BD™ MitoScreen

Flow Cytometry Mitochondrial Membrane Potential Detection Kit

Instruction Manual

Catalog No. 551302

BD

Table of Contents

Introduction

Researching Mitochondrial Roles in Apoptosis and Other Cellular Processes

There is a burgeoning interest in the scientific community to fully define the roles of mitochondria in cellular processes, particularly apoptosis (***reviewed in 1***). Apoptosis is a complex process that can be induced by many different factors, which, in turn, act through various cell death signaling pathways. The role of the mitochondria could potentially vary and may be dependent on a variety of factors including mode of apoptosis induction, cell type, or cell status with respect to the cell cycle, state of differentiation, development, normalcy or pathology. Two views of the mode of action are emerging. For example, there is an abundance of data suggesting that mitochondria play a critical role in apoptosis by releasing cytochrome c and other proteins that are essential for the activation of pro-caspase-9 and the execution of apoptosis. In this scenario, mitochondrial-activated caspase-9 activates caspase-3. Caspase-3 is often referred to as the primary executioner of apoptosis because it cleaves multiple downstream proteins leading to a loss of cellular structure and function, and ultimately cell death. Hence, one hypothesis supports the view that mitochondria are the primary triggers of cell death rather than the caspases.

Other data suggest that mitochondria act more as facilitators rather than essential players of apoptosis. For example, some signals may route to caspase activation without first involving the mitochondria, and thereby the activated caspases may target the mitochondria along with other cellular components. In this model, the caspases would be the primary triggers of cell death, and mitochondria, along with mitochondrial-linked caspase-9, would contribute to cellular demise rather than being essential for it. Given the complexity of apoptosis, it is likely that there are a number of mechanisms available to the cell for carrying out the process of apoptosis (***reviewed in 2***). Assays designed to evaluate the functional status of mitochondria are emerging as useful tools for helping to elucidate mitochondrial roles in apoptosis, the cell cycle, and other cellular processes.

Particular focus has recently been given to assays designed to study the mitochondrial membrane potential ($\Delta\psi$) during apoptosis (***reviewed in 3***). Energy released during the oxidation reactions in the mitochondrial respiratory chain is stored as a negative electrochemical gradient across the mitochondrial membrane and the $\Delta\psi$ is referred to as being polarized. Collapse of the $\Delta\psi$ results in a depolarized $\Delta\psi$, and is often, but not always, observed to occur early during apoptosis. For example, collapse of the $\Delta\psi$ during apoptosis has been reported in a number of studies, leading to a generalization that depolarization of the mitochondria is one of the first events occurring during apoptosis and may even be a prerequisite for

cytochrome c release. However, this generalization is now a matter of debate and there is data indicating that collapse of the $\Delta\psi$ does not always occur during apoptosis. Thus, depolarization of the $\Delta\psi$ may be a cause of or be associated with apoptosis in some, but not all systems. This is consistent with the concept that there are different mechanisms available for cells to carry out the process of apoptosis. In addition to apoptosis, changes in the $\Delta\psi$ have also been described during necrosis (depolarization)[4] and cell cycle arrest (hyperpolarization).[5] Knowledge of the $\Delta\psi$ and how it changes during apoptosis, necrosis, and the cell cycle may help to clarify the role of the mitochondria in these and other cellular processes.

The BD™ MitoScreen Kit: Application of JC-1 for Flow Cytometry

Flow cytometry has emerged as the technique of choice for analysis of the $\Delta\psi$ in whole cells.[3] Membrane-permeable lipophilic cationic fluorochromes are used as probes of $\Delta\psi$; they penetrate cells and their fluorescence is a reflection of $\Delta\psi$. JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) is a lipophilic fluorochrome that is used to evaluate the status of the $\Delta\psi$ (***reviewed in 6***). JC-1 stands for 1st J-aggregate-forming cationic dye found to be sensitive to the $\Delta\psi$.

The fluorescence emission spectrum of JC-1 is dependent on its concentration which, in turn, is determined by the status of the $\Delta\psi$. JC-1 can exist in two different states, aggregates or monomers, each with a different emission spectra. JC-1 forms monomers at low dye concentrations, and aggregates at higher concentrations. Both JC-1 aggregates and monomers exhibit fluorescence in the green end of the spectrum which is measured in the Green (FL-1) channel on flow cytometers.

