

Assessment of Cell Viability

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ABSTRACT

Cell viability may be judged by morphological changes or by changes in membrane permeability and/or physiological state inferred from the exclusion of certain dyes or the uptake and retention of others. This unit presents methods based on dye exclusion, esterase activity, and mitochondrial membrane potential, as well as protocols for determining the pre-fixation viability of fixed cells either before or after fixation with amine-reactive dyes suitable for a range of excitation wavelengths. Membrane-impermeable dead cell and live cell dyes as well as dye-exclusion procedures for microscopy are also included. *Curr. Protoc. Cytom.* 64:9.2.1-9.2.26. © 2013 by John Wiley & Sons, Inc.

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INTRODUCTION

The method used to determine cell viability (and to a degree, the definition of viability) is often related to the phenomenon studied. Frequently, cell viability is thought of in somewhat negative terms. That is, one needs to exclude dead cells because they generate artifacts as a result of nonspecific binding and/or uptake of fluorescent probes. In addition to simple enumeration of live or dead cells present, there is a broad range of biologically relevant cytometric procedures that are related to the physiological state of the cells measured. For example, one may wish to measure cell morbidity in apoptosis, cell survival as a result of cytotoxicity, the potential of bacteria or microalgae to survive environmental insult and grow once normal conditions have been restored, or the potential of intracellular protozoa to undergo division cycles as part of the infective process.

Cell viability may be judged by morphological changes or by changes in membrane permeability and/or physiological state inferred from the exclusion of certain dyes or the uptake and retention of others. Here, methods are presented for staining nonviable cells by dye exclusion (indicative of an intact membrane) using the fluorescent, DNA-binding probes propidium iodide (PI; see Basic Protocol 1) and 7-amino actinomycin D (7-AAD; see Alternate Protocol 1). These probes may also be used in cells labeled with phycoerythrin (PE)-conjugated antibodies (see Alternate Protocol 2). The next two protocols present different aspects of physiological state that can be used to assess viability; one is based on esterase activity (see Alternate Protocol 3) and the other on mitochondrial membrane potential (see Alternate Protocol 4). For fixed cells, the state of viability prior to fixation can be determined using DNA-binding probes either before (see Alternate Protocols 5 and 6) or after (see Alternate Protocol 7) fixation. In addition, amine-reactive dyes (see Basic Protocols 2 and 3) can be used with fixation and provide choices of excitation lasers. For assessment by microscopy, methods are provided that are based on the esterase activity of viable cells (see Basic Protocol 4) or simultaneous detection of live and dead cells (see Alternate Protocols 8 and 9). In contrast, the final method (see Alternate Protocol 10) is a dye-exclusion procedure for microscopy using trypan blue and a hemacytometer (*APPENDIX 3A & 3B*). Each of the above protocols requires a basic understanding of cell handling and flow cytometry/microscopy.

ASSESSMENT OF CELL VIABILITY USING PROBES FOR MEMBRANE INTEGRITY

Live cells with intact membranes are distinguished by their ability to exclude dyes that easily penetrate dead or damaged cells. Staining of nonviable cells with propidium iodide (PI) has been performed on most cell types. Its broad application is most likely due to ease of use: the procedure is very simple, and the stained cells are bright red and easy to identify. Alternatively, the longer-wavelength emission of 7-amino actinomycin D (7-AAD) makes it easier to use simultaneously with phycoerythrin (PE) as a surface marker, as detailed in Alternate Protocol 2, or with fluorescein isothiocyanate (FITC). Although 7-AAD is not as bright as PI, permeable cells are easily distinguishable from live cells.

BASIC PROTOCOL 1

Propidium Iodide Staining of Nonviable Cells

Materials

- 2 mg/ml propidium iodide (PI) in PBS (store wrapped in foil ≤ 1 month at 4°C)
- Cell suspension
- PBS (APPENDIX 2A)
- 13 × 100–mm polystyrene culture tubes

CAUTION: Propidium iodide is a suspected carcinogen and should be handled with care. In particular, be careful of particulate dust when weighing out the dye. Use gloves when handling it.

1. Add 1 μ l of 2 mg/ml propidium iodide (2 μ g/ml final) to approximately 10^6 washed cells suspended in 1 ml PBS in 13 × 100–mm polystyrene culture tubes.

To use this procedure with cells that are also labeled with PE-conjugated antibodies, see Alternate Protocol 2.

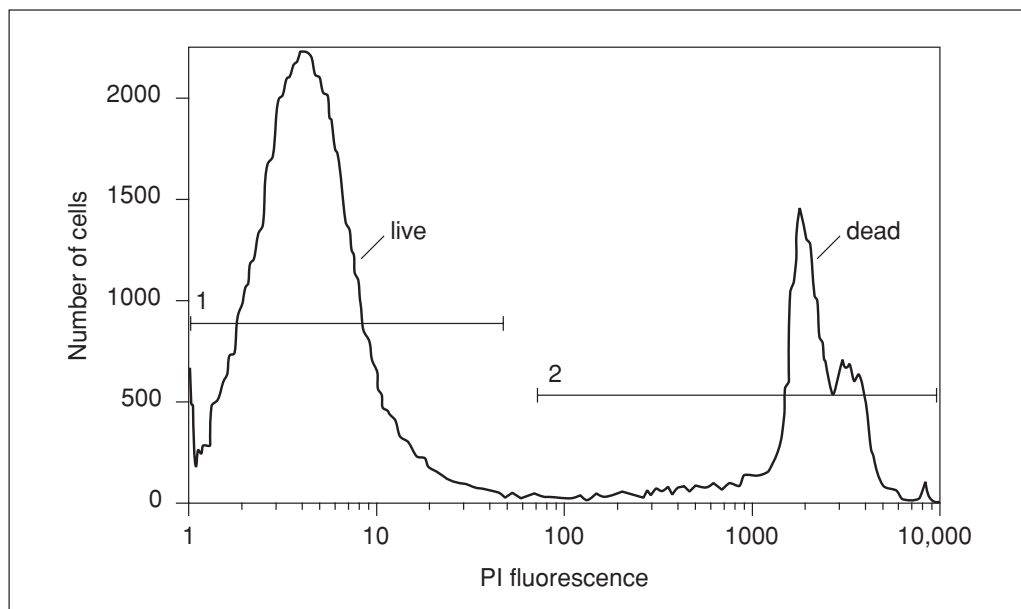


Figure 9.2.1 Identification of nonviable cells with propidium iodide (PI). Nonviable cells are more than two decades brighter than the unstained, viable cells. Gating on a one-parameter histogram is sufficient to identify the viable population. Region 1: viable cells; region 2: nonviable cells.

2. Incubate ≥ 5 min in the dark on ice.
3. Analyze on flow cytometer with excitation at 488 nm and emission collected at > 550 nm.

PI is easily excited at 488 nm. The dye has a broad fluorescence emission and can be detected with photomultiplier tubes (PMTs) normally used for phycoerythrin (~ 585 nm) or at longer wavelengths (≥ 650 nm).

Amplify the PMT signal logarithmically to distinguish populations of permeable (and presumed dead) cells from viable cells. Adjust the PMT high voltage such that bright cells are well separated from dim, viable cells. It is often easy to identify nonviable cells on a bivariate plot of forward light scatter (see Fig. 9.2.1).

Dead cells can be live-gated, but unless one is absolutely sure of the viable population, it is always much better to collect un-gated listmode data and perform gating after the raw data files are collected. Populations that may not be obvious on the flow cytometer display will be seen during subsequent data analysis. Moreover, any gating can be done and redone without losing cells.

7-AAD Staining of Nonviable Cells

Additional Materials (also see Basic Protocol 1)

1 mg/ml 7-amino actinomycin D (7-AAD; see recipe)

1. Add 1 μ l of 1 mg/ml 7-AAD (1 μ g/ml final) to approximately 10^6 washed cells suspended in 1 ml PBS in 13×100 -mm polystyrene culture tubes.

To use this procedure with cells that are also labeled with PE-conjugated antibodies, see Alternate Protocol 2.

2. Incubate ≥ 30 min in the dark on ice.
3. Analyze on flow cytometer with excitation at 488 nm. Collect fluorescence emission with a 650-nm long-pass or a 670 ± 20 -nm band-pass filter.

7-AAD is easily excited at 488 nm. The fluorescence emission of the dye has a peak at ~ 670 nm.

Use logarithmic amplification to distinguish permeable and bright cells from nonpermeable cells. Adjust the PMT high voltage to resolve a population of viable cells in the first decade of the 7-AAD fluorescence histogram as shown in region 1 of Figure 9.2.2A; nonviable cells are in region 2 of Figure 9.2.2A.

Use of PI or 7-AAD for Cells Labeled with PE-Conjugated Antibodies

Additional Materials (also see Basic Protocol 1)

PE-labeled cell suspension (UNIT 6.2)

1. Stain and incubate PE-labeled cells with PI (see Basic Protocol 1, steps 1 and 2) or 7-AAD (see Alternate Protocol 1, steps 1 and 2).
2. Analyze on flow cytometer with excitation at 488 nm. Use a 585 ± 20 -nm band-pass filter for detection of PE fluorescence and a 650-nm long-pass filter for PI or 7-AAD.

PI, 7-AAD, and PE are all excited by 488-nm light. Despite the separation by two detection filters, there is substantial overlap between the PE and PI or 7-AAD, requiring compensation between the two detectors. This problem is illustrated for PE and 7-AAD in Figure 9.2.2 (parts B and C), though the same procedure is used with PI. Note the typical discrimination of viable/nonviable cells. If one were to set a gate region on the viable cells in region 1 and use that for analysis in the absence of proper compensation, most PE-positive cells would be eliminated as nonviable (see Fig. 9.2.2B).

