1. INTRODUCTION

Mitochondria play a central role in the biochemical processes associated with the life and death stages of eukaryotic cells\(^\text{1}\). Under normal physiological conditions, a membrane-based proton pump generates an electrochemical gradient, enabling the production of ATP to drive cellular energy dependent processes\(^\text{2}\). 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 \((\Delta \Phi_m)\) of -80 to -120 mV and a pH gradient of 0.5-1.0 pH units\(^\text{3, 4}\).

Depolarization of the inner mitochondrial membrane can lead to an opening of the mitochondrial permeability transition pore (PTP)\(^\text{5}\). This results in the leakage of intermembrane proteins, including cytochrome c, that facilitate the induction of apoptosis through apoptosome formation\(^\text{1}\). Caspase activation has been shown to accelerate the process of \(\Delta \Phi_m\) loss\(^\text{6}\). Moreover, a feedback mechanism that results in the generation of reactive oxygen species (ROS) further accelerates the rate of cell death\(^\text{7}\). Because mitochondrial dysfunction has been closely tied to such neurodegenerative diseases as Alzheimer’s, Parkinson’s, and amyotrophic lateral sclerosis, mitochondria remain an important organelle of study\(^\text{8}\).

Loss of mitochondrial \(\Delta \Phi_m\), indicative of apoptosis, can easily be detected using lipophilic, cationic fluorescent redistribution dyes such as ICT’s MitoPT\(^\text{\textregistered}\) reagents: tetramethylrhodamine ethyl ester (TMRE), tetramethylrhodamine methyl ester (TMRM), and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1)\(^\text{9}\). 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\(^\text{9-11}\). They redistribute across cell membranes according to the Nernst equation in a voltage-dependent manner\(^\text{9-11}\). 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\(^\text{11}\). The MitoPT dyes exhibit very low toxicity and display rapid and reversible membrane equilibration properties\(^\text{11}\).

ICT’s TMRE and TMRM assay kits easily distinguish between healthy, 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\(^\text{10}\). When the mitochondrial \(\Delta \Phi_m\) collapses in apoptotic cells, TMRE or TMRM no longer accumulates 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.

TMRE and TMRM 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 dye solution into each sample and control. Incubate the cells for 15-30 minutes at 37°C to allow MitoPT to equilibrate within the polarized mitochondria. If cells are not undergoing some form of metabolic or apoptotic stress, the mitochondrial \(\Delta \Phi_m\) 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 \(\Delta \Phi_m\) 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.

MitoPT\(^\text{\textregistered}\) is for research use only. Not for use in diagnostic procedures.

Learn more about all of ICT’s products at [www.immunochemistry.com](http://www.immunochemistry.com) or call 1-800-829-3194.
2. PROTOCOL OVERVIEW

ImmunoChemistry Technologies’ MitoPT TMRE and TMRM mitochondrial membrane potential assay kits make it easy to screen cells with a fluorescence microscope, plate reader, or flow cytometer.

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 the 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) outputs of orange fluorescence. 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. Mitochondria in apoptotic cells have a reduced ΔΨm that results in lower levels of the potentiometric dye within these organelles; such cells will exhibit reduced fluorescence compared to cells with healthy, polarized mitochondria. TMRE and TMRM have been used concurrently with other fluorophores in multi-parametric analyses to measure mitochondrial depolarization, caspase activation, phosphatidylserine exposure, and/or cell viability within a single cell population.

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, washed, and 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 cell morphology (Dr. Brian Lee, ICT).
3. KIT CONTENTS

**MitoPT TMRE Assay, catalog #9103:**
- 1 vial MitoPT TMRE Reagent (500 Tests) #6254
- 2 bottles 10X Assay Buffer (2 x 125 mL) #6259
- 1 vial CCCP, 50 mM (600 µL) #6258

**MitoPT TMRM Assay, catalog #9105:**
- 1 vial MitoPT TMRM Reagent (500 Tests) #6256
- 2 bottles 10X Assay Buffer (2 x 125 mL) #6259
- 1 vial CCCP, 50 mM (600 µL) #6258

4. 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 up to 12 months protected from light and thawed no more than twice.

5. SAFETY DATA SHEETS (SDS)

Safety data sheets are available online at www.immunochemistry.com or by calling 1-800-829-3194 or 952-888-8788.