When live cells are incubated with JC-1, JC-1 penetrates the plasma membrane of cells as monomers. Uptake of JC-1 into mitochondria is driven by the $\Delta\psi$. The $\Delta\psi$ of normal, healthy mitochondria is polarized and JC-1 is rapidly taken up by such mitochondria. This uptake increases the concentration gradient of JC-1 leading to the formation of JC-1 aggregates (known as J-aggregates) within the mitochondria. JC-1 aggregates show a red spectral shift resulting in higher levels of red fluorescence emission which is measured in the Red (FL-2) channel on most flow cytometers.[7]

Although, JC-1 fluorescence is seen in both the FL-1 and FL-2 channels in healthy cells, the pattern of JC-1 staining may vary between cell type or cell line. For example, the mitochondria of cardiac muscle cells are more polarized than those in bladder epithelial cells and have brighter red fluorescence. In addition to mitochondrial heterogeneity between cell lines, intercellular heterogeneity within a cell population may exist.[6] Therefore, the level of brightness in the FL-2 channel can vary both between and within cell types and lines.

JC-1 does not accumulate in mitochondria with depolarized $\Delta\psi$ and remains in the cytoplasm as monomers. These monomers do not have the red spectral shift, and therefore have lowered fluorescence in the FL-2 channel. The formation of JC-1 aggregates is reversible. Thus, in mitochondria undergoing a transition from polarized to depolarized $\Delta\psi$ (due to apoptosis or other physiological events), JC-1 leaks out of the mitochondria into the cytoplasm as monomers resulting in a decrease of red fluorescence.

The majority of cells in healthy cultures will have a polarized $\Delta\psi$, and hence show JC-1 fluorescence in both the FL-1 and FL-2 channels (***Figure 1, A-C***). It is likely that there will be a small population of cells that has significantly reduced FL-2 fluorescence. JC-1 that fluoresces in the FL-1 channel and lacks fluorescence in the FL-2 channel is indicative of depolarized $\Delta\psi$. Depolarized $\Delta\psi$ indicates altered mitochondrial function which may be due to apoptosis or other cellular processes. The percentage of cells showing reduced fluorescence in the FL-2 channel may vary depending on the cell type and culture system. However, a large percentage of cells with reduced fluorescence in the FL-2 channel in normal or control cultures may indicate that the health of the culture has been compromised.

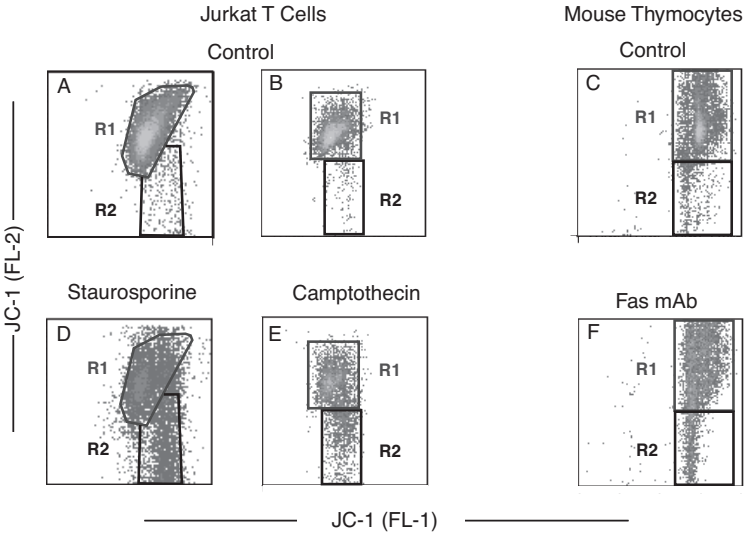

Figure 1. JC-1 Staining in Control and Apoptotic Cells. Cells (1×10^6 cells/ml) were left untreated (vehicle only, A-C) or treated with staurosporine (1µm, 4 h), camptothecin (4 µm, 4 h), or Fas mAb [clone Jo2, Cat. No. 554254 (2 µg/ml) and Protein G* (2 µg/ml), 1.5 h] to induce apoptosis (D-F). Cells were stained with JC-1 according to the protocol and analyzed on a BD FACSCalibur™ as described in the section Methods for Staining Cells with JC-1 and Analyzing by Flow Cytometry. (A-C) JC-1 fluorescence is seen in both the FL-2 and FL-1 channels (R1) in the control (untreated) cell populations. A small percentage of the population shows decreased fluorescence in the FL-2 channel (R2). (D-F) There is a significant increase in the number of cells with lowered red fluorescence [FL-2 (R2)], indicative of a change in the $\Delta\psi$, in the populations induced to undergo apoptosis. JC-1 that fluoresces in both the FL-2 and FL-1 channels is considered to correspond to mitochondria with a polarized $\Delta\psi$. JC-1 that fluoresces in the FL-1 channel and lacks fluorescence in the FL-2 channel is considered to correspond to mitochondria with a depolarized $\Delta\psi$. Thus, the data indicates that apoptosis induction was associated with depolarization of the $\Delta\psi$.