**ALTERNATE
PROTOCOL 1**

**ALTERNATE
PROTOCOL 2**

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9.2.3

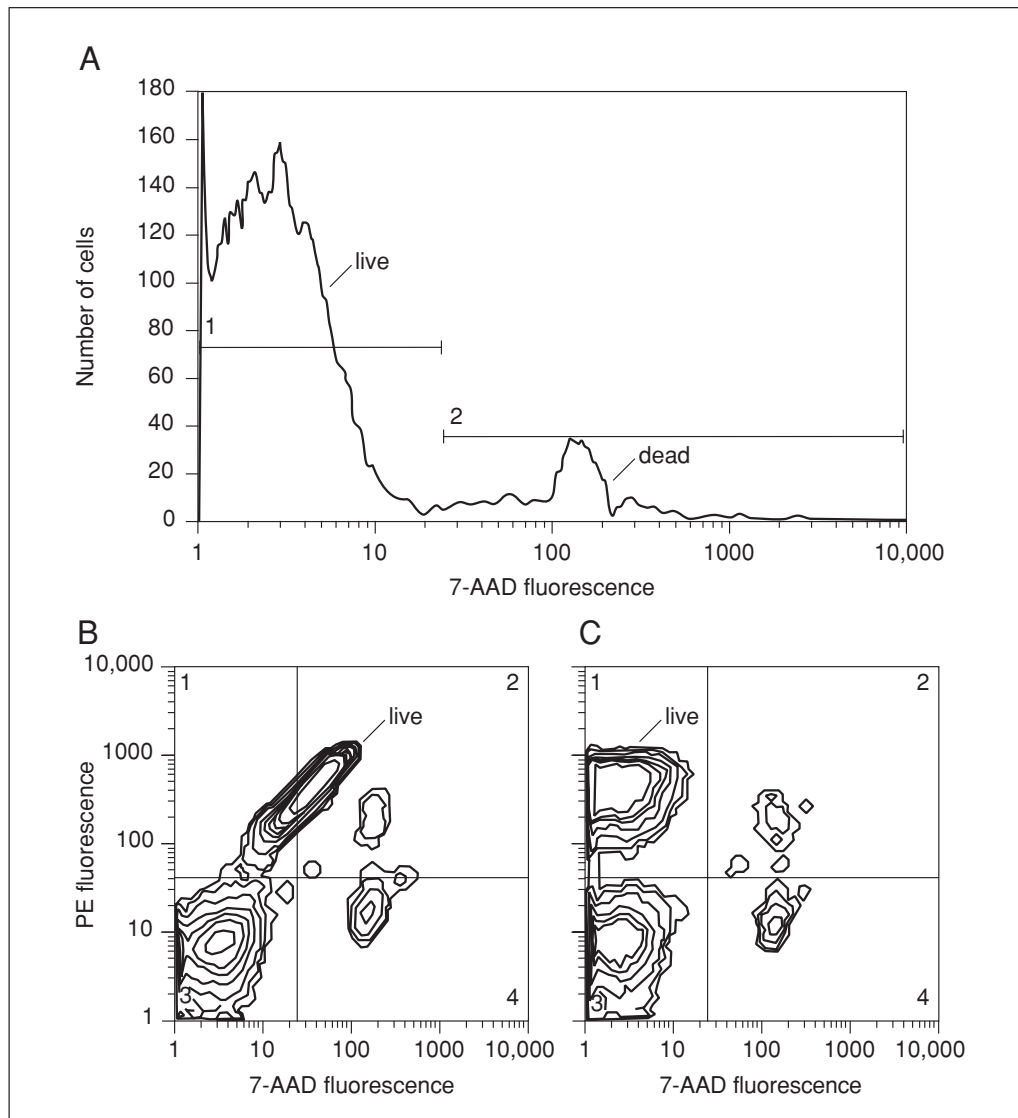


Figure 9.2.2 Effects of gating and compensation with 7-AAD. **(A)** Gating discriminates live cells. One-parameter histogram of logarithmically amplified 7-AAD fluorescence using a 650-nm long-pass filter. Mouse spleen cells are labeled only with 7-AAD. Note the peak of dead cell population in region 2 at a relative brightness between 100 and 200 (about ten-fold dimmer than what is expected for propidium iodide). The live cell population that occupies the first decade in the histogram (region 1) is 7-AAD negative and constitutes the majority of the cells in the population. **(B)** Uncompensated phycoerythrin fluorescence in the presence of 7-AAD. A bivariate plot of mouse spleen cells labeled with 7-AAD and a PE-labeled antibody to a cell surface antigen. Note the two small populations of dead cells in regions 2 and 4 and the large population of live cells that occupies region 1. If gating was performed before adequate compensation was achieved, then most of the viable PE-positive cells could be lost. **(C)** Compensation of PE with 7-AAD. Distribution of cells from same sample as in B. Note the dead cells are in the same location as in B, but the live cells are now clearly resolved from 7-AAD positive populations. At this point, the live cell gate defined in region 2 of A is valid.

Using a bivariate histogram of log 7-AAD versus log PE fluorescence, adjust the PE-7-AAD compensation such that PE-positive, 7-AAD-negative cells are above the PE-negative, 7-AAD-negative population (see Fig. 9.2.2C). Once proper compensation of the long-wavelength component of PE is subtracted from the output of the detector for 7-AAD, the problem disappears.

ASSESSMENT OF CELL VIABILITY USING PROBES OF PHYSIOLOGICAL STATE

These protocols describe the use of probes that require a specific cellular function in addition to an intact membrane. Alternate Protocol 3 describes the use of fluorescein diacetate (FDA), which requires cellular esterase activity, and Alternate Protocol 4 describes the use of rhodamine 123 as a probe for mitochondrial membrane potential.

Fluorescein Diacetate Staining of Viable Cells

Cell viability can be assessed directly through the presence of cytoplasmic esterases that cleave moieties from a lipid-soluble nonfluorescent probe to yield a fluorescent product. The product is charged, and thus is retained within the cell if membrane function is intact. Hence, viable cells are bright and nonviable cells are dim or nonfluorescent. Typical probes include fluorescein diacetate (FDA, described here), carboxyfluorescein, and calcein. Variations in uptake or retention of the dye among individual cells or under different conditions affect the efficacy of particular probes.

Additional Materials (also see Basic Protocol 1)

1 mg/ml fluorescein diacetate (FDA; prepare fresh in acetone in a 13-mm glass culture tube and cover with foil)

Cell suspension in culture medium appropriate for the cell type

1. Add 2 μ l of 1 mg/ml FDA (2 μ g/ml final) to approximately 10^6 cells in 1 ml medium in a 13 \times 100-mm polystyrene culture tube.
2. Vortex to mix and incubate 15 min at 37°C.
3. Analyze on flow cytometer immediately with excitation at 488 nm. Collect fluorescence using a 530 \pm 20-nm band-pass filter.

FDA is excited by 488-nm light and fluoresces green. Filters used for measuring fluorescein (e.g., 530 \pm 20-nm band-pass) are sufficient to visualize the nonfluorescent cells on the same scale. Use logarithmic amplification of the PMT output. Cells that take up and retain free fluorescein are very bright (approximately two decades brighter on a logarithmic scale) and should be easily distinguishable from nonfluorescent, nonviable cells.

Unless one is absolutely sure about the location of viable cells in the histograms, it is preferable to collect listmode data files of all samples and perform gating after the raw data files are collected, to avoid the danger of inadvertent loss of viable cells.

Rhodamine 123 Staining of Viable Cells

Another property of viable cells is the maintenance of electrochemical gradients across the plasma membrane. Functional subsets of this general phenomenon include the maintenance of pH and other ion gradients as well as the capacity for energy-yielding metabolism in mitochondria. These physiological processes can be exploited to distinguish viable from nonviable cells. One of the most commonly used probes for identifying viable cells is rhodamine 123, a cationic lipophilic dye that partitions into the low electrochemical potential of mitochondrial membranes. Active mitochondria in viable cells are stained bright green; loss of gradients within nonviable cells results in loss of the dye.

Additional Materials (also see Basic Protocol 1)

1 mg/ml rhodamine 123 (prepare fresh in distilled water)

Cell suspension in culture medium appropriate for the cell type

1. Add 5 μ l of 1 mg/ml rhodamine 123 (5 μ g/ml final) to approximately 10^6 cells in 1 ml medium in a 13 \times 100-mm polystyrene culture tube.
2. Vortex to mix and incubate 5 min at 37°C; return to room temperature.

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ALTERNATE PROTOCOL 4

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- Analyze immediately with excitation at 488 nm. Collect fluorescence using a 530 ± 20 -nm bandpass filter.

Rhodamine 123 absorbs 488-nm light and fluoresces green. Collect fluorescence through a filter that transmits at ~ 530 nm, as for FITC. PMT output should be amplified logarithmically.

Viable cells are brighter than nonviable cells, though with some cell types there may be some overlap (see Fig. 9.2.3A). A bivariate plot of forward scatter versus rhodamine 123 fluorescence can be useful to distinguish viable from nonviable cells (see Fig. 9.2.3B).

Unless one is absolutely sure about the location of viable cells in the histograms, it is preferable to collect listmode data files of all samples and perform gating after the raw data are collected, to avoid the danger of inadvertent loss of viable cells.

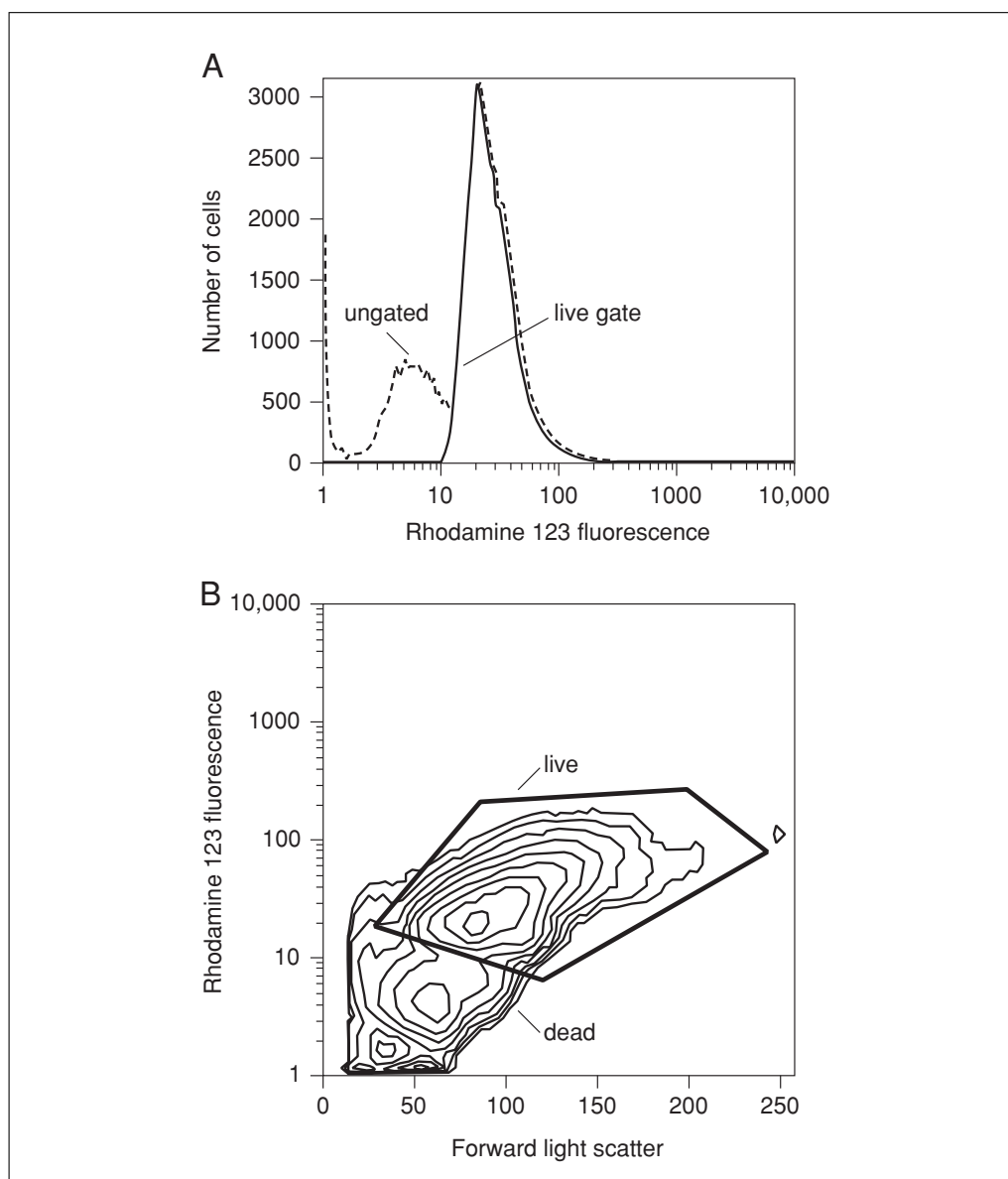


Figure 9.2.3 Effects of gating with rhodamine 123. **(A)** Identification of live cells after gating. Rhodamine 123 may not always completely resolve viable from nonviable cells as indicated in the un-gated histogram (dotted line). Gating on forward light scatter versus rhodamine 123 fluorescence helps separate both populations. Note the histogram of the gated population of viable cells (solid line) overlaid on the ungated population. **(B)** A bivariate plot of forward light scatter versus rhodamine 123 fluorescence helps to resolve live (rhodamine 123–bright) and dead (rhodamine 123–dim) populations. Debris is gated out at the same time.

