MitoPT®
TMRE & TMRM
Assay Kits
500 tests

RESEARCH USE ONLY

INTRODUCTION

Mitochondria play a central role in the biochemical processes associated with the life and death stages of eukaryotic cells[1]. Under normal physiological conditions, a membrane-based proton pump generates an electrochemical gradient, enabling the production of ATP to drive cellular energy dependent processes[2]. The oxidation of glucose and fatty acids by enzymes associated with the mitochondrial respiratory chain establishes a proton and pH gradient across the mitochondrial inner membrane, resulting in a transmembrane electrical potential gradient ($\Delta\Psi_m$) of -80 to -120 mV and a pH gradient of 0.5-1.0 pH units[3,4].

Depolarization of the inner mitochondrial membrane can lead to an opening of the mitochondrial permeability transition pore (PTP)[5]. This results in the leakage of intermembrane proteins, including cytochrome c, that facilitate the induction of apoptosis through apoptosome formation[6]. Caspase activation has been shown to accelerate the process of $\Delta\Psi_m$ loss[7]. Moreover, a feedback mechanism that results in the generation of reactive oxygen species (ROS) further accelerates the rate of cell death[7]. Because mitochondrial dysfunction has been closely tied to such neurodegenerative diseases as Alzheimers, Parkinsons, and amyotrophic lateral sclerosis, mitochondria remain an important organelle of study[8].

Loss of mitochondrial $\Delta\Psi_m$, indicative of apoptosis, can easily be detected using slow, lipophilic, cationic fluorescent redistribution dyes such as ICT's MitoPT® reagents: tetramethylrhodamine ethyl ester (TMRE), tetramethylrhodamine methyl ester (TMRM), and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1)[9]. These dyes have a delocalized positive charge dispersed throughout their molecular structure, and yet their lipophilic solubility enables them to be readily membrane permeant and penetrate living cells[9-11]. They redistribute across cell membranes according to the Nernst equation in a voltage-dependent manner[9-11]. Accordingly, they possess a low membrane partition coefficient: a low tendency to non-specifically associate with intracellular organelles and macromolecules.

These excellent potentiometric dyes also exhibit minimal self-quenching, low cytotoxicity, and are reasonably photostable[11]. The MitoPT® dyes exhibit very low toxicity and display rapid and reversible membrane equilibration properties[11].

ICT's MitoPT® TMRE and TMRM assay kits easily distinguish between non-apoptotic cell populations and cell populations that are transitioning into an apoptotic state. Inside a healthy, non-apoptotic cell, the lipophilic TMRE or TMRM dye, bearing a delocalized positive charge, enters the negatively charged mitochondria where it accumulates and fluoresces orange upon excitation[10]. When the mitochondrial $\Delta\Psi_m$ collapses in apoptotic cells, MitoPT® TMRE or TMRM no longer accumulate inside the mitochondria, instead becoming more evenly distributed throughout the cytosol. When dispersed in this manner, overall cellular fluorescence levels drop dramatically. Healthy cells fluoresce orange, whereas cells with depolarized mitochondria exhibit lower levels of orange fluorescence.

MitoPT® kits can be used in conjunction with existing research protocols. Grow cells following the usual cell cultivation protocol. If using an apoptosis induction model system, induce apoptosis according to the existing procedure, reserving a non-induced population of cells as a control. Once apoptosis has been induced or the mitochondrial membrane has been depolarized by a known method, such as using CCCP (included), spike MitoPT® TMRE or TMRM dye solution into each sample and control. Incubate the cells for 20 minutes at 37ºC to allow MitoPT® to equilibrate within the polarized mitochondria. If cells are not undergoing some form of metabolic or apoptotic stress, the mitochondrial $\Delta\Psi_m$ will remain intact, and MitoPT® will accumulate within the slightly negative/alkaline environment of the mitochondria and fluoresce brightly upon excitation. If the cells are apoptotic, the mitochondrial $\Delta\Psi_m$ will break down, causing MitoPT® to disperse throughout the cell cytosol. This results in a dramatic reduction in the fluorescence of the affected mitochondria, and as a result, overall cellular fluorescence is diminished significantly.

