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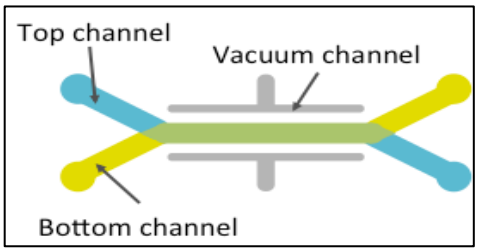
1. Materials

⇒ **Microfluidic cell culture platform (from Emulate, USA.)**

- Zoë-CM-1™ flow module
- Orb-HM1™ Hub Module
- UV lamp
- Pod™ portable modules for chips
- Chips (CHIP-S1™)



⇒ Cells

Primary human esophageal epithelial/tumor cells (organoids) and fibroblasts were isolated from consented EAC patients' derived tumor and adjacent normal tissues.

2. Reagents

a) For organoid expansion, culture and Chip medium

- Expansion media (see Table 1 and 2 below)
- Differentiation media (See Table 2 below)
- ROCK inhibitor: Y27632
- Matrigel (Corning - reduced growth factors) (thaw on ice or 4 ° C, always keep in ice when handling)
- 24-well plates (Cornings)

b) Chip activation and coating

- 1X PBS (pH: 7.4)
- ER1 and ER2 (Emulate USA)
- Rat tail collagen I (BD Corning Ca#: 354236)

⇒ Table 1. Propagation media for primary cells

Fibroblast propagatin medium	
Component	Final Conc.
DMEM+Glutamax-1	
Gentamicin	50ug/mL (1:200)
EGF	5ng/mL (1:20,000)

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Insulin	5ug/mL (1:800)
BPE	1:400
Normocin	100ug/mL (1:500)
FBS	10%

AD-DF+++ medium (base for esophagus conditioned media and DM)	
Component	Final Conc.
Advanced DMEM/F12	
Glutamax-1	2mM (1:100)
HEPES	10mM (1:100)
Pen/Strep	100U/mL

IntestiCult medium	
Component	Final Conc.
Component A	50%vol/vol
Component B	50%vol/vol
Pen/Strep	100U/mL (1x)
ROCK inhibitor	10uM

⇒ Table 2: Expansion (EM) and Differentiation (DM) media composition (for organoid derived tumor/epithelial cell culture in microfluidic chips.

Components	EM	DM	Final conc
IntestiCult Medium with ROCK inhibitor	+	-	50%vol/vol
Esophagus conditioned medium (cell line: HEK293) (Recombinant ligands: Wnt3a, R-Spondin, Noggin)	+	-	50%vol/vol
AD-DF+++ medium	+	+	-
B27	+	+	1x
N2	+	+	1x

Nicotinamide	+	-	10 mM
N AC (N-acetyl-l-cysteine	+	-	1.25 mM
Primocin	+	+	100 µg/ml
mEGF	+	+	50 ng/ml
hFGF10	+	-	200ng/ml
LeuGastrin	+	+	10 nM
A8301	+	+	500 nM
SB202190	+	-	10 µM

