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Title: Two Flow Cytometric Approaches of NKG2D Ligand Surface Detection to Distinguish Stem Cells from Bulk Subpopulations in Acute Myeloid Leukemia

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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. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interview Statements are read by JoVE's voiceover talent.

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

Current Protocol Length

Number of Steps: 10
Number of Shots: 25

Introduction

1. Introductory Interview Statements

NOTE to VO Talent: Please record all introduction and conclusion statements.

Videographer: Please skip the introduction and conclusion, authors have elected to have the VO talent to deliver these statements.

1.1. This protocol shows two distinct ways of detecting NKG2DLs on the surface of AML cells.

1.1.1. *Suggested: 3.4.2.*

1.2. The technique provides a fast, user friendly staining method to detect all known and possibly unknown NKG2DLs.

1.2.1. *Suggested: Figure 1.*

1.3. This method allows for separation of leukemic stem cells from bulk AML cells, which makes it possible to further characterize these cells.

1.3.1. *Suggested: 3.5.2.*

Ethics Title Card

1.4. Procedures involving human subjects have been approved by the Ethics Review Board of the University Hospitals of Basel and Tuebingen.

Protocol

2. Biotinylation of the NKG2D Fusion Protein and Thawing of Primary AML Cells

2.1. Begin by thawing the biotin and the NKG2D fusion protein tubes at room temperature **[1]**. Quickly spin down the NKG2D-Fc *(spell out 'N-K-G-2-D-F-C')* powder **[2]**, then add 500 microliters of PBS to reconstitute the powder and mix thoroughly using a P1000 micropipette **[3]**.

2.1.1. WIDE: Establishing shot of talent taking the tubes out of the freezer.

2.1.2. ~~Talent spinning down the NKG2D-Fc powder~~. NOTE: This was filmed, but leave it out of the video

2.1.3. Talent adding the PBS to the powder and mixing with the pipette.

2.2. Add 100 microliters of the NKG2DL fusion protein to a biotin tube to obtain a final concentration of 10 micrograms per milliliter **[1]** and mix the solution thoroughly with a P100 micropipette **[2]**.

2.2.1. Talent adding the fusion protein to the biotin tube.

2.2.2. Talent pipetting the solution up and down.

2.3. To thaw the AML cells, remove the cryovial containing primary AML from the liquid nitrogen storage **[1]** and immediately place it in a 37-degree Celsius water bath **[2]**. Gently move the tube back and forth in the water **[3]**, allowing the contents of the vial to thaw until there is only a small ice crystal left **[4]**. *Videographer: This step is difficult and important!*

2.3.1. Talent removing the cells from the storage.

2.3.2. Talent placing the cells in the water bath.

2.3.3. Talent moving the tube back and forth.

2.3.4. Thawed cells.

2.4. Immediately transfer the thawed cells into the medium-containing tube **[1]** and rinse the vial using 1 milliliter of medium **[2]**. Centrifuge the cells at 300 *x g* for 10 minutes **[3]** and discard the supernatant without disturbing the pellet **[4]**. *Videographer: This step is important!*

2.4.1. Talent transferring the cells to the tube with medium.

2.4.2. Talent rinsing the vial.

2.4.3. Talent putting the cells in the centrifuge and closing the lid.

2.4.4. Talent discarding the supernatant.

2.5. Wash the cells with 5 milliliters of RPMI medium containing 10% FCS **[1]** and repeat the centrifugation **[2]**.

2.5.1. Talent adding medium to the cells.

2.5.2. Talent putting the tube in the centrifuge.

3. Staining of Primary AML Cells with the Biotinylated NKG2D Fusion Protein

3.1. Resuspend the cell pellet with staining buffer to a final concentration of 0.5 x 10^7 cells per milliliter **[1]**, then transfer 100 microliters of the cell suspension to a cell culture 96-well U-bottom plate **[2]** and centrifuge the plate at 300 *x g* for 10 minutes **[3]**. Discard the supernatant without disturbing the pellet **[4]**.