PROTOCOL OVERVIEW

ImmunoChemistry Technologies' MitoPT® TMRE and TMRM mitochondrial permeability transition kits make it easy to screen cells with a fluorescence microscope, plate reader, or flow cytometer. The MitoPT® TMRE and TMRM dyes excite optimally at 549 nm and 548 nm, respectively, and exhibit emission maximums at 574 nm and 573 nm, respectively. Each 0.5-1.0 mL sample can be stained at a MitoPT® concentration of 20-200 nM, depending upon analysis method and user requirements for cell brightness. Protocols using MitoPT® TMRE or TMRM at concentrations greater than 50 nM should include a single wash step to minimize background fluorescence.

When viewing MitoPT® TMRE- or TMRM- stained cells under a fluorescence microscope, a 100-200 nM dye concentration is recommended. Non-apoptotic cells will have orange fluorescent spots from the MitoPT® TMRE or TMRM dye accumulating within polarized mitochondria. In contrast, apoptotic and metabolically stressed cells will have fewer bright fluorescent mitochondria and more dim or non-fluorescent mitochondria. The overall brightness of such cells will be visibly reduced as a result of the mitochondrial depolarization event.

When cells stained with MitoPT® TMRE or TMRM are to be analyzed with a fluorescence plate reader, each sample requires a dye concentration of 100-200 nM. The instrument will measure the total amount of orange fluorescence emitted from the cell population in the microtiter plate well. Healthy control cells, bearing mitochondria with normal electrochemical gradients, will concentrate the potentiometric dye to a greater extent than apoptotic cell populations and, therefore, generate higher relative fluorescence unit (RFU) ouputs of orange fluorescence[12-13]. The difference in fluorescence output of these two populations can be easily distinguished in black 96-well plates using filter tandems set to 540±10 nm excitation and 570±10 nm emission.

When a flow cytometer is used to analyze cells stained with MitoPT® TMRE or TMRM, samples can be stained at a concentration of 20-200 nM. The instrument will detect the presence of these potentiometric dyes by measuring orange fluorescence intensity (FL-2) relative to the negative control population[14-17]. Mitochondria in apoptotic cells have a reduced $\Delta\Psi_m$ that results in lower levels of the potentiometric dye within these organelles; such cells will exhibit reduced or no fluorescence. TMRE and TMRM have been used concurrently with other fluorophores in multi-parametric analyses to measure mitochondrial depolarization, caspase activation, phosphatidyl serine exposure, and/or cell viability within a single cell population[14, 18-19].

MitoPT® is for research use only. Not for use in diagnostic procedures.

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To learn more about all of ICT's products, please visit www.immunochemistry.com or call 1-800-829-3194.

KIT CONTENTS

MitoPT® TMRE Assay Kit, catalog #9103:

- 1 vial MitoPT® TMRE reagent (500 tests) #6254
- 2 bottles 10X Assay Buffer (125 mL) #6259
- 1 vial CCCP, 50 mM (600 µL) #6258
- Manual

MitoPT® TMRM Assay Kit, catalog #9105:

- 1 vial MitoPT® TMRM reagent (500 tests) #6256
- 2 bottles 10X Assay Buffer (125 mL) #6259
- 1 vial CCCP, 50 mM (600 µL) #6258
- Manual

STORAGE

Store the kit at ≤-20°C. Once the kit is opened, the 10X Assay Buffer may be stored at 2-8°C until the expiration date. CCCP, MitoPT® TMRE, and MitoPT® TMRM reagents should be stored frozen. Once reconstituted with DMSO, dilute and use MitoPT® immediately, or store at ≤-20°C for 12 months protected from light and thawed no more than twice.

MSDS

MSDS are available at www.immunochemistry.com.