3. Protocols:

A. Tissue processing to generate patient matched organoids and fibroblats

⇒ Tissues from consented patients were received following diagnostic endoscopy in transport media (RPMI 1640, Gentamicin (50 ug/mL), Pen/Strep (100 U/mL), Fungizone (2.5 ug/mL)).
⇒ In laboratory, at first each tissue sample was washed with sterile AD-DF+++ medium.
⇒ Following pieces were processed with a sterile razor blade
a) 1 small piece from tumor sample for FFPE sample
b) 1 small piece from tumor sample for WES sequencing.
c) Larger pieces from tumor or normal samples for processing organoid and fibroblast derivation
⇒ Mince tissues using sterile razor blades.
⇒ Transfer these tissues into 5 mL of tissue dissociation mix (Collagen III (2 mg/mL), Hyaluronidase (100 U/mL) and Primocin (10µg/mL) in AD-DF+++ media) prepared into a gentle MACS-C tube (Milltenyi Biotec. Cat no.130096334).
⇒ Dissociate tissues in GentleMACSTM Octo Dissociator with heaters (Miltenyi Biotec.) using Program A: 2x15 mins for fresh biopsy tissue and followed by Program B: 1 min.
⇒ Add 10mL of PBS + 1mM DTT to the MACS-C tubes and mix well.
⇒ Filter out undissociated tissues/debris through 100 um cell strainers (Fisherbrand, Cat No. 22363549) and collect the mix into a fresh 50mL tube.
⇒ Transfer the sample to 15mL tube.
⇒ Centrifuge at 500 rcf for 5 min at 4°C.
⇒ Remove supernatants and resuspend pellets into 1mL of 0.25% trypsin and incubate in 37°C water bath for 5 min.
⇒ Add 9mL PBS/10% FBS + 1mM DTT and resuspend well.
⇒ Centrifuge at 500 rcf for 5 min at 4°C.
⇒ Remove supernatant and resuspend in 4mL AD-DF+++.
⇒ Let stand for 10min, then transfer 2mL of the upper part of the supernatant to a new 15mL conical tube (this portion is used for fibroblast culture).
⇒ Centrifuge both fractions at 500 rcf for 5 min at 4 degrees.

B. For fibroblast propagation:

⇒ Remove supernatant and resuspend cell pellet into 1mL fibroblast medium.
⇒ Transfer the mix into Type I collagen precoated (working dilution: 1:30 in DPBS) 24 well plate.
⇒ Incubate the plate in the 37°C/5% CO2/3% O2 Tri-gas incubator.
⇒ Replace media after 72 h.

C. For organoid propagation:

⇒ Remove supernatant and place tube on ice to cool down.
⇒ Add 80uL Matrigel to cell pellets and mix well.
⇒ Seed cell-Matrigel mix as a dome into a new pre-warmed 24-well plate.
⇒ Transfer it to the 37°C/5% CO2/3% O2 Tri-gas incubator for 12-15 mins to allow the Matrigel to turn into a solid dome.
⇒ Add 600uL of EM (1:1 mix of IntestiCult and home-made Esophagus conditioned medium).
⇒ Return plate to Tri-gas incubator

D. Passaging and freezing organoids

⇒ Prepare Matrigel digestion mix: (1X Collagenase/Dispase in AD-DF+++ medium).
⇒ Remove existing medium and add 500uL Matrigel digestion mix in each organoid containg
⇒ Break the Matrigel domes.
⇒ Digest for about 1h at 37°C in Tri-gas incubator.
⇒ Collect mix in a 15mL tube and centrifuge at 500 rcf, 5 min.
⇒ Carefully remove supernatant, add 1mL of 0.25% Trypsin and incubate for 10 mins.
⇒ Vortex vigorously to break up the organoids.
⇒ Add 4mL of 5% FBS-PBS to inactivate the trypsin and Spin at 500 rcf, 5 min.
⇒ Remove supernatant, add 1mL AD-DF+++ medium to wash.
⇒ Count and prepare two fractions.
⇒ Seed 35000 per new well for ongoing propagation (as described above in Protocol.C).
⇒ Refresh media 3 days. Passage every 10-14 days depending on the growth rate of organoids.
⇒ Slow-freeze (1C/min) the rest of the cells in CryoStor CS10 (StemCells, Cat No. 07930).
⇒ Transfer the tubes in -80°C freezer. Kept overnight.
⇒ Next tranfer the tubes to liquid N2 storage.

