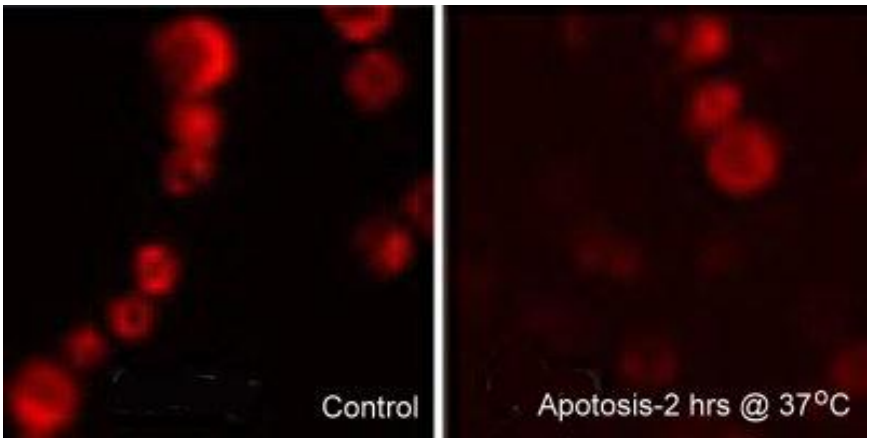
NEUROMICS

MitoPT™ TMRE 100 Test Kit-KF17358-100
MitoPT™ TMRE 500 Test Kit-KF17358-500
MitoPT™ TMRM 100 Test Kit- KF17359-100
MitoPT™ TMRM 500 Test Kit-KF17359-500

MitoPT ΔΨm

TMRE/TMRM Mitochondrial Membrane Potential Assessment Kit



non-apoptotic cells - high fluorescence
apoptotic cells - low or reduced fluorescence

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1. 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 for use in driving the cells' 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 (ΔΨm) of -80 to -120 mV (3), and a pH gradient of 0.5-1.0 pH units (4).

Depolarization of the ΔΨm leads to an opening of the mitochondrial permeability transition pore (PTP) (5). This leads to 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 ΔΨm 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 disease as Alzheimers, Parkinsons, and Amyotrophic Lateral Sclerosis to name a few, it remains an important organelle of study (8).

Loss of mitochondrial ΔΨm, indicative of apoptosis, can easily be detected using slow, lipophilic, cationic fluorescent redistribution dyes such as tetramethylrhodamine ethyl (TMRE) and methyl (TMRM) esters 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 allows them to be readily membrane permeant and penetrate living cells (9-11). The dyes redistribute across cell membranes according to the Nernst equation in a voltage dependent manner (9-11). Accordingly, they possess a low membrane partition coefficient; meaning a low tendency to non-specifically associate with intra-cellular organelles and macromolecules. These excellent potentiometric dyes also exhibit minimal self quenching, low cytotoxicity, and are reasonably photostable (11).

MitoPT™ TMRE/TMRM ΔΨm depolarization detection kits are easy to use for screening cells by flow cytometry, fluorescence microscopy, and fluorescence plate reader assay formats. 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 in an inner-membrane potentialdependent manner (10). The MitoPT potentiometric dyes exhibit very low toxicity and display rapid and reversible membrane equilibration properties (11). When the mitochondrial ΔΨm collapses in apoptotic cells, the MitoPT TMRE and TMRM potentiometric dyes no longer accumulate inside the mitochondria and become more evenly distributed throughout the cytosol. When dispersed in this manner, overall cellular fluorescence levels drop dramatically and this event can easily be visualized by fluorescence microscopy or quantitated by flow cytometry or fluorescence plate reader analysis techniques. The MitoPT ΔΨm depolarization detection kit easily distinguishes between healthy/non-apoptotic cell populations, and those cell populations that are transitioning into an apoptotic state.

The MitoPT TMRE or TMRM kit can be used in conjunction with your existing research protocols. Grow your cells using your usual cell cultivation protocol. If using an apoptosis induction model system, simply induce apoptosis according to your existing procedure (reserving a non-induced population of cells as a control). Once you have induced apoptosis in your cells, spike enough MitoPT dye solution into each test sample, plus your positive and negative control samples, to give a 20-200 nM dye concentration. Incubate the cells for 20 minutes at 37º C to allow the MitoPT potentiometric dyes to equilibrate

within the polarized mitochondria. If the cells are not undergoing some form of metabolic or apoptotic stress, the mitochondrial ΔΨm will remain intact, resulting in the concentration of the MitoPT dye within the
negative/alkaline environment of the mitochondria. If the cells are apoptotic, the mitochondrial ΔΨm will be breaking down, causing the MitoPT dye to be dispersed throughout the cell cytosol. This MitoPT dye dispersion results in a dramatic reduction in the overall fluorescence of the affected mitochondria in particular, and the entire cell in general.

MitoPT TMRE or TMRM ΔΨm Depolarization Detection Kits can evaluate apoptosis using flow cytometry, fluorescence microscopy, or a fluorometric plate reader. Following the flow cytometer and fluorescence microscope protocols, each sample can be stained in a 0.5-1.0 mL cell culture volume containing from 20-200 nM

MitoPT dye concentration, depending upon user requirements for cell brightness, and if a wash step is necessary. Protocols using MitoPT TMRE or TMRM at concentrations > 50 nM should generally include a single wash step to minimize background fluorescence issues. The MitoPT ΔΨm 500 Test Kit contains enough TMRE or TMRM reagent to evaluate 500 flow cytometry or fluorescence microscopy cell samples at a 200 nM dye concentration. If using a fluorescence plate reader analysis protocol, each 1 mL cell culture sample requires a concentration of 100-200 nM MitoPT dye. Accordingly, the MitoPT ΔΨm 500 Test Kit will process 500 samples spiked at a 200 nM dye
concentration, or 1000 samples spiked at 100 nM dye concentration.

When cells stained with MitoPT TMRE or TMRM are run through a flow cytometer, the instrument will measure apoptosis by monitoring the loss of orange (574 nm) fluorescence intensity (FL2 emission spectrum) relative to the normal (negative) control population. Apoptotic cell mitochondria have a reduced ΔΨm that results in lower levels of the potentiometric dye within these organelles. TMRE and TMRM have been used concurrently with other fluorophores in multi-parametric, flow cytometry based analyses measuring mitochondria depolarization, caspase activation, phosphatidyl-serine exposure, and/or cell viability within a single cell population (12-14). MitoPT TMRE and TMRM excite optimally at 549 nm and 548 nm, respectively, but also yield excellent results using the common Argon blue line (488nm) laser of most flow cytometers (12, 15-17). Optimal emission from the TMRE and TMRM, MitoPT Reagents lies in the FL2 emissions region (574nm and 573 nm, respectively). Orange emission filters (570nm +/- 10 nm) were utilized to detect the presence of these potentiometric dyes using flow cytometry-based protocols (12, 15 - 17). Representative sample data can be seen in Section 18.

