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Protocol

Analysis of the Killing Activity of Immune Cells with Tumor Organoids

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The tumor microenvironment (TME) is considered a key factor in the occurrence and development of tumors. It not only exists and affects the structure, function, and metabolism of the tissue where the tumor is located, but also relates to the intrinsic environment of the tumor cells themselves. Positive responses to immunotherapy often depend on the interaction between tumor cells and the immunoregulation within the TME. Under these interactions, the TME plays a significant role in either suppressing or enhancing immune responses. However, there are currently no mature research methods for characterizing or modeling the TME.

Organoids are in vitro three-dimensional cell culture systems that can replicate the parental tissue or organ, featuring complex spatial structures of self-assembly, including cell-to-cell and cell-to-extracellular matrix interactions. Ideally, they also include interactions between cells and immune cells. However, the limitation of tumor organoids derived from patient tumor tissues is that, although a lower proportion of immune cells can be detected in the initial few generations (usually the first 1-2 generations) of organoids, these immune cells' quantity and composition hardly represent the composition of the tumor immune microenvironment *in situ*.

Co-culturing immune cells with tumor organoids further explores the interaction between tumors and the immune system. However, the co-culture system of tumor organoids and immune cells cannot fully characterize the TME. By co-culturing specific cells within the TME with tumor organoids, some characteristics of the TME are partially simulated. Co-culturing tumor organoids with different immune cells (including TILs, CAR-T, CIK, or specific components of the TME) allows for the direct detection of the infiltration and killing effect of immune cells on the organoids, which is significant for research on

tumor immunotherapy and cell therapy products.

This protocol provides the side-by-side steps for co-culturing lung cancer organoids and cytokine-induced killer cells (CIK) cells using MasterAim® co-culture related products*. Researchers can refer to this protocol to co-culture other types of tumor organoids and immune cells and carry out further detection and analysis.

*The co-culture medium has been verified for the culture of immune cells such as TILs, NK cells, and CIK cells. The genetically engineered cells, including CAR-T cells and CAR-M cells have not been verified with this medium.

Reagents and consumables used in this protocol

- MasterAim® Lung Cancer Organoids and Immune Cell Co-culture Kit (#10-100-492)
- MasterAim® Lung Cancer Organoid Kit (#10-100-060)
- MasterAim® Tissue Preservation Medium (#100-049)
- MasterAim® Tissue Dissociation Medium I (#100-047)
- MasterAim® Tissue Dissociation Medium II (#100-048)
- MasterAim® Anti-Adherence Solution (#100-291)
- MasterAim® primary enhancer (#100-008)
- Fetal Bovine Serum (FBS) (#100-236)
- Dulbecco's Modified Eagle Medium (DMEM, #100-134)
- Live cell dyes (depend on experiential requirement, dyes used in this protocol are for references only)
- Green cell dyes recommended: Calcein-AM, CMFDA, Caspase3/7
- Red cell dyes recommended: CMTPX, Mito-Red
- Dulbecco's Phosphate-Buffered Saline (DPBS, no calcium, no magnesium, no phenol red) Buffer (#100-183)
- Red Cell Lysis Buffer (#B541001-0100, Sangon Biotech)
- Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (#356231, Corning)
- Pen-Strep Solution (#C3423-0100, Vivacell)
- TrypLE Express Enzyme (1X), Phenol Red (#12605-010, Gibco)
- 96-well Clear Ultra-Low Attachment Multiple Well Plates (#3474, Corning)
- 24-well Clear Ultra-Low Attachment Multiple Well Plates (#3473, Corning)
- Costar® 24-well Clear TC-treated Multiple Well Plates (#3524, Corning)

- Costar® 6-well Clear TC-treated Multiple Well Plates (#3516, Corning)
- X-VIVO15 Serum-free Medium with Gentamicin and Phenol Red (#04-418Q, Lonza)
- Recombinant Human IL-2 Protein (#GMP-TL777, T&L Biotechnology)
- Anti-Human CD3 mAb (#GMP-TL101, T&L Biotechnology)
- Anti-Human CD8 mAb (#GMP-TL102, T&L Biotechnology)
- Ficoll (#LTS1077, TBD Science)

Protocol

Part I Establishment of Lung Cancer Organoids

1. Reagents Preparation

1.1 Washing solution: Add 1% Pen-Strep (PS) Solution to DPBS buffer, named as DPBS-PS, pre-cool before using.

1.2 One day ahead, place MasterAim® Lung Cancer Organoid Culture Complete Medium: Place the MasterAim® Lung Cancer Organoid Basal Culture Medium and the MasterAim® Lung Cancer Organoid Culture Medium Supplement at 4°C to thaw. Transfer all the supplement into the basal medium, mix well, and it becomes the lung cancer organoid culture complete medium.