E. Passaging and freezing fibroblasts:

⇒ Discard the media from plates with fibroblasts (adhered at the bottom of collagen-coated plate) when cell confluency reaches 80-90%.
⇒ Wash cells once with 1mL 1X DBPS. Aspirate DPBS and add 500uL 0.25% trypsin, incubate 3-5 minutes at 37°C.
⇒ Add 1mL 1X DPBS with 5% FBS, mix well to detach the rest of adhered cells and transfer them in a 15mL tube.
⇒ Centrifuge 5 min, 500 rcf, then carefully remove supernatant.
⇒ Resuspend the pellet in 1mL Fibroblast media.
⇒ Transfer cells to a collagen-I pre-coated 10 cm petri dish.
⇒ Replenish media every 3 days untill 80-90% confluency is achieved.
⇒ Freeze fibroblasts following similar protocol as mentioned above.

F. Seeding cells and developing EAC-chips

❑ **Day 0**

⇒ *PDMS chip activation and coating:*

I. Inside the BSC, activate chips (CHIP-S1™) at room temperature in the dark using ER1/ER2 as manufacturer instructions.
II. Fill chip channels and make sure the entire channel is filled with ER1 uniformly without any bubble.
III. Roughly 100 µl of diluted ER1 solution required to fill each chip.

IV. Activate for 20 min under UV lamp under the hood.
V. ER1 Solution will change to a darker color (a sign of successful activation).
VI. Wash channels once with ER2 and thrice with 1X ice-cold DPBS.
VII. Prepare ice cold coating solution (100uL per chip): A) Mixture of 200ug/ml rat tail collagen I and 100ug/ml matrigel in 1X DPBS for top channel B) 200ug/ml rat tail collagen I in 1X DPBS for top channel for bottom channel.
VIII. Add coating solution to both channels.
IX. Remove air bubbles and excess coating solution on the chip.
X. Put chips into a petri dish (sterile moist chamber) and incubate for over night at 4°C.
XI. Takeout the chips and incubate at room temperature prior to cell seeding on next day.

❑ **Day 1**

⇒ *Prepare esophageal organoid fragments and seed.*
I. Prepare EM media and degas it.
II. Follow protocol D to dissociate Matrigel from organoids.
III. Prepare organoid fragments by incubating cells with 1x TrypLE™ (Gibco™ 12605010) for not more than1 min (extremely important step)
IV. Immediately add 1xDPBS with 10% FBS to deactivate TrypLE™.
V. Centrifuge at 500 rcf for 5 mins, discard supernatant.
VI. Add EM to break the pellet.
VII. Take out a small fraction and prepare 1:1 mix with trpan blue.
VIII. Count fragments.
IX. prepare fragmented organoids in EM with a final concentration 3X 10^6 cells/ml cell concentration.
X. Seed cells at the top channel.
XI. Add fresh EM media to the bottom channel.
XII. Aspirate coating solution out from both channels and wash twice with PBS before seeding the cells.
XIII. Add cell suspension to a top channel { aka tumor or epithelial channel} (about 30ul per chip). Make sure not making bubbles.
XIV. Place chips into petri dishes and put into an incubator for 4 h.
XV. Prime Pods according to the manufacturer's instructions and connect chips to pods, followed by load into Zoe.
XVI. Set the flow to 60ul/h (no stretch) and start cycle according to the manufacturer's instructions.
Inspect chips daily for bubbles and capture images.

❑ **Day 2**

❑ *Prepare patient matched fibroblasts and seed*
I. Check if the flow is even (same volume in both outlets)
II. Inspect top channel and make sure cells already form a layer and covering channel fully (no patches or breaks) and then proceed to seed fibroblasts.
III. Need to expand for fibroblast for 3 days in advance.
IV. Harvest fibroblasts as single cells as mentioned in protocol E.
XVII. Count and re-suspend cells in fresh fibroblast propagation medium with a final 3.5 X 10^6 cells/ml cell concentration.
V. After preparing cells, disconnect chips, aspirate medium out from bottom channel and wash with DPBS.