When cells stained with MitoPT TMRE or TMRM are analyzed with a fluorescence plate reader, the instrument will measure apoptosis by monitoring the amount of emitted orange fluorescence. Healthy control cells bearing mitochondria with normal electrochemical gradients will concentrate the potentiometric dye to a greater extent than will apoptotic cell populations (18-19). The difference in fluorescence output of these two populations can be easily distinguished on a fluorescence plate reader using 540 +/- 10 nm excitation / 570 nm +/- 10 nm emission filter tandems in black 96-well plates. (See Section 21 for sample data).

Looking at the cells under a fluorescence microscope, non-apoptotic cells will appear to have orange fluorescent spots (the MitoPT TMRE or TMRM dye accumulates) within healthy 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 these cells will also be visibly reduced as a result of the mitochondrial depolarization event. (See Section 25 for sample data).

2. Kit Contents

- MitoPT 100, TMRE KF17358-100 test or TMRM KF17359-100 test size kit. MitoPT 500, TMRE KF17358-500 test or TMRM KF17359-500-test size kit.
- 10X Assay Buffer 1 x 30 mL bottle in 100 test size kit or 1 x 125 mL bottle in 500 test size kit.
- 50 mM Carbonylcyanide *m* - chlorophenylhydrazone (CCCP) concentrate in DMSO, 1 x 125 µL vial in 100 test size kit or 1 x 600 µL vial in 500 test size kit.
- Assay Manual with protocols for 3 applications: Flow Cytometer; Fluorometer; and Fluorescence Microscope.

3. Recommended Materials and Equipment (not all are required):

- Cultured cells and media
- Protocol and reagents to induce metabolic stress, mitochondrial depolarization, or apoptosis
- 15 mL polypropylene centrifuge tubes
- 12 X 75 mm (5 mL) polypropylene tubes
- Clinical centrifuge adjustable to 100 – 300 X g
- Adjustable Pipette(s) capable of accurate delivery of 2 µL to 20 µL and 100 µL to 1000 µL volumes.
- Graduated cylinder
- Dimethyl Sulfoxide - DMSO (100 µL)
- Apoptosis induction agents such as Staurosporine or Camptothecin to induce **apoptotic** positive control cell population.
- Vortexer
- Amber vials or polypropylene tubes for storage of MitoPT TMRE and TMRM
- dye stock solutions at < –20°C
- Black round or flat bottom 96-well microtiter plates.
- Microscope Slides
- 37°C CO2 incubator
- Hemocytometer

4. Instrumentation (not all are required):

- Flow cytometer with excitation laser at 488 nm and emission filter at 570 +/- 10 nm.
- 96-well fluorescence plate reader with 540-550 nm excitation, and 580 +/- 10 nm emissions filters, with endpoint reading.
- Fluorescence microscope with 540-550 nm excitation filter plus >575 nm (long pass) filter tandem.

volumes, add 10 mL 10X assay buffer to 90 mL DI H_2O to yield 100 mL of 1X Assay Buffer.
3. Stir the 1X solution for at least 5 minutes.
4. If not using the 1X assay buffer the same day it was prepared, store it at 2°C to 8°C for up to 7 days. Warm the 1X Assay Buffer to RT prior to use.

5. Storage and Shelf-Life

- Store the unopened kit (and each unopened component) at 2°C to 8°C until the expiration date.
- Once reconstituted in DMSO, store the 1 mM and 0.2 mM stock concentrates frozen, in the dark, at < –20°C, for up to 12 months. The 1 mM and 0.2 mM. stock concentrates may be re-frozen up to 3 times without loss of performance activity.
- Once diluted, store the 1X Assay Buffer at 2°C to 8°C up to 7 days.

6. Safety Information

Use gloves while handling the MitoPT potentiometric dye reagents, CCCP depolarization reagent, and 10X assay buffer.

Dispose of all liquid components down the sink and flush with large amounts of water. Solid components may be tossed in standard trash bins. MSDS for kit components available by e-mailing mailto:pshuster@neuromics.com or by calling 1-866-350-1500.

7. Overview of the MitoPT ΔΨm Protocol

Staining cells with MitoPT TMRE or TMRM dye takes only about 20 minutes at 37ºC. However, the MitoPT ΔΨm kit is used with living cells that may take from several hours to several days to prepare. In addition, appropriate control cells must be induced to undergo apoptosis or oxidative-stress, that may also take several hours to complete.

The 50-500X (10 μM in assay/wash buffer) TMRE/TMRM reagent will maintain proper functionality during a 7 day storage period when stored at 2º to 8ºC, protected from light. It is recommended however, that the 1:100 dilution of the 1 mM (500 test size vial) or 1:20 dilution of the 0.2 mM (100 test size vial) TMRE / TMRM stock be prepared just prior to use. Here is a quick overview of the MitoPT ΔΨm protocol:

1. Culture cells to a density optimal for apoptosis or oxidative stress induction, according to your specific induction protocol, **but not to exceed 10^6 cells/mL.**
2. At the same time, culture a non-induced negative control cell population (at the same density as the induced population).
3. Induce apoptosis or purposely depolarize the mitochondria following your desired protocol (See Section 8, below).
4. Prepare 1X Assay/Wash Buffer. (Section 9).
5. Prepare the 1 mM or 0.2 mM MitoPT TMRE / TMRM stock (Section 10).
6. Prepare a 1:100 dilution of the 1 mM, or a 1:20 dilution of the 0.2 mM MitoPT TMRE / TMRM solution for spiking into cell culture samples (Section 11 or 13).

7. Stain cells using a final 20–200 nM concentration of MitoPT TMRE / TMRM solution (Section 14, 19, 22, or 23).
8. Analyze data (Section 15, 20, or 24).