*The addition of Protein G enhances the ability of Jo2 to induce apoptosis, presumably by cross-linking Fas receptors.

Apoptosis is frequently associated with depolarization of the $\Delta\psi$, resulting in increased numbers of cells with reduced JC-1 fluorescence in the FL-2 channel (***Figure 1, D-F)***. That is, the apoptotic population frequently presents a lower red fluorescence signal intensity (***FL-2 axis***) than the negative control population. In some apoptotic systems, changes in the level of green fluorescence measured in FL-1 has also been observed. It is not clear how these changes relate to changes in the level of membrane polarization or apoptosis.

The BD™ MitoScreen Kit contains JC-1, which has been optimized for use in flow cytometry, and 10× Assay Buffer. JC-1 is typically excited using the 488 nm line of an argon ion laser. JC-1 monomers emit maximally at 527 nm and aggregates at 590 nm. In the existing literature, a number of ways have been used to set up flow cytometers for measurement of the FL-1 and FL-2 channels. Factors which can be varied include the PMT settings for each channel and the amount of compensation.

Data may appear differently depending on how the machine is set up. In this manual we provide a standardized approach to allow for reproducible instrument set up. Representative data obtained using the approach described herein is shown in ***Figure 1***. A partial list of publications containing figures with additional JC-1 flow cytometry data is provided in the section JC-1 Bibliography. Given potential differences in instrument settings, data obtained using the standardized set up may appear to be different from previously published data.

General Information: Kit Contents, Usage, and Storage

The BD™ MitoScreen Kit is designed for use in flow cytometry. It consists of two reagents, JC-1 and 10× Assay Buffer. JC-1 excites at 488 – 490 nm. The monomeric form emits at 527 nm; J-aggregates emit at 590 nm. There are enough reagents to test 100 samples by flow cytometry using the protocol provided. Store the unopened vials of JC-1 and 10× Assay Buffer at 2°C – 8°C.

JC-1: 4 Amber Vials/Lyophilized

There is enough JC-1 in each vial for 25 flow cytometry tests. JC-1 is supplied in amber vials because it is light sensitive. JC-1 is reconstituted prior to use, first into a Stock Solution and then into a Working Solution.

10× Assay Buffer: 60 ml

The 10× Assay Buffer is supplied in excess of what is generally needed for 100 flow cytometry tests. The 10× Assay Buffer is diluted to 1× prior to use.

Please refer to the section Kit Reagent Preparation for details on reconstitution of JC-1 and dilution of 10× Assay Buffer, and to ***Diagram 1*** for an overview of reagent preparation and subsequent JC-1 staining.