ASSESSMENT OF CELL VIABILITY IN FIXED CELLS

For reasons of safety or convenience, it is frequently necessary to fix cells prior to analysis. Data analysis is less ambiguous if nonviable or damaged cells can be eliminated, but the methods discussed above will not work, because fixation will render all cells permeable. There are, however, DNA probes that penetrate and stain dead or damaged cells and that can withstand fixation. Ethidium monoazide (EMA) is positively charged and penetrates the membranes of dead or damaged cells but not live ones. EMA can be photochemically cross-linked with short exposure to visible light; after the excess dye is washed away, the cells are fixed. Another DNA-binding fluorochrome used for staining nonviable cells is laser dye styryl-751 (LDS-751). The procedure is somewhat more simple, as staining is done after fixation and does not require cross-linking.

Recent advances in probe design have yielded live cell exclusion dyes of multiple spectral and chemical varieties, allowing for greater flexibility in experimental design. Among these newer amine-reactive dyes are the Live/Dead probes (Invitrogen/Life Technologies), Horizon Fixable Viability Stain 450 (Becton Dickinson), and the Fixable Viability Dye eFluor dyes (eBioscience). These probes adhere to intact membranes of live cells, resulting in weak staining, and diffuse into cells with compromised cell membranes, resulting in bright staining easily distinguishable from dead or unstained cells. The available probes provide a range of options in their excitation/emission properties. In addition, these probes are compatible with aldehyde-based fixation, allowing for viability analysis of fixed or non-fixed cells if staining is performed prior to fixation.

Carboxymethylfluorescein diacetate (Cell Tracker CMFDA, Invitrogen/Life Technologies) provides an alternative to the cell-exclusion dyes that is still reliant on membrane integrity. This dye freely enters cells and reacts with intracellular esterases to form a nondiffusible product. Cells with intact membranes thus retain the fluorescent probe while the probe diffuses from damaged cells. Positive staining thus identifies live cells while weak or absent staining designates damaged or dead cells. The brightness of the dye allow for easy distinction of these populations of cells. As with the Live/Dead fixable dyes, the Cell Tracker dyes are compatible with aldehyde-based fixation and are available in multiple spectral variants.

Live/Dead Far Red Fixable Staining of Cells for Assessment of Viability

Materials

- Live/Dead Far Red Dead stock solution (see recipe)
- Cell suspension, washed
- Phosphate-buffered saline (PBS; *APPENDIX 2A*)
- 100 mM digitonin (see recipe; optional/recommended).
- 37% formaldehyde (Sigma, cat. no. 252549; optional)
- PBS (*APPENDIX 2A*) with 1% (w/v) bovine serum albumin (BSA) or cell culture medium without phenol red supplemented with 1% BSA
- 13 × 100–mm culture tubes or any suitable container for delivering cells to the flow cytometer for analysis
- Tabletop centrifuge
- Flow cytometer

1. Add 1 μ l of Far Red Dead fixable cell stain stock to approximately 10^6 washed cells suspended in 1 ml PBS in 13 × 100–mm polystyrene culture tubes or alternate container.

**BASIC
PROTOCOL 2**

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If multiple cell samples will be compared prepare the Far Red Dead staining solution prior to resuspending cells in PBS by diluting the dye in PBS at a 1:1000 ratio, preparing 1 ml of solution per sample.

- Optional (recommended): Prepare a positive control for dead cells by adding 1 μl of 100 mM digitonin to one 1-ml aliquot of cells containing approximately 10^6 cells and mixing by gently pipetting up and down.
- Incubate protected from light for 20 to 30 min at room temperature or on ice.
- Optional: Fix cells by adding 100 μl of 37% formaldehyde and incubating for 15 min at room temperature.
- Wash cells by centrifuging 5 min at 700 to 1000 $\times g$, room temperature, in a tabletop centrifuge, removing the supernatant by gentle pipetting, and resuspending in PBS with 1% (w/v) BSA or cell culture medium with 1% (w/v) BSA.
- Resuspend cells in PBS with 1% (w/v) BSA or cell culture medium with 1% (w/v) BSA. Store, protected from light, in polystyrene culture tubes on ice.
- Analyze on flow cytometer with excitation at 635 nm. Collect fluorescence emission with a 650-nm long-pass or a 670 ± 20 -nm band-pass filter.

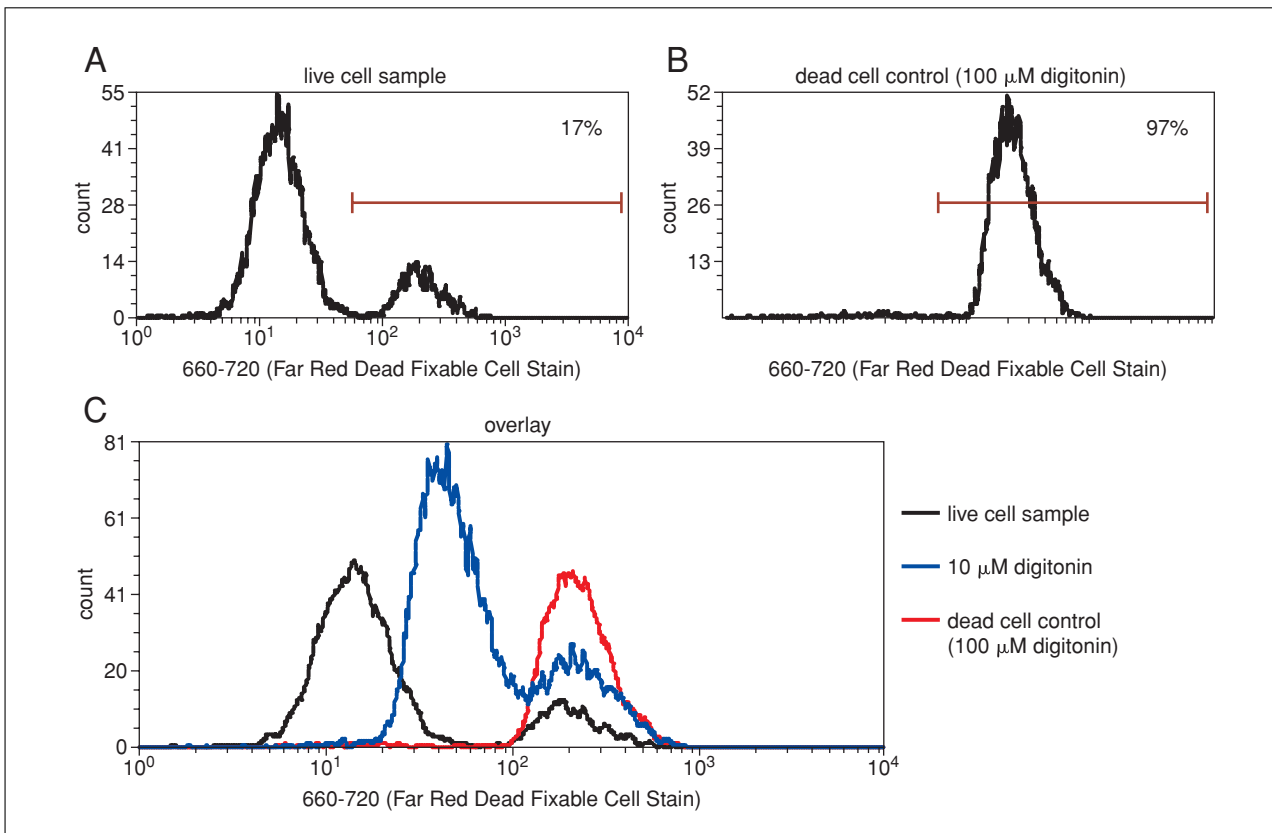


Figure 9.2.4 Example of staining and flow cytometry analysis of live/dead populations using fixed cells stained that had been stained with Live/Dead Deep Red. **(A)** Sample plot of a confluent population of cells. Live cells stain weakly, while dead cells, in this case representing 17% of the culture population, stain brightly. **(B)** A positive control for dead cells produced by treating cells with 100 μM digitonin during staining. **(C)** An overlay of the sample, dead cell control, and an intermediate concentration of digitonin demonstrating that partial staining may occur if cells are partially permeabilized. This was only observed when cells were permeabilized using digitonin and does not occur in normal cell populations.

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Far Red Dead fixable cell stain has an excitation peak at 650 nm and is easily excited at 633/655 nm.

The bright staining of dead/permeable cells is easily distinguished from the weak staining of impermeable cells (see Fig. 9.2.4). A positive control for dead cells is recommended and can be used to aid in setting an optimal cytometer gain and providing a population on which to set a dead-gate if desired. 100 μ M digitonin added to a cell aliquot during the staining step provides a simple positive control for the bright staining of dead cells.

Lightly permeabilized cells may stain with an intensity intermediate to live or dead cells (see Fig. 9.2.4C). These cells were not observed under normal culture conditions but were found to be a product of low-dose digitonin treatment. As with all live/dead dyes, viability should be carefully defined as part of the experimental design prior to running any samples, and suitable positive and negative controls should be included to allow for unambiguous distinction of defined viable and nonviable populations.

Live/Dead fixable dyes are available in a number of spectral variations, providing flexibility in cytometer choice, wavelength combinations, and experimental design.

Violet-Excited Fixable Staining of Cells for Assessment of Viability

Materials

Phosphate-buffered saline (PBS; APPENDIX 2A), azide-free and serum/protein-free

Cell suspension ($\sim 1 \times 10^6$ cells/ml)

100 mM digitonin (see recipe; optional/recommended).

Fixable Viability Dye eFluor 506 (eBioscience; supplied as a pre-diluted solution in anhydrous DMSO; protect from light and moisture; store at $\leq -70^\circ\text{C}$ with desiccant; may be freeze-thawed up to 20 times)

37% formaldehyde (Sigma, cat. no. 252549; optional)

PBS (APPENDIX 2A) with 1% (w/v) bovine serum albumin (BSA) or cell culture medium without phenol red supplemented with 1% BSA

Culture tubes (13 \times 100–mm) or any alternative suitable container for delivering cells to the flow cytometer for analysis

Flow cytometer

1. Place 1 ml of an $\sim 1 \times 10^6$ cell/ml suspension in a 13 \times 100–mm polystyrene culture tube, or equivalent container. Wash cells twice in azide-free and serum/protein-free PBS, each time by centrifuging 5 min at $1000 \times g$, room temperature. Remove the supernatant from the final wash.
2. *Optional/recommended:* Prepare a positive control for dead cells by adding 1 μ l of 100 mM digitonin to one 1-ml aliquot of $\sim 1 \times 10^6$ cells and mixing by gentle pipetting.

A dead control is recommended if the percentage of dead cells is expected to be less than 5%.
3. Resuspend cells at $1\text{--}10 \times 10^6$ /ml in azide-free and serum/protein-free PBS.
4. Add 1 μ l of eFluor 506 fixable dye per 1 ml of cells and vortex immediately.

Allow vial to equilibrate to room temperature before opening.
5. Incubate for 30 min at $^\circ$ to 8°C ; protect from light.
6. Wash cells one to two times with PBS.
7. *Optional:* Fix cells by adding 100 μ l of 37% formaldehyde and incubating for 15 min at room temperature.

