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Model of Chemotherapy-associated Mucositis and Oral Opportunistic Infections

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[Abstract] Oral mucositis is a common complication of cancer chemotherapy treatment. Because of the lack of relevant oral mucositis experimental models, it is not clear how chemotherapeutic agents injure the oral mucosa and if commensal microorganisms accelerate tissue damage. We developed an organotypic oral mucosa model that mimics cellular responses commonly associated with cytotoxic chemotherapy. The organotypic model consists of multilayer oral epithelial cells growing over a collagen type I matrix, with embedded fibroblasts. Treatment of organotypic constructs with the chemotherapeutic agent, 5-fluorouracil (5-FU), leads to major histopathologic changes resembling mucositis, such as DNA synthesis inhibition, increased apoptosis and cytoplasmic vacuolation. *Candida albicans* formed mucosal biofilms on these tissues and augmented the inflammatory responses to 5-FU. This model can be used in further mechanistic studies of oral mucositis and associated opportunistic infections.
Keywords: Oral mucositis, Organotypic, *Candida*, Chemotherapy, BrdU, Caspase 3

[Background] *Candida albicans* is a fungal commensal of the upper and lower gastrointestinal tract implicated in opportunistic mucosal and disseminated infections in immunocompromised hosts (Westbrook *et al.*, 2013). Clinical studies have shown that risk factors associated with candidemia go beyond the frequently described use of central venous catheters; they are also associated with the use of high dose chemotherapeutic agents. Under this scenario, the mucosal barrier integrity is decreased, resulting in life threatening systemic dissemination (Nucci *et al.*, 1998; Lalla *et al.*, 2014). We have recently started to explore the role of cytotoxic chemotherapy in *Candida albicans* mucosal infection. We showed that this fungus can amplify the proinflammatory epithelial response to the cytotoxic drug 5-FU *in vitro,* (Sobue *et al.*, 2018) and it can promote a dysbiotic state *in vivo,* that causes increased oral mucosal barrier breach (Bertolini *et al.*, 2019). 5-fluorouracil is a chemotherapeutic agent used for treatment of a variety of metastatic cancers including breast, gastrointestinal tract and head & neck. In this patient population oral thrush and/or candidemia with disseminated multi-organ infection are prevalent and most frequently attributed to *C. albicans* (Teoh and Pavelka, 2016).

Thus far organotypic models of oral mucositis have relied on cost-prohibitive commercially available constructs (Sonis *et al.*, 2017) or primary epithelial cultures which vary in life span and other phenotypic characteristics, and are associated with ethical considerations for harvest (Tobita *et al.*, 2010). Our in-house organotypic construct is based on an oral epithelial cell line which readily forms a multilayer epithelial structure and responds to 5-FU in a manner similar to oral mucosa *in vivo* (Sobue *et al.*, 2018; Bertolini *et al.*, 2019). This model will facilitate further mechanistic studies on oral mucositis and the

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complex relationships with fungal and bacterial organisms that overgrow on the oral mucosa during chemotherapy-driven immunosuppression. This model could lead to novel future interventions designed to prevent the initiation of oral mucositis in chemotherapy treated patients and better understand the effect of resident microbiota in aggravating oral mucosal lesions.

Materials and Reagents

A. Plates/Inserts/tubes/pipettes
   1. 6-well Falcon Deep Well Plate (Corning, catalog number: 355467)
   2. Transwell 3414 (24 mm diameter inserts, 3.0 µm pore size, Corning, catalog number: 3413)
   3. 50 ml polypropylene tube (Fisher Scientific, catalog number: 05-539-8)
   4. 25 ml polystyrene pipette (Fisher Scientific, catalog number: 13-678-11)
   5. 10 ml polystyrene pipette (Fisher Scientific, catalog number: 13-678-11E)
   6. 9 inch glass pasteur pipette (Fisher Scientific, catalog number: 13-678-20C)
   7. Tissue embedding cassettes (Fisher Scientific, catalog number: B1000729BL)
   8. Tweezers (Fisher Scientific, catalog number: 12-000-132)
   9. 75 cm^2 Cell culture flask (Corning, catalog number: 430641U)
   10. 100 mm Petri Dish (Fisher Scientific, catalog number: FB0875713)

B. Cells

   Note: Use freshly thawed vial of cells from Liquid Nitrogen. Sub-culture when cells are 70-80% confluent. Sub-culture one week after starting culture.
   1. Fibroblast: 3T3 cell line (ATCC, catalog number: CRL-1658)
   2. Epithelial cells: SCC15 cell line (ATCC, catalog number: CRL-1623)