8. Induction of Apoptosis or Mitochondrial Depolarization

The MitoPT TMRE/TMRM ΔΨm kit is compatible with any apoptosis or mitochondrial assessment protocol. An apoptotic positive control can routinely be established in Jurkat leukemic T cells using either a 2 µg/ml camptothecin or 1 µM staurosporine induction for 3-4 hours at 37ºC. Depolarized mitochondria containing (positive control) cell populations can easily be prepared by incubating cells with 5-50 µM CCCP for 30 to 60 minutes at 37ºC. (50 mM CCCP stock included in kit). This proton gradient uncoupling agent quickly reduces the electrochemical potential across the inner mitochondrial membrane, resulting in a rapid intra-cellular mitochondrial depolarization event (20-21). To generate a CCCP-induced positive control population, follow the brief protocol listed below:

1. Pre-determine when you intend to run the mitochondrial depolarization analysis on your control and experimental (treatment) cell populations. Approximately 75 minutes prior to performing this analysis using the MitoPT ΔΨm kit, plan to generate a positive and negative mitochondrial depolarization control sample set using the CCCP mitochondria depolarizing agent that is included in this kit.
2. Gently warm the 50 mM CCCP stock reagent vial to RT. Be sure to mix or gently vortex the CCCP stock vial contents prior to adding it to the cell cultures.
3. Spike the designated and labeled positive control population with enough CCCP de-polarizing reagent to obtain a 10–50 µM CCCP concentration in the cell culture media. If using a 50 µM CCCP concentration for mitochondrial depolarization, simply spike in 1 µL of 50 mM CCCP stock per mL of cell suspension/overlay media. If using a lower concentration for induction, make a 1:10 dilution of an aliquot of the 50 mM CCCP stock in tissue culture grade DMSO, so as to be able to accurately dispense the CCCP into the cell culture tube or flask.
4. Prepare a negative control cell population that was spiked with the same volume of tissue culture grade DMSO as was used to spike the CCCP cell population.
5. Incubate the negative and positive control cell populations for 30–60 minutes at 37ºC in the CO_2 incubator to allow for the depolarization process to proceed.

9. Preparation of 1X Assay Buffer

The Assay Buffer is formulated for use as a reaction buffer and as a cell suspension medium for analyzing the cells in flow cytometry, fluorescence microscopy, and fluorescence plate reader analysis techniques (it is used in Sections 8, 10, 12, 18, 22, and 23). The 500 Test Kit contains one 125 mL bottle of Assay Buffer. The 100 test size kit contains a 30 mL bottle of Assay Buffer. This Assay Buffer is supplied as a 10X concentrate that must be diluted to 1X with DI H_20 prior to use.

If cells can not be analyzed within a 2-3 hour time frame, it is recommended that a reagent grade BSA powder be added to the 1X Assay Buffer to a 1% final concentration. This will assure an extended cell viability environment until analysis can be completed.

1. If necessary, gently warm the 10X concentrate to completely dissolve any salt crystals that may have come out of solution.
2. Dilute the 10X assay buffer concentrate 1:10 in DI H2O. For example, add the contents of one 30 mL bottle to 270 mL of DI H2O. This will yield a 300 mL volume of 1X Assay Buffer for use as a wash or readout suspension medium. For smaller For smaller volumes, add 10 mL 10X assay buffer to 90 mL DI H2O to yield 100 mL of 1X Assay Buffer.
3. Stir the 1X solution for at least 5 minutes.
4. If not using the 1X assay buffer the same day it was prepared, store it at 2°C to 8°C for up to 7 days. Warm the 1X Assay Buffer to RT prior to use.

10. Reconstitution of the MitoPT TMRE / TMRM Reagent Vials

The MitoPT potentiometric dye reagent is supplied as a highly concentrated lyophilized powder. It must first be reconstituted, forming either a 1 mM (500 test vial) or 0.2 mM (100 test vial) stock concentrate in DMSO, and then diluted 1:100 or 1:20, respectively, to form a 10μM working solution. The 10 μM working solution must be prepared immediately prior to use; however, the reconstituted 1 mM or 0.2mM stock solution can be stored at –20°C for 6-12 months. Aliquots of the 1 mM or 0.2mM stock solution can be frozen, thawed, used, and refrozen up to 2 more times during a 6 month (frozen shelf–life) period.

- **The newly reconstituted 1 mM or 0.2 mM MitoPT TMRE / TMRM stock solution must be used, or frozen immediately after it is prepared, and protected from light during handling.**

1. Reconstitute the MitoPT TMRE / TMRM vials with 100 µL DMSO at room temperature (RT), forming either a 1 mM or 0.2 mM stock concentrate, depending upon vial size.
2. Re-cap the vial and invert or vortex the base of the vial 3-4 times to fully dissolve the MitoPT TMRE/TMRM dye reagent.
3. Immediately remove desired amount of the 1 mM or 0.2 mM stock concentrate that will be diluted 1:100 or 1:20 respectively, in the Assay Buffer to obtain the 10 μM working solution for the assay (Section 11, below).
4. Remaining 1 mM or 0.2 mM stock can be aliquoted into separate vials for future use (Section 12).

11. Preparation of 10 μM MitoPT TMRE / TMRM Working Solution

Using the freshly reconstituted 1 mM or 0.2 mM MitoPT TMRE / TMRM stock, prepare the 10 µM working solution by diluting the stock 1:100 or 1:20, respectively, with 1X Assay Buffer (you can substitute your own cell culture media in place of the 1X Assay Buffer). Cell culture samples should be stained with a 20 nM–200 nM final concentration of the TMRE or TMRM potentiometric dye.

- **The 10 µM Working Solution should be prepared the day it will be used and stored refrigerated in the dark until ready to spike into the cell culture samples.**

1. Calculate the amount of 10 µM working solution that will be needed to assess the apoptotic/ΔΨm status of each experimental cell population sample. For example, if you are using 1 mL sample volumes, either in suspension or overlaying an adherent layer, this requires a 2, 5, 10, or 20 µL per mL spike of the 10 µM working solution to obtain a final concentration of 20, 50, 100 or 200 nM, respectively.
2. Remove the volume of 1 mM or 0.2 mM MitoPT TMRE / TMRM stock that is needed to prepare the pre-calculated volume of 10 µM working solution. The 1 mM or 0.2 mM stock should be diluted using 1X Assay Buffer (or your own cell culture media if desired), to obtain the 10 µM working solution. For example, if you determine that you need a total of 2 mL of the 10 µM working solution to perform your analysis, simply dilute 20 µL of 1 mM stock concentrate into 1980 µL of 1X Assay Buffer. This 10 µM working solution can then be used to spike 100 individual 1 mL cell samples with 20 µL for a final staining concentration of 200 nM
TMRE/TMRM.
3. This 10 µM working solution is stable for up to 7 days when stored at 2 to 8ºC, protected from light.
4. Re-freeze any remaining 1 mM or 0.2 mM stock at < -20ºC, protected from light.
5. Go on to the staining protocols for the different analytical techniques (Sections 14, 19, 22, or 23).

