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Title: Monitoring Cancer Cell Invasion and T-Cell Cytotoxicity in 3D Culture

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **N**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

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Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Yuan-Na Lin**: These methods can provide mechanistic insights into immune cell-mediated tumor cell cytotoxicity and invasive behavior in a 3D setting **[1]**.

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

REQUIRED:

1.2. **Yuan-Na Lin**: The main advantage of this technique is that this 3D spheroid co-culture model does not require the transfer of spheroids for multiple subsequent assays **[1]**.

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

Introduction of Demonstrator on Camera

1.3. **Yuan-Na Lin**: Demonstrating the procedure will be Apsra Nasir, a PhD student from our Department **[1][2]**.

1.3.1. INTERVIEW: Author saying the above
1.3.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera

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Protocol

Videographer NOTE: Heads up that some of the close ups were shot in 4K as the crop on my sensor when shooting in 4K allows me to zoom in more to get more detail without getting so close so you will have to resize a few of the close up shots when importing into a 1080p timeline in editing. Also for a few shots we did multiple takes where the first shot was slightly wider and the second shot was closer up to give more detail if necessary. Most of the shots were called out verbally.

2. Spheroid Generation

2.1. To generate the spheroids, using aseptic technique in a cell culture hood **[1]**, add 500 microliters of molten agarose into an 81-microwell rubber mold, taking care to avoid bubbles **[2-TXT]**.

2.1.1. WIDE: Talent placing materials into hood

2.1.2. Talent adding agarose to well(s) **TEXT: Alternative: See text for 35-microwell rubber mold cast and culture details**

2.2. When the agarose has solidified, carefully flex the rubber mold to pop out the agarose casts **[1]** into individual wells of a 12-well plate **[2]**.

2.2.1. Mold being flexed *Videographer: Important step* **TEXT: Caution: Flex at multiple positions and do not over flex**

2.2.2. Cast being dropped into well Videographer NOTE: Use shot 2.2.2 take 4 (File 6H0A3898) as the cast fell into the well in the right orientation as opposed to the first three takes. *Videographer: Important step*

2.3. To equilibrate the casts, add 2.5 milliliters of cell culture medium supplemented with 10% fetal bovine serum to each well **[1-TXT]** and place the plate into a cell culture incubator for 1 hour **[2]**.

2.3.1. Talent adding medium to well **TEXT: See text for all medium preparation details**

2.3.2. Talent placing plate into incubator

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2.4. At the end of the incubation, tilt the plate to remove the cell culture medium **[1]** and carefully seed 190 microliters of the prepared tumor cell suspension drop-wise into each cell seeding chamber **[2-TXT]**

2.4.1. Talent removing medium from tilted plate *Videographer: Important step*
2.4.2. Tumor cells being added to chamber Videographer NOTE: We recorded Shot 2.4.2 after shot 2.5.1, this was the only shot in the protocol that was shot out of sequence *Videographer: Important step* **TEXT: See text for tumor cell preparation details**

2.5. After a 15-minute incubation in the cell culture incubator, add 2.5 milliliters of medium to the outside of the cast **[1]** and return the plate to the incubator for 48 hours **[2]**.

2.5.1. Medium being added to well, with medium container visible in frame
2.5.2. Talent placing plate into incubator

3. T Cell Co-Culture

3.1. To set up a T cell co-culture, resuspend the appropriate number of T cells in 190 microliters of an appropriate T cell culture medium per well **[1]** and tilt the 12-well plate to allow removal of the cell culture medium surrounding the agarose cast without removing the spheroids **[2]**.

3.1.1. WIDE: Talent resuspending T cells in medium, with medium container visible in frame *Videographer: Important step*
3.1.2. Talent tilting plate, then removing medium *Videographer: Important step*

3.2. Use a light microscope to check whether any spheroids have been aspirated **[1]** and, holding the P200-loaded pipette about half a centimeter above each cast **[2]**, carefully seed the T cells onto the casts in a drop-wise fashion without dislodging the spheroids **[3]**.

3.2.1. Talent at microscope, checking for spheroids *Videographer: Important step*
3.2.2. Pipette being positioned over cast *Videographer: Important step*
3.2.3. Droplets being added to cast *Videographer: Important step*

3.3. When all of the casts have been seeded, carefully return the plate to the cell culture incubator for 15 minutes **[1]** before adding fresh cell culture medium supplemented with fetal bovine serum to the outside of each cast for another 48-hour incubation **[2]**.