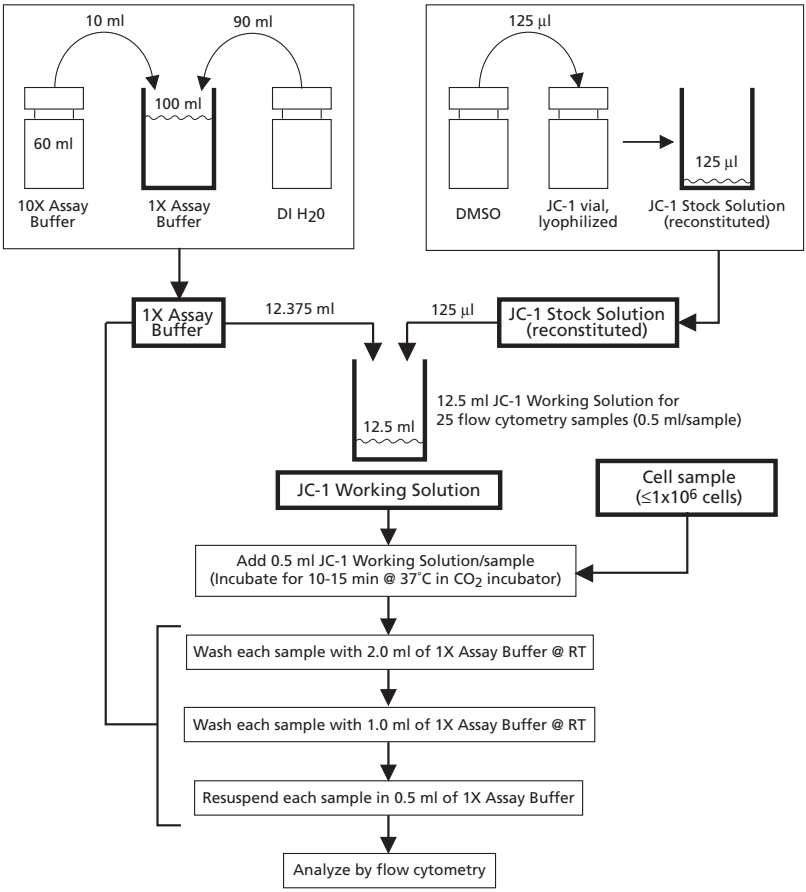

Diagram 1. Overview of Reagent Preparation and JC-1 Staining Protocol. This diagram summarizes the reconstitution of the BD™ MitoScreen Kit components and sample preparation. Please refer to the manual for details.

Usage

The BD™ MitoScreen Kit is compatible with a wide variety of cell model systems and treatment protocols. In flow cytometric applications utilizing intact cells, JC-1 has typically been used to study relationships between apoptosis and $\Delta\psi$. Most of the published literature utilizing the flow cytometry application has been focused on using JC-1 to study the effects of apoptosis on $\Delta\psi$.

There are numerous ways to induce apoptosis and each researcher may need to optimize protocols for their own experimental system. Researchers are strongly encouraged to refer to the extensive literature for information regarding different protocols that have been used to induce apoptosis. The optimal protocol for inducing apoptosis may be cell line dependent.

It is important to note apoptosis may be present in the absence of observed changes in JC-1 fluorescence. This is because depolarization of the $\Delta\psi$ does not always occur during apoptosis or may not be present at the time point examined. Researchers are encouraged to complement their JC-1 studies with other apoptosis assays to gain additional information about the status of their model system.

Kit Reagent Preparation

The 10× Assay Buffer is diluted to a 1× solution prior to use; and JC-1 is reconstituted into a Stock Solution and then diluted to a Working Solution prior to use (***Diagram 1***).

Dilution of 10× Assay Buffer

The Assay Buffer is formulated for use as reaction buffer, and for washing the cells. It is supplied as a 10× concentrate which must be diluted to 1× with DI H_20 prior to use.

1. To completely dissolve any salt crystals that may have come out of solution, gently warm the 10× Assay Buffer in a 37°C water bath.
2. Dilute the 10× Assay Buffer 1:10 in DI H_2O. For example, add 10 ml 10× Assay Buffer to 90 ml DI H_2O.
3. Stir the solution for 5 min.
4. Warm the 1× Assay Buffer to 37°C prior to use.

Note: We recommend diluting only the amount of 10× Assay Buffer that will be used in a given day. However, unused diluted (1×) assay buffer may be stored for up to 7 days at 2°C – 8°C.

Preparation of JC-1

JC-1 is supplied lyophilized. It is first prepared as a Stock Solution and then used as a Working Solution. The JC-1 Stock Solution may be aliquoted and stored at –20°C. The Working Solution, prepared from the Stock Solution, must be used immediately after preparation and cannot be stored.

Preparation of JC-1 Stock Solution (one vial)

1. Reconstitute the lyophilized JC-1 reagent at room temperature with 125 µl DMSO (per vial) to yield a JC-1 Stock Solution. Re-cap the vial and invert several times to fully dissolve the reagent.
2. The Stock Solution must be used immediately by diluting into a Working Solution (see ***Preparation of JC-1 Working Solution from Stock Solution***) or aliquoted into amber vials (to protect against light) and stored at –20°C (for up to six months).
3. Avoid repeated freeze/thaws of individual vials of JC-1 Stock Solution as this may compromise its integrity. Hence, we recommend that when making aliquots of JC-1 Stock Solution for freezing, that each aliquot contains only the amount that will likely be needed at the time of thawing.

