, and place on ice.
2. Place tubes containing 10X EMEM and 7.5% Na-bicarbonate on ice.
3. Place Matrigel and type I collagen on ice (40 ml will be needed for two OTC plates).
4. Pre-chill empty 50-ml tubes on ice for **Acellular layer (Label "A")**(one tube/1-4 OTC plates) and **cellular layer (Label "C")**(one tube per 1 OTC plate or two).

Label OTC plates (IMPORTANT) to **make an orientation**, label a corner or two on both lid and the bottom of OTC plates (6-well Transwell Carrier) inside the tissue culture hood with a lab marker (permanent, quick-drying, alcohol-resistant ink).

Place inserts (Corning Costar Transwell 3414) into each well (6 per plate) inside the tissue culture hood. To hold inserts, wear gloves and wipe fingers with paper towel wet with 70% EtOH.

Making acellular layer:

1. Add 10x EMEM, FBS, L-Glutamine, N-bicarbonate and type I collagen in this order (see Table below for the volume needed).
2. Mix gently using a 25-ml pipette to **avoid making bubbles** in a 50-ml tube on ice.
3. Pour 1 ml per insert using a 10-ml pipette.
4. Leave still inside the tissue culture hood while preparing fibroblasts and cellular layer.

acellular layer	1 plate	2 plates
10x EMEM	690 μl (x 1)	690 μl (x 2)
FBS	700 μl (x 1)	700 μl (x 2)
L-Glutamine	60 μl (x 1)	120 μl (x 1)
Na-bicarbonate	140 μl (x 1)	280 μl (x 1)
Type I collagen	5.6 ml (x 1)	11.2 ml (x 1)

x2, Take twice using P-1000 Pipetman

Prepare 6×10^5 /ml Fibroblast cell suspension:

1. Trypsinize, pellet, and resuspend in 0.4 ml DMEM-10% FBS-1% P/S.
2. Take 10 μ l to count with coulter counter (1000x dilution) and follow the example below:
3. If the count was 12,000, the actual cell conc. = $12\text{M/ml} = 120 \times 10^5/\text{ml}$ (and total #: 4.8M).
4. To prepare 8 ml of $6 \times 10^5/\text{ml}$ fibroblast cell suspension (this is good for up to 5 plates), use the following formula: $X \text{ (ml)} = (6 \times 10^5 \times 8) / (120 \times 10^5) = 6 \times 8 / 120 = 0.4 \text{ ml}$.
5. Add 0.4 ml of the cell suspension into 7.6 ml of DMEM-10% FBS-1% P/S.

Making cellular layer:

1. Add into a 50-ml tube on ice 10x EMEM, FBS, L-Glutamine, N-bicarbonate, type I collagen, Matrigel and fibroblasts in this order (Follow the Table below).
2. Mix gently using a 25-ml pipette to **avoid making bubbles**.
3. Pour 3 ml per insert using a 10-ml pipette.
4. Incubate for 30-45 min in the tissue culture incubator (37°C, 5% CO₂).
5. Add DMEM-10% FBS-1% P/S: 10 ml into the bottom of the wells, 2 ml into the insert.

cellular layer	1 plate	2 plates
10x EMEM	1.8 ml (x 1)	3.6 ml (x 1)
FBS	2 ml (x 1)	4 ml (x 1)
L-Glutamine	160 μ l (x 1)	320 μ l (x 1)
Na-bicarbonate	380 μ l (x 1)	760 μ l (x 1)
Type I collagen	11.4 ml (x 1)	11.4 ml (x 2)
Matrigel	3.8 ml (x 1)	7.6 ml (x 1)
$6 \times 10^5/\text{ml}$ fibroblasts*	1.6 ml (x 1)	3.2 ml (x 1)

x2, Take twice using P-1000 Pipetman.

Day 2 – Dislodge matrix and add 2 ml of DMEM-10% FBS-1% P/S into the insert.

Use a sterile glass Pasteur pipette to go around (2-3 times) the matrix along with the inner wall of the insert. Feel the friction at the tip on the transwell membrane, but try not to pierce it.

Watch the matrix getting contracted over the next few days. No need to change medium.

Wait **at least 4 days** before seeding epithelial cells.

Day 5 or later: Seeding epithelial cells

Grow epithelial cells (EPC2, EPC1/EPC2-hTERT, EPC2-hTERT derivatives, cancer cell lines, etc.) according to pertinent culture protocol for each cell line. A half million (5×10^5) of epithelial cells are seeded per insert/well. Prepare 3 million for 6 wells, 6 million for 12 wells (i.e. 2 plates). For example, ~80% confluent EPC2-hTERT cell derivatives grown in a 75-cm² flask yields about 4 million cells.