RECOMMENDED MATERIALS

- DMSO, ~100 µL
- DiH_2O, 2.25 L to dilute assay buffer
- Cultured cells or tissues treated with the experimental conditions, ready to be labeled
- Reagents to create controls by inducing metabolic stress, mitochondrial depolarization, or apoptosis
- Hemocytometer
- Centrifuge at <300g
- 15 mL polypropylene centrifuge tubes (1 per sample). Do not use polystyrene; TMRE and TMRM bind significantly to this form of plastic.
- Black round or flat bottom 96-well microtiter plates
- Microscope slides
- FACS tubes

DETECTION EQUIPMENT

- Fluorescence microscope with a 540-550 nm excitation filter and >575 nm (long pass) filter tandem
- Fluorescence plate reader with 540-550 nm excitation and 570 ± 10 nm emission filters with endpoint reading
- Flow cytometer with excitation laser at 488 nm and emission filter at 570 ± 10 nm (FL-2)

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PROTOCOLS

1. Experimental Preparation & Controls

MitoPT® TMRE and TMRM mitochondrial membrane potential assay kits are compatible with other apoptosis or mitochondrial assessment protocols. Because MitoPT® detects mitochondrial membrane depolarization, plan the experiment so that it will be diluted and administered at the time when this event is expected to occur in the cells. The recommended staining concentration of MitoPT® is 100-200 nM, but the amount may vary based on the experimental conditions and cell type. An initial experiment may be necessary to determine when and how much MitoPT® to use. It is highly recommended that 2 sets of controls be run:

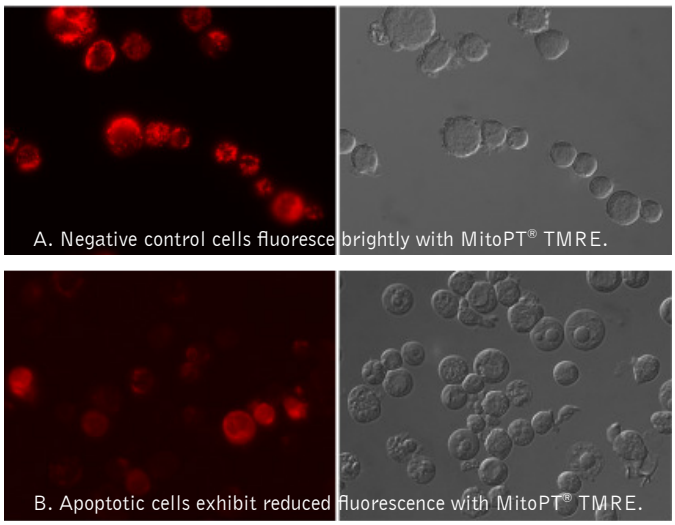
- A negative population of cells that were not exposed to the experimental conditions.
- A positive control population that was induced to undergo mitochondrial depolarization or apoptosis, such as:
  - A positive control exhibiting a reduced mitochondrial potential can be created using the CCCP included in the kit (Section 2).
  - An apoptotic positive control can be created by adding 2 μg/mL camptothecin (catalog #6210) or 1 μM staurosporine (catalog #6212) to Jurkat leukemic T-cells for 3-4 hours at 37°C (Figure 1).

2. Mitochondrial Depolarization with CCCP

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is a reversible proton gradient uncoupling agent that quickly reduces the electrochemical potential across the inner mitochondrial membrane, resulting in a rapid intracellular mitochondrial depolarization event[20-21]. CCCP can be used to create a positive control cell population containing depolarized mitochondria. ICT's CCCP reagent (#6257) is a liquid stock at 50 mM in DMSO that should be stored ≤-20°C. Use gloves when handling.

1. Determine when the mitochondrial depolarization analysis will be run. Approximately 75 minutes prior to analysis, generate positive and negative mitochondrial depolarization controls using CCCP and DMSO.
2. Gently warm CCCP to RT; mix or lightly vortex.
3. Spike with CCCP to obtain a 5–50 μM CCCP concentration in the cell culture media. The CCCP stock is at 50 mM; simply spike 1 μL CCCP stock per 1 mL cell suspension/overlay media. If using a lower concentration of CCCP, first dilute it with tissue culture grade DMSO.
4. Prepare a negative control population by spiking with the same volume of tissue culture grade DMSO as was used to spike the CCCP population.
5. Incubate the positive and negative control cell populations for 30–60 minutes at 37°C in a CO_2 incubator to allow time for the depolarization process to occur.
6. Follow the staining protocol. If cells are washed, read the controls immediately; the mitochondria may revert to a polarized state once the CCCP is removed.

