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Title: Establishment and Histological Analysis of Esophageal Organoids Modeling the Progression from Normal to Cancerous Tissues

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No.**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No.**

3. **Filming location:** Will the filming need to take place in multiple locations? **No.**

Current Protocol Length
Number of Steps: 17
Number of Shots: 38

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Rucheng Liu:** Our research focuses on tumor initiation and early tumorigenesis. We examine how normal cells transform into tumor cells and how these cells modify their microenvironment to facilitate tumorigenesis **[1]**.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:4.1*

What technologies are currently used to advance research in your field?

1.2. **Rucheng Liu:** At present, there are many technologies used to promote research, including stem cell technologies and genee diting technologies, advanced 3D culture systems & biomaterials and single-cell and spatial omics technologies **[1]**.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What are the current experimental challenges?

1.3. **Rucheng Liu:** The biggest challenge we face is keeping organoids free from bacteria and fungi. These contaminants sneak in during tissue handling and transport, often ruining our cultures **[1]**.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:2.11*

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Institutional Review Board (IRB) at the Cancer Hospital, Chinese Academy of Medical Sciences

Protocol

2. Generation of Human Esophageal Organoids for Imaging and Analysis

Demonstrator: Rucheng Liu

2.1. To begin, thaw the basement membrane matrix and human esophageal organoid culture medium at 4 degrees Celsius **[1]**. Transfer the tissue sample into 5-milliliter centrifuge tubes **[2]**, then wash the sample three times with room temperature wash buffer **[3-TXT]**.

2.1.1. WIDE: Talent placing frozen tubes of basement membrane matrix and H-EOCM into a 4 degrees Celsius refrigerator.

2.1.2. Talent placing the sample into centrifuge tubes with forceps.

2.1.3. Shot of wash buffer being added to the tubes. **TXT: Wash Buffer: PBS containing Anti-Anti and 0.15 mM HEPES**

2.2. Using sterile scissors, mince the tissue into 1-millimeter cubed fragments in 1.5-milliliter centrifuge tubes **[1].** ~~Transfer them into 1.5-milliliter centrifuge tubes **[2].**~~ NOTE: VO is removed for the deleted shot.

2.2.1. Talent cutting tissue with scissors.

~~2.2.2. Talent transferring fragments into labeled centrifuge tubes.~~ Author's NOTE: Shot not filmed.

2.3. Now, suspend the tissue fragments in 1 milliliter of digestion buffer **[1].** Shake the mixture at 37 degrees Celsius at 50 to 100 revolutions per minute for 10 to 20 minutes to digest the tissue **[2]**.

2.3.1. Shot of the fragments being suspended in 1 mL digestion buffer.

2.3.2. Talent placing the tube on a shaker set to 37 degrees Celsius and starting the digestion process.

2.4. Next, centrifuge the mixture at 400 *g for* 5 minutes at 4 degrees Celsius **[1].** Then discard the supernatant **[2]**.

2.4.1. Talent loading the tube into a centrifuge and setting conditions.

2.4.2. Talent discarding the supernatant after the run.

2.5. Resuspend the pellet in 500 microliters of 0.025% trypsin-EDTA *(E-D-T-A)* **[1].** Incubate

the suspension at 37 degrees Celsius for 10 minutes **[2]**. Then add 1 milliliter of DMEM *(D-M-E-M)* supplemented with 10% fetal bovine serum to stop enzymatic activity **[3]**.

2.5.1. Talent adding trypsin-EDTA to the tube.

2.5.2. Talent placing the tube in a 37 degrees Celsius incubator.

2.5.3. Talent adding DMEM with fetal bovine serum to the tube after incubation.

2.6. Next, pass the suspension through a 70-micrometer sterile filter **[1]** and collect the filtrate into a 1.5-milliliter centrifuge tube **[2]**.

2.6.1. Talent filtering the cell suspension.

2.6.2. Shot of the filtrate in the 1.5 mL centrifuge tube.

2.7. Centrifuge the filtrate for 5 minutes at 400 *g* at 4 degrees Celsius **[1]**. Resuspend the cells in 100 microliters of H-EOCM *(H-E-O-C-M)* after discarding the supernatant **[2-TXT]**.

2.7.1. Talent placing the filtrate in a centrifuge.

2.7.2. Talent pipetting H-EOCM to resuspend the cell pellet. **TXT: H-EOCM: Human Esophageal Organoid Culture Medium**

2.8. For organoid seeding, gently resuspend cells in 50 to 100 microliters of basement membrane matrix **[1]**. NOTE: VO is modified by the author for the removed shot.

~~2.8.1. Talent aspirating the supernatant.~~ NOTE: Shot removed by the author.