1. Make DMEM (-)/F12 (3:1)(See table below) to pre-saturate the OTC matrix.

DMEM/F12 Media (3:1)	2 Plate (144 ml+)
DMEM (-)	120 ml
Ham's F12	40 ml

2. Remove old medium from the OTC plates/inserts.
3. Add DMEM+F-12 (3:1) – add 10 ml to the bottom well and 2 ml into the insert.
4. Incubate for **1 hr** to equilibrate the matrix.
5. Trypsinize and count epithelial cells to be seeded.
6. Make 1×10^7 /ml epithelial cell suspension in the regular medium used to grow epithelial cells (e.g. KSFM for EPC2-hTERT).
7. Remove DMEM+F-12 (3:1).
8. Seed epithelial cells by applying 50 μ l (5×10^5) of cell suspension per well onto the center of the surface of contracted matrix.
9. Incubate for **2 hrs** in the tissue culture incubator without medium.
10. Make EP2 (see table below). Store at 4°C. This will be used also for Day 6.
11. Add EP2 to plate - 10 ml into the bottom well and 2 ml into the insert.

For two plates	EP2 (300ml)/4°C 24 ml/well, 72 ml/plate for 2 plates
DMEM (-)	218 ml
F12	72 ml
L-Glutamine (LQ)	6 ml
Hydrocortisone (H)	600 μ l
ITES	600 μ l
O-phosphorylethanolamine (O)	600 μ l
Adenine (A)	600 μ l
Progesterone (P)	600 μ l
Triiodothyronine (T)	600 μ l
Newborn Calf Serum (NBCS)	300 μ l
Gentamycin	300 μ l

Day 7 (or 2 days after seeding epithelial cells): Medium change with EP2

Day 9 (or 4 days after seeding epithelial cells): medium change with EP3

1. Make EP3 (see table below). Store at 4°C. This will be used also for Day 10.
2. Remove old medium from both inserts and the bottom well.
3. Add 7.5 ml of EP3 into the bottom wells only.

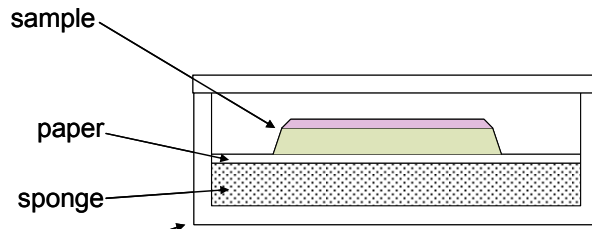
For two plates	EP3 (200ml) 15 ml/well, 90 ml/plate for 2 plates
DMEM (-)	95 ml
F12	95 ml
L-Glutamine (LQ)	4 ml
Hydrocortisone (H)	400 µl
ITES	400 µl
O-phosphorylethanolamine (O)	400 µl
Adenine (A)	400 µl
Triiodothyronine (T)	400 µl
Newborn Calf Serum (NBCS)	4 ml
Gentamycin	200 µl

Day 11 (or 6 days after seeding epithelial cells): medium change with EP3

1. Remove old medium from both inserts and the bottom well.
2. Add 7.5 ml of EP3 into the bottom wells only.

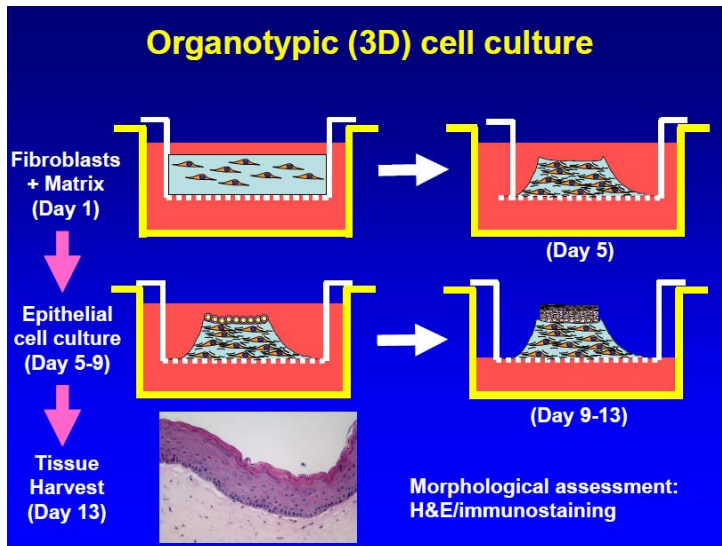
Day 14 (or 8 days after seeding epithelial cells): Harvesting OTC 3D culture**Fixation for OTC**

- 1) Fix with 10% neutralized buffered formaldehyde in the plate at 4 °C for 1 hr.
Do not fix too long (Over fixation kills certain antigens such as E-cadherin)!
- 2) Cut the membrane of the insert and set the sample to a cassette as shown below.
- 3) Wash cassettes twice in 1xPBS at RT for 10 min or 4 °C for 15min.
- 4) Put the cassettes in 70% EtOH and keep at 4°C until processed to make paraffin blocks, etc./H&E staining/IHC/IF (morph core).