FIGURE 1: MICROSCOPY ANALYSIS OF APOPTOTIC SUSPENSION CELLS



Jurkat cells were treated with 1 µM staurosporine for 2 hours to induce apoptosis, or with DMSO as the negative control. Cells were stained with MitoPT® TMRE at 150 nM for 20 minutes at 37°C, then washed. Cells were photographed using a Nikon Eclipse E800 photomicroscope equipped with differential interference contrast (DIC) phase, and fluorescence optics using a green excitation filter at 510-560 nm in tandem with a 570-620 nm emission filter.

Normal healthy cells, containing mitochondria with polarized inner membranes, concentrate MitoPT® TMRE and fluoresce bright orange (A). Apoptotic cells, bearing depolarized mitochondria, exhibit a reduced orange fluorescence relative to the healthy cell population (B). Depolarized mitochondria will no longer concentrate MitoPT® TMRE, leading to a dramatic reduction in fluorescence intensity. Each fluorescence photo is accompanied by a corresponding DIC image to visualize apoptotic cells with reduced fluorescence (Dr. Brian Lee, ICT).

3. Preparation of 1X Assay Buffer

1. 10X Assay Buffer may form precipitates during cold storage. If this happens, gently warm it until all crystals have dissolved. Do not boil.
2. Dilute 10X Assay Buffer 1:10 in diH_2O. For example, add 60 mL 10X Assay Buffer to 540 mL diH_2O for a total of 600 mL. 1X Assay Buffer may be stored at 2-8°C and used within 1 week, or it may be frozen and used within 12 months.

4. Preparation of MitoPT®

MitoPT® is a lyophilized powder that may be slightly visible as an iridescent or faint red sheen inside the vial. The reconstituted stock should be diluted immediately or frozen for future use. Protect from light and use gloves when handling.

1. Reconstitute MitoPT® with 100 µL DMSO to form a 1 mM stock solution. The stock solution should be slightly red in color. Once reconstituted, it may be stored at ≤-20°C for 12 months. Protect the stock from light and thaw no more than twice during that time.
2. Create the 10 µM MitoPT® working solution by diluting the stock solution 1:100 (e.g., 20 µL into 1980 µL buffer). 1X Assay Buffer, cell culture media, or PBS may be used to dilute the stock. Protect from light. Discard any unused MitoPT® working solution.

5. Microscopy Staining Protocol for Suspension Cells

Suspension cells can readily be evaluated using fluorescence microscopy. An optional wash step may be necessary after staining with MitoPT® (Step 5).

1. Prepare experimental and control cell populations. Cell concentration should be at least 5 X 10^5 cells/mL but should not exceed 10^6 cells/mL; cells cultivated in excess of this concentration may become overcrowded and transition into apoptosis. Expose cells to the experimental conditions.
2. Transfer 0.5-1 mL cells into fresh tubes. The volume of cells and amount of MitoPT® should be adjusted to accommodate each particular cell line and research conditions.
3. Add an appropriate volume of MitoPT® working solution (Section 4) to achieve the final staining concentration and mix by gently flicking the tubes. A 100-200 nM staining concentration is recommended. To achieve a 200 nM staining concentration, add the 10 µM MitoPT® working solution at 1:50. For example, add 20 µL to a 980 µL sample.
4. Incubate 15-20 minutes at 37°C, protected from light.
5. If the staining concentration is <50 nM, a wash step is optional as the background fluorescence from the free dye is low enough for good resolution of the cells. If staining at >50 nM, wash the cells to remove any free dye in the supernatant that may interfere with the analysis:
   a. Centrifuge at <300 X g for 5 minutes at RT to pellet the cells.
   b. Carefully remove and discard supernatant.
   c. Add 1X Assay Buffer (Section 3) and gently mix to resuspend cells. Note: If samples cannot be analyzed immediately, add >0.1% BSA to the assay buffer, or use cell culture media or Dulbecco's PBS to store the samples. Protect from light.
6. To easily observe multiple cells in a single viewing field, concentrate cells to >2 x 10^6 cells/mL in 1X Assay Buffer.
7. Place 50-100 µL on a clean microscope slide and add a cover slip.
8. Observe cells under a fluorescence microscope with excitation in the green wavelength of 510-540 nm and emission in the orange wavelength of 570-620 nm (Figure 1).