2.8.2. Talent resuspending cells in the matrix using a pipette.

NOTE: Move step 2.9 before 2.8

2.9. After resuspending cells, determine the cell density by using a cell counter **[1]**. Then transfer 5,000 to 15,000 cells per milliliter into a fresh 1.5-milliliter tube **[2].**

2.9.1. Talent places the cell suspension into a cell counter.

2.9.2. Shot of cell suspension being transferred to a fresh 1.5 mL tube.

2.10. Pipette 50 microliters of the matrix-cell mixture to the center of each well of a 24-well plate **[1]**. Incubate at 37 degrees Celsius for 30 minutes to polymerize the basement membrane matrix **[2]**.

2.10.1. Talent carefully dispensing matrix-cell mixture into wells.

2.10.2. Talent placing plate in an incubator for polymerization.

2.10. Now pipette 500 microliters of pre-warmed H-EOCM to each well to cover the matrix **[1]**. Then incubate the plate in a humidified incubator with 5% carbon dioxide at 37 degrees Celsius **[2]**.

2.10.3. Talent pipetting warm H-EOCM into each well.

2.10.4. Talent placing the plate in a CO_2 incubator.

2.11. To replace the medium, aspirate the spent medium **[1].** Replenish each well with 500 microliters of fresh H-EOCM pre-warmed to 37 degrees Celsius **[2]**.

2.11.1. Talent aspirating old medium.

2.11.2. Talent adding fresh H-EOCM into the wells.

3. Multiplex Immunofluorescence Staining of Cultured Organoids

Demonstrator: Lingxuan Zhu

3.1. To perform multiplex immunofluorescence staining on reparaffinized and rehydrated organoids **[1]**, add sheep serum-blocking solution to cover the organoids on a slide placed in a humidity chamber and incubate the slide at room temperature for 30 minutes **[2-TXT].**

3.1.2. Talent placing the slide in a humidity chamber. NOTE: 3.1.2 is placed before 3.1.1 (VO does not need swapping). The slating may/may not have changed. The video sequence should be 1) placing the slide in the humidity chamber/shot of the slide in the chamber, 2) Adding blocking solution to the slide kept in the chamber.

3.1.1. Talent applying sheep serum-blocking solution on the slide. **TXT: Apply blocking solution after antigen retrieval and peroxidase blocking.**

3.2. Wash the slide with PBS containing Tween for 2 minutes **[1]**.

3.2.1. Talent adding PBST over the slide.

3.3. Next, drop diluted primary antibody to cover the organoid area **[1]**. Incubate in a humidity chamber according to antibody requirements **[2-TXT]**. After incubation, wash the slides in PBST two times for 2 minutes at 80 rpm in a thermostable chamber **[3]**.

3.3.1. Shot of diluted primary antibody being dropped over the sample.

3.3.2. Shot of the slide in a humidity chamber. **TXT: Incubation: 2 h at RT or 8 - 14 h at**

4 °C

3.3.3. Talent washing slides in PBST in a thermostable chamber, at 80 rpm.

3.4. Now drop secondary antibody solution onto the organoid area **[1]** and incubate in a humidity chamber at room temperature for 20 minutes **[2]**. After incubation, wash the slides in PBST two times for 2 minutes at 80 rpm *(R-P-M)* in a thermostable chamber **[3].**

3.4.1. Talent pipetting secondary antibody.

3.4.2. Talent placing the slide in a humidity chamber.

3.4.3. Talent places the slides in PBST in a thermostable chamber, at 80 rpm.

3.5. Pipette fluorescent dye solution over the washed slide to cover the organoid area and incubate again **[1-TXT]**. After incubation, wash the slides in PBST two times **[2].** For multi-staining, repeat antigen retrieval and antibody blocking **[3].**

3.5.1. Talent applying dye and placing the slide in chamber. **TXT: Incubation: Humidity chamber at RT, 10 - 20 min; Protect from light from this step onward**

3.5.2. Talent places the slides in PBST in a thermostable chamber, at 80 rpm.

3.5.3. Shot of antibodies being added over the stained slides.

3.6. Then drop DAPI *(Dah-pee)* solution to cover the organoid area **[1]**. Immerse the slides in sterilized water for 2 minutes to wash away the excess stain **[2]**.

3.6.1. Talent adding DAPI to organoid area.

3.6.2. Talent immersing slide in sterilized water.

3.7. Add antifade mounting medium **[1]** and apply coverslip before acquiring digital images **[2]**.

3.7.1. Talent mounting slide with antifade medium.

3.7.2. Talent placing a coverslip.

Results

4. Results

4.1. Organoid structures became increasingly disorganized from normal to carcinoma stages, as visualized by KRT6A *(K-R-T-Six-A)* staining **[1].**

4.1.1. LAB MEDIA: Figure 2. *Video editor: Please highlight the grayscale images of the third row from left to right*

4.2. Expression of PD-L1 *(P-D-L-One)* progressively increased from normal esophageal mucosa to esophageal squamous cell carcinoma **[1].**

4.2.1. LAB MEDIA: Figure 2. *Video editor: Please highlight the green fluorescence images of the first row from left to right*