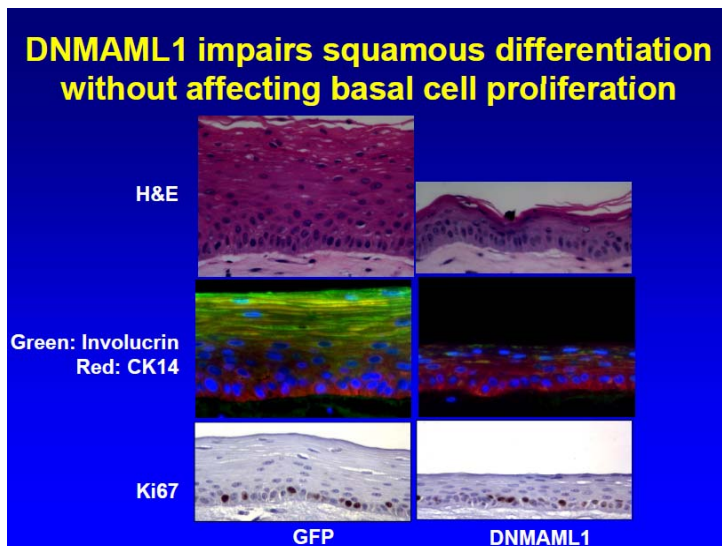
•Paper (Fisher brand® Chromatography Paper; 05-714-4)
→ **only bottom of the sample**

•Cassette → a white large cassette



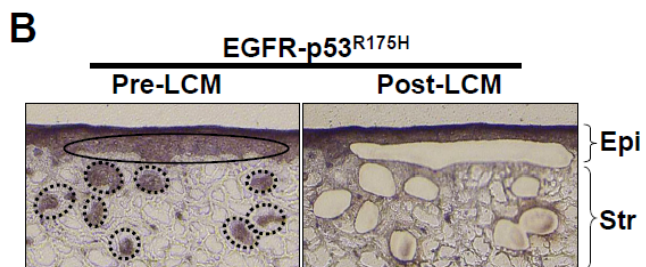
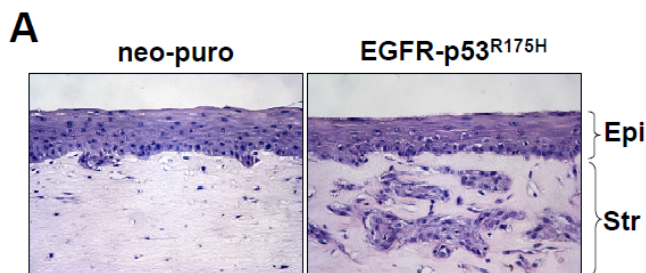
Summary of the entire OTC process

Matrix is prepared along with FEF3 fibroblasts on Day 1. In this example, EPC2-hTERT epithelial cells were seeded on Day 5, grown in a submerged condition in EP2 medium (Day 5-9), and then exposed to the liquid-air interface (Day 9-13) to trigger epithelial stratification with a reduced volume of medium (EP3). The epithelial cells and the underlying matrix compartment are fixed as a whole and embedded into a paraffin block for morphological analyses.



OTC example 1: Assessment of squamous differentiation and cell proliferation.

Impact of Notch inhibition upon epithelial formation was determined using EPC2-hTERT cells expressing DNAMAML1, a genetic pan-Notch inhibitor. Note that DNAMAML1 impaired epithelial stratification (H&E) with loss of Involucrin (a marker for early squamous differentiation)(IF), without affecting basal cell marker expression (CK14-IF) and basal cell proliferation (Ki67-IHC). GFP, control cells.



OTC example 2:

Assessment of invasive growth of human esophageal cells transformed by EGFR and mutant p53 using empty vectors-transduced control cells.

Laser captured microdissection was done to isolate cells in the basal compartment and those in the stromal compartment for real-time RT-PCR.

Epi, epithelium; Str, stroma.