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8. Wash cells once in PBS with 1% BSA or cell culture medium with 1% BSA, each time by centrifuging 5 min at 700 to 1000 × g, room temperature. Remove the supernatant from the final wash.
9. Resuspend cells in PBS with 1% BSA or cell culture medium with 1% BSA. Store, protected from light on ice.
10. Analyze on flow cytometer with excitation at 405 nm. Collect fluorescence emission with a 510 ± 50-nm band-pass filter.

Staining with eFluor 506 fixable dye must be done in azide-free and serum/protein-free PBS. For consistent staining of cells, do not stain cells in less volume than 0.5 ml.

Cells may be stained with eFluor 506 fixable dye before or after surface staining. After staining with eFluor 506 fixable dye, cells may also be cryopreserved for analysis at a later time.

The dye is titrated for use with mouse thymocytes. It is recommended that you titrate the dye concentration for use with your cells.

A range of fixable eFluor dyes is available with excitation from violet to red.

ALTERNATE PROTOCOL 5

Carboxymethylfluorescein Diacetate (Cell Tracker CMFDA) Staining of Cells for Assessment of Viability by Flow Cytometry

Additional Materials (also see Basic Protocol 1)

5 mM carboxymethylfluorescein diacetate (see recipe)

1. Add 1 μl of 5 mM CMFDA (5 μM final) to approximately 10⁶ washed cells suspended in 1 ml PBS in 13 × 100-mm polystyrene culture tubes.

Recommended: include one unstained sample to provide an unstained control (see Fig. 9.2.5).

2. Incubate 15 min in the dark at room temperature.
3. Wash once in PBS (centrifuging 5 min at 700 to 1000 × g, room temperature) and incubate 5 min in the dark at room temperature to destain.

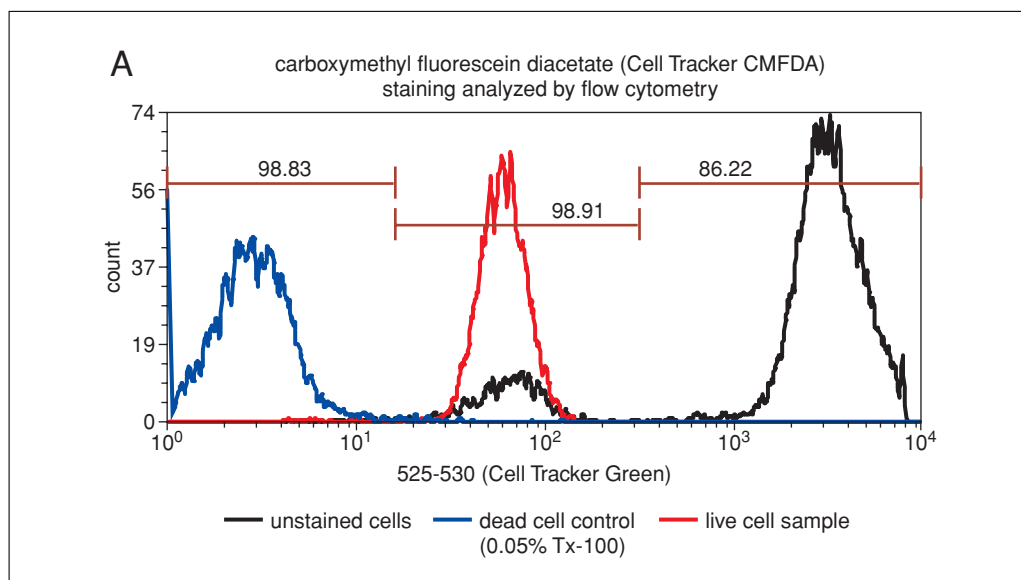


Figure 9.2.5 Sample staining using Cell Tracker CMFDA. Stained dead cells are brighter than unstained cells while stained live cells are >100 fold brighter than dead cells and easily distinguished from the dead population.

4. *Optional*: Fix cells by adding 100 μ l of 37% formaldehyde and incubating for 15 min at room temperature.
5. Resuspend cells in PBS with 1% BSA or cell culture medium with 1% BSA. Store, protected from light, in polystyrene culture tubes on ice.
6. Analyze on flow cytometer with excitation at 488 nm. Collect fluorescence emission using a filter appropriate for 520 nm emission.

CMFDA is a very bright dye and live cells are easily distinguished from dead cells.

Ethidium Monoazide Staining of Nonviable Cells Prior to Fixation

Additional Materials (also see *Basic Protocol 1*)

50 μ g/ml ethidium monoazide (EMA; see recipe)

1% (w/v) paraformaldehyde in PBS (see *APPENDIX 2A* for PBS; store mixture \leq 1 week at 4°C and discard if precipitate forms)

40-W fluorescent light

1. Add 10 μ l of 50 μ g/ml EMA (\sim 5 μ g/ml final) to approximately 10^6 washed cells suspended in 100 μ l PBS in a 13 \times 100-mm polystyrene culture tube.

Preparations of EMA dye vary. The exact concentration needed may range from 1 to 5 μ g/ml.

2. Place tubes on ice \sim 18 cm beneath a 40-W fluorescent light for 10 min.

EMA diffuses into dead cells and intercalates into DNA. When exposed to light, EMA is then covalently bound to the DNA

3. Wash and fix cells by adding 1 ml μ l of 1% paraformaldehyde to the cell pellet. Incubate 1 hr at room temperature.

4. Analyze on flow cytometer with excitation at 488 nm. Collect fluorescence emission using a \geq 630-nm long-pass filter; amplify PMT output logarithmically.

EMA excites with 488-nm light and fluoresces well into the red region of the spectrum. EMA does not fluoresce as brightly as propidium iodide, so discrimination of nonviable, EMA-bright cells from viable cells may be less obvious. A bivariate plot of forward light scatter versus EMA fluorescence may aid in distinguishing viable (EMA-negative) cells.

Dead cells can be live-gated, but unless one is absolutely sure of the viable population, it is always better to collect un-gated listmode data and perform gating after the raw data files are collected. Populations that may not be obvious on the flow cytometer display will be seen during subsequent data analysis. Moreover, any gating can be done and redone without losing cells.

LDS-751 Staining of Previously Nonviable Cells After Fixation

Additional Materials (also see *Basic Protocol 1*)

1% (w/v) paraformaldehyde in PBS (see *APPENDIX 2A* for PBS; store mixture \leq 1 week at 4°C and discard if precipitate forms)

2 μ g/ml LDS-751 (laser dye styryl-751) working solution (see recipe)

1. Wash approximately 10^6 cells in PBS in a 13 \times 100-mm polystyrene culture tube.
2. Fix cells by adding 1 ml μ l of 1% paraformaldehyde to the cell pellet. Incubate 1 hr at room temperature.
3. Add 10 μ l of 2 μ g/ml LDS-751 working solution to 1 ml fixed cells at a concentration of approximately 10^6 cells per ml.

**ALTERNATE
PROTOCOL 6**

**ALTERNATE
PROTOCOL 7**

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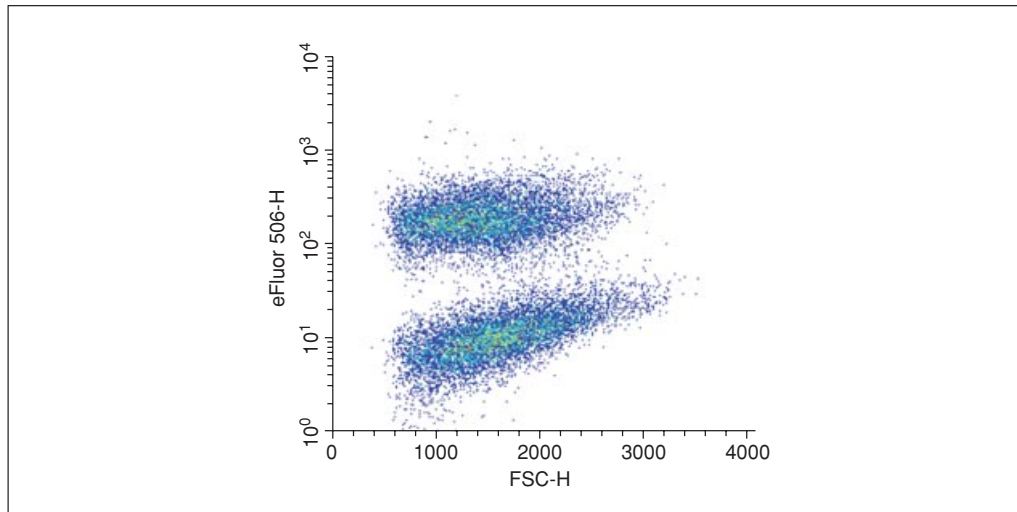


Figure 9.2.6 Identification of dead cells with eFluor 506. The bright dead population is clearly distinguished from unstained cells. While the excitation was with 405-nm light, the detection filter, (530/30), was slightly suboptimal for detection.

4. Incubate overnight at room temperature in the dark.
5. Analyze on flow cytometer with excitation at 488 nm. Collect fluorescence emission using a 650-nm long-pass filter.

LDS-751 is excited with 488-nm light and emits in the red portion of the spectrum; the 650-nm long-pass filter is adequate to separate red fluorescence from other fluorochromes and scattered laser light. Amplify the PMT output logarithmically to distinguish bright, nonviable cells from dim, viable cells and from nonfluorescent red cells or debris. A bivariate plot of forward scatter versus log LDS-751 fluorescence can help identify populations.

Dead cells can be live-gated, but unless one is absolutely sure of the viable population, it is always much better to collect un-gated listmode data and perform gating after the raw data files are collected. Populations that may not be obvious on the flow cytometer display will be seen during subsequent data analysis. Moreover, any gating can be done and redone without losing cells.

ASSESSMENT OF CELL VIABILITY BY MICROSCOPY

Determination of cellular viability by microscopy provides data complementary to that obtained using flow cytometry analysis but also allows for simultaneous examination of cell morphology, examination of subcellular structures using fluorescent proteins or vital dyes, or, when fixable viability dyes are used, analysis of viability at the time of fixation combined with immunologically based staining. Microscopy may also be the preferred method of viability assessment in cells that are not easily amenable to flow cytometry analysis due to culture conditions or cellular properties. Analysis of viability by fluorescence microscopy has become increasingly versatile in recent years, with the development of many spectral alternatives in both fixable and nonfixable dyes. Determining viability of a population of cells in culture is relatively easy, but usually requires the addition of a viability-independent cell stain. Flow cytometry may be the preferred method for viability determination in some cases, given that each cell is recorded as an event and a viability-independent counterstain is not necessary.

Carboxymethylfluorescein diacetate (Cell Tracker CMFDA), described in Basic Protocol 1, is a bright, cell membrane integrity-sensitive dye that provides a clear distinction between intact and damaged or dead cells (Fig. 9.2.7). CMFDA is easily combined with Hoechst 33342 to provide a cell count with which to determine percent viability.