- **To avoid photo-bleaching and degradation of the dye, protect the MitoPT TMRE / TMRM reagent from bright light while handling.**

12. Storage of 1 mM and 0.2 mM MitoPT TMRE / TMRM Stock for Future Use

If not all of the DMSO solubilized, 1 mM or 0.2 mM MitoPT stock concentrates will be used at the time they are reconstituted, the unused portion may be stored at < -20°C, for up to 6 months. During that time, the MitoPT TMRE / TMRM stock concentrates may be thawed and re-frozen twice. After the second thaw, discard any remaining 1 mM or 0.2 mM MitoPT TMRE / TMRM stock. If you anticipate using the stock more than 3 times, make smaller aliquots in amber vials or polypropylene tubes and freeze.

13. Preparation of 10 µM Working Solution from a Frozen 1 mM or 0.2 mM Stock Aliquot

Aliquots of the 1 mM or 0.2 mM MitoPT TMRE / TMRM stock stored at < –20°C may be freeze/thawed 2 times within 6 months.

1. Thaw the 1 mM or 0.2 mM stock solutions; keep protected from light.
2. Once the aliquot has become liquid, dilute the 1 mM MitoPT TMRE / TMRM stock 1:100 in 1X Assay Buffer (or cell culture media) to obtain the 10 µM working solution. The 0.2 mM stock solution will only need a 1:20 dilution to achieve the 10 µM working strength concentration.
3. If the 1 mM or 0.2 mM stock solutions were frozen immediately after reconstitution and were never previously thawed, they can be safely returned to the freezer and refrozen for future use. If the stock solutions have been thawed twice before, discard any stock reagent that was not used up in this latest ΔΨm assay.

4. Vortex the 10 μM working solution thoroughly.
5. Use this 10 μM working solution to stain cells using any of the three protocols described in this kit insert.

14. Flow Cytometry Staining Protocol

Following a standard flow cytometry staining protocol, each 0.5–1 mL cell sample requires only 20–200 nM MitoPT TMRE / TMRM potentiometric dye. No wash step is necessary if using low (20-30 nM) concentrations of TMRE or TMRM dye. Otherwise, using a 100–200 nM concentration of dye with a single wash step gives excellent resolution of depolarized versus charged mitochondria in flow based assays. The MitoPT ΔΨm 500 test assay kit provides enough potentiometric dye reagent to analyze > 500 x1 mL cell culture samples. This translates into a potential volume of 500 mL at 200 nM MitoPT TMRE / TMRM staining solution or a 1000 mL volume if the staining concentration used is 100 nM. Correspondingly, a 100 test size kit will provide enough TMRE/M reagent for > 100 x1 mL cell culture assays.

1. As discussed in Section 7, culture cells to a density optimal for your induction of apoptosis or ΔΨm disruption model experiments, according to your specific cell culture protocol.

- **Cell density in the cell culture flasks typically should not exceed 10^6 cells per mL. Cells cultivated in excess of this concentration may begin to naturally enter apoptosis. Optimal cell concentration will vary depending on the cell line used. Density can be determined by counting cell populations on a hemocytometer.**

2. Generate your experimental apoptosis or ΔΨm disrupted cells using your preferred protocol.
3. Create a non-induced negative control cell population as well as a positive control population where either a known apoptosis inducing agent is added, or the cells are exposed to a common uncoupling reagent such as carbonyl cyanide 3- hlorophenylhydrazone (CCCP) provided in the kit (Section 8).
4. After the induction process, transfer 0.5-3mL of each cell suspension into a properly labeled tube.
5. Concentrate cells if desired, using a brief, gentle centrifugation step (5-8 minutes at < 300 x g, 15-25ºC). If concentration is not necessary, proceed directly to the staining steps below (steps 8 – 9).
6. Remove enough supernatant from the cell pellets such that their final volume is between 0.5–1 mL total cell sample volume.
7. Gently vortex or re-suspend the cell pellets to disrupt any cell-to-cell clumping that can occur with some cell types.
8. Spike the cell suspension (or supernatant for adherent cells) with either a 10 μL or 20 μL/mL volume of the 10 μM working solution that was prepared earlier (Section 11 or 13, above). This will give a 100 nM and 200 nM MitoPT TMRE / TMRM staining solution, respectively. Use of the 100 nM potentiometric dye concentration has been shown to give good mitochondrial staining using both the TMRE and TMRM ΔΨm dyes.
9. If using a lower (20-30 nM) potentiometric dye concentration, flow cytometry may be performed without a wash step prior to the analysis. This method will discriminate between apoptotic and non-apoptotic, or mitochondrial membrane compromised versus healthy cell populations. A greater level of mitochondrialdepolarization detection sensitivity can be obtained however, if a

100-200 nM dye concentration and wash step is employed. When choosing this no-wash staining option, go directly to the flow cytometry set up sections (Section 15-18) to continue
with the analysis.

10. Incubate the cells for 15-20 minutes in a 37°C CO_2 incubator.
11. Wash the TMRE / TMRM dye stained cells by centrifuging at < 300 x g for 5 minutes at RT. Discard the supernatant.
12. Add 1 mL of 1X Assay Buffer to each tube and vortex gently to resuspend the cell pellet.
13. Store samples protected from light until they can be analyzed on the flow cytometer (Sections 15-18).

15. Flow Cytometer Set Up

MitoPT TMRE and TMRM excite optimally at 549 nm and 548 nm, respectively, but also yield excellent results using the common Argon blue line (488 nm) laser that is present in most flow cytometers (Sections 12, 15-17). Optimal emission from the TMRE and TMRM MitoPT reagents lies in the FL2 emissions region (574 nm and 573 nm, respectively). Orange emission filters (570 nm +/- 10 nm) were utilized to detect the presence of these potentiometric dyes using flow cytometry-based protocols (Sections 12, 15-17). MitoPT TMRE and TMRM measure apoptosis by monitoring the loss of fluorescence intensity relative to the normal (negative/healthy) control population.

16. Single-Parameter Analysis Using a Flow Cytometer

1. Generate a log FL2 (X-axis) versus relative cell number (Y-axis) histogram.
2. Adjust the FL2 PMT voltage to allow the peak to fall within the third log decade when running the more brightly fluorescent, negative control sample.
3. When running the induced positive samples, use the same adjusted PMT voltage as was determined for the negative control. The histogram peak should still be observable on the X-axis. If not, increase PMT voltage slightly to achieve positive control staining within the first decade of the log scale.
4. Observe the mean fluorescence intensity of both the apoptotic/mito-depolarized positive cell population, as well as the mean fluorescence of the negative control cell population and all test samples using the same voltage settings.

