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Title: Analysis of Side Population in Solid Tumor Cell Lines

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No, but screen needs to be filmed for 2 shots.**

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes, Lab and Core Facility.**

   If **Yes**, how far apart are the locations? **300 meters apart.**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Xiaoli Dong:** Analysis of side population cells is one of the commonly used methods to isolate and identify cancer stem cells from tumor tissues and tumor cell lines. This assay is convenient, fast, and cost-effective.

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

OPTIONAL:

1.2. **Yingying Wei:** This method can contribute to a better understanding of the effect of genes or other extracellular and intracellular signals on stemness properties of tumor cells.

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

1.3. **Yingying Wei:** Many factors can influence the percentage of SP cells in this analysis, so it is critical to explore the proper conditions before the formal experiment.

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

Protocol

2. Cell Preparation

2.1. Seed tumor cells in a 6-well plate **[1]** and incubate them in a 37-degree Celsius incubator supplied with 5% carbon dioxide **[2]**. When the cell density reaches about 90%, aspirate the culture medium **[3]** and wash the cells twice with 3 milliliters of PBS **[4]**.

2.1.1. WIDE: Establishing shot of talent seeding cells on a plate.

2.1.2. Talent putting the plate in the incubator and closing the door. *Videographer: Obtain multiple usable takes of this shot because it will be reused in 2.2.2.*

2.1.3. Talent aspirating the culture medium.

2.1.4. Talent adding PBS to cells.

2.2. Then, add 500 microliters of 0.25% trypsin-EDTA to each well **[1]** and incubate the plate at 37 degrees Celsius for 1 to 3 minutes **[2]**. Gently tap the plate to detach the cells **[3]**, add 3 milliliters of PBS supplemented with 2% FBS to each well, and pipette the cells up and down 3 to 5 times to disperse the cell clumps **[4]**.

2.2.1. Talent adding trypsin-EDTA to a few wells.

2.2.2. *Use 2.1.2.*

2.2.3. Talent tapping the plate.

2.2.4. Talent adding PBS supplemented with 2% FBS to the cells and pipetting them up and down.

2.3. Examine the cell suspension under the microscope **[1]**. If cell clumps are present, pass the suspension through a 70-micrometer strainer **[2]**. Transfer the cells to a 15-milliliter centrifuge tube **[3]** and centrifuge them at 200 *x g* for 5 minutes **[4]**.

2.3.1. Talent using the microscope.

2.3.2. Talent filtering the cells.

2.3.3. Talent transferring the cells into a 15-mL tube.

2.3.4. Talent putting the tube in the centrifuge and starting it.

2.4. Remove the supernatant **[1]** and resuspend the cells in 3 milliliters of PBS supplemented with 2% FBS, pipetting the cells up and down 3 to 5 times to mix **[2]**.

2.4.1. Talent removing the supernatant.

2.4.2. Talent adding PBS supplemented with 2% FBS to the cells and pipetting them up and down.

3. Cell Staining

3.1. Count cells under the microscope using a hemocytometer **[1]** and dilute them in PBS supplemented with 2% FBS to a final concentration of 1 x 10^6 cells per milliliter **[2]**. Prepare two 5-milliliter polystyrene round bottom test tubes and add 1 milliliter of the cell suspension to each tube **[3].** Label one tube as a test tube and the other as a blocker control tube **[4]**. *Videographer: This step is important!*

3.1.1. Talent counting the cells using the hemocytometer and microscope.

3.1.2. Talent diluting the cells in PBS supplemented with 2% FBS.

3.1.3. Talent adding cell suspension to each of the 5-mL polystyrene round bottom test tubes.

3.1.4. Talent labeling two sample tubes with cells.

3.2. Prepare a solution of blocker by diluting it to the appropriate concentration **[1]**. Add it to the blocker control tube and mix well **[2]**, then incubate the tube at 37 degrees Celsius for 30 minutes **[3]**. Shake the tube every 10 minutes **[4].** *Videographer: This step is important!*

3.2.1. Talent diluting the blocker.

3.2.2. Talent adding the blocker to the blocker control tube with cells and mixing.

3.2.3. Talent putting the tube with the blocker into the incubator and closing the door.

3.2.4. Talent shaking a tube.

3.3. Prepare a solution of Hoechst 33342 *(pronounce like this)* by diluting it to the appropriate concentration **[1]**. Add it to the test tube and blocker control tube separately, and mix well **[2],** then incubate the tubes at 37 degrees Celsius for 60 minutes **[3]**. Shake the tubes every 10 minutes **[4].** *Videographer: This step is important!*

3.3.1. Talent diluting the Hoechst 33342.

3.3.2. Talent adding Hoechst 33342 to the test tube and the blocker control tube separately and mixing.

3.3.3. Talent putting the tubes into the incubator and closing the door.

3.3.4. Talent shaking the tubes.

3.4. After the incubation, centrifuge the cells for 5 minutes at 200 *x g* and 4 degrees Celsius **[1]** and carefully aspirate the supernatant **[2]**. Resuspend the cells in each tube with 1 milliliter of ice-cold PBS supplemented with 2% FBS, then pipette the cells up and down to mix **[3]**. Add 1 microliter of propidium iodide, or PI, to each tube and mix **[4]**. *Videographer: This step is important!*

3.4.1. Talent putting the tubes with the cells in the centrifuge and closing the lid.

3.4.2. Talent aspirating the supernatant.

3.4.3. Talent adding PBS supplemented with 2% FBS to the cells and pipetting them up and down.

3.4.4. Talent adding PI to the cells and mixing.

4. Flow Cytometry

4.1. Click the "Parameters" tab in the flow cytometer software. First, choose the parameters of FSC, SSC, Hoechst Blue, Hoechst Red and PI, respectively. Second, choose the logarithmic scale for the PI parameter. Finally, choose area and width scales for the FSC, SSC, Hoechst Blue, Hoechst Red and PI parameters, respectively **[1]**.