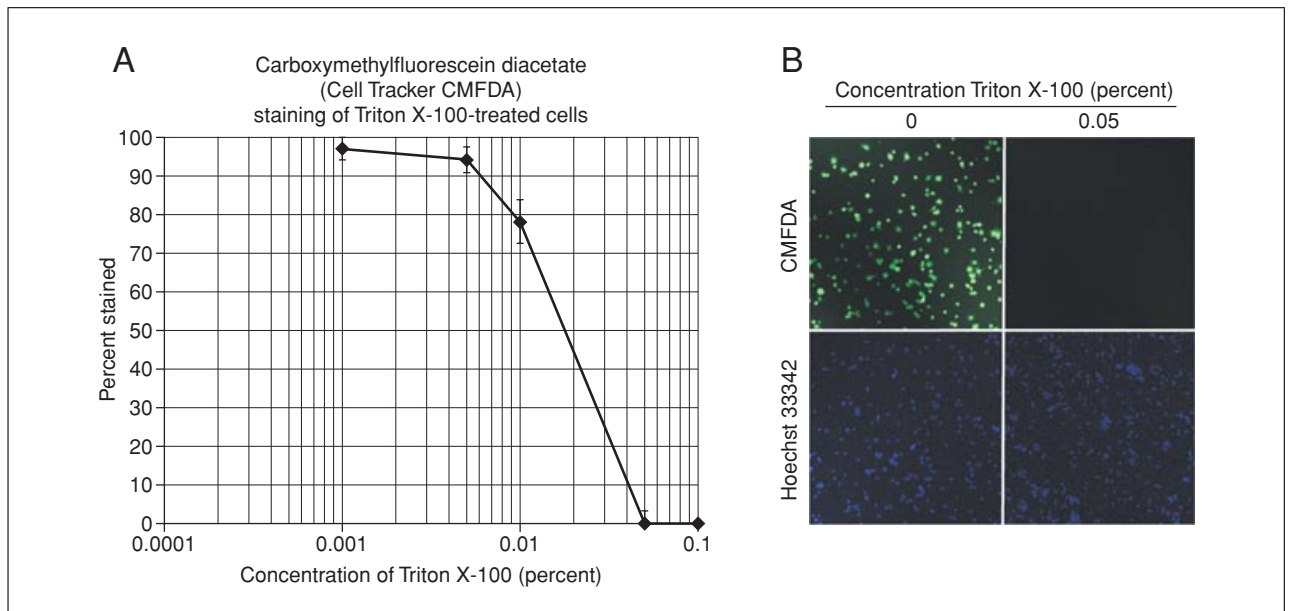


Figure 9.2.7 Example of viability analysis using Cell Tracker CMFDA and microscopy. **(A)** Cells are efficiently permeabilized using Triton X-100 as a positive control and CMFDA shows a steep dose response curve indicating high specificity for permeabilized cells. **(B)** Live cells are extremely bright and easily distinguished from dead cells. A counterstain is necessary to present data as a percent of total.

CMFDA is also fixable and alternative spectral variants are available (including CMAC, see Alternate Protocol 2), though each spectral variant is likely to have a unique sensitivity to membrane integrity.

Combined use of membrane-impermeable dead cell dyes (those that only enter damaged or dead cells) and live cell dyes (those that are retained in living cells but diffuse out of damaged or dead cells) allows for detection of viability using multiple measures simultaneously. These combinations allow for a more specific definition of viability in situations where viability status may be ambiguous using only one dye. Calcein AM and EtHD-1 (Alternate Protocol 1) make one such dye combination. Figure 9.2.8 demonstrates the differences in sensitivity of these dyes, with low-dose Triton X-100 producing cells that do not accumulate either Calcein AM or EtHD-1, thus staining neither viable nor nonviable cells. A stringent viability cutoff could designate only cells that are both negative for EtHD-1 and positive for Calcein AM as viable.

Sytox AADvanced (a derivative of 7-AAD) and chloromethylaminocoumarin (Cell Tracker Blue CMAC) provide an alternative combined set of live/dead dyes. Sytox and Cell Tracker are both available in multiple spectral variants, allowing for selection of dyes compatible with the available microscopy equipment. Cell Tracker dyes are fixable, as discussed, allowing for fixation post-imaging and further analysis.

Finally, assessment of cell viability may be accomplished with a simple microscope, using dyes that mark nonviable cells by dye exclusion. The most commonly used dye is trypan blue, but others may be used as well; see Background Information for details on other useful stains. Viable cells have intact membranes and exclude the dye; nonviable cells are labeled with the dye and are visible with brightfield optics. As well as being useful as a means of assessing functional integrity, trypan blue exclusion is widely used as an objective method of determining viable cell count prior to using cells; a simple protocol for this application is presented, along with other basic cell culture techniques, in *APPENDIX 3B*.

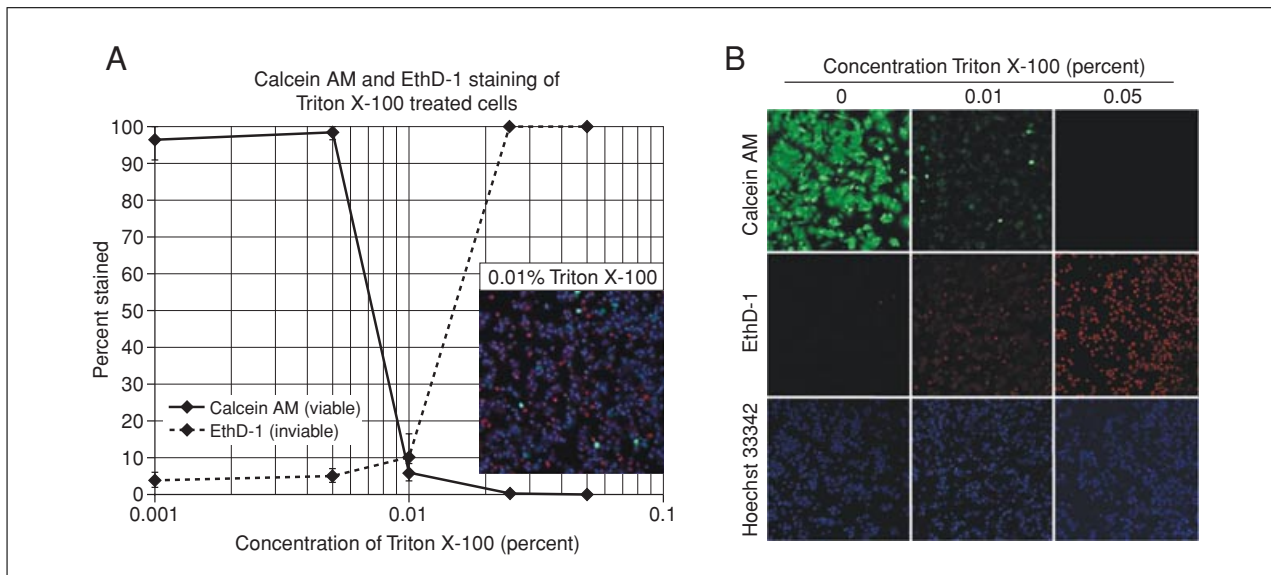


Figure 9.2.8 Example of dual staining with Calcein AM and EthD-1. **(A)** Analysis of dual-stained cells permeabilized with Triton X-100 demonstrates that while both dyes are highly specific for their target cells (they have steep drop-offs of staining in the dose response curve), they are slightly non-overlapping in their staining. Calcein AM is lost before EthD-1 stains cells (see inset). This analysis did not include weakly staining cells, and adjustment of brightness to include these cells may reduce the observed lack of complementation. **(B)** Cells stained with either dye are extremely bright and easy to distinguish from unstained cells, although intermediate permeabilization can result in a lack of either stain.

BASIC PROTOCOL 4

Carboxymethyl Fluorescein Diacetate (Cell Tracker CMFDA) Staining of Cells for Assessment of Viability by Microscopy

Materials

- 5 mM carboxymethylfluorescein diacetate (see recipe)
- 1 mM Hoechst 33342 (Sigma) in phosphate-buffered saline (PBS; *APPENDIX 2A*)
- Cell culture medium (prewarmed to 37°C)
- Cultured cells on chambered coverglass (Nunc, cat. no. 155379) or grown on coverslips (for fixed only)
- Phosphate-buffered saline (PBS; *APPENDIX 2A*)
- Fluorescence microscope with filters appropriate for FITC and DAPI

1. Add 1 μ l of 5 mM CMFDA (5 μ M final) and 10 μ l of 1 mM Hoechst 33342 (10 μ M final) in PBS per ml to pre-warmed cell culture medium, making enough to cover cells completely (1 ml per well in covered chamber slides). Do not add Hoechst if fixing cells (optional, step 4).
2. Aspirate medium from cells on chambered coverglass and replace with the medium containing dyes from step 1.
3. Incubate 15 min in the dark under cell culture conditions.
4. Wash once with warm PBS, replace medium, and incubate 10 to 15 min in the dark at cell culture conditions to destain.
5. *Optional:* Fix cells by adding 100 μ l of 37% formaldehyde per ml medium, gently mixing, and incubating for 15 min at room temperature. Wash once with PBS and store at 4°C in PBS.
6. Image cells by microscopy using filters appropriate for FITC (for CMFDA) and DAPI (for Hoechst).

If cells were fixed using optional step 4 DNA can be stained using any compatible DNA fluorophore, such as DAPI, to provide a counterstain.

CMFDA is a very bright dye and live cells are easily distinguished from dead cells (see Fig. 9.2.7). If fixed, cells can be used for additional staining.

Simultaneous Detection of Live and Dead Cells by Microscopy using Calcein AM, EthD-1, and Hoechst 33342

**ALTERNATE
PROTOCOL 8**

Materials

2 mM Calcein AM (see recipe)
4 mM ethidium homodimer 1 (EthD-1; see recipe)
Cell culture medium (prewarmed to 37°C)
Cultured cells on chambered covers glass (Nunc, cat. no. 155379) or grown on coverslips (for fixed cells only)
100 mM digitonin (see recipe) or 50% (v/v) Triton X-100 in H₂O; optional/recommended
Phosphate-buffered saline (PBS; APPENDIX 2A)
Fluorescence microscope with filters for FITC (for Calcein AM), Texas Red (for EthD-1), and DAPI (for Hoechst)

1. Add 1 µl of 2 mM Calcein AM (2 µM final), 1 µl of 4 mM EthD-1 (1 µM final), and 10 µl of 1 mM Hoechst 33342 (10 µM final) in PBS per ml to prewarmed cell culture medium, making enough to cover the cells completely (1 ml per well in covered chamber slides).
2. Aspirate medium from the cells and replace with medium containing dyes.
3. *Optional (recommended):* Add 1 µl of 100 mM digitonin in DMSO per ml medium, or add Triton X-100 to 0.05% (v/v; final concentration) to cells to create a positive control.
4. Incubate 15 min in the dark under cell culture conditions.
5. Wash once with warm PBS, replace with fresh medium, and incubate 10 to 15 min in the dark under cell culture conditions to destain.
6. Image cells by microscopy using filters appropriate for FITC (for Calcein AM), Texas Red (for EthD-1), and DAPI (for Hoechst).

Calcein AM and EthD-1 are both very bright, very sensitive dyes, but loss of Calcein AM staining can be observed in permeabilized cells, indicating that defining loss of viability may depend on the dye analyzed. Because of the slight lack of overlap between the two dyes, a nuclear dye should always be included so that the percent viable or nonviable cells is reflective of the total cell count, rather than the two fluorophores alone. Furthermore, viability should be carefully defined prior to the start of the experiment, such that it is predetermined whether cells lacking either stain are considered viable or not (see Fig. 9.2.8).

Triton X-100 provides a simple and cheap positive control but can, at high concentrations, lift cells from the surface of the plate and prevent imaging. Digitonin, on the other hand, is more specific for membrane permeabilization but is toxic and expensive. Triton should be prepared as a 50% stock prior to use to allow for pipetting of the viscous compound.