17. Multi-Parameter Analysis Using a Flow Cytometer

1. MitoPT TMRE and TMRM emissions are monitored on a log FL-2 axis. The other axis can be used for monitoring the fluorescence of any desired second fluorochrome (eg FL1 for fluorescein, FL3 for 7AAD, or FL4 for a second laser analyte). Create a log (X-axis) verses log (Y-axis) scatter plot, making sure that one axis is detecting FL2 emissions.
2. Use the appropriate controls for the second color analyte, as well as the positive and negative controls for the TMRE/TMRM dye, to set compensation levels and the quadrant staining gates.
3. The FL2 PMT voltage should be adjusted using the negative control sample (noninduced cells) so that the majority of the cell population falls within a positive staining quadrant.
4. Using the same PMT settings established for the non-induced negative control

sample in Step 3, run the induced positive sample. If a change in mitochondrial ΔΨm has occurred, an increase in the number of cells falling into a negative staining quadrant is observed.

18. Flow Cytometry Sample Data

When cells stained with MitoPT TMRE / TMRM are run through a flow cytometer, the instrument will measure apoptosis by monitoring the amount of orange fluorescence. Healthy cells fluoresce orange. As the mitochondrial ΔΨm collapses and cells enter apoptosis or some other oxidative stress driven mechanism, the amount of fluorescence will drop as the concentrated TMRE / TMRM dye equilibrates back out of the mitochondria and into the cytosol where it exists at a much lower dye concentration and the magnitude of dye fluorescence is drastically reduced. This cell population fluorescence shift is demonstrated by overlaying the histograms of both the normal (negative) cell population and the apoptosis-induced/depolarized cell population (Figure1).

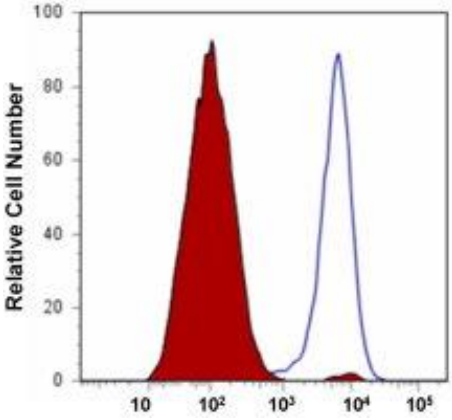

Figure 1: Jurkat cells were treated with either DMSO (open histogram) or with staurosporine (filled histogram) for 3 hours at 37°C and then labeled with MitoPT TMRM for 15-20 minutes. Cells were analyzed in a FACS Caliber Becton Dickinson Flow Cytometer. Apoptotic cells exhibit significantly less fluorescence compared to healthy control cells.

Dual labeling TMRM (FL-2) of staurosporine induced Jurkat cells using MitoPT TMRM potentiometric dye and a Fluorescence Labeled Inhibitors of Caspases (FLICA) probe FAM-VAD-FMK, allows for the simultaneous detection of both caspase enzymes and depolarized mitochondria in the same cell population (Figure 2). As the apoptotic cascade is initiated, caspase activation and mitochondrial depolarization become important factors in the propagation and imaging of the cell death process. Mitochondrial depolarization is evidenced by the drop in orange (FL2) fluorescence (Figure 2B). There is also a concurrent increase in cell-bound green fluorescence (FL1) that is associated with the covalent attachment of the FAM-VAD-FMK FLICA probe to apoptotic enzymes (Figure 2B).

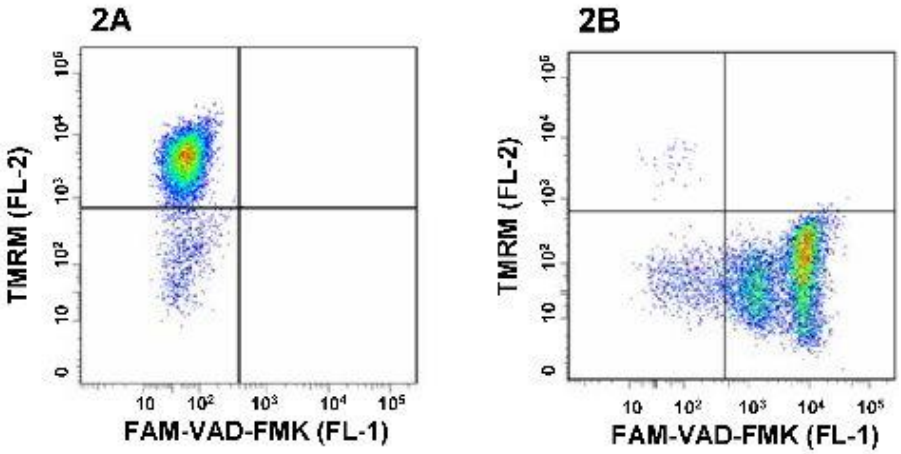

Figure 2: Jurkat cells were dually stained with TMRM and FAM-VAD-FMK. Healthy control cells (Figure 2A) are evident in UL Quad 3, Apoptotic cells are found in LR Quad 2 (Figure 2B).

19. 96-Well Fluorescence Spectroscopy Staining Protocol

When using a 96-well fluorescence plate reader for analysis, cell concentrations exceeding 1 x 105 cells/well are highly recommended. This corresponds to a spiked and washed cell suspension of > 1 x 106 cells/mL if 100 µL/well aliquots are read in black 96-well plates. Because a single wash step is required for adequate analysis in the plate reader format, we also recommend using a concentration of 100-200 nM TMRE/TMRM dye. If using 1 mL cell suspension volumes, this requires a spike of 10 to 20 µL of the 10 µM working solution. The MitoPT ΔΨ**m** 500 and 100 test assay kits can process up to 500 and 100, respectively, (1 mL samples) using a 200 nM dye concentration and 1000 samples if using a 100 nM dye concentration.

1. Culture cells to a density optimal for your induction of apoptosis or ΔΨ**m** disruption
model experiments, according to your specific cell culture protocol.

- **Cell density in the cell culture flasks should not exceed 106 cells per mL. Cells cultivated in excess of this concentration may begin to naturally enter apoptosis. Optimal cell concentration will vary depending on the cell line used. Density can be determined by counting cell populations on a hemocytometer.**

2. Generate your experimental apoptosis or ΔΨ**m** disrupted cells using your preferred protocol.
3. Create a non-induced negative control cell population as well as a positive control population where either a known apoptosis inducing agent is added or the cells are exposed to a common uncoupling reagent such as carbonyl cyanide 3-chlorophenylhydrazone (CCCP) provided in the kit (See Section 8).