6. RECOMMENDED MATERIALS

- DMSO, 100 µL to reconstitute MitoPT
- DiH2O, 2.25 L to dilute 10X 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 200 x g
- 15 mL polycarbonate 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

FOR RESEARCH USE ONLY.
Not for use in diagnostic procedures.

7. 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
- Flow cytometer with excitation laser at 488 nm and emission filter at 570 ± 10 nm (FL-2)

8. 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:

1. A negative population of cells that were not exposed to the experimental conditions.
2. A positive control population that was induced to undergo mitochondrial depolarization or apoptosis, such as:
   - An apoptotic positive control can be created by adding 2 µg/mL camptothecin (catalog #6210) or 1 µM staurosporine (catalog #6212) to cells for >3-4 hours at 37°C.
   - A positive control exhibiting a reduced mitochondrial potential can be created using the CCCP included in the kit (Section 9).

9. 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 is a liquid stock at 50 mM in DMSO that should be stored ≤-20°C. Use gloves when handling.

- Warning: CCCP contains DMSO, which is a combustible liquid, causes skin irritation, and causes serious eye irritation. In case of exposure, immediately flush eyes or skin with water. See SDS for further information.

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, respectively.
2. Gently warm CCCP to RT; mix or lightly vortex.
1. Prepare experimental and control cell populations. Cell culture media, or PBS may be used to dilute the stock. Protect the stock from light and thaw no more than twice during the time that may interfere with the analysis.

a. Centrifuge at 200 x g for 10 minutes at RT to pellet the cells.

b. Carefully remove and discard supernatants.

c. Add 1X Assay Buffer (Section 10) 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 medium 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 10⁶ 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).

13. MICROSCOPY ANALYSIS OF 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 medium overlaying the adherent cell monolayer with the appropriate volume of 10 µM MitoPT working solution (Section 11) 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 20 µL to a 980 µL sample.

12. MICROSCOPY ANALYSIS OF 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 10⁶ cells/mL but should not exceed 10⁶ 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 11) 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 15-30 minutes at 37°C, protected from light.

5. 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. Centrifuge at 200 x g for 10 minutes at RT to pellet the cells.

b. Carefully remove and discard supernatants.

c. Add 1X Assay Buffer (Section 10) 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 medium 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 10⁶ 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).

12. MICROSCOPY ANALYSIS OF 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 10⁶ cells/mL but should not exceed 10⁶ 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 11) 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 15-30 minutes at 37°C, protected from light.

5. 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. Centrifuge at 200 x g for 10 minutes at RT to pellet the cells.

b. Carefully remove and discard supernatants.

c. Add 1X Assay Buffer (Section 10) 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 medium 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 10⁶ 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).
3. Incubate 15-30 minutes at 37°C, protected 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 10) 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 medium 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).

14. FLUORESCENCE PLATE READER ANALYSIS

The loss of the electrochemical potential gradient across the inner membranes of depolarized mitochondria (ΔΨm) is easily detected by most fluorescence 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 (Sections 15 & 16), a higher concentration of cells is recommended. For best results, cell populations should be in excess of 3 x 10⁵ cells/well, which corresponds to a spiked and washed cell suspension of >3 x 10⁶ 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 10⁵ cells/mL but should not exceed 10⁶ cells/mL; cells cultivated in excess of this concentration may become overcrowded and naturally transition into apoptosis.

2. Remove a small aliquot and determine the cell density of each population. Concentrate cells to >3 x 10⁶ cells/mL:
   a. Centrifuge at 200 x g for 10 minutes at RT to pellet the cells.
   b. Carefully remove and discard supernatants.
   c. Add enough cell culture medium to achieve the >3 x 10⁶ 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 11) 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 15-30 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 200 x g for 10 minutes at RT to pellet the cells.
   b. Carefully remove and discard supernatants.
   c. Add 1 mL 1X Assay Buffer (Section 10) 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. If possible, use a 570 nm emission cut-off filter to reduce any plate noise from the excitation signal input (Figure 2).

15. FLOW CYTOMETRY ANALYSIS: SINGLE-COLOR

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.

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. Prepare experimental and control cell populations. Cells should not exceed the threshold where spontaneous apoptosis occurs.