Simultaneous Detection of Live and Dead Cells by Microscopy using Chloromethylaminocoumarin (Cell Tracker Blue CMAC) and Sytox AADvanced

**ALTERNATE
PROTOCOL 9**

Materials

1 mM CMAC (see recipe)
1 mM Sytox AADvanced (see recipe)

**Studies of Cell
Function**

9.2.15

Cell culture medium (prewarmed to 37°C)

Cultured cells on chambered covers glass (Nunc, cat. no. 155379) or grown on coverslips (for fixed cells only)

100 mM digitonin (see recipe) or 50% (v/v) Triton X-100 in H₂O; optional/recommended

Phosphate-buffered saline (PBS; APPENDIX 2A)

Fluorescence microscope with filters appropriate for 353 nm absorption/466 nm emission (CMAC) and 546 nm absorption/647 emission (for Sytox AADvanced)

1. Add 1 μ l of 1 mM CMAC (1 μ M final) and 1 μ l of 1 mM Sytox AADvanced to prewarmed cell culture medium, making enough to cover cells completely (1 ml per well in covered chamber slides).
2. Aspirate medium and replace with medium containing dyes.
3. *Optional (recommended)*: Add 1 μ l of 100 mM digitonin in DMSO per ml medium or Triton X-100 to 0.05% (v/v; final concentration) to cells to create a positive control.
4. Incubate 15 min in the dark under cell culture conditions.
5. Wash once with warm PBS, replace with fresh medium, and incubate 10 to 15 min in the dark under cell culture conditions to destain.
6. Image cells by microscopy using filters appropriate for 353 nm absorption/466 nm emission (CMAC) and 546 nm absorption/647 emission (for Sytox AADvanced).

Calcein AM and EthD-1 are both very bright, very sensitive dyes. These dyes are highly complementary in their staining (see Fig. 9.2.9) with cells generally staining positively for one of the two dyes. A spectrally compatible nuclear dye may be included to provide a total cell count, as in the previous protocols.

This protocol can be easily adapted depending on the spectral requirements of the experiment. The variety of Cell Tracker and Sytox probes available provides flexibility in dye combinations and allows for selection of dyes that are spectrally compatible with additional probes.

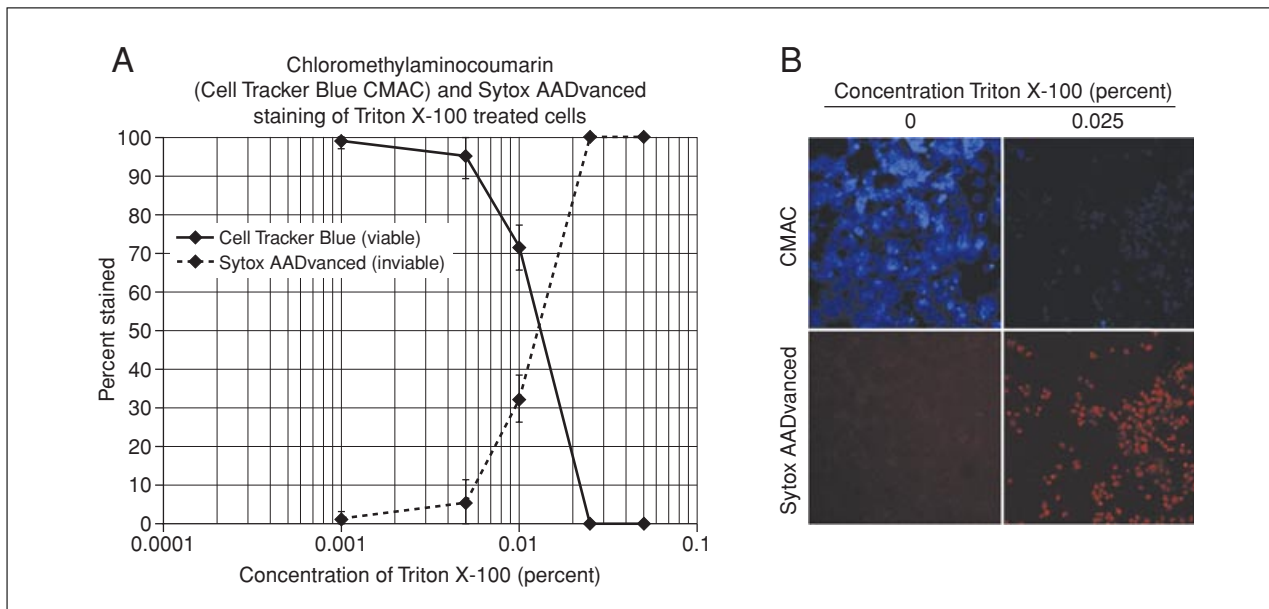


Figure 9.2.9 Sample staining using Cell Tracker Blue CMAC and Sytox AADvanced. **(A)** Dose response curve for staining and permeabilization with Sytox AADvanced and CMAC. **(B)** These dyes appeared to be highly complementary, with no apparent intermediate dose where cells lacked staining for either dye, although no total cell staining dye was included in this analysis.

9.2.16

Trypan Blue Staining**Additional Materials** (also see *Basic Protocol 1*)

0.4% (w/v) trypan blue in PBS (store up to 1 year at room temperature in the dark; filter if a precipitate forms; for PBS, see *APPENDIX 2A*)

Serum-free culture medium (*APPENDIX 3B* optional)

Additional materials for cell counting with a hemacytometer (*APPENDIX 3A*)

1. Add an equal volume of 0.4% trypan blue to a cell suspension at a concentration of approximately 10^6 cells per ml.

Use PBS or a serum-free medium for the cell suspension. Serum proteins may stain with trypan blue, resulting in falsely depressed viable counts.

2. Incubate at room temperature for ~3 min and load into a hemacytometer. Using brightfield optics, count cells in three separate fields (see *APPENDIX 3A* for use of hemacytometer). Count nonviable, deep blue cells as well as viable, clear cells.

3. Calculate viability: % viable = (number viable cells/number total cells) \times 100.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

7-AAD (7-amino actinomycin D), 1.0 mg/ml

Dissolve 1.0 mg 7-AAD in 50 μ l of dimethyl sulfoxide (DMSO), and then add 950 μ l of PBS (*APPENDIX 2A*). Store \leq 1 month in the dark at 4°C.

As 7-AAD is not water soluble, an organic solvent is required. Although the mutagenicity of 7-AAD is unknown, caution should be exercised when handling the dye.

Calcein AM (calcein acetoxymethyl ester), 2 mM

Spin down a tube of 50 μ g desiccated Calcein AM powder (Invitrogen, cat. no. C3100MP) to collect dye at the bottom of the tube. Dissolve in 5 μ l of DMSO. Spin again to ensure that all powder is collected at bottom of tube. Store protected from light and moisture up to 1 year at -20°C.

Cell Tracker Blue CMAC (chloromethylaminocoumarin), 1 mM

Dissolve 5 mg of chloromethylaminocoumarin (Invitrogen, cat. no. C2110) in 238.5 μ l of DMSO. Aliquot and store protected from light and moisture at -20°C.

CMFDA (carboxymethylfluorescein diacetate), 5 mM

Dissolve 1 mg of CMFDA (Invitrogen/Life Technologies, cat. no. C2925) in 420 μ l of sterile DMSO. Aliquot and store protected from light and moisture at -20°C.

Digitonin, 100 mM

Spin down a tube of 100 mg desiccated digitonin (Sigma-Aldrich, cat. no. D141-100MG). Add 813.5 μ l DMSO and dissolve by gently pipetting up and down. Store at -20°C.

EMA (ethidium monoazide), 50 μ g/ml

Dissolve ethidium monoazide at 5 mg/ml in PBS (*APPENDIX 2A*). Dilute to 50 μ g/ml (working strength) and divide into 0.5-ml aliquots. Wrap in aluminum foil and store \leq 6 months at -20°C. Thaw immediately before use. Discard unused working-strength dye.

EMA is very light sensitive; keep wrapped in foil or in the dark.

EtHD-1 (ethidium homodimer 1), 4 mM

Dissolve 1 mg of EtHD-1 (Invitrogen/Life Technologies cat. no. E1169) in 275 μ l of DMSO. Aliquot and store protected from light and moisture at -20°C .

LDS-751 (laser dye styryl-751) working solution, 2 μ g/ml

Stock solution: Dissolve LDS-751 (Exciton Corp.) at 0.2 mg/ml in methanol. Store up to 1 month at 4°C in the dark.

Working solution: Add 0.5 ml stock solution to 50 ml PBS (*APPENDIX 2A*; 2 μ g/ml final). Store up to 1 week in the dark at 4°C .

Live/Dead Far Red Dead stock solution

Prepare a fresh stock solution of Live/Dead Far Red Dead fixable cell stain (Invitrogen/Life Technologies, cat. no. L10120) in DMSO by combining components A and B from the kit according to the manufacturer's instructions.

Desiccated dye powder can be stored at -20°C >6 months, while it is recommended that dye in DMSO solution be stored at -20°C and used within 2 weeks of preparation.

Sytox AADvanced, 1 mM

Add 100 μ l of DMSO to one tube of Sytox AADvanced (Invitrogen/Life Technologies, cat. no. S10349) to prepare a 1 mM stock solution. Store protected from light and moisture at -20°C for 1 year avoiding freeze-thaw (manufacturer recommends use immediately after preparation of stock solution).

COMMENTARY

Background Information

Assessments of viability depend on one or both of two cellular properties: (1) the intactness of the cell membrane, and (2) the physiological state of the cell.

Dye-exclusion methods are based on the fact that only intact membranes are impermeable to large or charged molecules. Intact membranes also maintain cytoplasmic gradients with respect to the surrounding medium, thus retaining intracellular concentrations of ions and small molecules. This latter property also reflects the physiological state of the cells in that energy is required to maintain gradients. Thus, methods that assay physiological properties of the cell also are dependent upon and indicative of an intact membrane.

Probes for membrane integrity

Permeability of the cytoplasmic membrane is commonly exploited to mark cells that are moribund or dead. The reagents most often used for this purpose are dyes such as trypan blue or a variety of fluorochromes that will penetrate only damaged permeable membranes of nonviable cells. These are then easily identified visually by the presence of blue color (with trypan blue) in a simple brightfield microscope, or by bright fluores-

cence seen by fluorescence microscopy or flow cytometry.

Fluorescent probes include a wide range of dyes that label DNA of membrane-damaged cells. Tetrabromofluorescein (eosin Y) is a fluorescein derivative that has been used to identify nonviable *Candida* blastospores (Costantino et al., 1995). In addition, the intracellular penetration of enzymes such as DNase or trypsin can indicate the loss of membrane integrity and thus nonviability (Frankfurt, 1990; Darzynkiewicz et al., 1994; Johnson, 1995). Along the same lines, the penetration of probes for cytoplasmic markers (actin, tubulin, or cytokeratin) has been used to identify cells with damaged plasma membranes (O'Brien and Bolton, 1995).