C. Common reagents
   1. 10% normal goat serum (Jackson ImmunoResearch, catalog number: 005-000-121)
   2. 100%, 95%, 70% and 50% Ethanol (EtOH) (Pharmo, catalog number: 111000200)
   3. 30% H_2O_2 (Sigma-Aldrich, catalog number: H1009-100ML)
   4. Phosphate buffered saline (PBS, Corning, catalog number: 21-031-CV)
   5. Triton X-100 (Sigma-Aldrich, catalog number: T8787)
   6. Xylene (Fisher Scientific, catalog number: X3P-1GAL)
   7. 1 N Sodium Hydroxide (NaOH, Sigma-Aldrich, catalog number: S2770)
   8. Dimethyl sulfoxide (DMSO, Sigma-Aldrich, catalog number: 276855)
   9. 0.05 % Trypsin-EDTA (Gibco, catalog number: 25300-054)
   10. 20x Citrate buffer (Thermo Fisher Scientific, catalog number: 005000, see Recipes)
   11. 16% Paraformaldehyde (Electron Microscopy Science, catalog number: 15710, see Recipes)

D. Fibroblast Culture Media: DMEM-10% FBS-1% Pen/Strep

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1. Dulbeccos Modified Eagle Medium (DMEM) liquid (high glucose) without Pyruvate (Gibco, catalog number: 11965-084)
2. Fetal bovine serum (FBS, Gibco, catalog number: 16000044)
3. Penicillin/streptomycin (Pen/Strep, Gibco, catalog number: 15140-122)

E. Epithelial Cell Culture Media

KSFM with Bovine Pituitary Extract (BPE) (1 tube/each bottle), 0.2 ng/ml human epithelial growth factor (hEGF) (Invitrogen, catalog number: 17005-042), 0.4 mM $CaCl_2$ (Sigma-Aldrich, catalog number: C-3306) and Pen/Strep (1:100)

F. Matrix and organotypic cell culture media components

1. Type I Collagen (Organogenesis, catalog number: 200/50)
2. Matrigel Basement Membrane Matrix (BD Biosciences, catalog number: 354234)
3. Newborn Calf Serum (HyClone, catalog number: SH 3011802)
4. DMEM (-) (Gibco, catalog number: 11965-084)
5. Hams F12 (Gibco, catalog number: 11765 -054)
6. 10x EMEM (BioWhittaker, catalog number: 12-684F)
7. 7.5% Sodium-Bicarbonate (Na-Bicarbonate, BioWhittaker, catalog number: 17-613E)
8. FBS (Gibco, catalog number: 16000044)
9. L-Glutamine (Cellgro, catalog number: 25-005CI)
10. Gentamicin, 50 mg/ml (Cellgro, catalog number: MT30-0005-CR)
11. ITES - Insulin, Transferrin, Ethanolamine and Selenium (BioWhittaker, catalog number: 17839Z)
12. Hydrocortisone (Sigma-Aldrich, catalog number: H0888, see Recipes)
13. *O*-phosphorylethanolamine (Sigma-Aldrich, catalog number: P0503, see Recipes)
14. Adenine (Sigma-Aldrich, catalog number: A9795, see Recipes)
15. Progesterone (Sigma-Aldrich, catalog number: P8783, see Recipes)
16. Triiodothyronine (Sigma-Aldrich, catalog number: T5516, see Recipes)
17. 5-Fluorouracil, 5-FU (Sigma-Aldrich, catalog number: F6627, see Recipes)
18. 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside, BrdU (Sigma-Aldrich, catalog number: B4252, see Recipes)

G. Microorganisms used and microbiological media

1. *Candida albicans* strain SN425 (reference strain, derived from SC5314, provided by Dr. Clarissa J. Nobile, University of California, Merced, CA)
2. YPD medium and Agar (see Recipes)
   a. Yeast Extract (Sigma-Aldrich, catalog number: Y1625)
   b. Peptone (BD, catalog number: 211677)
   c. Dextrose (J. T. Baker, catalog number: 1616-05)
   d. Agar (BD, catalog number: 214530)

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H. Immunofluorescence stain
1. Coplin Staining Jar (Thermo Scientific, catalog number: 19-4)
2. Rat anti BrdU monoclonal antibody (Abcam, catalog number: ab6326)
3. Goat anti rat Alexa Fluor 555 conjugated secondary antibody (Thermo Fisher scientific, catalog number: A21434)
4. Goat anti rabbit anti active Caspase-3 antibody (Abcam, catalog number: Ab32042)
5. Alexa Flour 568 conjugated secondary antibody (Thermo Fisher scientific, catalog number: A11011)
6. Rabbit FITC conjugated anti *Candida albicans* polyclonal antibody (Meridian Life Science, catalog number: B65411F)
7. Hoechst 33258 (10 mg/ml, Thermo Fisher scientific, catalog number: H3569)
8. Fluoro-Gel (Electron Microscopy Sciences, catalog number: 17895-11)