Note:

(1) The complete organoid culture medium does not contain antibiotics. Add the antibiotics (i.e. PS) before use.

(2) It is recommended to aliquot the complete medium after preparation. Keep the un-used aliquots at -20°C. After thawing, keep at 4°C, and use up within 2 weeks. Avoid repeated freeze-thaw cycles.

(3) For the establishment of primary organoids, 1X MasterAim® Primary Enhancer can be added to the culture medium to increase the culture efficacy. After passaging, organoids can be directly cultured in MasterAim® Lung Cancer Organoid Complete Culture Medium with antibiotics.

2. Tissue Processing

Note:

*For all steps processing with cells or tissues, all tubes and pipette tips **MUST** be pre-rinsed with MasterAim® Anti-Adhesion Solution (#100-291) to reduce cell loss.*

2.1 Tissue should be kept in cold MasterAim® Tissue Preservation Medium upon arrival at the lab.

2.2 Depending on the size of the tissue, place the tissue into a 15/50 mL falcon tube. Wash with 5/15 mL

DPBS-PS for 3 - 5 times until the supernatant is clear. The washing solution and tissue preservation medium can be collected, centrifuged, and observed for the presence of cells. If present, the cells can be washed, centrifuged, and collected separately.

3. Tissue Dissociation

3.1 Transfer the cleaned tissue to an EP tube, add 200 μL of DPBS-PS or MasterAim® Tissue Dissociation Medium I (#100-047), and chop the tissue with scissors to 0.5-1 mm^3 fragments, ensuring the chopped tissue pieces appear uniform.

3.2 Use a pre-rinsed Pasteur pipette to transfer the chopped tissue fragments from the EP tube to a 15 mL falcon tube, add 4 mL MasterAim® Tissue Dissociation Medium I (#100-047). Place the tube on a horizontal shaker for 30 - 45 mins, 120 rpm, at 37°C. Observe under a microscope every 15 mins. Stop the digestion once significant number of cells leaking from the tissues under observation. Usually, digestion of lung cancer samples should not exceed 1 h.

Note:

After step 3.1, observe for cell leaking out under microscope. If significant number of cells leak, it indicates the tissue digests easily, and digestion time might need to be shortened; if the tissue is highly fibrotic, the cell yield may be lower, and digestion time could be extended.

3.3 After digestion with MasterAim® Tissue Dissociation Medium I, add 8 mL DPBS-PS to stop the digestion, centrifuge at 300 g for 5 mins, discard the supernatant. Add 2 mL MasterAim® Tissue Dissociation Medium II (#100-048) to the pellet, then digest on a horizonal shaker for 10 - 15 mins, 120 rpm, at 37°C. Observe under a microscope every 5 mins. Stop the digestion when most cell clumps are comprised of 5 - 20 cells.

4. Primary organoids initiation

4.1 After digestion, add 4 mL DPBS-PS to stop the digestion, centrifuge at 300 g for 5 mins, and collect the cells.

4.2 Discard the supernatant and resuspend the cells in 2 mL DPBS-PS.

4.3 (Optional, small biopsy samples may choose not to filter) Pre-rinse a 100 μM cell strainer, filter the cell suspension through the 100 μM filter into a new 50 mL pre-rinsed falcon tube. Wash the cell suspension tube with DPBS-PS several times for filtration. Centrifuge the filtrates at 300 g for 5 mins.

4.4 Discard the supernatant, avoiding contact with the cell pellet. Use a pipette tip to remove the bottom liquid.

4.5 (Optional step) If cell pellet appears red, add 1 - 2 mL red blood cell lysis solution (volume can be adjusted based on the size of cell pellet), use 1 mL pipette tip to suspend the pellet thoroughly, incubate at room temperature for 6 min. Add 4 - 6 mL DPBS-PS to stop the lysis, then centrifuge at 300 g for 5 mins.

Note:*Lysis time should not exceed 8 min.*

4.6 Cell Counting: Resuspend the cell pellet obtained in the previous step with 1 mL DPBS-PS, and count the number of cell clusters using an automatic cell counter or a manual counting plate.