3.3.1. Talent placing plate into incubator
3.3.2. Medium being added to wall of well

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4. 3D Co-Culture Embedding

4.1. To embed the 3D co-cultures in type one collagen, dilute stock collagen with serum free base medium to a final working concentration of 3 milligrams/milliliter **[1]** and add 11 microliters of 10x PBS **[2]** and 1.2 microliters 1-molar sodium hydroxide per 100 microliters of collagen **[3]**.

4.1.1. WIDE: Talent adding collagen to container, with collagen container visible in frame
4.1.2. Talent adding PBS to container, with PBS container visible in frame
4.1.3. Talent adding NaOH to container, with NaOH container visible in frame

4.2. After neutralizing the collagen solution on ice for 1 hour **[1]**, remove the cell culture medium surrounding and within each cast as demonstrated **[2]**.

4.2.1. Talent placing collagen on ice
4.2.2. Plate being tilted/surrounding medium being removed

4.3. Carefully add the neutralized collagen one mixture to each cast in a dropwise fashion **[1]** and immediately return the plate to the cell culture incubator for 5 minutes **[2]** before inverting the plate for an additional 1 hour of incubation **[3]**.

4.3.1. Collagen being added to cast Videographer NOTE: FILE 6H0A3925 is shot 4.3.1 I miscalled it verbally as shot 4.1.1
4.3.2. Talent placing plate *Videographer: Important step*
4.3.3. Talent inverting plate *Videographer: Important step*

4.4. At the end of the incubation, place the plate right side up in the biosafety hood **[1]** and slowly add fresh cell culture medium down the side of each well **[2]**.

4.4.1. Talent placing plate into hood
4.4.2. Talent adding medium to well

4.5. Then return the plate to the cell culture incubator for 48 hours **[1]**.

4.5.1. Talent placing plate into incubator

5. Immunofluorescence Staining

5.1. For immunofluorescence staining of the embedded 3D co-cultures, fix each entire agarose cast, including the spheroids **[1]**, in 5.4% formalin overnight at room temperature in a humidified chamber **[2]**.

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5.1.1. WIDE: Talent adding formalin to well(s), with formalin container visible in frame
5.1.2. Talent placing plate into chamber

5.2. The next day, remove the formalin surrounding the agarose cast **[1]** and use sleek-tip tweezers to grasp the corner of each collagen matrix **[2]** to allow the casts to be peeled from the seeding chambers in a single, fluent movement **[3]**.

5.2.1. Formalin being removed *Videographer: Important step*
5.2.2. Matrix corner being grasped Videographer NOTE: FILE 6H0A3938 is probably the best take of SHOT 5.2.2 *Videographer: Important step*
5.2.3. Cast being removed *Videographer: Important step*

5.3. Place each collagen patch into a single well of an 8-well chamber slide **[1]** and add 250 microliters of 0.5% octoxynol to each well **[2]**.

5.3.1. Talent placing patch into slide
5.3.2. Talent adding octoxynol to well, with octoxynol container visible in frame

5.4. After 1 hour at room temperature, replace the octoxynol with 250 microliters of blocking solution **[1]**.

5.4.1. Blocking solution being added, with blocking solution container visible in frame

5.5. After 1 hour at room temperature, add 250 microliters of the primary antibody cocktail of interest to each well **[1-TXT]** and place the slide in a dark humidified chamber overnight at room temperature **[2]**.

5.5.1. Talent adding cocktail to well(s), with antibody and stain solution containers visible in frame **TEXT: *e.g.*, anti-keratin 8, Phalloidin 546, Hoechst**
5.5.2. Talent placing slide into a dark humidified chamber

5.6. The next morning, remove the primary antibody from each well **[1]** and wash the wells three times with 300 microliters of IF (I-F) buffer for 5 minutes at room temperature per wash **[2]**.

5.6.1. Antibody solution being aspirated
5.6.2. Talent adding buffer to well(s), with buffer container visible in frame

5.7. After the last wash, add 250 microliters of an appropriate secondary antibody solution to each well for **[1]** a 1-hour incubation at room temperature protected from light **[2-added]**.

5.7.1. Talent adding solution to well(s), with solution container visible in frame
5.7.2. Added: Talent placing slide into a dark humidified chamber.

5.8. At the end of the incubation, remove the antibody **[1]** and wash each sample three times with IF buffer as demonstrated **[2]**.

5.8.1. Antibody solution being aspirated
5.8.2. Talent adding buffer to well(s), with buffer container visible in frame

5.9. After the last wash, rinse the samples with 300 microliters of PBS per well **[1]** and carefully remove the walls of the chamber slide so that only the glass slide at the bottom remains **[2]**.

