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Viability Dye Staining

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Introduction

Viability staining is an essential component of any flow cytometry experiment. Dead cells can compromise the integrity of the data by non-specifically binding antibodies; therefore it is essential that dead cells be excluded from analysis.

Protocol A: Staining Dead Cells with Propidium Iodide (PI) or 7-amino-actinomycin D (7-AAD)

Propidium iodide and 7-AAD can be used to stain dead cells so that they may be excluded from analysis in standard live cell surface staining protocols. These dyes cannot pass through intact cell membranes, but may freely enter cells with compromised cell membranes. Upon entering dead cells, propidium iodide will intercalate into double-stranded DNA or double-stranded RNA in a stoichiometric manner, while 7-AAD will intercalate preferentially with double-stranded DNA. Because this intercalation is mediated by noncovalent forces, these dyes must remain present in the buffer used to resuspend cells for data acquisition so that dead cells will remain labeled.

General Notes

NOTE: *Neither Propidium Iodide nor 7-AAD can be used to discriminate live and dead cells when intracellular staining is desired. Please see "Staining Dead Cells with Fixable Viability Dyes (FVD), Protocol C" below) for staining dead cells with viability dyes that are compatible with intracellular staining protocols.*

Materials

- Viability Dyes
  - Propidium Iodide Staining Solution (cat. no. 00-6990)
  - 7-AAD Viability Staining Solution (cat. no. 00-6993)
- Flow Cytometry Staining Buffer (cat. no. 00-4222)
- 12 x 75 mm round-bottom tubes

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Experimental Procedure

1. After staining cells for surface antigens, wash cells 1-2 times with Flow Cytometry Staining Buffer.
2. Resuspend cells in an appropriate volume of Flow Cytometry Staining Buffer.
3. Add 5 µL of Propidium Iodide Staining Solution or 7-AAD Staining Solution per 100 µL of cells.
4. Incubate for 5–15 minutes on ice or at room temperature. Do not wash cells.

NOTE*: Propidium Iodide and 7-AAD must remain in the buffer during acquisition. Do not wash cells after the addition of Propidium Iodide or 7-AAD.*

5. Analyze samples by flow cytometry.

NOTE*: Cells should be analyzed within 4 hours after the initial incubation period due to adverse effects on the viability of cells left in the presence of propidium iodide or 7-AAD for prolonged periods. Store at 2–8°C and protect from light until ready for analysis.*

Protocol B: Staining Live Cells with Calcein Dyes

Calcein AM, Calcein Violet AM, and Calcein Blue AM labeling dyes cross the cell membrane easily, selectively labeling live cells for analysis by flow cytometry or fluorescent microscopy; apoptotic and dead cells with compromised cell membranes do not retain calcein. Co-staining with Annexin V or 7-AAD is recommended to allow the greatest resolution between live and dead/apoptotic cells.

General Notes

- Calcein dyes are lyophilized and should be reconstituted in anhydrous DMSO before use. Reconstituted dye should be used within a short period of time after reconstitution. For short-term storage, store at –20°C with dessicant. Avoid freeze-thawing and allow the vial to equilibrate to room temperature before opening.
- Staining with calcein dyes may be done before or after surface staining with antibodies. It is recommended that the dyes be titrated by each investigator for optimal performance in the assay of interest. Because calcein dyes are not retained in cells with compromised cell membranes, they are not compatible with intracellular staining protocols. Please see "Staining Dead Cells with Fixable Viability Dyes (FVD), Protocol C" below) for staining dead cells with viability dyes that are compatible with intracellular staining protocols.

Materials

- Calcein Dyes
  - Calcein AM (UltraPure Grade) (cat. no. 65-0853)
  - Calcein Violet 450 AM (cat. no. 65-0854)
  - Calcein Blue AM (cat. no. 65-0855)

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- DMSO, anhydrous
- [Optional] Propidium Iodide Staining Solution (cat. no. 00-6990) or 7-AAD Viability Staining Solution (cat. no. 00-6993)
- Flow Cytometry Staining Buffer (cat. no. 00-4222)
- 12 x 75 mm round-bottom tubes

Experimental Procedure

1. Prepare cells as desired for single-cell suspension and/or surface staining.
2. Resuspend 1–5 x 10^6 cells in an appropriate volume of Flow Cytometry Staining Buffer (0.1–1 mL).
3. Add calcein dye at the desired concentration and mix well. (Please see technical data sheet for the specific calcein dye of interest for a recommended concentration range.)