4.1.1. SCREEN: Parameters being set. *Videographer: Film the screen for this step.* Ask talent to narrate their actions as they work.

4.2. Run the cells stained with Hoechst 33342 first, then run the cells stained with blocker and Hoechst 33342 using the same voltages and gates **[1]**. Collect 20 to 100 thousand events from each sample to analyze the percentage of SP cells **[2]**.

4.2.1. Talent putting tubes with cells on the flow cytometer.

4.2.2. SCREEN: Events being collected. *Videographer: Film the screen for this step.*

Results

5. Results: SP Analysis

5.1. This method was used to detect the proportion of SP cells in the MDA-MB-231 human breast cancer cell line **[1-TXT]**. The dot plot of FSC-A versus PI-A showed a population of dead cells **[2]**, cell debris **[3]**, and the main population of PI-negative cells **[4]**, which was subjected to further analysis **[5]**.

5.1.1. LAB MEDIA: Figure 1, just the first plot (FSC-A vs PI-A) in both A and B. *Video Editor: Label A "***Untreated***", label B "***Treated with Reserpine***".*

5.1.2. LAB MEDIA: Figure 1 A, just the first plot (FSC-A vs PI-A) in both A and B. *Video Editor: Emphasize the PI positive/dead cells.*

5.1.3. LAB MEDIA: Figure 1 A, just the first plot (FSC-A vs PI-A) in both A and B. *Video Editor: Emphasize the cell debris.*

5.1.4. LAB MEDIA: Figure 1 A, just the first plot (FSC-A vs PI-A) in both A and B. *Video Editor: Emphasize the main population (enclosed with a border).*

5.1.5. LAB MEDIA: Figure 1. *Video Editor: Just show the whole figure here.*

5.2. A single-cell population gated from the dot plot of FSC-A versus FSC-W **[1]** and the dot plot of SSC-A versus SSC-W **[2]** was used to analyze the proportion of SP cells **[3]**, which was about 0.9% in untreated cells **[4]** and about 0.09% in cells treated with Reserpine **[5]**.

5.2.1. LAB MEDIA: Figure 1. *Video Editor: Emphasize the FSC-A vs FSC-W plot on both A and B.*

5.2.2. LAB MEDIA: Figure 1. *Video Editor: Emphasize the SSC-A vs SSC-W plot on both A and B.*

5.2.3. LAB MEDIA: Figure 1, just the last plot for both A and B (Hoechst Red vs Hoechst Blue).

5.2.4. LAB MEDIA: Figure 1, just the last plot for both A and B (Hoechst Red vs Hoechst Blue). *Video Editor: Emphasize the population of cells enclosed with the black border on A.*

5.2.5. LAB MEDIA: Figure 1, just the last plot for both A and B (Hoechst Red vs Hoechst Blue). *Video Editor: Emphasize the population of cells enclosed with the black border on B.*

5.3. Different staining concentrations of Hoechst 33342 *(pronounce like this)* were tested on MDA-MB-435 cells **[1]** and it was determined that 2 micrograms per milliliter was optimal for SP analysis **[2]**. Low concentrations made it difficult to distinguish SP cells from other populations **[3]**, while high concentrations caused SP cells to disappear **[4]**.

5.3.1. LAB MEDIA: Figure 2 A.

5.3.2. LAB MEDIA: Figure 2 A. *Video Editor: Emphasize the plot with 2 µg/mL Hoechst concentration.*

5.3.3. LAB MEDIA: *Figure 2 A. Video Editor: Emphasize the plots with 0.5, 1, and 1.5 µg/mL Hoechst concentrations.*

5.3.4. LAB MEDIA: Figure 2 A. *Video Editor: Emphasize the plots with 2.5, 3, 3.5, 4 µg/mL Hoechst concentrations (second row).*

5.4. Verapamil and Reserpine were tested to determine the proper blocker for the MDA-MB-435 cells **[1]**. About 0.4% of cells expelled the dye after Verapamil treatment **[2]**, but the ratio dropped to about 0.1% after Reserpine treatment, demonstrating that Reserpine is a more appropriate blocker for this cell line **[3]**.

5.4.1. LAB MEDIA: Figure 2 B and C. *Video Editor: Label B "***Verapamil***" and C "***Reserpine***".*

5.4.2. LAB MEDIA: Figure 2 B and C. *Video Editor: Emphasize B.*

5.4.3. LAB MEDIA: Figure 2 B and C. *Video Editor: Emphasize C.*

5.5. This protocol was also used to determine the proportion of SP cells in A549 human lung adenocarcinoma cells treated with the STAT3 *(pronounce "stat-3')* activator-Colivelin, **[1]** and in T47D human breast cancer cells treated with the FRA1 inhibitor-SKLB816 **[2]**.

5.5.1. LAB MEDIA: Figure 3.

5.5.2. LAB MEDIA: Figure 4.

Conclusion

6. Conclusion Interview Statements

6.1. **Xiaoli Dong:** This assay provides clues to the regulatory effect of signal pathways on the features of cancer stem cells and facilitates the discovery of new mechanisms, which can ultimately guide the targeted therapy of tumors. NOTE: Author did not like how she pronounced the words during the video shoot and is requesting that we use an audio recording instead: Voice recording-part 6.1.m4a. You can use shots from 4.1 or 4.2 as visuals or shot 2.1.1.

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