3.1.1. Talent resuspending the cells.

3.1.2. Talent transferring the cells to a plate.

3.1.3. Talent putting the plate in the centrifuge.

3.1.4. Talent discarding the supernatant.

3.2. Prepare a master mix of biotinylated NKG2D fusion protein so that cells are resuspended in a final volume of 50 microliters per well with a final NKG2D concentration of 10 micrograms per milliliter per well **[1]**. *Videographer: This step is important!*

3.2.1. Talent preparing the master mix.

3.3. Add the prepared master mix and resuspend the cell pellets with a 300-microliter multichannel pipette. Incubate and centrifuge the plate as described in the text manuscript **[1]**.

3.3.1. Talent adding the master mix and resuspending the cells.

3.4. Prepare a master mix using Streptavidin-PE so that the cells are resuspended in a final volume of 50 microliters **[1]**. Add the master mix to the cells and resuspend the pellets with a 300-microliter multichannel pipette **[2]**. *Videographer: This step is important!*

3.4.1. Talent preparing the Streptavidin-PE master mix.

3.4.2. Talent adding the master mix and resuspending the cells.

3.5. After centrifuging the plate and discarding the supernatant, use a 300-microliter multichannel micropipette to resuspend the cell pellets in 200 microliters of staining buffer + 7-AAD *(spell out '7-A-A-D')* **[1]**, then analyze the cells using a flow cytometry device **[2]**. *Videographer: This step is important!*

3.5.1. Talent resuspending the cells in staining buffer.

3.5.2. Talent using the flow cytometer. *Videographer: Please obtain several usable takes of this shot because it will be used as coverage for some of the interview statements.*

Results

4. Results: Gating Strategy and Staining Protocol Comparison

4.1. The analyzed AML samples are positive for CD34 and NKG2DL, but negative subpopulations also exist, with four different populations in total **[1]**. The typical gating strategy starts with the selection of the main population of cells via their FSC and SSC **[2]**.

4.1.1. LAB MEDIA: Figure 1.

4.1.2. LAB MEDIA: Figure 1 A. *Video Editor: Emphasize the FSC vs SSC plot.*

4.2. Doublets and dead cells are excluded in the downstream analysis **[1]**. The gates are adjusted using FMO controls to ensure proper identification of positive cells **[2]**.

4.2.1. LAB MEDIA: Figure 1 A. *Video Editor: Emphasize the Single Cells and Viable Cells plots.*

4.2.2. LAB MEDIA: Figure 1 B.

4.3. The fluorescence intensities of cells positive for CD34 versus NKG2DL are highlighted here. AML cells that are positive for CD34 show a lower surface expression of NKG2DL, indicating that NKG2DL expression is associated with lack of stemness **[1]**.

4.3.1. LAB MEDIA: Figure 1 C.

4.4. Three primary AML samples demonstrated 19.8, 49.8, and 89.4% positive events for the fusion protein staining **[1]** versus 20.4, 50.4, and 90.6% for the pooled anti-NKG2DL antibody staining **[2]**.

4.4.1. LAB MEDIA: Figure 2 A. *Video Editor: Emphasize the black bars.*

4.4.2. LAB MEDIA: Figure 2 A. *Video Editor: Emphasize the white bars.*

4.5. On the other hand, single ligand staining showed a range of positive events up to 92%, with the percentages varying based on the ligand **[1]**.

4.5.1. LAB MEDIA: Figure 2 B.

Conclusion

5. Conclusion Interview Statements

NOTE to VO Talent: Please record all introduction and conclusion statements.

5.1. When attempting this method, it is very important to be quick when thawing the primary AML cells because they are fragile and can die easily during this step.

5.1.1. *Suggested: 2.3.1, 2.3.2, 2.3.3, 2.3.4*

5.2. After performing this protocol, a cell sort based on the NKG2DL signal can be performed to further investigate the populations.

5.2.1. *Suggested: 3.5.2.*