Equipment

1. Fluorescence microscope (Zeiss Axio Imager M1)
2. Objective lens (EC Plan-Neofluar 20x/0.5)
3. Tissue culture incubator (Thermo Forma, catalog number: 3110)
4. Biological safety cabinet (Thermo Forma, catalog number: 1284)
5. Autoclave
6. Orbital Shaker (New Brunswick Scientific, catalog number: C1A88IC-C1)
7. Centrifuge machine (Thermo Scientific, catalog number: CL2)
8. Hemocytometer (Hausser Scientific, Fisher Scientific, catalog number: 0267110)

Software

1. AxioVision LE64 (Zeiss)

Procedure

A. Organotypic constructs (OTC)

The organotypic oral mucosal model has been described previously (Dongari-Bagtzoglou and Kashleva, 2006a; Dongari-Bagtzoglou and Kashleva, 2006b; Sobue *et al.*, 2016). The protocol was recently modified based on work by Nakagawa and colleagues (Nakagawa *et al.*, 2015). The organotypic model consists of human immortalized oral keratinocytes (squamous cell carcinoma) SCC15 and type I collagen matrix-embedded mouse fibroblasts, 3T3. Mucosal constructs take approximately 2 weeks to form, resulting in a non-keratinizing, multilayer squamous epithelium which is then exposed to the chemotherapy agent 5-FU (Figure 1).

1. **A week ahead:** Start growing 3T3 cells

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a. Thaw a frozen vial in 9 ml of DMEM with 10% FBS 1% Pen/Strep in a 50 ml tube.
b. Centrifuge the tube 3 min with 670 *x g* in a centrifuge machine.
c. Aspirate supernatant from the tube.
d. Add 10 ml of DMEM with 10% FBS 1% Pen/Strep into 50 ml tube and resuspend gently.
e. Seed 10 ml cell suspension on 75 cm^2 cell culture flask and incubate the flask in the incubator with 37 °C and 5% CO_2.
f. Change the culture medium every 2 to 3 days.
g. Grow until they reach 80% confluent in DMEM with 10% FBS 1% Pen/Strep.

2. **Day 0:** Thaw Matrigel overnight (one vial for 2.5 OTC plates) at 4 °C.
3. **Day 1:** Matrix preparation

15-30 min prior to matrix preparation:

a. Place FBS, L-Glutamine, 10x EMEM and 7.5% Na-bicarbonate, Matrigel and type I collagen on ice.
b. Place transwell inserts into each well in 6-well Falcon Deep Well Plate.

Making acellular layer

a. Add 10x EMEM, FBS, L-Glutamine, Na-bicarbonate and type I collagen in this order in a 50 ml tube on ice (Table 1).
b. Mix gently using a 25 ml pipette in a 50 ml tube on ice.
Note: When making acellular and cellular layers, mix components gently on ice to avoid bubbles.
c. Pour 1 ml per insert using a 10 ml pipette.
d. Leave inside the tissue culture hood while preparing fibroblasts and cellular layer.

Table 1. Acellular layer media

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)

Making cellular layer

a. Aspirate condition medium from cell culture flask.
b. Wash cells with 10 ml PBS and aspirate PBS. Repeat one more time.
c. Add 1 ml 0.05% Trypsin/EDTA to cell culture flask. Tap the flask gently and keep in the incubator for up to 2 min.
d. Once cells detach from the bottom of flask, add 9 ml of DMEM with 10 % FBS 1% Pen/Strep and suspend cells with a 10 ml pipette.

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e. Collect cell suspension and place to a new 50 ml tube.
f. Centrifuge the tube 3 min with 670 *x g* in a centrifuge machine.
g. Aspirate supernatant and resuspend the cell pellet with 10 ml of DMEM with 10 % FBS 1% Pen/Strep.
h. Count cell number with a hemocytometer and prepare cell suspension (6 x 10^5/ml, 2 ml for each plate).
i. Add 10x EMEM, FBS, L-Glutamine, Na-bicarbonate, type I collagen, Matrigel and fibroblasts in this order into a new 50 ml tube on ice (Table 2).
j. Mix gently using a 25 ml pipette in the 50 ml tube on ice.

Note: When making acellular and cellular layers, mix components gently on ice to avoid bubbles.

k. Pour 3 ml per insert using a 10 ml pipette.
l. Incubate for 30-45 min in the tissue culture incubator (37 °C, 5% CO_2).
m. Add DMEM-10% FBS-1% Pen/Strep: 10 ml into the bottom of the wells, 2 ml into the insert.