2. Spike samples with the appropriate volume of 10 µM MitoPT working solution (Section 11) 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 15-30 minutes at 37°C, protected 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. Centrifuge at 200 x g for 10 minutes at RT to pellet the cells.
   b. Carefully remove and discard supernatants.
   c. Add 0.5-1 mL 1X Assay Buffer (Section 10) per sample and gently mix to resuspend cells. Note: If samples cannot be
15. FLOW CYTOMETRY ANALYSIS: MULTI-COLOR

MitoPT® TMRE & TMRM Assays

analyzed immediately, add >0.1% BSA to the assay buffer, or use cell culture medium or Dulbecco’s PBS to store the samples. Protect from light until analysis.

5. 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 the cells of interest are displayed on scale and can be gated as desired.

6. 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.

7. 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.

8. 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.

9. Observe the mean fluorescence intensity of all controls and samples at the adjusted settings.

16. REFERENCES


FOR RESEARCH USE ONLY.
Not for use in diagnostic procedures.
FIGURE 2: FLUORESCENCE PLATE READER ANALYSIS

Jurkat cells were exposed to DMSO as the negative control (left, dark orange bars) or 50 µM CCCP depolarizing agent (right, light orange bars) for 15 minutes at 37°C. Samples were subsequently incubated with MitoPT TMRE or TMRM for 20 minutes at 37°C and washed. Aliquots (100 µL) 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 was measured by the plate reader. Healthy cells in the DMSO control populations exhibited a high level of orange fluorescence; metabolically stressed cells in the CCCP-stimulated samples exhibited a reduced level of orange fluorescence after the mitochondria became depolarized. As the membrane potential gradient collapses, TMRE and TMRM equilibrate out of the mitochondria and into the cytosol, causing cells to lose their orange fluorescence (Ms. Tracy Hanson, ICT).

FIGURE 3: FLOW CYTOMETRY ANALYSIS

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

Apoptotic cells (left), bearing depolarized mitochondria, exhibit significantly less orange fluorescence intensity compared to healthy, negative control cells (right). Cells in the negative control population contain mitochondria with polarized inner membranes; these cells concentrate MitoPT TMRE and fluoresce bright orange. (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 the green FLICA® poly caspase probe FAM-VAD-FMK (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). Samples were then incubated with FLICA and MitoPT, washed, and cells were analyzed on a flow cytometer.

Mitochondrial depolarization is evidenced by the decrease 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 left quadrant of the negative control population (A). Apoptotic cells were found in the lower right quadrant (B). Treatment with staurosporine induced apoptosis in about 90% of cells (Ms. Tracy Hanson, ICT).
11. Rasola, A. & Geuna, M. A flow cytometry assay simultaneously
detects independent apoptotic parameters. Cytometry 45,
12. Wong, A. & Cortopassi, G. A. High-throughput measurement of
mitochondrial membrane potential in a neural cell line using a
fluorescence plate reader. Biochem Biophys Res Commun 298,
750-754 (2002).
13. Toescu, E. C. & Verkhratsky, A. Assessment of mitochondrial po-
larization status in living cells based on analysis of the spatial
heterogeneity of rhodamine 123 fluorescence staining. Pflugers
analysis of HA14-1-induced apoptosis in follicular lymphoma
monitoring of mitochondrial depolarisation: from fluorescence
intensities to millivolts. J Photochem Photobiol B 78, 99-108,
16. Huang, S. G. Development of a high throughput screening
assay for mitochondrial membrane potential in living cells.
J Biomol Screen 7, 383-389, doi:10.1089/108705702320351240
(2002).
17. Russell, J. W. et al. High glucose-induced oxidative stress and
mitochondrial dysfunction in neurons. FASEB J 16, 1738-1748,
18. Jayaraman, S. A novel method for the detection of viable hu-
man pancreatic beta cells by flow cytometry using
fluorophores that selectively detect labile zinc, mitochondrial
membrane potential and protein thiols. Cytometry A 73,
19. Jayaraman, S. Flow cytometric determination of mitochondrial
membrane potential changes during apoptosis of T lympho-
cytic and pancreatic beta cell lines: comparison of tetrameth-
yrhodamine ethylester (TMRE), chloromethyl-X-rosamine
(H2-CMX-Ros) and MitoTracker Red 580 (MTR580). J Immunol
20. Kasianowicz, J., Benz, R. & McLaughlin, S. The kinetic mecha-
nism by which CCCP (carbonyl cyanide m-chlorophenylhydra-
zeine) transports protons across membranes. J Membr Biol 82,
21. Lim, M. L., Minamikawa, T. & Nagley, P. The protonophore
CCCP induces mitochondrial permeability transition without
cytochrome c release in human osteosarcoma cells. FEBS Lett