4.7 Centrifuge at 300 g for 5 mins. According to the counting results, resuspend the cell pellet obtained in the previous stage in appropriate volume of complete organoid medium, the recommended seeding density is 4*10^6 cells/mL. Add 1.5 volume of Matrigel to the suspension to mix the cell fragments, try not involving bubbles by only pressing to the first stop of the pipettor. This step **MUST** be operated on ice to prevent Matrigel solidify.

Note: *This step requires the use of ice-cold pipette tips and pre-cold medium, Matrigel* ***MUST*** *be thawed on ice in advance.*

4.8 Pipette 50 μL cell suspension to the center of a pre-warmed 24-well plate. To prevent bubbles, only press the pipettor to the first stop when using, and a solidified droplet (domes) should form in the center of each well.

Note: *The culture plate* ***MUST*** *be pre-warmed in a 37°C incubator for more than half an hour. The pipette tip* ***MUST*** *not touch the bottom of the culture plate.*

4.9 Leave the plate at 37°C for 5 mins, then invert the plate and leave it for 25 mins to allow the Matrigel to solidify.

4.10 Gently add 500 μL of pre-warmed MasterAim® Lung Cancer Organoid Culture Medium to each well along the side walls of the wells, avoiding direct addition to the domes.

4.11 Add DPBS-PS to other wells without cells to maintain humidity during culture.

Note*: Do not seed organoids into the outermost wells.*

4.12 Cover the culture plate and cultivate at 37°C and 5% CO_2.

4.13 Change the culture medium every 2 - 4 days, observe the growth, and record with photos until passaging.

4.14 Generally, organoids can be observed forming 3 - 4 days after seeding. Before passaging, ensure most organoids are larger than 100 μM. Generally, the P0 generation will undergo the first passaging after 7 - 21 days.

Part II Preparation of CIK Cells from human peripheral blood mononuclear cells (PBMCs)

1. Obtain PBMCs from freshly collected human whole blood samples using density gradient centrifugation with Ficoll. At least 10 mL of fresh whole blood is recommended for the experiment.

2. Resuspend PBMCs in X-VIVO15 medium supplemented with 10% FBS at a concentration of 1-2*10^6/mL. Add IL-2 (6000IU/mL) and mix well, then transfer the cells to a 24-well/6-well TC-treated plate based on the volume of the culture medium.

3. After three days, transfer the cell suspension to a new 24-well/6-well TC-treated plate, and add CD3 antibody (500 ng/mL) and CD28 antibody (500 ng/mL). Culture in a 37°C, 5% CO_2 incubator.

Note: *During the early activation phase of the culture process, a significant number of cells will adhere to the well. It is appropriate to collect the culture by pipetting. Discard the fully adhered cells.*

4. When the culture medium turns yellow or the cell concentration approaches saturation (i.e., greater than 2*10^6/mL), add medium to adjust the cell concentration to between 1-2*10^6/mL. Depending on the amount of medium, transfer to a larger cell culture vessel.

Note: *After 7 days of step 3, the cells can be used for co-culture experiments. For optimal results, it is recommended to use CIKs with a viability greater than 80% for co-culture. Due to immunocyte exhaustion, long-term expansion and cryopreservation are not feasible. It is suggested that CIKs cultured for more than 30 days should not be used for co-culture experiments. Below is a comparison image before and after activation.*

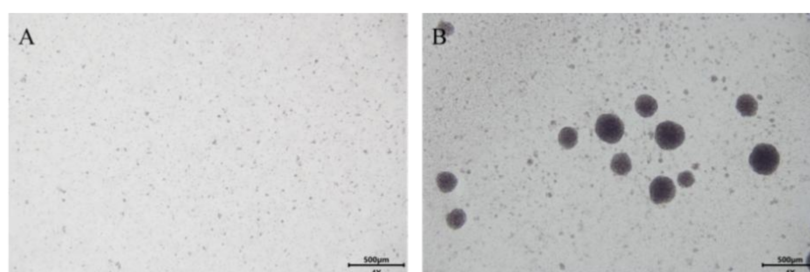

Figure 1. Bright field images of CIK cell before and after activation. A. PBMCs before activation B. After adding CD3/CD28 for 7 days, activated CIKs form colonies.

Part III. Using Lung Cancer Organoids to Evaluate the Cytotoxicity of CIK Cells

1. Reagents Preparation

1.1 One day ahead, place the MasterAim® Lung Cancer Organoid and Immune Cell Co-Culture Basal Medium (#100-493), MasterAim® Lung Cancer Organoid and Immune Cell Co-Culture Supplement 1 (#100-494), and MasterAim® Lung Cancer Organoid and Immune Cell Co-Culture Supplement 2 (#100-495) at 4°C to thaw.