Note: The kit contains 4 vials of lyophilized JC-1 reagent and we recommend reconstituting only the amount of vials needed for a given experiment

Preparation of JC-1 Working Solution from Stock Solution

1. Warm the 1× Assay Buffer to 37°C.
2. Prepare a 1× JC-1 Working Solution by diluting the JC-1 Stock Solution 1:100 with prewarmed 1× Assay Buffer. For example, add 125 µl JC-1 stock to 12.375 ml of prewarmed assay buffer. 0.5 ml JC-1 Working Solution is required for each sample (cell pellet containing $< 1 \times 10^6$ cells). Therefore, 12.5 ml JC-1 Working Solution would be enough for 25 flow cytometry samples.
3. Vortex the JC-1 Working Solution thoroughly.
4. After vortexing, particulate matter consisting of JC-1 aggregates may be present. This should not interfere with flow cytometric analysis. However, if desired, the solution can be clarified by centrifugation at 13,000 × g in a microfuge for 3 min, or 15 min in a centrifuge at 1,000 × g at RT. Transfer the clarified supernatant to a clean tube and discard particulates.
5. The JC-1 Working solution must be used immediately after preparation. Proceed to the section Methods for Staining Cells with JC-1 and Analyzing by Flow Cytometry for JC-1 staining and analysis protocols. Discard leftover JC-1 Working Solution, do not store.

Methods for Staining Cells with JC-1 and Analyzing by Flow Cytometry

An overview of staining cells with JC-1 for use in flow cytometry is illustrated in ***Diagram 1***. Details for staining and flow cytometric analysis follow.

Staining Cells with JC-1

1. Culture cells to an optimal density. Typically, cell density in the cell culture flasks should not exceed 1×10^6 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. Concentration can be determined by counting cell populations on a hemocytometer.
2. Proceed with treatment of choice (eg, apoptosis induction) or leave untreated.
3. At the end of the treatment period, transfer 1 ml of each cell suspension into a sterile 15 ml polystyrene centrifuge tube.
4. Centrifuge cells at 400 × g for 5 minutes at RT. Carefully remove and discard the supernatant.
5. Add 0.5 ml of freshly prepared JC-1 Working Solution to each pellet. Gently resuspend cells in the JC-1 Working Solution. Vortex or use a pipette to disrupt any cell-to-cell clumping.
6. Incubate the cells in JC-1 Working Solution for 10 – 15 min at 37°C in a CO_2 incubator. Wash cells twice following incubation (see below; ***wash steps are performed at RT***).
7. 1st wash: Add 2 ml of 1× Assay Buffer to each tube and gently resuspend cells. Vortex or use a pipette to disrupt any cell-to-cell clumping.
8. Centrifuge cells at 400 × g for 5 min.
9. Carefully remove and discard supernatant.
10. 2nd wash: Add 1 ml of 1× Assay Buffer to each tube and gently resuspend cells. Vortex or use a pipette to disrupt any cell-to-cell clumping.
11. Centrifuge the cells at 400 × g for 5 min.
12. Gently resuspend each cell pellet in 0.5 ml of 1× Assay Buffer. Vortex or use a pipette to disrupt any cell-to-cell clumping.
13. Analyze cells by flow cytometry (see ***Multi-Parameter Flow Cytometry Analysis of JC-1***).

Flow Cytometer Setup

The Cytometer setup information in this section is designed for the BD FACScan™, BD FACSort™, and BD FACSCalibur flow cytometers. The BD FACSComp™ software is used for setting up the flow cytometers. Compensation for FL-1/FL-2 should be set up using BD CaliBRITE™ beads (Cat. No. 349502).

Instrument Setup with BD CaliBRITE™ Beads

- Start-up the instrument
- Perform flow check
- Prepare tubes of BD CaliBRITE™ beads
- Launch BD FACSComp™ software
- Run BD FACSComp™ using the lyse/no wash procedure.

Note: For detailed information on using BD FACSComp with BD CaliBRITE beads to set up the flow cytometer, refer to the BD FACSComp Software User's Guide and the BD CaliBRITE Beads Package Insert.

