Submission ID #: 68346
Scriptwriter Name: Sulakshana Karkala
Project Page Link: https://review.jove.com/account/file-uploader?src=20845578

Title: Establishment of a Co-Culture System of Patient-Derived Colorectal Tumor Organoids and Tumor-Infiltrating Lymphocytes (TILs)

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Author Questionnaire

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Current Protocol Length

Number of Steps: 28
Number of Shots: 57

Introduction

1.1. **ZeShuo Feng:** The research aims to explore Tumor-Infiltrating Lymphocytes' interactions with Colorectal Cancer organoids and assess the therapeutic potential of TIL-based therapy for personalized Colorectal Cancer treatment.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:6.5*

What advantage does your protocol offer compared to other techniques?

1.2. **ZeShuo Feng:** Our protocol simplifies Tumor-Infiltrating Lymphocytes production from limited samples and enables their co-culture with Colorectal Cancer organoids, overcoming prior efficiency and compatibility limitations.

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

Ethics Title Card

This research has been approved by the Ethics Committee of Zhongshan Hospital affiliated with Dalian University

Protocol

2. Preparation of Single-Cell Suspensions from Primary Colorectal Cancer Tissue for Downstream Applications

Demonstrator: ZeShuo Feng

2.1. To begin, prepare the experimental reagents and labware for use **[1-TXT]**. Then rinse the primary colorectal cancer tumor tissue three times with 5 milliliters of PBS followed by tissue wash buffer **[2]**.

2.1.1. WIDE: Talent arranging scissors, forceps, AdDMEM^{++++} medium, and CRC digestion buffer on a bench. **TXT: AdDMEM^{++++}: Advanced DMEM with 1% penicillin-streptomycin, 1% HEPES, 1% glutamine, and 5 mM Y-27632**

2.1.2. Talent rinsing the tissue with phosphate-buffered saline or tissue wash buffer three times.

2.2. With tissue scissors, mince the tissue into small fragments **[1]**. Transfer the minced tissue into the CRC *(C-R-C)* digestion buffer **[2-TXT]**. Incubate the sample in a water bath set to 37 degrees Celsius for 30 minutes until the tissue is fully dissociated **[3]**.

2.2.1. Talent mincing the tissue into small pieces using tissue scissors.

2.2.2. Talent transferring minced tissue into a tube containing CRC digestion buffer. **TXT: CRC: Colorectal Cancer**

2.2.3. Talent placing the tube into a 37 degrees Celsius water bath.

2.3. Then add an equal volume of AdDMEM^{++++} *(Add-D-M-E-M)* to the digested sample to neutralize the reaction **[1]**. Centrifuge the suspension at 380 *g* for 5 minutes at 25 degrees Celsius **[2]**.

2.3.1. Talent adding AdDMEM^{++++} to the tube containing digested tissue.

2.3.2. Talent placing the tube into a centrifuge.

2.4. Now, discard the supernatant from the centrifuged sample using a pipette **[1-TXT]**. Pipette 1 to 3 milliliters of pre-warmed erythrocyte lysis buffer to the pellet **[2]** and incubate the sample at 25 degrees Celsius for 10 minutes **[3]**.

2.4.1. Talent discarding the supernatant with a pipette. **TXT: Observe the number of erythrocytes in the pellet**

2.4.2. Talent adding erythrocyte lysis buffer

2.4.3. Talent placing the tube at 25 °C for incubation.

2.5. Add an equal volume of AdDMEM^{++++} to terminate lysis **[1]**. Centrifuge the tube at 380 *g* for 5 minutes at approximately 25 degrees Celsius **[2]**.

2.5.1. Talent adding AdDMEM^{++++} to the lysed sample

2.5.2. Talent placing the tube into the centrifuge.

2.6. After discarding the supernatant, resuspend the cell pellet in an appropriate volume of AdDMEM^{++++} **[1]**.

2.6.1. Talent resuspending the pellet in AdDMEM^{++++}.

2.7. Add trypan blue to the suspension to quantify viable cells and obtain a single-cell suspension of the tumor tissue **[1]**.