1.2 Transfer all the Supplement 1 into the basal culture medium, mix well to create the partially complete co-culture medium.

1.3 Supplement 2 and MasterAim® primary enhancer is added before use, with a ratio of 120 μL of the Supplement 2 per 10 mL of the partially complete co-culture medium. It is suggested to add 0.5X MasterAim® primary enhancer throughout the entire co-culture process of organoids and immune cells.

Note:

(1) This medium does not contain antibiotics; please add the antibiotics according to experimental requirements.

(2) It is recommended to aliquot appropriately for storage at -20°C. After opening, store at 4°C and use within 2 weeks. Avoid repeated freeze-thaw cycles.

2. Cytotoxicity Analysis of CIK Cells with Lung Cancer Organoids

Note*: The following protocol uses a 96-well plate culture system as an example. Adjust the culture vessel with required cell numbers and culture volumes accordingly. This protocol uses IncuCyte's real-time imaging for the analysis of immune cell cytotoxicity, refer to the IncuCyte operation manual for specific procedures. Or choose different analysis methods according to experimental needs.*

2.1 Preparation of lung cancer organoids for co-culture:

One day ahead, disperse the cultured organoids with cold DPBS-PS. Try to be gentle to maintain organoid integrity. Typically, resuspend 3 - 4 50 μL domes of lung cancer organoids in 1 mL DPBS-PS, approximately 800 organoids, which can be used for 1 mL co-culture cell suspension. After centrifugation at 300 g for 5 mins, resuspend the organoids in 1 mL MasterAim® lung cancer organoid complete culture medium and culture in a 24-well clear Ultra-Low Attachment plate.

2.2 The next day, stain and count the suspended organoids and immune cells.

Note:*Current literature mentions an effector to target ratio between 1:5 to 1:20 for immune cells and organoids; this experiment uses a 1:10 ratio, which can be adjusted for different experimental purposes to achieve optimal results.*

2.2.1 Lung cancer organoid counting process: Considering that the organoids need to be intact during co-culture, they should not be digested into single cells. Therefore, at least 100 μL of organoid suspension needs to be used for cell counting, which will not be used in co-culture. Use TrypLE to digest the organoid suspension into single cells for counting. Based on the number of single cells in the 100 μL system, calculate the number of cells in organoids from step 2.1. The required cell number for 96-well

plate is 10^4 per well, and 2*10^5 for 24-well plate. Calculate the total required number of organoids for different experimental groups.

2.2.2 Lung cancer organoids' staining: Centrifuge at 300 g for 5 mins and collect the needed lung cancer organoids in a 1.5 mL EP tube, with the staining quantity ranging from 800 to 5000 organoids per tube. Discard the supernatant, resuspend the cells in 1 mL 37°C pre-warmed DMEM, add CMTPX to a final concentration of 2 μM, incubate in a 37°C incubator in the dark for 30 mins. After staining, centrifuge the cells at 300 g for 5 mins, wash the cells 2 - 3 times with 1 mL DPBS. After the last wash, suspend in 1 mL MasterAim® lung cancer organoid culture medium in a 24-well clear Ultra-Low Attachment plate for 4 hours, then wash the cells 2 - 3 times with 1 mL DPBS, and collect the organoids.

2.2.3 Counting of CIK cells: 4 hours after staining the organoids, calculate the required number of CIK cells according to the cell number of organoids in step 2.2.1, and transfer the required number of cells to a 1.5 mL EP tube.

2.2.4 CIK cells' staining: Centrifuge at 300 g for 5 mins and collect the CIKs in a 1.5 mL EP tube, the appropriate number of cells for staining per tube is 5 - 10*10^5 cells. Discard the supernatant, resuspend the cells in 1 mL 37°C pre-warmed DMEM, add CMFDA to a final concentration of 2 μM, incubate in a 37°C incubator in the dark for 15 mins. After staining, centrifuge the cells at 300 g for 5 mins, wash the cells 3 - 5 times with 1 mL DPBS, and collect the cells.

Note: *Steps 2.2.2 and 2.2.4 are fluorescence staining steps. The laboratory can adjust the staining scheme and the type of live cell dye according to needs. If fluorescence staining is not performed, proceed directly to step 2.3 and subsequent steps.*

2.3 After staining the lung cancer organoids and CIK cells, resuspend an appropriate amount of MasterAim® lung cancer organoids and immune cells co-culture complete culture medium according to the experimental group. In general, it is recommended to mix the volume of organoids and immune cells at 10:1. The total culture volume per well in a 96-well plate is recommended to be 100 - 150 μL per well.