Table 2. Cellular layer media

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 µl (x 1)	320 µl (x 1)
Na-bicarbonate	380 µl (x 1)	760 µl (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 x 10^5/ml fibroblasts	1.6 ml (x 1)	3.2 ml (x 1)

Start growing SCC15 cells

a. Thaw a frozen vial in 9 ml of KSFM with BPE, 0.2 ng/ml hEGF, 0.4 mM and 1% Pen/Strep in a 50 ml tube.
b. Centrifuge the tube for 3 min with 670 *x g* in a centrifuge machine at room temperature.
c. Aspirate supernatant from the tube.
d. Add 10 ml of KSFM with BPE, 0.2 ng/ml hEGF, 0.4 mM and 1% Pen/Strep into the 50 ml tube and resuspend gently.
e. Seed 10 ml cell suspension on 75 cm^2 cell culture flask and incubate the flask in the incubator with 37 °C and 5% CO_2.
f. Change the culture medium every 2 to 3 days.
g. Grow until they reach 80% confluent in KSFM with BPE, 0.2 ng/ml hEGF, 0.4 mM and 1% Pen/Strep.

4. **Day 2:** Dislodge matrix and add 2 ml of DMEM-10% FBS-1% Pen/Strep into the insert.
   a. Use a sterile glass Pasteur pipette to go around (2-3 times) the matrix along the inner wall of the insert. Feel the friction at the tip on the transwell membrane, but try not to pierce it.

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Note: The matrix will contract over the next few days. Additional 2 ml needs to submerge the OTC.

b. No need to change medium. Wait at least 4 days before seeding epithelial cells.

5. **Day 5 or later (up to Day 7)**: Seeding epithelial cells
   a. Make DMEM (-)/F12 (Table 3) to pre-saturate the OTC matrix.
   b. Remove old medium from the OTC plates/inserts.
      Note: Gentle aspiration is recommended to reduce the risk of distracting OTC.
   c. Add DMEM (-)/F12. Add 10 ml to the bottom well and 2 ml into the insert.
   d. Incubate for 1 h to equilibrate the matrix in the tissue culture incubator (37 °C, 5% CO_2).
   e. Aspirate condition medium from the SCC15 cell culture flask.
   f. Wash cells with 10 ml PBS and aspirate PBS. Repeat one more time.
   g. Add 1 ml 0.05% Trypsin/EDTA to cell culture flask. Tap the flask gently and keep in the incubator (37 °C, 5% CO_2) up to 2 min.
   h. Once cells detach from the bottom of flask, add 9 ml of DMEM with 10% FBS 1% Pen/Strep and suspend cells with a 10 ml pipette.
   i. Collect cell suspension and place to a new 50 ml tube. Count cell number with hemocytometer.
   j. Centrifuge the tube 3 min with 670 *x g* in a centrifuge machine at room temperature.
   k. Aspirate supernatant and resuspend cell pellet with KSFM with BPE, 0.2 ng/ml hEGF, 0.4 mM and 1% Pen/Strep. The volume of medium needs to be adjusted based on the counting cell number in Step A5i.
      Note: Prepare 1 x 10^7/ml epithelial cell suspension (300 µl per plate). 5 x 10^5 epithelial cells are seeded per insert/well. One ~80% confluent 75 cm^2 flask yields about 2-3 x 10^6 SCC15 cells.
   l. Remove DMEM (-)/F12 from the OTC plates/inserts.
   m. Seed epithelial cells by adding 50 µl (5 x 10^5) of cell suspension per well onto the center of the surface of contracted matrix.
   n. Incubate for 2 h in the tissue culture incubator without medium.
   o. Make EP2 media. Mix all cell culture media components seen in Table 4 and store at 4 °C.
   p. Add EP2 media to the plate–10 ml into the bottom well and 2 ml into the insert. And incubate the plate in the tissue culture incubator (37 °C, 5% CO_2).

Table 3. DMEM/F12 Media

DMEM/F12 Media (3:1)	2 Plates (144 ml+)	3 Plates (216 ml+)
DMEM (-)	120 ml	180 ml
Ham's F12	40 ml	60 ml

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Table 4. EP2 Media

EP2	2 plates (300 ml)	3 plates (450 ml)
DMEM (-)	218 ml	327 ml
F12	72 ml	108 ml
L-Glutamine (LQ)	6 ml	9 ml
Hydrocortisone (H)	600 µl	900 µl
ITES	600 µl	900 µl
O-phosphorylethanolamine (O)	600 µl	900 µl
Adenine (A)	600 µl	900 µl
Progesterone (P)	600 µl	900 µl
Triiodothyronine (T)	600 µl	900 µl
Newborn Calf Serum (NBCS)	300 µl	450 µl
Gentamicin	300 µl	450 µl

6. **Day 7** (**or 2 days after seeding epithelial cells):** Medium change with EP2 media.
   a. Remove old medium from the OTC plates/inserts.
   b. Add EP2 media: add 10 ml to the bottom well and 2 ml into the insert.
   c. Incubate the plate in the tissue culture incubator (37 °C, 5% CO_2).
7. **Day 9 (or 4 days after seeding epithelial cells, Air Lifting):** Replace EP2 media with EP3 media.
   a. Make EP3 media. Mix all cell culture media components seen in Table 5 and store at 4 °C.
   b. Remove old medium from both inserts and the bottom well.
   c. Add 7.5 ml of EP3 media into the bottom wells only.
   d. Incubate the plate in the tissue culture incubator (37 °C, 5% CO_2).