NOTE: *It may be necessary to make a more dilute working solution of the dye, which may be done with flow cytometry staining buffer or PBS*

4. Incubate for 30 minutes at room temperature. Protect from light.
5. Add 2 mL of Flow Cytometry Staining Buffer and centrifuge at 400–600 x g for 5 minutes at room temperature. Discard supernatant.
6. Repeat Step 5.
7. [Optional] Stain cells for surface markers. Refer to "Staining Cell Surface Targets, Protocol A" found in our Best Protocols.
8. Analyze samples by flow cytometry.

Protocol C: Staining Dead Cells with Fixable Viability Dyes (FVD)

Fixable Viability Dyes (FVDs) brightly stain cells with compromised membranes and covalently cross-link to cellular proteins, irreversibly labeling dead cells from all species. This allows the samples to undergo cryopreservation, fixation, and permeabilization procedures without the loss of staining intensity of the dead cells, ensuring that they can be excluded from subsequent analysis; thus improving data quality. FVDs are available in a range of colors suitable for use with UV, violet, blue, and red lasers.

General Notes

Best practices when using FVDs

- FVDs are supplied as pre-diluted solutions prepared in highquality, anhydrous DMSO. They should be protected from light and moisture. Store at ≤–70°C with dessicant. They may be freeze-thawed up to 20 times.
- Allow vial of FVD to equilibrate to room temperature before opening.

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- For the brightest staining, it is best to stain with FVD in azide and protein-free PBS.
- Cells may be stained with FVD before or after surface staining. After staining with FVD, cells may also be cryopreserved for analysis at a later time. It is recommended that each investigator determine the optimal concentration for the assay of interest.
- FVD may be used in combination with fixation, permeabilization, and intracellular staining. FVD can also be used in experiments using live, unfixed cells.
- For compensation, it is recommended to use a sample of the cells of interest stained with the FVD only.
- If the percentage of dead cells is expected to be less than 5%, then it is recommended to take a small aliquot of cells and heat them at 65°C for 1 minute, then immediately place on ice for 1 minute. After this treatment, the heat-killed cells can be combined 1:1 with live cells and then stained with FVD.

Alternative Protocols (Protocols C3–C5)

- Protocols C3, C4, and C5 are modifications for ease of use, which may result in reduced staining intensity of the dead cells. These alternative staining protocols should be avoided if maximum staining intensity is desired. It is recommended that each investigator determine whether these protocol modifications provide sufficient staining intensity of dead cells.
- It is possible to stain unlysed whole blood with FVD. See "Protocol C3" (below) for details.
- It is possible to stain in azide-free, but protein-containing PBS. This method may result in a small reduction in the staining intensity of the dead cell population. See "Protocol C4" (below).
- It is also possible to stain in azide- and protein-containing PBS, such as Flow Cytometry Staining Buffer (cat. no. 00-4222). This method may result in a significant decrease in the staining intensity of the dead cell population and/or an increase in background staining of the live cell population. See "Protocol C4" (below).
- It is possible to add the FVD to an antibody cocktail prior to adding it to the cells. The FVD should spend as little time as possible in the cocktail prior to staining. It is best to use an azide-free, protein-containing buffer for dilution of the antibody cocktail and FVD. See "Protocol C5" (below).

Materials

- Fixable Viability Dye eFluor® 455 (UV) (cat. 65-0868) –off the UV laser
- Fixable Viability Dye eFluor® 450 (cat. 65-0863) –off the violet laser
- Fixable Viability Dye eFluor® 506 (cat. 65-0866) –off the violet laser
- Fixable Viability Dye eFluor® 520 (cat. 65-0867) –off the blue laser
- Fixable Viability Dye eFluor® 660 (cat. 65-0864) –off the red laser
- Fixable Viability Dye eFluor® 780 (cat. 65-0865) –off the red laser

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Experimental Procedure

C1. Standard staining in 12 x 75 mm Tubes

Materials

- Phosphate-buffered saline (PBS), azide- and protein- free
- Flow Cytometry Staining Buffer (cat. no. 00-4222)
- 12 x 75 mm round-bottom tubes

1. Prepare cells in 12 x 75 mm tubes.
2. Wash cells 2 times in azide-free and protein-free PBS.
3. Resuspend cells at 1–10 x 10^6/mL in azide-free and serum/protein-free PBS.