2.4 First, add organoids to a 96-well clear Ultra-Low Attachment plate, wait for 30min in a 37°C incubator, then slowly drop CIK cells along the liquid interface and use the pipette tip to gently stir, mixing the entire culture system.

2.5 Place the cell culture plate in an incubator or imaging microscope for subsequent experiments. The experimental data for part IV is obtained through real-time imaging with IncuCyte S3, with image collection interval set to 2 hours, totaling 72 hours.

Part IV AimingMed Internal Data

1. MasterAim® Lung Cancer Organoid and Immune Cell Co-culture Kit (#10-100-492) Data

Using lung cancer organoids and T cells as example, AimingMed evaluated the effects of lung cancer organoid culture medium, T cell culture medium, and MasterAim® lung cancer organoid and immune cell co-culture medium (hereinafter referred to as "co-culture medium") on T cells and lung cancer organoids. As shown in Fig 2A and 2B, the co-culture medium can maintain the viability of T cells, whereas T cells cultured in the lung cancer organoid medium group showed significantly lower viability than those cultured in the immune cell medium ($p<0.01$). This result indicates that the tumor organoid medium is not suitable for the culture of T cells, while the co-culture medium can maintain the viability of T cells during the experimental window.

Next, we tested the effect of the various culture media on the viability of lung cancer organoids. As observed in Fig 2C, lung cancer organoids began to disintegrate after 8 hours of culture in the T cell culture medium, indicating that the T cell culture medium is not suitable for the growth of organoids. Meanwhile, using the Incucyte S3, we evaluated the impact of the co-culture medium on the survival of lung cancer organoids. As shown in Fig 2D/E, compared to MasterAim® lung cancer organoid culture medium, there was no significant difference in growth rate with the MasterAim® lung cancer organoid and immune cell co-culture medium. In summary, the MasterAim® organoid and immune cell co-culture kit, upon testing, can maintain the viability of both cells within the experimental window, providing more reliable data.