2.7.1. Talent mixing cells with trypan blue and loading them into a hemocytometer for counting.

3. Parallel Establishment of Organoids and Immune Cultures from Colorectal Tumor Single-Cell Suspensions

3.1. Divide the single-cell suspension of tumor tissue into two portions**.** Use one portion for establishing the patient-derived organoid model **[1]** and the other for culturing tumor-infiltrating lymphocytes **[2].**

3.1.1. Talent dividing the cell suspension into two tubes.

3.1.2. Talent labeling one tube for PDO and the other for TILs culture.

3.2. After counting the cells, mix 80,000 cells per well with Matrigel at a 1 to 1 ratio for organoid culture **[1].** Then resuspend the remaining cell suspension in complete medium in a 24-well plate **[2-TXT].**

3.2.1. Talent pipetting 80,000 cells per well, and mixing with Matrigel in equal volume.

3.2.2. Talent pipetting the remaining cells into a 24-well plate and adjusting the concentration using complete medium. **TXT: Final concentration: 1 x 10^6 cells/mL**

3.3. Collect the tumor-infiltrating lymphocytes into a centrifuge tube **[1].** Rinse the well plate with fresh medium to recover any remaining cells, transfer them to the same centrifuge tube and centrifuge **[2-TXT].**

3.3.1. Talent transferring TILs to a centrifuge tube.

3.3.2. Talent rinsing the well plate and adding the rinse to the centrifuge tube. **TXT:**

Centrifugation: 125 x *g*, 5 min, 25 °C

3.3.3. Talent placing the tube in the centrifuge and initiating spin.

3.4. After discarding the supernatant, wash the pellet with 2 milliliters of PBS and centrifuge again **[1].**

3.4.1. Talent adding 2 mL phosphate-buffered saline to the pellet and mixing.

3.4.2. Shot of the suspension being placed in the centrifuge. **TXT: Perform cell counting after centrifugation**

3.5. Next, use CD3-positive magnetic beads to sort and isolate CD3-positive T cells **[1].** Place the sorting column into the magnetic stand **[2].** Then transfer the cell suspension to the column and allow magnetic bead-labelled cells to remain in the column **[3].**

3.5.1. Talent adding CD3 magnetic beads to the cell suspension and mixing gently.

3.5.2. Talent inserting the column into the magnetic stand.

3.5.3. Talent applying the cell suspension into the column and waiting for flow-through.

3.6. Once sorting is complete, remove the sorting column from the magnet **[1].** Then add buffer to elute the retained positive cells **[2].** Resuspend the eluted cells in 2 milliliters of PBS and centrifuge **[3-TXT].**

3.6.1. Talent removing the column from the magnetic stand.

3.6.2. Talent adding buffer into the column and collecting eluate.

3.6.3. Talent resuspending cells in 2 mL PBS. **TXT: Centrifugation: 195 x *g*, 5 mins, 25 °C**

3.7. Discard the supernatant using a pipette **[1].** Resuspend the cells in a 12-well plate with fresh EM medium at a concentration of 1 million cells per milliliter **[2].** Add CD3 antibody to a final concentration of 5 micrograms per milliliter **[3].**

3.7.1. Talent removing supernatant from the tube.

3.7.2. Talent adding EM medium to the well plate and resuspending cells.

3.7.3. Talent pipetting CD3 antibody into the medium.

4. Immune Profiling of Colorectal Cancer Organoids and Tumor-Infiltrating Lymphocytes

4.1. Add interferon-gamma to the organoid medium to enhance antigen presentation **[1]**.

Incubate the culture at 37 degrees Celsius with 5 percent carbon dioxide for 24 hours **[2]**.

4.1.1. Talent pipetting interferon-gamma into the organoid medium.

4.1.2. Talent placing the culture into a 37 degrees Celsius incubator with 5 percent carbon dioxide.

4.2. Dilute the anti-CD28 *(C-D-Twenty-Eight)* antibody with PBS to a concentration of 2 micrograms per milliliter **[1]**. Coat each well of a 96-well plate by adding 50 microliters of the antibody solution **[2]**. Then seal the plate with sealing film and incubate **[3-TXT]**.