NOTE: For consistent staining of cells, it is not recommended to stain in less than 0.5 mL.

4. Add 1 µL of FVD per 1 mL of cells and vortex immediately.
5. Incubate for 30 minutes at 2–8°C; protect from light.
6. Wash cells 1–2 times with Flow Cytometry Staining Buffer or equivalent.
7. Continue with experiment, as desired.

C2. Standard staining in 96-well Plates

Materials

- Phosphate-buffered saline (PBS), azide- and protein- free
- Flow Cytometry Staining Buffer (cat. no. 00-4222)
- 96-well assay plates

1. Prepare cells as desired in 96-well plates.
2. Wash cells 2 times in azide-free and serum/protein-free PBS. Completely decant supernatant.
3. Prepare a working stock solution of the FVD by diluting it 1:1,000, in azide- and serum/protein-free PBS. Make enough for 100 µL/well. As an example, for 96 wells, add 10 µL of FVD to 10 mL of PBS.
4. Add 100 µL of the working stock solution of the FVD to each well and mix immediately by pipetting or gentle vortexing.
5. Incubate for 30 minutes at 2–8°C; protect from light.
6. Wash cells 1–2 times with Flow Cytometry Staining Buffer or equivalent.
7. Continue with experiment, as desired.

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C3. Standard staining in 96-well Plates

Materials

- Phosphate-buffered saline (PBS), azide- and protein- free
- Flow Cytometry Staining Buffer (cat. no. 00-4222)
- Red blood cell lysis buffer, such as
  - 1X RBC Lysis Buffer (cat. no. 00-4333),
  - 10X RBC Lysis Buffer (Multi-species) (cat. no. 00-4300), or
  - 1-step Fix/Lyse Solution (10X) (cat. no. 00-5333)
- 12 x 75 mm round-bottom tubes

1. Add unlysed whole blood to 12 x 75 mm tubes.
2. Add 1 µL of FVD per 100 µL of whole blood.
3. Add other surface staining antibodies after addition of the FVD.

NOTE: Alternatively, FVD may be added directly to the surface staining antibody cocktail at 1 µL per sample to be stained. This cocktail should be made just prior to addition to whole blood samples.

4. Incubate for 30 minutes at 2–8°C, protect from light.
5. Wash samples 1–2 times with Flow Cytometry Staining Buffer.
6. Lyse red blood cells and continue with experiment, as desired.

C4. Staining with FVD in Azide and/or Protein-containing Staining Buffers

Materials

- Flow Cytometry Staining Buffer (cat. no. 00-4222)
- 12 x 75 mm round-bottom tubes

1. Prepare cells in 12 x 75 mm tubes at 1–10 x 106/mL in Flow Cytometry Staining Buffer.
2. Add 1 µL of FVD per 1 mL of cells and vortex immediately.
3. Incubate for 30 minutes at 2–8°C; protect from light.
4. Wash cells 1–2 times with Flow Cytometry Staining Buffer.
5. Continue with experiment, as desired.

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C5. Staining with FVD in an Antibody Cocktail

Materials

- Phosphate-buffered saline (PBS), azide- and protein- free
- Flow Cytometry Staining Buffer (cat. no. 00-4222)
- 12 x 75 mm round-bottom tubes

1. Prepare cells in 12 x 75 mm tubes at 1–10 x 106 in 100 µL buffer (see following note).

NOTE: To maximize FVD brightness, cells may be resuspended in azide-free and serum/protein-free PBS (as in "Protocol C1: Standard Staining in 12 x 75 mm Tubes" [see above]). If maximal brightness is not critical, cells may be resuspended in Flow Cytometry Staining Buffer ("Protocol C4: Staining with FVD in Azide and/or Protein-containing Staining Buffers" [see above]).

2. Prepare desired antibody cocktail in Flow Cytometry Staining Buffer.
3. Immediately prior to addition to cells, add FVD to antibody cocktail at 0.5–1 µL per sample to be stained.
4. Add FVD/antibody cocktail to cell samples.
5. Incubate for 30 minutes at 2–8°C; protect from light.
6. Wash cells 1–2 times with Flow Cytometry Staining Buffer.
7. Continue with experiment, as desired

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