4. After the induction process, transfer 1-5 mL of each cell suspension into properly labeled centrifuge tubes.
5. Take out a small aliquot to determine the concentration of both the induced (treatment group) and non-induced (normal control group) cell populations. It is desirable for the concentrations of all the cell suspension samples to be approximately equal.
6. Concentrate cells if required, to achieve the desired cell density range of > 1–2 x 10^6 cells/mL. This can be achieved by a brief, gentle centrifugation step (5–8 minutes at < 300 x g, 15-25ºC). If concentration is not necessary, proceed directly to the staining step 9 below.

- **When concentrated, cells should be at least 1-2 X 10^6 cells/mL.**

7. Remove the supernatant from the cell pellets and replace with a volume of cell culture media that gives the desired cell concentration range stated above in **bold font**. Gently vortex or re-suspend the cell pellets to disrupt any cell-to-cell clumping that can occur with some cell types.
8. Remove a 1 mL aliquot from each of the concentration-equalized cell samples and place these cell suspension aliquots into properly labeled 15 mL polypropylene centrifuge tubes.

- **Note, you may substitute any appropriately sized polypropylene tube for these incubations and wash procedures but do not use polystyrene types of plastic due to their propensity to non-specifically bind the TMRE/TMRM dyes.**

9. Spike the cell suspension (or supernatant for adherent cells) with either a 5 µL or 10 µL/mL volume of the 10 µM working solution prepared earlier (Section 11 or 13, above). This will give a 50 nM and 100 nM MitoPT™ TMRE / TMRM staining solution; respectively. Use of a 50-100 nM potentiometric dye concentration has been shown to give good mitochondrial staining and induced apoptotic population resolution using both the TMRE and TMRM **ΔΨm** potentiometric dyes.
10. Incubate the cells with the MitoPT TMRE / TMRM dye reagent for 15-20 minutes in a 37°C CO2 incubator.
11. Wash the TMRE / TMRM dye stained cells by centrifuging at < 300 x g for 5 minutes at RT. Discard the supernatant.
12. Add 1 mL of 1X Assay Buffer to each tube and vortex gently to resuspend the cell pellet.
13. Store protected from light until the cells can be analyzed using a 96-well fluorescence plate reader.

- **A minimum of 1 X 105 cells/well is recommended to generate an adequate fluorescence signal using most 96-well plate readers.**

14. For each sample to be tested, dispense 100-200 µL of stained cell suspension
volumes into triplicate wells of a black round or flat bottom 96-well microtiter plate.

- **An opaque black 96-well microtiter plate is highly recommended for cell samples in a fluorescence plate reader. Clear or white plates will cause a drop in assay sensitivity and increased background problems.**

15. Analyze cells using a fluorescence plate reader (Section 20).

20. 96-Well Fluorescence Plate Reader Set Up

1. Set the plate reader to perform an endpoint read.
2. Set the excitation wavelength at 540 nm.
3. Set the emission wavelengths to 575 nm for orange fluorescence. If possible, utilize a 570 nm emissions cutoff filter to reduce any plate noise from the excitation signal input.
4. Prepare a plate map template to ensure that the identity of the replicate samples is properly documented to avoid confusion during the data analysis process.
5. Read the samples and print out a copy of the fluorescence (RFU) output. Save the data electronically for future reference.

21. 96-Well Fluorescence Spectroscopy Sample Data

When cells stained with MitoPT TMRE or TMRM are analyzed with a fluorescence plate reader, the instrument will measure the amount of orange fluorescence. Healthy cells will give a high reading of orange fluorescence; apoptotic and metabolically stressed cells will exhibit a reduced level of orange fluorescence intensity. By comparing the average 575 nm fluorescence signal in stimulated versus nonstimulated sample wells, loss of $\Delta\Psi_m$ can be detected. As the $\Delta\Psi_m$ collapses (commonly an early stage of apoptosis), and the TMRE or TMRM dye equilibrates out of the mitochondria and into the cytosol, more and more cells will fade, losing their orange fluorescence (Figure 3). The loss of the electrochemical gradient across the inner membrane of the depolarizing mitochondria is easily detectable in most fluorescent plate reader formats.

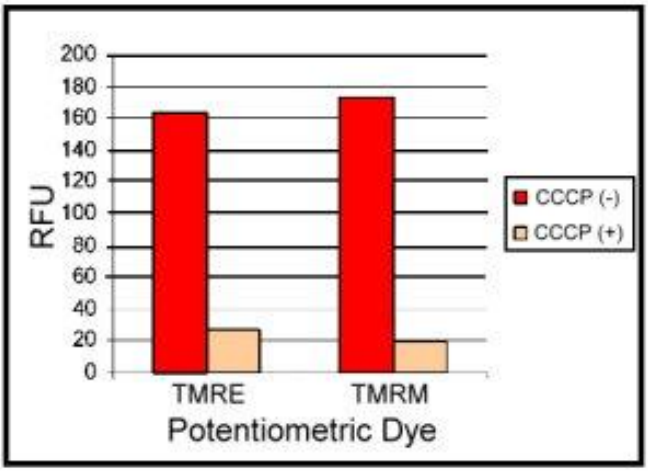

Figure 3. Detection of mitochondrial depolarization in Jurkat cells using 100 nM TMRE or TMRM potentiometric dyes. Jurkat cells were exposed to 50 µM CCCP depolarizing agent or DMSO control for 15 min at 37°C, and subsequently incubated with either TMRE or TMRM potentiometric dyes for 20 min at 37°C. Cells were then washed once in MitoPT assay buffer, aliquoted into 100 µL triplicate wells in a black 96-well plate, and read using a fluorescence plate reader set for 550 nm excitation, 580 nm emission, and a 570 nm electronic cutoff setting.

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22. Fluorescence Microscopy Staining Protocol for Adherent Cells

When analyzing cells using a fluorescence microscope, adherent cell monolayers can readily be stained using a concentration of 100–200 nM MitoPT TMRE / TMRM potentiometric dye followed by a single wash step using 1X Assay Buffer. The wash step is optional when staining with dye concentrations < 40 nM as the background fluorescence from free dye present in the supernatant is low enough to still allow for good resolution of the polarized mitochondria containing cells. The MitoPT ΔΨm 500 test and 100 test assay kits can process 500 and 100 respectively, (1 mL cell supernatant samples) using a 200 nM dye concentration and 1000 and 200 samples respectively, if using a 100 nM dye concentration.

1. Culture cells on a sterile coverslip or chamber-slide to a cell density that will not lead to spontaneous apoptosis induction due to over-crowding on the monolayer surface. Adherent cell lines will vary as to their propensity to spontaneously transition into an apoptotic phase once confluent monolayers have become established.