4.2.1. Talent preparing the antibody dilution in a microcentrifuge tube.

4.2.2. Talent pipetting 50 microliters of diluted antibody into each well of the 96-well plate.

4.2.3. Talent sealing the plate. **TXT: Incubation: 4 °C, 24 h**

4.3. After 24 hours, use TrypLE *(Trip-L-E)* to dissociate the organoids into single cells and count them **[1]**. Then wash the cells twice with PBS **[2]**.

4.3.1. Talent adding TrypLE to the organoids for dissociation.

4.3.2. Talent performing two washes of the cells with phosphate-buffered saline.

4.4. Collect the paired tumor-infiltrating lymphocytes **[1]**, count the cells using a cell counter or hemocytometer **[2]**, and wash them twice with PBS **[3]**.

4.4.1. Talent collecting TILs into a tube.

4.4.2. Talent counting TILs with a cell counter.

4.4.3. Talent washing TILs twice with phosphate-buffered saline.

4.5. Inoculate organoids into each well at 10,000 cells per well **[1]**. Inoculate tumor-infiltrating lymphocytes at 100,000 cells per well to achieve an effector-to-target ratio of 10 to 1 **[2]**.

4.5.1. Talent pipetting 10,000 organoid cells into each well.

4.5.2. Talent adding 100,000 TILs per well to the same wells.

4.6. Perform the co-culture in supplemented RPMI 1640 medium **[1-TXT]**. Incubate the co-culture for 3 days **[2]** and capture images for observation and documentation **[3]**.

4.6.1. Talent preparing and adding the supplemented RPMI 1640 medium to the co-culture wells. **TXT: RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin, 3000 U/mL IL-2, and 20 µg/mL anti-PD-1 blocking antibody**

4.6.2. Talent placing the plate into the incubator for 3 days.

4.6.3. Talent capturing images of the co-culture wells using a microscope or imaging system.

5. Surface Marker Labelling of Co-Cultured Tumor and Immune Cells for Cytometric Analysis

5.1. At the end of the co-culture period, collect the patient-derived organoids and tumor-infiltrating lymphocytes into a centrifuge tube **[1]**.

5.1.1. Talent transferring the contents of co-culture wells into a centrifuge tube.

5.2. Centrifuge the collected cells twice in PBS at 300 *g* for 5 minutes each time **[1-TXT]**.

5.2.1. Talent centrifuging the tube at 300 g for 5 minutes. **TXT: Discard supernatant after each wash**

5.3. Then prepare the antibody cocktail containing CD3-FITC *(C-D-Three-Fit-C)*, CD4-APC/Cyanine7 *(C-D-Four-A-P-C-Sya-Nin-Seven)*, CD8-APC *(C-D-Eight-A-P-C)*, CD107a-PE-Cyanine7 *(C-D-One-Zero-Seven-A-P-E-Sy-ah-nin-Seven)*, CD279-PE *(C-D-Two-Seventy-Nine-P-E)*, and 7-AAD *(Seven-A-A-D)* **[1]**.

5.3.1. Talent preparing a labelled tube with the specified antibodies, pipetting each reagent into the mix.

5.4. Resuspend the washed cells in 100 microliters of the antibody cocktail **[1]** and incubate the tube at room temperature for 20 minutes while protecting it from light **[2]**.

5.4.1. Talent adding 100 microliters of antibody cocktail to the cell pellet.

5.4.2. Talent placing the tube under a cover or in a drawer for light protection during incubation.

5.5. Add 1 milliliter of PBS to wash the cells **[1]**. Then centrifuge the tube at 300 *g* for 5 minutes **[2].** Discard the supernatant using a pipette **[3]**.

5.5.1. Talent adding phosphate-buffered saline to the labelled tube and gently mixing.

5.5.2. Talent placing the tube in a centrifuge.

5.5.3. Talent removing the supernatant with a pipette.

5.6. Finally, resuspend the cell pellet in 500 microliters of PBS before performing flow cytometric analysis **[1]**.

5.6.1. Talent gently pipetting to resuspend cells in phosphate-buffered saline.

Results

6. Results

6.1. Patient-derived organoids showed visible expansion over a 12-day culture period, with small clusters observed on Day 1 **[1]**, larger and more numerous structures by Day 6 **[2]**, and well-defined, mature organoids by Day 12 **[3]**.