6. Microscopy Staining Protocol for Adherent Cells

Adherent cell monolayers can readily be evaluated using fluorescence microscopy. An optional wash step may be necessary after staining with MitoPT® (Step 4).

1. Prepare experimental and control cell populations by culturing cells on a sterile coverslip or chamber slide. Cells should not exceed the threshold where spontaneous apoptosis or cell sloughing occurs. Expose cells to the experimental or control conditions.
2. Spike the cell culture supernatant overlaying the adherent cell monolayer with the appropriate volume of 10 µM MitoPT® working solution (Section 4) to achieve the desired final staining concentration. To yield a 200 nM staining concentration, add the 10 µM MitoPT® working solution at 1:50. For example, add 10 µL to a 490 µL sample.
3. Incubate 15-20 minutes at 37°C and protect from light.
4. If the MitoPT® staining concentration is <50 nM, a wash step is optional as the background fluorescence from the free dye is low enough for good resolution of the cells. If staining at >50 nM, wash the cells to remove any free dye in the supernatant that may interfere with the analysis:
   a. Gently remove the cell culture supernatant.
   b. Add enough 1X Assay Buffer (Section 3) to cover the cell surface and dilute any remaining free MitoPT® dye.
   c. Incubate ~10 minutes at 37°C, protected from light.
   d. Remove the 1X Assay Buffer and replace with enough fresh 1X Assay Buffer to cover the cell surface. Note: If samples cannot be analyzed immediately, add >0.1% BSA to the assay buffer, or use cell culture media or Dulbecco's PBS to store the samples. Protect from light.
5. Cover with a cover slip.
6. Observe cells under a fluorescence microscope with excitation in the green wavelength of 510-540 nm and emission in the orange wavelength of 570-620 nm (Figure 1).

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7. 96-Well Fluorescence Spectroscopy Staining Protocol

The loss of the electrochemical gradient across the inner membrane of depolarized mitochondria ($\Delta\Psi_m$) is easily detected and measured by most fluorescent plate readers by comparing the average 574 nm signal in stimulated versus non-stimulated samples. As fluorescence plate reader spectroscopy is generally less sensitive than flow cytometry (Section 8), a higher concentration of cells is recommended. For best results, cell populations should be in excess of 3 x 10^5 cells/well, which corresponds to a spiked and washed cell suspension of >3 x 10^6 cells/mL in 100 µL/well aliquots. MitoPT® TMRE or TMRM should be used at a staining concentration of 100-200 nM (Step 4).

1. Prepare experimental and control cell populations. Cell concentration should be at least 5 X 10^5 cells/mL but should not exceed 10^6 cells/mL; cells cultivated in excess of this concentration may become overcrowded and transition into apoptosis. Expose cells to the experimental conditions.
2. Remove a small aliquot and determine the cell density of each population. Concentrate cells to >3 x 10^6 cells/mL:
   a. Centrifuge at <300 x g for 5-8 minutes.
   b. Remove the supernatant.
   c. Add enough cell culture media to achieve the >3 x 10^6 cells/mL target concentration. Gently vortex to resuspend cells.
3. Place 0.5-1 mL per sample into fresh 15 mL polypropylene centrifuge tubes.
4. Add an appropriate volume of 10 µM MitoPT® working solution (Section 4) to achieve a final staining concentration of 100-200 nM. Mix gently by flicking the tubes. To yield a 200 nM final staining concentration, add the 10 µM MitoPT® working solution at 1:50. For example, add 20 µL to a 980 µL sample.
5. Incubate 15-20 minutes at 37°C, protected from light.
6. Wash cells to remove any free dye in the supernatant that may interfere with the analysis:
   a. Centrifuge at <300 x g for 5 minutes to pellet the cells.
   b. Carefully remove and discard supernatants.
   c. Add 1 mL 1X Assay Buffer (Section 3) per sample and gently mix to resuspend cells. Protect from light until analysis.
7. Pipette 100-200 µL of each sample (in triplicate) into a black round or flat-bottom 96-well microtiter plate. Do not use clear or white plates, as this will diminish sensitivity and increase background noise.
8. Analyze with a fluorescence plate reader set to perform an end-point read with excitation at 540 nm and emission in the orange wavelength at 574 nm. Use a 570 nm emission cut-off filter to reduce any plate noise from the excitation signal input (Figure 2).