Table 5. EP3 Media

EP3	2 plates (200 ml)	3 plates (300 ml)
DMEM (-)	95 ml	142.5 ml
F12	95 ml	142.5 ml
L-Glutamine (LQ)	4 ml	6 ml
Hydrocortisone (H)	400 µl	600 µl
ITES	400 µl	600 µl
O-phosphorylethanolamine (O)	400 µl	600 µl
Adenine (A)	400 µl	600 µl
Triiodothyronine (T)	400 µl	600 µl
Newborn Calf Serum (NBCS)	4 ml	6 ml
Gentamicin	200 µl	300 µl

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8. **Day 11 (or 6 days after seeding epithelial cells):** Medium change with EP3 media, prepared on Day 9.
   a. Remove old medium from both inserts and the bottom well.
      Note: After air lifting, further significant contraction will be seen. Contraction can be better assessed during medium change.
   b. Add 7.5 ml of EP3 media into the bottom wells only.
   c. Incubate the plate in the tissue culture incubator (37 °C, 5% CO_2).
9. **Day 13 (or 8 days after seeding epithelial cells):** Medium change with EP3 media, prepared on Day 9.
   a. Remove old medium from both inserts and the bottom well.
   b. Add 7.5 ml of EP3 media into the bottom wells only.
   c. Incubate the plate in the tissue culture incubator (37 °C, 5% CO_2).
10. **Day 14 (or 9 days after seeding epithelial cells):** Three-dimensional mucosal organotypic culture is now ready to use for 5-FU treatment followed by microbial inoculation.

B. 5-FU treatment of organotypic construct and confirmation of the drug effect on cell proliferation
   1. Prepare EP3 media with 10 µM 5-FU (2 ml for each well).
   2. Transfer inserts to a new 6 well dish with sterile tweezers.
   3. Add 2 ml EP3 media with 5-FU to basolateral side only to expose the submucosal compartment to the drug.
   4. Add 1 ml of fresh cell culture media without 5-FU to the apical mucosal surface.
   5. Add 100x concentrated BrdU (final concentration: 10 µM) on both apical mucosal surface (100 µl) and basolateral (200 µl) sides.
      Note: BrdU labeling is needed to confirm the anti-proliferative effect of 5-FU. Incorporation should be allowed for a minimum of 16 h since most of epithelial cells are slowly proliferating after the multilayer construct is formed.
   6. Incubate mucosal tissues in a transwell plate for 16 h at 37 °C with 5% CO_2.

C. *Candida albicans* infection of 5-FU treated organotypic construct
   1. *Candida albicans* (*C. albicans*) was inoculated flasks from colonies growing on YPD Agar plates into 10 ml YPD broth and incubate aerobically with gentle agitation, at room temperature, on a shaker.
   2. Remove all cell culture media from both sides of transwells (apical and basolateral) after 16 h incubation with 5-FU.
   3. Add EP3 media to both sides of transwells and incubate for 1 h at 37 °C with 5% CO_2. Repeat twice more in total 3 h to wash out 5-FU from mucosal tissues.
      Note: Removing 5-FU prior to microbial inoculation is necessary because it is toxic to most organisms.
   4. Count cell number of *C. albicans* with a hemocytometer and prepare cell suspension (10^6 fungi

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cells in 20 µl EP3 media for each well).

5. Remove all cell culture media from both sides of the transwell plate.
6. Add the inoculum of *C. albicans* on mucosal surface side and leave for 45 min at room temperature.

   Note: Place the inoculum gently on the center of mucosal surface; to avoid the excessive spread of the liquid media to the sides use a small volume (up to 20 µl) and ensure microorganisms are inoculated on the surface.
7. Add 1.5 ml of EP3 media to basolateral side only and incubate for 2 h at 37 °C with 5% CO_2.
8. Add 0.5 ml of EP3 media to mucosal surface side and incubate the plate for 16 h at 37 °C with 5% CO_2.