The most widely used group of fluorescent probes are those that label nucleic acids (for further discussion of nucleic acid stains, see *UNIT 4.3*). The most straightforward labeling methods use propidium iodide (PI), 7-amino actinomycin D (7-AAD), or DAPI to identify dead cells, which are hundreds or thousands of times brighter than viable cells. Propidium iodide is in widespread use with many mammalian cell types (Jacobs and Pipho, 1983; Massaro et al., 1989; Coco-Martin et al., 1992; Darzynkiewicz et al., 1994; Stewart and

Stewart, 1994; O'Brien and Bolton, 1995), bacteria (Vesey et al., 1994; Nebe-von Caron and Badley, 1996), and protozoa (Armstrong et al., 1991; Humphreys et al., 1994). 7-AAD is a useful alternative to PI. Like PI, 7-AAD penetrates only dead cells, but 7-AAD fluorescence is both less intense and at a longer wavelength (~670 nm, versus ~610 nm for PI). These latter two properties make 7-AAD preferable as a viability marker when FITC and PE are used to label surface antigens (Schmid et al., 1992). It has been reported that the dye can be used for fixed cells as well (Fetterhoff et al., 1993); see section on viability assays of fixed cells for further discussion. If an instrument has the proper excitation options (UV, near UV, or 405nm laser), DAPI provides a simple alternative and with flow cytometers, circumvents compensation issues of single laser systems (Muczynski et al., 2003). Alternatively, hydroxystilbamide (Invitrogen/Life Technologies), also marketed as or Fluoro-Gold (Fluorochrome, LLC; <http://www.fluorochrome.com/>), is a UV- or near UV-excited DNA-binding dye excluded from viable cells. Its brightness is similar to that of 7-AAD (Barber et al., 1999).

SYBR-14, a DNA stain from Invitrogen/Life Technologies, penetrates viable cells (Garner et al., 1994). The dye can be used in conjunction with propidium iodide to unambiguously differentiate viable from dead or moribund sperm cells (Garner et al., 1994; Christensen et al., 2004). The method was used to analyze semen from boars, rams, rats, rabbits, humans, and turkeys (Christensen et al., 2004). SYTO13 has been used in combination with 7-AAD and the leukocyte marker, CD45 to determine the viability of nucleated cells (Shenkin et al., 2007). Another dye that can differentiate apoptotic and nonapoptotic cells is SYTO 16 (Invitrogen/Life Technologies), of the SYTO series of dyes (Frey, 1995). The dyes YOYO-1 and TOTO-1 (Invitrogen/Life Technologies; both derivatives of thiazole orange) have also been used successfully in viability assays (Becker et al., 1994). As with other membrane exclusion/DNA binding probes, the dyes do not penetrate viable cells, and remain nonfluorescent until they bind to DNA. Variants of these dyes, YO-PRO-1, TO-PRO-1, or TO-PRO-3 (Invitrogen/Life Technologies), have even higher affinities for nucleic acids and have been used successfully with mammalian or bacterial cells (Vesey et al., 1994; O'Brien and Bolton, 1995; Mathioudakis et al., 2002; see Haugland, 1994, for details of dyes). The use of YO-PRO-1 for the identi-

fication of apoptotic cells seems to have the advantage of preserving the proliferation capacity of living cells (Idziorek et al., 1995; for further details on microbial viability, see *UNIT 11.3* and Davey, 2011).

Ethidium bromide (EB) used with low concentrations of acridine orange (AO) identifies normal (AO at high fluorescence level, EB low), early apoptotic (AO low, EB low), and late apoptotic/necrotic (AO low, EB high) cells (Liegler et al., 1995; Olivier, 1995). Ethidium bromide staining due to loss of membrane integrity identifies the population of necrotic cells; the mechanism of decreased AO staining is not clear but may be related to loss of DNA integrity (Liegler et al., 1995).

Probes of physiological state

Viable cells can be identified directly using fluorescent probes that identify properties of normal cells. Two principal properties are the integrity of the plasma membrane and the presence of metabolic processes. Cell viability can be assessed based on the presence of cytoplasmic esterases that cleave moieties from a lipid-soluble, nonfluorescent probe to yield a charged fluorescent product that is retained within the cell if membrane function is intact. Hence, viable cells are bright and nonviable cells are dim or nonfluorescent. The most common of these probes is fluorescein diacetate (FDA). It has been used with bacteria (Diaper et al., 1992; Diaper and Edwards, 1994; Vesey et al., 1994; Nebe-von Caron and Badley, 1996), protozoa (Armstrong et al., 1991; Humphreys et al., 1994), phytoplankton (Yentsch and Pomponi, 1994), plants (Galbraith, 1994; Brigham et al., 1995; Kodama and Komamine, 1995), and a variety of mammalian cells (Coco-Martin et al., 1992; Darzynkiewicz et al., 1994; Johnson, 1995). Once FDA diffuses into cells, non-specific esterases in the cell cytoplasm generate free fluorescein. The dye works well in some instances, but the rate at which fluorescein diffuses out of cells varies greatly. To circumvent this problem, dye variants such as BCECF (Invitrogen/Life Technologies) and carboxyfluorescein diacetate, which require energy-dependent efflux of the fluorescent dye (Massaro et al., 1989; Breeuwer et al., 1994), have been developed.

Another approach to minimize dye loss is to use the acetoxymethyl ester of calcein (calcein AM). The ester group facilitates uptake of the dye and is cleaved in the cytoplasm to give free calcein. The fluorochrome has an increased

retention time (a 3-hr half-life is reported) and less sensitivity to pH (Haugland, 1994; Holló et al., 1994). Invitrogen/Life Technologies provides the dye as a kit in conjunction with ethidium homodimer (EukoLight, now LIVE/DEAD Cytotoxicity Kit). Only live cells retain calcein and are labeled green, while dead cells are labeled red because their permeable membranes allow the ethidium homodimer to penetrate and label DNA. In addition, Invitrogen/Life Technologies has produced a variety of kits for determining viability in bacteria (BacLight) or fungi (LIVE/DEAD Funga Light Yeast Viability Kit and Funga Light CFDA-SE/PI Yeast Vitality Kit, and FUN1 and FUN2 dyes).

Dihydroethidium is taken up by viable cells and cleaved by esterases to generate ethidium monomer, which binds to DNA and is retained in the nucleus (Bucana et al., 1986). Dead cells do not produce the monomer and remain nonfluorescent. Viable intracellular parasites (*Babesia bovi*) can be identified with flow cytometry (Wyatt et al., 1991). The dye has been used in conjunction with carboxyfluorescein diacetate to identify viable populations of sperm from frozen samples (Ericsson et al., 1989). Questions regarding the potential toxicity of the dye were raised when the dye was found to inhibit the oxygen uptake of sperm cells stained with dihydroethidium (Downing et al., 1991).

Another dye that seemed to have promise is Vita Blue (apparently withdrawn from the market; Lee et al., 1989). This dye is excited by red light, thereby making possible the simultaneous use of green and orange emitting dyes that are excited by 488-nm light. TO-PRO-3 provides an alternative for use with red excitation.

Viable cells maintain electrochemical gradients across the plasma membrane. Functional subsets of this general phenomenon include maintenance of pH and other ion gradients as well as the capacity for energy-yielding metabolism in mitochondria. These physiological processes can be exploited to distinguish viable from nonviable cells. Fluorochromes useful in viability assays include those used to measure membrane potential and intracellular pH. These probes are typically lipophilic, charged molecules that preferentially partition into cells having a negative potential difference with respect to the surrounding environment, so that the dye becomes concentrated in the cytoplasm or internal organelles.

One of the most commonly used probes for identifying viable cells is rhodamine 123. This is a cationic lipophilic dye that partitions into mitochondria because of their low potential. Active mitochondria in viable cells are stained bright green; loss of gradients within the cell results in loss of the dye. Rhodamine 123 can indicate viable cells among bacteria (Diaper et al., 1992; Diaper and Edwards, 1994; Vesey et al., 1994; Porter et al., 1995a,b; Nebe-von Caron and Badley, 1996) or a variety of mammalian cells (Darzynkiewicz et al., 1994; Johnson, 1995). The presence of aliphatic side chains on fluorochromes may strongly influence retention within the cells. For example, side chains of the cyanine dye DiOC₆ (3) cause the probe to partition into the mitochondrial membranes, resulting in increased concentration and brightness—the brightness related to better fluorescence in a lipid environment (Sims et al., 1974). A possible disadvantage is that increased affinity for membranes may also slow the loss of the dye if mitochondria lose their metabolic capacity.

Any of the assays employing dye uptake to label viable cells can be coupled with assays using dyes to label nonviable cells. Thus, one may use rhodamine 123 with, for example, PI or ethidium bromide. A novel example employs the combination of FluoZin-3, tetramethylrhodamine ethylester [TMRE], and monochlorobimane [mBcl] for the determination of the viability and function of islet pancreatic cells (Jayaraman, 2008). In combination, the zinc-specific dye and the indicators of mitochondrial function and thiol levels estimate the beta cell viability and function.

Instead of viable cells being labeled with cationic dyes, nonviable cells can be labeled with the lipophilic anionic dye oxonol; this has been done in bacteria (Deere et al., 1995; Nebe-von Caron and Badley, 1996) and protozoa (Humphreys et al., 1994). Loss of negative potential with respect to the outside causes accumulation of oxonol within dead cells. It has been reported, however, that starved bacteria can contain populations of oxonol-positive bacteria that are PI-negative (Nebe-von Caron et al., 1996).

In some cells, dyes that accumulate in live cells may be pumped out by the glycoprotein pump (Holló et al., 1994; Shapiro, 1995). Thus, cells that do not stain brightly with a dye such as rhodamine 123 may still be viable. It has also been reported that some non-specific staining of rhodamine 123 in natural

particulate environments may complicate the identification of live bacteria (Porter et al., 1995b). Conversely, cells in the presence of glutathione may have hyperpolarized mitochondria and hence enhanced uptake of dye into mitochondria (Pieri et al., 1992). Valinomycin has been used to hyperpolarize bacteria to enhance their dye uptake (Porter et al., 1995b). There is some indication that the toxicity of rhodamine is low (Downing et al., 1991).

Probes for fixed cells

Often, cells must be fixed or permeabilized before analysis. In both cases, analysis of other markers is less ambiguous if nonviable or damaged cells can be identified and rejected. Several methods have been successful in the labeling of dead or damaged cells prior to fixation. A modification of Hoechst/PI staining methods permits the use of ethanol as a fixative (Pollack and Cianco, 1990). Prior to fixation, dead cells are labeled with PI. After ethanol fixation, all cells label with Hoechst, but the PI in dead cells quench Hoechst fluorescence; viable cells are reported to have insignificant PI fluorescence. Other DNA probes that penetrate and stain dead or damaged cells brightly are ethidium monoazide (EMA) and laser dye styryl-751 (LDS-751).

Ethidium monoazide is positively charged and penetrates the membranes of dead or damaged cells but not live cells. EMA intercalates into DNA and can be photochemically cross-linked with short exposure to visible light (Riedy et al., 1991). In contrast, LDS-751 penetrates both damaged and live cells, but labels the DNA of damaged or dead cells much more brightly (Terstappen et al., 1988).

A novel use of 7-AAD for labeling nonviable fixed cells involves addition of a molar excess of actinomycin D (AD) while cells are being fixed (Fetterhoff et al., 1993). It is thought that the higher concentration of AD prevents binding of 7-AAD to viable cells; for the short term, nonviable cells retain bound 7-AAD.

A different approach is based on the penetration of large molecules into dead or damaged cells. Streptavidin-Tricolor (SA-TR; Invitrogen/Life Technologies) irreversibly penetrates nonviable cells, staining them bright red; subsequent washing, fixation, and permeabilization do not result in significant dye loss (Levelt and Eichmann, 1994). The mechanism for this retention is not clear. This technique permits prelabeling damaged cells prior to labeling intracellular antigens.