8. Flow Cytometry Protocol for Single-Color Analysis

MitoPT® can readily be used to evaluate suspension cells by flow cytometry. MitoPT® TMRE excites optimally at 549 nm, and MitoPT® TMRM excites optimally at 548 nm; both yield excellent results using the common argon blue laser at 488 nm for excitation. Optimal emissions lie in the FL-2 region: 574 nm for TMRE and 573 nm for TMRM. Orange emission filters (570 nm ± 10 nm) detect the presence of these potentiometric dyes.

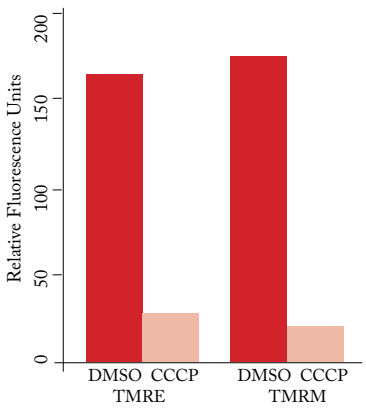
Excellent resolution of depolarized versus charged mitochondria is achieved in flow cytometry using MitoPT® at 100-200 nM in 0.5–1 mL cell samples with a single wash step. If MitoPT® is used at <50 nM, no wash step is necessary.

1. Follow Section 5 (Steps 1-5) to yield a final sample volume of 0.5-1 mL.
2. Run an unstained cell sample as an autofluorescence control and generate a FSC (forward scatter) versus SSC (side scatter) plot. Adjust detector settings so that cells of interest are displayed on scale and can be gated as desired.
3. While gating on the cell population of interest, adjust FL-2 detector settings so that autofluorescence background is roughly within the first decade of the log scale on the fluorescence intensity histogram.
4. Run the brightly fluorescent negative control sample. Generate a histogram with log FL-2 on the X-axis versus the number of cells on the Y-axis. On the histogram, there will appear two cell populations represented by two peaks (Figure 3, orange peaks). The majority of the stained negative control cells should lie within the higher log fluorescence output decades of the FL-2 (X-axis), whereas the depolarized cell population will appear as a separate peak or as a shoulder of the larger peak, showing decreased fluorescence intensity in the lower log output decades. If possible, adjust the FL-2 PMT voltage to allow the peak of the fluorescent negative control to fall within the third log decade.

FIGURE 2: FLUORESCENCE PLATE READER ANALYSIS

Jurkat cells were exposed to DMSO (dark orange bars) or 50 µM CCCP depolarizing agent (light orange bars) for 15 minutes at 37°C. Samples were subsequently incubated with either MitoPT® TMRE or MitoPT® TMRM for 20 minutes at 37°C and washed once. 100 µL aliquots were analyzed in triplicate in a black 96-well plate using a Molecular Devices Gemini XS fluorescence plate reader set at 550 nm excitation and 580 nm emission using a 570 nm cut-off filter.

The amount of orange fluorescence, indicating cells with polarized mitochondria, was measured by the plate reader. The DMSO population exhibited a high level of orange fluorescence; metabolically stressed cells in the CCCP-stimulated samples exhibited a reduced level of orange fluorescence. When the mitochondrial membrane potential gradient collapses, TMRE or TMRM equilibrates out of the mitochondria and into the cytosol, causing cells to exhibit a loss of orange fluorescence (Ms. Tracy Hanson, ICT).



5. Run the depolarized positive control sample using the same adjusted PMT voltage as determined for the negative control. The histogram peak should still be observable on the X-axis (Figure 3, white peak). If not, increase PMT voltage slightly to achieve positive control staining that falls at least as bright as the first decade of the log scale.
6. Observe the mean fluorescence intensity of all controls and samples at the adjusted settings.

9. Flow Cytometry Protocol for Multi-Color Analysis

MitoPT® TMRE and TMRM can be readily combined with other fluorochromes to measure multiple parameters in a single cell population. MitoPT® TMRE and TMRM emissions are monitored on a log FL-2 axis; therefore, in a multi-color analysis, the other axis can be used to detect a different fluorochrome, e.g., FL-1 for fluorescein-labeled probes (Figure 4), FL-3 for 7-AAD live/dead stain (catalog #6163), or FL-4 for FLICA® 660 caspase assays.