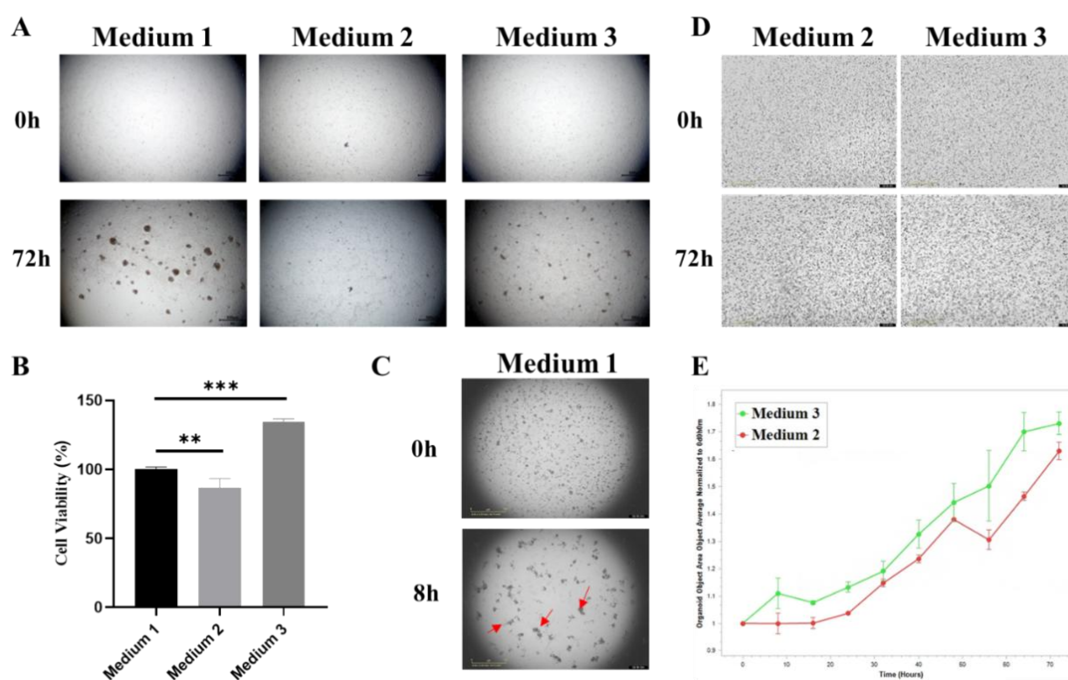

Figure 2. The impact of different culture systems on immune cells and lung cancer organoids. (A) Bright-field images of the effects of different culture media on T cells for 72h; (B) CCK-8 assay statistical differences in growth among different groups. Medium 1 - T cell culture medium, Medium 2 - MasterAim® lung cancer organoid culture medium; Medium 3 - MasterAim® lung cancer organoid and immune cell co-culture medium. t-test, $^{**}p < 0.01$, $^{***}p < 0.001$; (C) Bright-field images of lung cancer organoids cultured in T cell medium for 8h, with red arrows indicating apoptotic organoids; (D) Bright-field observations of the impact of different culture media on lung cancer organoids at 0 hours and 72h; (E) Statistical curve of the growth area of organoids in different groups.

2. MasterAim® Colon Cancer Organoid and Immune Cell Co-culture Kit (#10-100-504) Data

Using this protocol, colon cancer organoids and CIK cells are co-cultured at an effector-to-target ratio of 1:10 to observe the killing effect of CIK cells on the organoids within 72 hours. AimingMed utilizes the Incucyte® S3 live-cell imaging system for real-time observation of CIK cell infiltration and killing of the organoids, facilitating the selection of the optimal experimental endpoint for evaluating their killing efficacy.

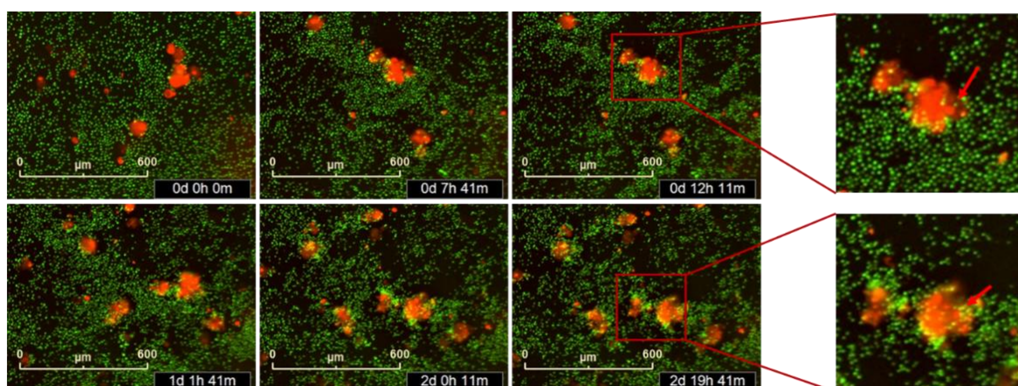

Figure 3. Detection of CIK cell-mediated killing and infiltration of colon cancer organoids using the Incucyte® S3 real-time imaging system. Fluorescence images of colorectal cancer organoids (red) and CIK cells (green) after 72 hours of co-culture. Before co-culture, organoids and CIK cells were labeled with red and green probes, respectively. 12 hours after co-culture, CIK cells can be observed aggregating around and infiltrating (red arrows) the colon cancer organoids.

Part V. Tips

1. It is recommended to culture cancer organoids derived from primary tissues using MasterAim® cancer organoid culture medium, as demonstrated in this experimental protocol.

2. The co-culture system used in this protocol is a suspension culture, which seems to allow immune

cells to attack and kill the organoids more easily compared to methods involving a matrix gel.

3. The suspension culture method does not allow for medium changes. Due to medium consumption, the optimal window for the entire culture system is 72 hours; do not exceed 96 hours, as the organoids and immune cells may undergo apoptosis due to insufficient nutrients.

4. The density of co-cultured organoids can significantly vary depending on the culture generation and certain parameters of the source organoids, so seeding density is flexible; it can be optimized based on the initial sample and growth conditions. If there is no experience with co-culture, the seeding density provided in this protocol can be used, with subsequent experiments optimized based on the results.

5. It has been verified that the MasterAim® lung cancer organoid and immune cell co-culture medium is suitable for immune cells including CIK, T cells, TILs, etc.

6. This experimental protocol only presents a co-culture protocol for immune cells and tumor organoids. If evaluating the efficacy of immune checkpoint inhibitors, an additional group of immune cells + tumor organoids + PD-1/PD-L1 can be added to compare with the immune cells + tumor organoids group.

7. Immune kill efficacy with tumor organoids can be evaluated not only by fluorescence labeling for invasion and killing effects but also any established detection methods for cell killing indicators, include FACS, qPCR, ELISA, etc.