Perhaps the largest choice of reagents for assessing cell viability with subsequent fixation is the family of amine reactive dyes. There are dyes from several manufacturers that are compatible with most laser lines from violet to red. Weak cell surface staining of viable cells is easily distinguished from the strong intracellular staining of dead cells. Further, the dyes are compatible with standard formaldehyde fixation. For multi-color experiments, there is a kit available to aid in compensation when using LIVE/DEAD Fixable dead cell stains from Invitrogen/Life Technologies. It has been emphasized that the dyes should be titrated for use with adequate numbers of dead cells to clearly determine optimal dye concentration. For additional details, see *UNIT 9.3*.

Finally, the method of mass cytometry can employ a probe-exclusion method based on the drug cisplatin. When exposed briefly to cisplatin, viable cells exclude the drug but dead cells retain enough of the platinum-containing drug to be identified by mass spectroscopy (Feinberg et al., 2012).

Methods for microscopy

In determining the viability of cells, one should not overlook the obvious. Much information can be obtained by direct observation of the cells under a microscope. In many cases, obviously misshapen or bloated cells or cells that have lost refractility in phase contrast indicate severe problems that obviate more sophisticated approaches. In other cases, morphological changes are very useful in following physiological processes. For example, certain morphological changes are hallmarks of apoptosis (Darzynkiewicz et al., 1994). In plant cells, changes in shape are easily detectable, and the loss of metabolically driven processes such as cytoplasmic streaming can indicate the loss of viability (Brigham et al., 1995). Aniline blue stain in lactophenol has also been used for the detection and counting of viable pollen grains (Mudd and Arathi, 2012).

Assessment of cell viability under the microscope can be accomplished with stains that mark nonviable cells or viable cells with active metabolism. Fluorescent probes in common use for flow cytometry can also be used in the microscope. These include FDA (Humphreys et al., 1994), YO-PRO-1 (Idziorek et al., 1995), and dihydroethidine (Bucana et al., 1986). For methods with fresh tissue, see *UNIT 9.39*.

Other dyes include the very common trypan blue (McGahon et al., 1995), nigrosin (Johnson, 1995), and erythrosin B (Bochner et al., 1989). Viable cells have intact membranes and

exclude the dyes. Nonviable cells are labeled and are visible with brightfield optics.

Critical Parameters and Troubleshooting

Positive and negative controls

A variety of alternative methods exist for producing dead cells including heat shock and detergent-based permeabilization. Digitonin and Triton X-100 provide two alternate means of producing control populations for dead cell staining using membrane-impermeable or live cell-accumulating dyes. These compounds may produce artifacts at low concentrations with cells staining at intermediate intensities to live or dead populations (see 10 μ M digitonin in Fig. 9.2.4). Triton X-100, a non-ionic detergent, is a nontoxic and extremely inexpensive option, but may detach cells in culture and degrade cells used for flow cytometry. Triton should be used at no higher than 0.1% (0.05% is sufficient to permeabilize cells, see Figs. 9.2.6 to 9.2.8) and mixing should be performed gently to avoid destroying cells. Digitonin will not degrade or detach cells at concentrations suitable for creating permeabilized controls, but is toxic and expensive.

Using PI or 7-AAD with phycoerythrin (PE)

Although the emission spectrum of PE overlaps the shorter-wavelength end of PI and 7-AAD emission spectra, PE can be used in conjunction with PI or 7-AAD for dead cell discrimination. Using a gate for PI- or 7-AAD-negative cells allows for the examination of PE label on presumed viable cells.

It is important that there be appropriate compensation between PE and PI or 7-AAD detectors, as both fluorochromes will be detected by both detectors (for illustrations of compensation, see Fig. 9.2.2 and comments in Alternate Protocol 2). To get compensation for dual-labeled cells, prepare a tube without PI or 7-AAD. Adjust the compensation of PE into the PI/7-AAD channel such that PE-positive cells are in the range of PI- or 7-AAD-negative or viable cells (see Fig. 9.2.2C).

If expected nonviable cells are not found, demonstrate the efficacy of the dye by heating. Take one tube of cells ready for analysis and heat 10 min in a 45°C water bath. Cool to room temperature and reanalyze. All cells are now nonviable and should be a brighter red.

7-AAD should also be usable with dyes that emit in the longer-wavelength region if they are excited by a second laser that is not colinear with the 488-nm laser. For example, a

helium-neon (HeNe) laser emitting at 633 nm, a krypton (Kr) laser emitting at 647 nm, or a diode laser operating in the same region would not excite 7-AAD. One may also be able to use a HeNe laser that is colinear with a 488-nm beam if the filters in front of the PMT that detects 7-AAD emission exclude the 633-nm laser line.

Probes of physiological state

Cells must be kept under optimal conditions for assays that reflect their physiological state. That is, some cells may survive well in PBS or HBSS, but others may require serum supplementation or other factors to remain healthy.

Because FDA can leak from cells, it is important to analyze immediately after the incubation period. When FDA is used together with cell surface markers labeled with PE, the FDA fluorescence overlaps the PE emission range. Thus, setting proper compensation is crucial. Compensation should be checked with samples labeled with each fluorochrome.

The glycoprotein pump in some cells may pump rhodamine 123 out of live cells (Holló, 1994; Shapiro, 1995). Thus, cells that do not stain brightly may be viable. When in doubt, try an alternate reagent for nonviable cell identification, such as PI or 7-AAD.

Probes for fixed cells

Although EMA emission can be used in conjunction with surface-labeled antibodies, the emission of PE overlaps that of EMA; hence, appropriate compensation is required (see Alternate Protocol 2 for further discussion of compensation). LDS-751 stains all nucleated cells, so all will be positive. Dead or damaged cells are about ten-fold brighter than viable cells. If nonviable cells are present, then two populations should be present; mature red cells will be negative (see Terstappen et al., 1988, for illustrations). Artifacts in cell surface labels have been observed in formaldehyde-fixed cells labeled with LDS-751 (McCarthy et al., 1994). If alteration of the surface labeling pattern is suspected, compare the staining pattern of unfixed, labeled cells stained with LDS-751.

Relative sensitivities of Live/Dead dyes

Cell viability probes each have a unique threshold for determination of viability status (see Fig. 9.2.8). Some dyes, under certain conditions, such as weak permeabilization, may show intermediate staining (see Fig. 9.2.4). These qualities do not detract from the utility of the dyes presented here, but highlight the importance of proper controls and careful

definition of viability in the context of experimental design. Dual staining using viable and nonviable cell probes in combination (see Alternate Protocols 8 and 9) allow for measurement of viability by simultaneous measures. Using multiple probes allows for a greater stringency in determining viable or nonviable populations.

Methods for microscopy

Cells stained with calcein AM and EtHD-1 should generally be imaged soon after staining (within 30 min), as Calcein AM may slowly leak from cells. The Cell Tracker probes are designed for maximum retention by viable cells and are compatible with long-term staining, and cells stained with these dyes can be imaged hours after initial staining. EtHD-1 and the Sytox probes are high-affinity DNA-binding probes, and cells stained with these dyes can be imaged hours after staining, though replacing the dye solution with fresh medium is crucial, as the dyes can slowly leak into and stain living cells if present in solution.

After staining with trypan blue, count cells within 5 min. On sitting, some viable cells may become permeable and take up dye, appearing as false nonviable cells.

Anticipated Results

Probes for membrane integrity. Nonviable cells are stained bright red and viable cells are nonfluorescent. With PI, there should be about a 2-log difference in brightness between viable and nonviable cells; 7-AAD-positive cells are dimmer. Discrimination of viable cells, nonviable cells, and debris can be done easily on a bivariate plot of forward scatter (typically linear scale) versus log PI or 7-AAD fluorescence (see Fig. 9.2.1).

Probes of physiological state. FDA and rhodamine 123 are both excited by 488-nm light and fluoresce green. Filters used for measuring fluorescein (e.g., 530 ± 20-nm band-pass) are adequate to detect fluorescence of either dye. With logarithmic amplification of the fluorescence signal, viable FDA-positive cells are very bright, but viable rhodamine 123-positive cells will be dimmer. A bivariate plot of forward scatter versus rhodamine 123 fluorescence may make it simpler to distinguish rhodamine 123-positive viable cells from nonviable cells (see Fig. 9.2.3B).

Assessment of cell viability by flow cytometry using fixable dyes. Intact cells stain weakly and dead or damaged cells brightly with Live/Dead Far Red or eFluor 506 and fall into distinct populations (see Figs. 9.2.4 and

9.2.5). Cell Tracker Green CMFDA weakly stains permeable cells and brightly stains viable cells (see Fig. 9.2.6).

Probes for fixed cells. Because the fluorescence of EMA is not as bright as that of propidium iodide, discrimination of nonviable, EMA-bright cells from viable cells may be less obvious. A bivariate plot of forward light scatter versus EMA fluorescence may aid in distinguishing viable (EMA-negative) cells. When stained with LDS-751, viable cells can be identified on a bivariate plot of light scatter and red fluorescence as a population of intermediate brightness. Dead or damaged cells stain more brightly, and enucleate red blood cells are unstained.

Methods for microscopy. The trypan blue procedure is very straightforward, but phase-contrast optics can aid in the identification of viable cells that do not stain with trypan blue. Nonviable cells are blue and phase dense; viable cells are phase bright.

Assessment of cell viability by microscopy using fluorescent probes. CMFDA brightly stains viable cells and weakly stains dead or damaged cells (see Fig. 9.2.7). Calcein AM also brightly stains viable cells with weak staining of nonviable cells while EtHD-1 brightly stains nonviable cells but can leak into and weakly stain living cells (see Fig. 9.2.8). Calcein AM and EtHD-1 have slightly non-overlapping staining and a subset of cells may lack or show only weak staining from both dyes. CMAC and Sytox AADvanced both brightly stain their respective targets with CMAC staining viable cells and Sytox staining dead or damaged cells (See Fig. 9.2.9).

Time Considerations

Probes for membrane integrity. Cell staining should take <5 min with PI or 30 min with 7-AAD. Instrument setup (and compensation, if employed) should take 5 min. If data are collected as listmode files, then subsequent analysis may require another 5 to 10 min depending on the complexity of the gating and the degree of automation of the analysis software employed.

Probes of physiological state. Cell staining should take 5 min with rhodamine 123 or 15 min with FDA. Instrument setup (and compensation, if employed) should take 5 min. If data are collected as listmode files, then subsequent analysis may require another 5 to 10 min depending on the complexity of the gating and the degree of automation of the analysis software employed.

Probes for fixed cells. Using fixable dyes, preparation of cell suspensions (not described here) should take 10 min. Subsequent staining should take less than 45 min with either dye set, slightly longer if fixation is employed (up to 1 hr). Instrument setup (and compensation, if employed) should take 5 to 10 min. Data analysis will take another 5 to 10 min depending on the software used.

EMA labeling of cells takes ~10 min to stain and cross-link the dye, followed by ~30 min for washing and fixing. Labeling of cells with LDS-751 takes ~20 min for staining and incubation. Instrument setup (and compensation, if employed) should take 5 min. If data are collected as listmode files, then subsequent analysis may require another 5 to 10 min depending on the complexity of the gating and the degree of automation of the analysis software employed.

Methods for microscopy. Trypan blue staining and counting cells can be done in 5 to 10 min.

Assessment of cell viability by microscopy using fluorescent probes. Cell staining should take less than 45 min with each of the dye sets. Microscope startup and imaging setup will take 5 min and can be performed while cells are staining. Imaging time will vary depending on the number of sample imaged and quality of images desired but will generally be less than 5 min per sample (can be as low as 1 min per sample if depending on image capture method).

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