6.1.1. LAB MEDIA: Figure 2. *Video editor: Highlight the Day 1 column images*

6.1.2. LAB MEDIA: Figure 2. *Video editor: Highlight the Day 6 column images*

6.1.3. LAB MEDIA: Figure 2. *Video editor: Highlight the Day 12 column images*

6.2. Organoids exhibited similar histological architecture to colorectal cancer tissue, as shown by hematoxylin and eosin staining **[1]**.

6.2.1. LAB MEDIA: Figure 3. *Video editor: Please show only the first column HE and the emphasize the "Organoid" image in the column.*

6.3. Immunostaining revealed cytokeratin 7 expression in both tissue and organoids, confirming epithelial identity **[1]**, with cytokeratin 20 also showing strong expression in both sample types **[2]**.

6.3.1. LAB MEDIA: Figure 3. *Video editor: Please show only the second column CK7*

6.3.2. LAB MEDIA: Figure 3. *Video editor: Please show the last column CK20*

6.4. Similar expression patterns were also observed for Ki-67, a proliferation marker, and caudal type homeobox 2, an intestinal differentiation marker **[1]**.

6.4.1. LAB MEDIA: Figure 3. *Video editor: Please highlight images of the column Ki67 and CDX2*

6.5. Tumor-infiltrating lymphocytes showed progressive expansion from sparse distribution on Day 1 **[1]**, to noticeable clustering by Day 14 **[2]**, and dense aggregation by Day 22 **[3]**.

6.5.1. LAB MEDIA: Figure 4. *Video editor: Highlight the Day 1 column images*

6.5.2. LAB MEDIA: Figure 4. *Video editor: Highlight the Day 14 column images*

6.5.3. LAB MEDIA: Figure 4. *Video editor: Highlight the Day 22 column images*

6.6. Immunofluorescence staining showed strong CD45 and CD3 signals in Tumor-infiltrating lymphocytes, confirming the immune origin of the cells **[1]**.

6.6.1. LAB MEDIA: Figure 5. *Video editor: Highlight the green signal for CD45 in the leftmost panel and the red signal for CD3 in the middle image*

6.7. Absence of EpCAM staining in the Tumor-infiltrating lymphocytes population confirmed that the expanded cells were free of epithelial tumor cells **[1]**.

6.7.1. LAB MEDIA: Figure 5. *Video editor: Please show rightmost image*

Pronunciation Guide:

1. Colorectal

- **Pronunciation Link**: https://www.merriam-webster.com/dictionary/colorectal
- **IPA**: /ˌkoʊ.loʊˈrɛk.təl/
- **Phonetic Spelling**: koh-loh-REK-tuhlmerriam-webster.commerriam-webster.com+2merriam-webster.com+2merriam-webster.com+2

2. Organoid

- **Pronunciation Link**: https://www.merriam-webster.com/medical/organoid
- **IPA**: /ˈɔːr.gə.nɔɪd/
- **Phonetic Spelling**: OR-guh-noidmerriam-webster.com+7merriam-webster.com+7merriam-webster.com+7

3. Lymphocyte

- **Pronunciation Link**: https://www.merriam-webster.com/dictionary/lymphocyte
- **IPA**: /ˈlɪm.fəˌsaɪt/
- **Phonetic Spelling**: LIM-fuh-sytemerriam-webster.com+7merriam-webster.com+7merriam-webster.com+7

4. Trypan Blue

- **Pronunciation Link**: https://www.merriam-webster.com/medical/trypan%20blue
- **IPA**: /ˈtrɪp.æn bluː/
- **Phonetic Spelling**: TRIP-an bloomerriam-webster.com+7merriam-webster.com+7merriam-webster.com+7merriam-webster.com+8merriam-webster.com+8merriam-webster.com+8merriam-webster.com+1merriam-webster.com+1

5. Cytokeratin

- **Pronunciation Link**: https://www.merriam-webster.com/medical/cytokeratin
- **IPA**: /ˌsaɪ.toʊˈkɛr.ə.tɪn/
- **Phonetic Spelling**: SY-toh-KER-uh-tinmerriam-webster.com+9merriam-webster.com+9merriam-webster.com+9