1. Follow Section 5 (Steps 1-5) to yield a final sample volume of 0.5-1 mL. Stain with the second fluorochrome according to your specific protocol. Be sure to create single-stain controls for each fluorochrome.
2. Follow Section 8 (Steps 2-5) using the unstained and single-stain controls to adjust settings for FL-2 and for the particular detector corresponding to the second fluorochrome.
3. Create a 2-color dot plot (Figure 4). Set compensation levels and quadrant staining gates based on the positive and negative MitoPT® controls and the appropriate controls for the second fluorescent reagent.
4. Observe the mean fluorescence intensity of all controls and samples at the adjusted settings.

FIGURE 3: FLOW CYTOMETRY ANALYSIS

Jurkat cells were treated with 1 µM staurosporine as an apoptosis-inducing agent (left, white histogram) or DMSO as a negative control (right, orange histogram) for 3 hours at 37°C, then stained with 30 nM MitoPT® TMRM for 15-20 minutes. Cells were analyzed with a BD FACSCalibur™ flow cytometer.

Apoptotic cells (left), bearing depolarized mitochondria, exhibit significantly less orange fluorescence intensity compared to negative control cells (right). Cells in the negative control group fluoresce bright orange (right); such cells contain mitochondria with polarized inner membranes, in which the MitoPT® TMRM is concentrated (Ms. Tracy Hanson, ICT).

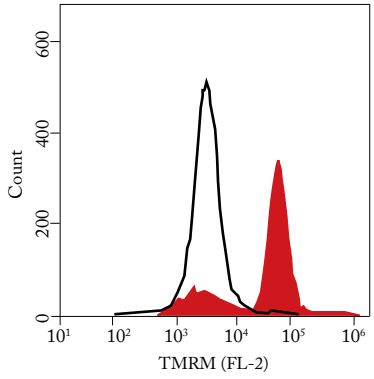
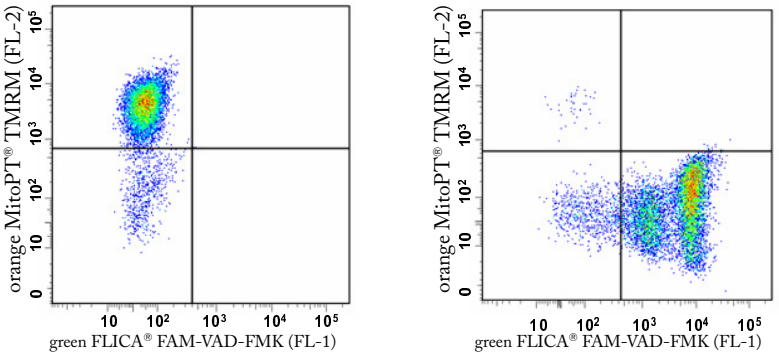


FIGURE 4: MULTI-COLOR FLOW CYTOMETRY ANALYSIS

As the apoptotic cascade is initiated, caspase activation and mitochondrial depolarization become important factors in the cell death process. To monitor this process, Jurkat cells were dually stained with orange MitoPT® TMRM to measure mitochondrial depolarization and green FLICA® FAM-VAD-FMK poly caspase probe (catalog #92) to measure caspase activity in the same cell population. Jurkat cells were treated with DMSO as a negative control (A) or staurosporine to induce apoptosis (B). Cells were then incubated with FLICA® and MitoPT® TMRM. Following a single wash step, cells were analyzed on a flow cytometer.

Mitochondrial depolarization is evidenced by the drop in orange fluorescence from MitoPT® TMRM (FL-2). In the staurosporine-treated population (B), there is a concurrent increase in cell-bound green fluorescence (FL-1) that is associated with the covalent attachment of the FLICA® probe to activated poly caspase enzymes inside the cell. Healthy, unaffected cells were evident in the upper quandrant of the negative control population (A). Apoptotic cells were found in the lower right quadrants (B). Treatment with staurosporine induced apoptosis in about 90% of cells (Ms. Tracy Hanson, ICT).



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