- **Cell density should not exceed the threshold where cell sloughing occurs.**

2. Generate your experimental apoptosis or ΔΨm disrupted cells using your preferred protocol.
3. Create a non-induced negative control cell population, as well as a positive control population where either a known apoptosis inducing agent is added, or the cells are exposed to a common uncoupling reagent such as carbonyl cyanide 3- chlorophenylhydrazone (CCCP) provided in the kit (See Section 8).
4. Spike the cell culture supernatant floating over the adherent cell monolayer, with a 10-20 µL/mL volume of the 10 µM working solution that was prepared earlier in section 11 or 13, above. This will give a final concentration of 100-200 nM MitoPT TMRE / TMRM staining solution, respectively. Use of a 150 nM potentiometric dye concentration has been shown to give good mitochondrial staining using both the TMRE and TMRM ΔΨm dyes.
5. Incubate the cells with the MitoPT TMRE / TMRM dye reagent, for 15-20 minutes in a 37°C CO2 incubator.
6. Gently remove the cell culture supernatant and add enough 1X Assay Buffer to cover the cell surface and dilute out any remaining free TMRE or TMRM dye.
7. Remove this wash Assay Buffer from the cell supernatant and replace with enough 1X Assay Buffer to cover the cells (50–100 µL).
8. Cover the stained cells with a cover slip and observe using a fluorescence microscope (See Sections 24 and 25).

23. Fluorescence Microscopy Staining Protocol for Suspension Cells

Suspension cells can readily be evaluated using fluorescence microscopy in a manner similar to that used in staining adherent cells. MitoPT TMRE / TMRM potentiometric dye concentrations of 100–200 nM typically require a single rinse/wash step using the 1X Assay Buffer. A wash step is optional when staining with dye concentrations < 40 nM as the background fluorescence from free dye present in the supernatant is low enough to still allow for good resolution of the polarized mitochondria containing cells. The MitoPT ΔΨm 500 test and 100 test assay kits can process 500 and 100 1 mL cell suspension samples, respectively, using a 200 nM dye concentration.

1. Culture cells to a cell density that will not lead to spontaneous apoptosis induction due to over-crowding in the suspension media. Suspension cell lines will vary in their tolerance to high cell concentration environments. This can lead to some suspension cell lines transitioning into an apoptotic cell cycle mode at lower cell concentration densities than others will tolerate. As a general rule, cell culture concentrations should not exceed 1 x 106 cells/mL for extended time periods (>12 hours) unless it is known that they can tolerate this higher cell density without becoming spontaneously apoptotic.

- **Optimal cell densities will vary with the cell line. Cell concentrations may be determined using a hemocytometer.**

2. Induce apoptosis or ΔΨm disruption using your preferred experimental design. MitoPT TMRE/TMRM ΔΨm kits are compatible with any apoptosis induction or mitochondrial potential assessment protocol.
3. Create a non-induced negative control cell population, as well as a positive control population where either a known apoptosis inducing agent is added or the cells are exposed to a common uncoupling reagent such as carbonyl cyanide 3- chlorophenylhydrazone (CCCP) (See Section 8, above).
4. Spike the cell cultures with a 10 -20 µL/mL volume of the 10 µM working solution that was prepared earlier in section 11 or 13, above. This yields a final concentration of 100-200 nM MitoPT TMRE / TMRM staining solution, respectively. Use of a 150 nM potentiometric dye concentration has been shown to give good mitochondrial staining using both the TMRE and TMRM $\Delta\Psi_m$ dyes.
5. Incubate the cells with the MitoPT TMRE / TMRM dye reagent, for 15-20 minutes in a 37°C CO_2 incubator.
6. Pellet the TMRE / TMRM dye stained cells by centrifuging at < 300 x g for 5 minutes at RT and discard the supernatant.
7. Add 1 mL of 1X Assay Buffer to each tube and re-suspend the cells, allowing for the dispersion/dilution of any cell membrane-associated dye molecules in the TMRE / TMRM dye stained samples.
8. Concentrate these cells by centrifugation, if necessary, to achieve a relatively dense cell suspension concentration (>1 x 10_6 cells/ml) that will allow easy observation of multiple cells in a single microscopic observation field.
9. Remove a 50–100 µL aliquot of cells and place under a cover slip for observance using the fluorescence microscope (Sections 24 and 25).

24. Fluorescence Microscope Set Up

TMRE or TMRM stained cells should be visually examined using a fluorescence microscope capable of excitation in the green wavelength range (510-560 nm) and with an emissions filter in the orange-red fluorescence range (570-620 nm).

25. Fluorescence Microscopy Sample Data

The MitoPT TMRE or TMRM dye accumulates within the mitochondria of healthy cells, due to their lipophilic and de-localized positive charge, leading to bright orange fluorescent mitochondrial organelle bodies within non-apoptotic cells. The MitoPT TMRE/TMRM dye will not accumulate in the mitochondria of cells where the mitochondrial membrane electrochemical gradient has been compromised. This results in the dye being evenly dispersed and an overall reduction in orange fluorescence in cells or cell populations bearing the depolarized mitochondrial

bodies. Apoptotic or metabolically stressed cells exhibit a partial to complete loss in orange fluorescence.
The number of observable fluorescent mitochondria within individual cells may also diminish with the onset of apoptosis or metabolic and oxidative stress.