Multi-parameter Flow Cytometry Analysis of JC-1

As noted earlier there are a large number of factors that can affect JC-1 fluorescence emission, both with respect to absolute intensity in the Green (FL-1) and Red (FL-2) channels and to the ratio of the two channels. Figure 1 shows the results of 3 different experiments done on different days each analyzed using the instrument setup described in the section Flow Cytometer Setup. Each experiment consists of a healthy control (untreated) population and a population induced to undergo apoptosis. Major populations (R1) are identified as FL-1 bright, FL-2 bright in the healthy controls, indicative of polarized $\Delta\psi$. Minor populations are identified that have decreased fluorescence in the FL-2 channel (R2) and are FL-1 bright, FL-2 dull, indicative of depolarized $\Delta\psi$. These observations are consistent with the notion that mitochondria in control cells are primarily healthy and functioning normally (polarized $\Delta\psi$). The small percentage of the control population of cells with depolarized $\Delta\psi$ may reflect a basal level of apoptosis or presence of other cellular processes that are associated with depolarized $\Delta\psi$.

The fluorescence pattern of JC staining differs dramatically even on healthy control cells (***Figure 1, A-C***). For example, a comparison of the major populations (R1) in ***Figure 1***A (human Jurkat cells) and C (primary mouse thymocytes) shows that the control Jurkat cells show lower fluorescence intensity in both the FL-1 and FL-2 channels. The pattern can even be different using the same cell type on different days (***compare A and B***).

There is a significant increase in the number of cells with lowered red fluorescence [FL-2 (R2)] in the apoptotic compared to the corresponding control populations (***compare Figure 1, D-F to A-C, respectively***). These results are consistent with the notion that apoptosis is often associated with a transition from polarized to depolarized $\Delta\psi$.

Guidelines for Setting Gates

As the pattern of JC-1 staining can vary, it is not possible to provide users with universal guidelines for how to set gates for healthy, non-apoptotic {FL-1bright, FL-2bright; R1} and apoptotic {FL-1bright, FL-2dull; R2} cells. Each user will have to define the appropriate gates for their experimental model system of apoptosis. The following are some guidelines that will help set appropriate gates.

1. Given the variability it is critical that whenever possible, positive (induced apoptotic) and control (untreated) samples be included in every experiment for every cell type. In preliminary experiments it is strongly recommended that the presence (in the induced sample) or absence (in the control sample) of apoptotic cells be supported by an alternative apoptosis assay such as Annexin V or Active Caspase 3. These products are available in a number of formats; please visit www.bdbiosciences.com for more information on our full line of apoptosis products.

2. The R1 (non-apoptotic cell) gate should be set to encompass the major population(s) in the control sample. ***Figure 1*** A-C show the gates set for three different experiments. The shape and placement of the gate will vary from cell type to cell type. Generally, there is clearly a single major distinct population of FL-1 bright, FL-2 bright cells as in ***Figure 1*** A and B. However, because the shape and size of the R1 gate can vary on different days, direct comparisons between similar cell types using this approach should be carried out on the same day.

In some cases the boundaries of the major population may be harder to define, making the division between FL-1 bright, FL-2 bright and FL-1 bright, FL-2 dull more arbitrary, as is shown in C. In these cases, it is particularly critical to define the approximate percentage of apoptotic cells through alternative assays.

3. Since apoptotic cells typically lose JC-1 fluorescence in the FL-2 channel the R2 (apoptotic cell) gate is drawn to encompass the area directly below the R1 gate as shown in ***Figure 1*** A-C. The accuracy of these gates is best confirmed using the positive induced sample with demonstrable apoptotic cells (***Figure 1, D-F***).

4. Once the appropriate R1 and R2 gates have been determined using the control and treated samples they should be maintained throughout all samples of equivalent cell types for that experiment on a given day.

5. As the user becomes familiar with their experimental system they may wish to set up gating templates that can be individually adjusted for each experiment. It should always be kept in mind, that the actual $\Delta\psi$ JC-1 staining profile seen in a given population, control or treated, may depend on a variety of factors, including the model system, the cell type, the overall health of the cell culture, and treatment type or time.

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The following is a partial list of publications containing figures with cells stained with JC-1 and analyzed by flow cytometric analysis.

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   Model system: U937 and K562 cells treated with valinomycin

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   Model system: mouse thymocytes treated with dexamethasone

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   Model system: U937 cells treated with ouabain, KCl, valinomycin, or FCCP

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   Model system: human peripheral blood mononuclear cells treated with 2-chloro-adenosine and 2-chloro-2'-deoxy-adenosine

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   Model system: HL-60 cells treated with etoposide

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Model system: rat cardiomyocytes

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Model system: acute myeloblastic leukemia cell line (OCI/AML-2) subclones treated with etoposide

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Model system: HL60 cells treated with staurosporine

Notes

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