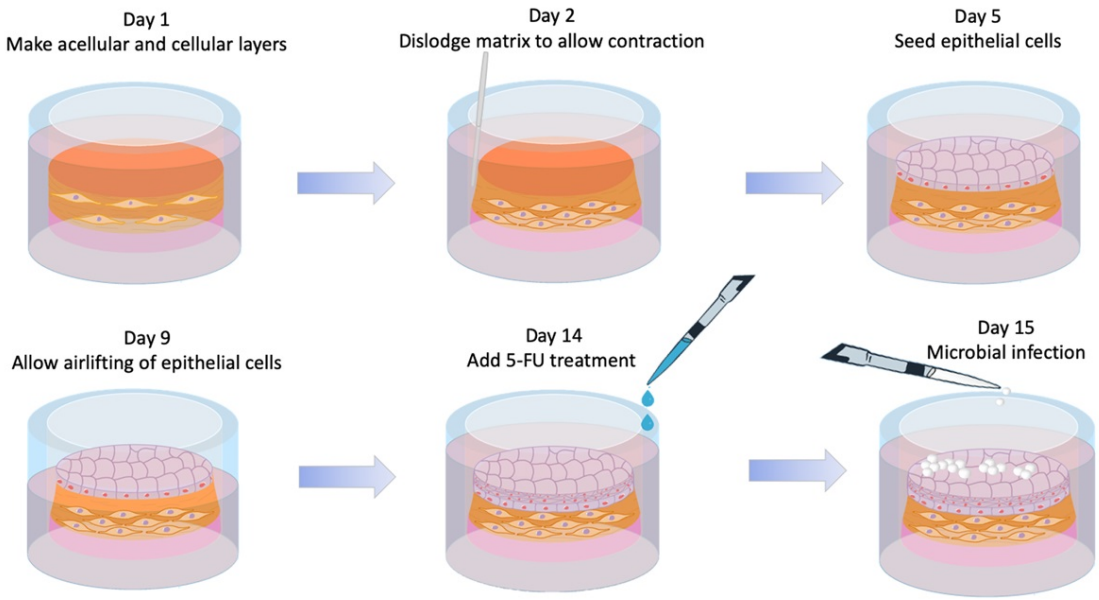

Figure 1. Organotypic Oral Mucosa model

D. Preparation for analysis

1. Submerge mucosal tissues in a new 6 well cell culture plate with 4% paraformaldehyde for fixation. Keep culture plates for 2 h at 4 °C.

   Note: Do not over-fix. It may mask certain antigens. Two hours are sufficient for fixation with 4% paraformaldehyde.
2. Wash mucosal tissues with 4 ml of PBS twice for 3 min each at room temperature.
3. Remove mucosal tissues from cell culture inserts and place the tissue into tissue embedding and processing cassettes.
4. Process ordinal dehydration and paraffin embedding steps and cut 5 µm thick section for histological and Immunofluorescence analysis.

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E. Immunofluorescence analysis

1. Deparaffinize by immersion in xylene, rinse in xylene and rehydrate in a series of ethanol washes.
2. Antigen retrieval with 70 ml of Citrate buffer, pH 6.0 in Coplin jar for 10 min at 94 °C.
   Note: To maximize antigen-antibody binding, antigen retrieval step is necessary for unmasking epitope.
3. Leave the slides at room temperature for 20 min.
4. Wash with 70 ml of PBS for 3 min and repeat twice more, total 3 times in Coplin jar at room temperature.
5. Immerse the slides in 70 ml of 0.05% H_2O_2 in PBS for 30 min in Coplin jar at room temperature.
6. Wash with 70 ml of PBS for 3 min and repeat twice more, total 3 times in Coplin jar at room temperature.
7. Incubate the slides in 300 µl of 10% normal goat serum, 0.2% triton X-100 in PBS (blocking buffer) for 30 min at room temperature.
8. Incubate the slides with 300 µl of anti BrdU antibody (1:40 in blocking buffer) or anti active Caspase-3 antibody (1:100 in blocking buffer) overnight at 4 °C. To stain *C. albicans*, incubate with 300 µl of anti *C. albicans* antibody (1:20 in PBS) for 2 h in the dark at room temperature.
9. Wash with 70 ml of PBS for 3 min and repeat twice more, total 3 times in Coplin jar at room temperature.
10. For BrdU and active Caspase-3 stains, incubate with 300 µl of secondary antibody (A21434 for BrdU and A11011, 1:100 in PBS) for 1 h in the dark at room temperature.
11. Wash with 70 ml of PBS for 3 min and repeat twice more, total 3 times in Coplin jar at room temperature.
12. Counter staining with 300 µl of Hoescht 33258 (1:5000 in PBS) for 30 min in the dark at room temperature.
13. Rinse with 70 ml of PBS and distilled water 3 min each and add a few drops of mounting solution, Fluoro-Gel, with coverslip.
14. Sections can be observed under fluorescence microscope with 20x objective lens and further analysis can be conducted with AxioVision software.