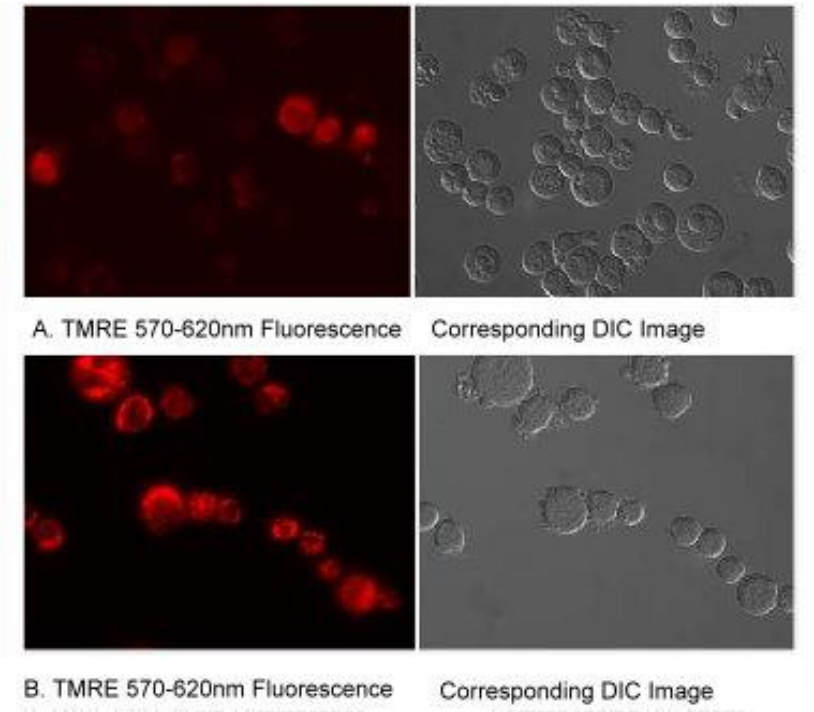

Figure 5: Fluorescence microscopy detection of the loss of ΔΨm in staurosporine induced, apoptotic Jurkat cells. MitoPT TMRE (Tetramethylrhodamine ethyl ester) potentiometric dye was incubated at a 150 nM concentration (20 minutes, 37ºC) with a normal healthy (negative control) population of Jurkat cells as well as a staurosporineinduced, apoptotic (positive control) Jurkat cell population. Following a single wash step, the cells were photographed using a Nikon Eclipse E800 photomicroscope equipped with Differential Interference Contrast (DIC), phase, and fluorescence optics. MitoPT TMRE stained Jurkat cells were excited using a green excitation filter (510- 560 nm) in tandem with a 570-620 nm emissions filter. Apoptotic cells, bearing depolarized mitochondria **(A)**, exhibit a reduced orange fluorescence relative to a corresponding healthy cell population **(B)**. Depolarized mitochondria will no longer concentrate the MitoPT TMRE potentiometric dye leading to a dramatic reduction in fluorescence intensity. Normal healthy cells, containing mitochondria with polarized inner membranes, concentrate the MitoPT TMRE dye and exhibit a bright orange fluorescence **(B).** Each fluorescence photo is accompanied by a corresponding DIC image. These were included to help visualize the location of reduced fluorescence (apoptotic) cells that are present in the 2 hour staurosporine-induced fluorescence
images.

26. References

1. Kroemer, G. and J.C. Reed. 2000. Mitochondrial control of cell death. *Nature Med.* **6(5)**: 513-519.
2. Mitchell, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature* **191**: 144-148.
3. Hoek, J.B., D.G. Nicholls and J.R. Williamson. 1980. Determination of the mitochondrial protonmotive force in isolated hepatocytes. *J. Biol. Chem*. **255(4)**: 1458-1464.
4. Nicholls, D.G. and S.L. Budd. 2000. Mitochondria and neuronal survival. *Physiol. Rev*. **80**: 315-360.
5. Budd, S.L., L. Tenneti, T. Lishnak and S.A. Lipton. 2000. Mitochondrial and extramitochondrial apoptotic signaling pathways in cerebrocortical neurons. *PNAS* **97(11)**:6161-6166.
6. Peng, L., D. Nijhawan, I. Budihardjo, S. Srinivasula, M. Ahmad, E. S. Alnenri, and X. Wang. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**: 479-489.
7. Ricci, J., R.A. Gottlieb and D. R. Green. 2003. Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis. *J. Cell Biol.* **160 (1)**: 65 – 75.
8. Plasek, J. and K. Sigler. 1996. Slow fluorescent indicators of membrane potential: a survey of different approaches to probe response analysis. *J. Photochem. Photobiol. B*: *Biol*. **33**: 101-124.
9. Ehrenberg, B., V. Montana, M. Wei, J.P. Wuskell, and L.M. Loew. 1988. Membrane potential can be determined in individual cells from the nernstian distribution of cationic dyes. *Biophys*. *J*. **53**: 785-794.
10. Farkas, D.L., M. Wei, P. Febbroriello, J.H. Carson, and L.M. Loew. 1989. Simultaneous imaging of cell and mitochondrial membrane potentials. *Biophys*. *J*. **56**: 1053-1069.
11. Rasola, A. and M. Geuna 1989. A flow Cytometry assay simultaneously detects independent apoptotic parameters. *Cytometry* **45**:151-157.
12. Wlodkowic, D., J. Skommer, and J. Pelkonen. 2006. Multiparametric analysis of HA14-1 induced apoptosis in follicular lymphoma cells. *Leukemia Res*. **30**:1187-1192.
13. Jayaraman S. 2008. A novel method for the detection of viable human pancreatic beta cells by flow Cytometry using fluorophores that selectively detect labile zinc, mitochondrial membrane potential and protein thiols. *Cytometry* **73A**: 615-625.
14. Jayaraman S. 2005. Flow cytometric determination of mitochondrial membrane potential changes during apoptosis of T lymphocytic and pancreatic beta cell lines: Comparison of tetramethylrhodamineethylester (TMRE), chloromethyl-Xrosamine
(H2-CMX-Ros) and mitotracker red 580 (MTR580). *J. Immunol. Methods* **306**: 68-79.
15. Plasek, J., A. Vojtiskova, and J. Houstek. 2005. Flow cytometric monitoring of mitochondrial depolarization: from fluorescence intensities to millivolts. *J. Photochem. Photobiol. B*: *Biol***. 78**:99-108.
16. Huang, S. 2002. Development of a high throughput screening assay for mitochondrial membrane potential in living cells. *J. Biomol. Screen.* **7(4)**:383-389.

17. Russell, J.W., D. Golovoy, A. M. Vincent, P. Mahendru, J.A. Olzmann, A Mentzer, and E. L. Feldman. 2002. High glucose induced oxidative stress and mitochondrial dysfunction in neurons. *FASEB J.* **16**:1738-1748.
18. Wong, A. and G. A. Cortopassi. 2002. High-throughput measurement of mitochondrial membrane potential in a neural cell line using a fluorescence plate reader. *Biochem. Biophys. Res. Comm.* **298**:750-754.
19. Toescu, E.C.and A. Verkhratsky. 2000. Assessment of mitochondrial polarization status in living cells based on analysis of the spatial heterogeneity of rhodamine 123 fluorescence staining. *Pflugers Arch*. **440**:941-947.
20. Kasianowicz, J., R. Benz and S. McLaughlin. 1984. The kinetic mechanism by which CCCP (Carbonyl cyanide m-chlorophenylhydrazone) transports protons across membranes. *J. Membrane Biol*. **82**:179-190.
21. Lim, M. L. R., T. Minamikawa, and P. Nagley. 2001. The protonophore CCCP induces mitochondrial permeability transition without cytochrome c release in human osteosarcoma cells. *FASEB J.* **503**:69-74.

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