5.9.1. PBS being added/aspirated
5.9.2. Wall(s) being removed

5.10. Add 200 microliters of mounting medium to the glass slide **[1]** and carefully drop the coverslip onto the sample without creating bubbles **[2]**.

5.10.1. Talent adding mounting medium to glass slide, with mounting medium container visible in frame NOTE: 5.10.1 – 5.10.2 combined
5.10.2. Coverslip being placed onto slide

5.11. Then store the mounted samples in a dark, dry place overnight **[1]** before imaging by confocal fluorescence microscopy **[2]**.

5.11.1. Talent placing slide into storage box
5.11.2. LAB MEDIA: Figures 4G-J

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Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.2., 2.4., 3.1., 3.2., 4.3., 5.2.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
3.1. Risk of removing spheroids – gently target the pipette to one corner of the seeding chamber while keeping the well-plate tilted.
3.2. Risk of flushing out the spheroids – Add the T-cells very slowly and about 0.5 cm above the seeding chamber.

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Results

6. Results: Representative Cancer Cell Invasion Monitoring

6.1. Here typical experimental setups for the assays **[1]** and the yield of representative and analyzable samples at the end of the experiment for each protocol can be observed **[2]**.

6.1.1. LAB MEDIA: Table 1 *Video Editor: please emphasize typical setup column*
6.1.2. LAB MEDIA: Table 1 *Video Editor: please typical yield column*

6.2. Embedding the 3D culture in type one collagen within the microwells of an agarose cast allows monitoring and analysis of the invasion by Image J **[1]**.

6.2.1. LAB MEDIA: Figure 2A *Video Editor: please emphasize red area*

6.3. In this analysis of two primary murine pancreatic cancer cell lines **[1]**, different spheroid shapes **[2]** and invasive behaviors were observed **[3]**.

6.3.1. LAB MEDIA: Figure 2B *Video Editor: please add/emphasize cell line 1 and cell line 2 rows of images*
6.3.2. LAB MEDIA: Figure 2B *Video Editor: please emphasize spheroid images*
6.3.3. LAB MEDIA: Figure 2B *Video Editor: please emphasize invasion images*

6.4. The first cell line 1 demonstrated a more compact spheroid formation **[1]** and "spiky" invasion, similar to that observed for single cell invasions **[2]**, while the second cell line formed spheroids that were more loose **[4]** and demonstrated a collective invasion pattern **[4]**.

6.4.1. LAB MEDIA: Figures 2B and 2C *Video Editor: please emphasize cell line 1 spheroid image and relative invasion area black data bar*
6.4.2. LAB MEDIA: Figures 2B and 2C *Video Editor: please emphasize cell line 1 invasion image and number of spikes/spheroid black data bar*
6.4.3. LAB MEDIA: Figures 2B and 2C *Video Editor: please emphasize cell line 2 spheroid image and relative invasion area white data bar*
6.4.4. LAB MEDIA: Figures 2B and 2C *Video Editor: please emphasize cell line 2 invasion image and number of spikes/spheroid black data bar*

6.5. Co-culture can be performed with two different tumor clonal cell lines seeded at the same time **[1]** or with tumor and T cells upon tumor spheroid formation for subsequent tumor-T cell interaction evaluations **[2]**.

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6.5.1. LAB MEDIA: Figures 3A-3E
6.5.2. LAB MEDIA: Figures 3F-3J

6.6. For a detailed assessment of the invasive behavior, immunofluorescence staining and confocal imaging can be performed in a high-throughput manner **[1]**.

6.6.1. LAB MEDIA: Figures 4C-4F *Video Editor: please sequentially add Figures 4D, 4E, and 4F*

6.7. The agarose cast allows embedding of the whole 3D culture system in paraffin for serial sectioning **[1]** and immunohistochemistry staining to quantify the spatial relationship between tumor and T cells **[2]**.

6.7.1. LAB MEDIA: Figures 5A-5C *Video Editor: please emphasize Figure 5A*
6.7.2. LAB MEDIA: Figures 5A-5C *Video Editor: please Figures 5B and 5C*

6.8. For example, T cell infiltration can be identified and characterized by cell surface marker staining **[1]**.

6.8.1. LAB MEDIA: Figures 5D and 5E *Video Editor: please brown staining in both figures*

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Conclusion

7. Conclusion Interview Statements

7.1. **Yuan-Na Lin**: This technique allows the analysis of patient samples as well as the use of stimulating or blocking drugs to probe tumor cell-T cell interactions **[1]**.

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

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