Data analysis

1. Histological response to 5-FU treatment can also be observed in hematoxylin and eosin (H&E) stained organotypic constructs under microscope. Although no significant structural change of multiple epithelium layers is noted, there is extensive widening of epithelial intercellular spaces and cytoplasmic vacuolation in 5-FU treated tissues compared to control (Figure 2A), consistent with histopathologic characteristics of oral mucositis in humans (von Bültzingslöwen *et al.*, 2001).
2. To reveal the inhibitory effect of 5-FU on cell proliferation, organotypic constructs are labeled with BrdU during 5-FU treatment. In Figure 2B immunofluorescence stain of BrdU incorporation

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revealed that the untreated tissues contained multiple BrdU-positive cells (cells in S phase) in all epithelial layers, consistent with the squamous carcinoma phenotype of SCC15 cells. The BrdU positive cells were almost completely absent with 5-FU treatment, indicating lack of DNA synthesis (Figure 2B).

3. We also examined whether the treatment of 5-FU affected cell apoptosis in mucosal constructs. Immunofluorescence stain of active Caspase-3 revealed an increase in the number of apoptotic cells in the 5-FU-treated tissues, whereas minimal epithelial cell apoptosis was observed in untreated tissues (Figure 2C). Collectively these results showed that treatment of organotypic constructs for 16 h with 5-FU reproduced the major histopathologic characteristics of oral mucositis.

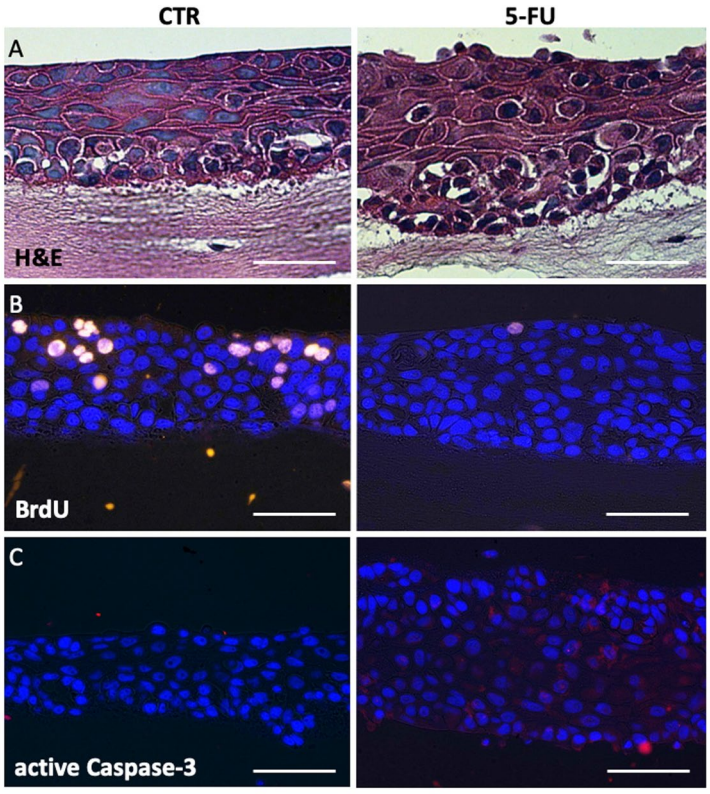

Figure 2. 5-FU treatment on organotypic oral mucosa model. A. H&E stain revealed 5-FU treatment resulted in a widening of epithelial intercellular spaces and cytoplasmic vacuolation. B. Immunofluorescence staining for BrdU incorporation revealed 5-FU treatment decreased DNA synthesis. C. Immunofluorescence staining for active Caspase 3 revealed 5-FU treatment increased apoptosis (Red spots, right panel). Bars = 50 μm.

4. We examined whether 5-FU-treated mucosal constructs promote fungal growth. Mucosal constructs were first exposed 5-FU for 16 h followed by infection with *C.albicans* for 16 h. H&E stain revealed *C.albicans* invasion on mucosal constructs regardless of 5-FU treatment (Figure 3A). 5-FU treatment increased *C.albicans* invasion in the submucosal area (Figures 3A and 3B),

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consistent with the increase in intercellular spaces (Figure 2A) and E-cadherin dissolution observed with 5-FU treatment (Sobue *et al.*, 2018).