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VI. Seed fibroblasts into the bottom channel.
VII. After adding cells, invert chips in petri dish (use tips beneath chips to keep them horizontally).
VIII. Incubate chips at 37°C/5% CO2/3% O2 Tri-gas incubator for 3h.
IX. Repleish media into pods.
X. Reconnect the chips to Zoe in the same manner as on day 1.
XI. Restart flow cycle at 60 µl/h.

⇒ **Day 3 onwards**

I. Inspect chips for bubble, flow rate and change media regularly.
II. EAC chips achieved human relevant epithelial tissue integrity within 5 days under dynamic fluid flow.

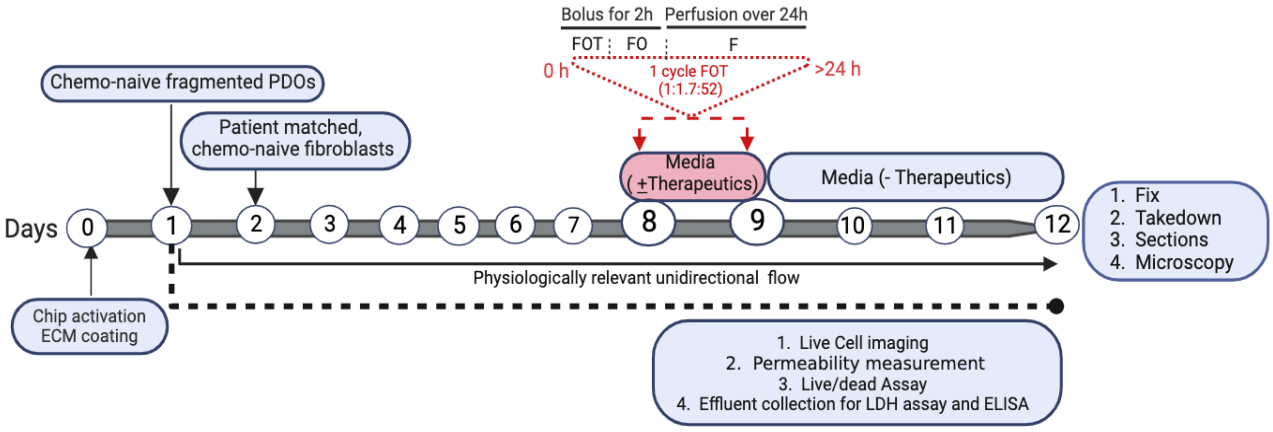
⇒ **Day 6**

III. The expansion media in the epithelial inlets were replaced with differentiation media.

⇒ **Day 8**

❑ *Start clinically relevant drug dosing*

I. Chips were removed from Pods.
II. Perform a flush cycle with a mix of triplet chemotherapeutic agents (FOT: 1:1.7:52) for1-2 mins with a flow rate of 1000 µl/h in the bottom channel and 0 µl/h in the top channel. This ensure proper distribution of the drugs only in the stromal microfluidic channel networks.
III. Reconnect chips with Pods.
IV. To mimic bolus IV-injection, a mixture of all three chemotherapeutics was administered at a flow rate of 100 µl/h (both channels) during first hour of cycle, followed by only a mixture of oxaliplatin (O) and 5FU (F) at the rate 60 µl/h for another hour. Subsequently, only 5FU (F) was perfused through stromal channel at a flow rate of 60 µl/h for a total of 24h.



Detailed schematic representation of the step-by-step procedure involved in the establishment and maintenance of Epithelial-Stromal Interactions in the microfluidic platform.

⇒ **Day 9**

❑ *Finish 1 cycle of triplet chemotherapy.*

I. Stop the flow and disconnect the chips from Pods.

II. Discard media containing chemotherapeutic agents and replenish inlets with fresh media without drugs. DM at the top channel and fibroblast propagation media at the bottom channel.

III. Reconnect chips with Pods, followed by Zoe.

IV. Restart flow (reverse cycle) at 60 µl/h.

V. *Maintain chips for 12 days,* followed by fix with 4% PFA and harvest for downstream applications.