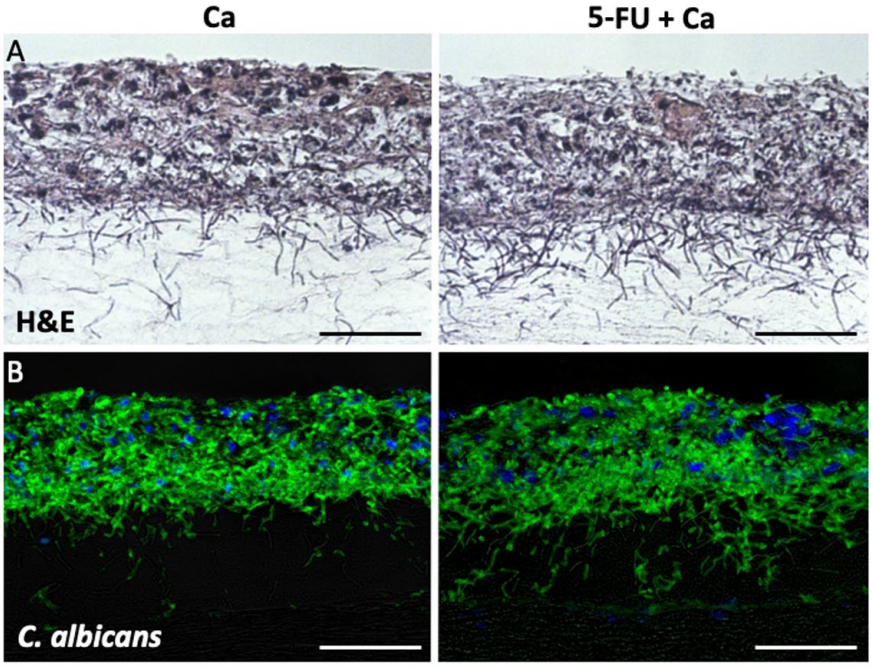

Figure 3. *Candida albicans* infection on 5-FU treated organotypic oral mucosa model. *Candida albicans* biofilms growing on untreated (Ca) or 5-FU treated (5-FU + Ca) mucosal constructs for 16 h. A: H&E stain. B: Immunofluorescence staining for *Candida albicans*. Bars = 50 µm.

Recipes

1. Hydrocortisone (MW = 362.46 g/mol)
   a. Dissolve 0.0269 g hydrocortisone in 2.5 ml EtOH
   b. Add into 97.5 ml DMEM (0.74 mM)
   c. Filter-sterilize, dispense into aliquots (2 ml) and store at -20 °C
2. *O*-phosphorylethanolamine
   a. Dissolve 0.705 g *O*-phosphorylethanolamine in 100 ml DMEM (50 mM)
   b. Filter-sterilize, dispense into aliquots (2 ml) and store at -20 °C
3. Adenine
   a. Dissolve 1.55 g adenine in 100 ml warm (37 °C) ddH_2O (0.09 M)
   b. Filter-sterilize, dispense into aliquots (2 ml) and store at -20 °C
4. Progesterone
   a. Dissolve 1 mg progesterone in 1 ml EtOH
   b. Add 14.7 ml ddH_2O
   c. Dilute 1ml of that in 100 ml DMEM (2.0 µM)

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   d. Filter-sterilize, dispense into aliquots (2 ml) and store at -20 °C
5. Triiodothyronine
   a. Dissolve 1 mg triiodothyronine in 1 ml 1 N NaOH
   b. Add 19 ml of DMEM (-)
   c. Dilute 4 µl of that in 31 ml plain DMEM (1 nM)
   d. Filter-sterilize, dispense into aliquots (2 ml) and store at -20 °C
6. YPD medium
   a. Dissolve 5 g of yeast extract, 10 g of peptone in 450 ml of distilled water
   b. Autoclave the mixture, YP medium for sterilization (121 °C, 15 lb/in² for 20 min)
   c. Mix 1 part of 20% Dextrose with 9 part of YP medium to make YPD medium
7. YPD Agar
   a. Dissolve 10 g of yeast extract, 20 g of peptone, 20 g of agar in 900 ml of distilled water
   b. Autoclave the mixture for sterilization (121 °C, 15 lb/in² for 20 min)
   c. Leave YPD Agar to cool down to 55 degree
   d. Add 100 ml of 20% Dextrose to the mixture to make YPD Agar
   e. Poor 10 ml each to 100 mm petri dish
8. 5-FU (10 µM)
   a. Stock solution (50 mg/ml): Resolve 50 mg of 5-FU powder in 1 ml DMSO. Keep it at 4 °C for 3 months
   b. Working solution (10 µM): Make fresh immediately before use. Dilute 10 µl of stock solution with 3830 µl of cell culture media to make 1 mM 5-FU. Dilute 200 µl of 1 mM 5-FU with 20 ml of cell culture media to make 10 µM 5-FU supplemented cell culture media
9. BrdU (1 mM)
   a. Stock solution (10 mg/ml): Resolve 10 mg of BrdU powder in 1 ml distilled water. Keep it at -20 °C
   b. Working solution (1 mM): Make fresh immediately before use. Dilute 3.1 µl of stock solution with 100 µl of EP3 media
10. Citrate buffer
   a. Dilute 5 ml of 20x concentrated Citrate buffer with 95 ml of distilled water
   b. Preheat the buffer to 95 °C
11. 4 % PFA
   a. Dilute 10 ml of 16% PFA with 30 ml of PBS
   b. Use 4 % PFA in one week and keep it at 4 °C

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Competing interests

No competing interests.

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