

JC-1 Mitochondrial Membrane Potential

Introduction

Background

Apoptosis is a cellular process involving a genetically programmed series of events leading to the death of a cell. During this process, several key events occur in mitochondria, including the release of caspase activators such as cytochrome c, changes in electron transport, and loss of mitochondrial transmembrane potential ($\Delta\Psi_m$).¹ For this reason, $\Delta\Psi_m$ is an important parameter of mitochondrial function and has been used as an indicator of healthy cells.

Variation of $\Delta\Psi_m$ has been previously studied by evaluating the changes in fluorescence intensity of cells stained with cationic dyes such as rhodamine-123 (Rh123) and DiOC₆(3).² However, data obtained by using these probes may not be reliable. For example, Rh123 is relatively insensitive to $\Delta\Psi_m$ changes and DiOC₆(3) cannot distinguish between depolarization of the plasma membrane and changes at $\Delta\Psi_m$ in several physiological or pathological conditions when both events can take place.³ More recently, a new cytofluorimetric, lipophilic cationic dye, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) has been developed. JC-1 has advantages over other cationic dyes in that it can selectively enter into mitochondria and reversibly change color from red to green as the membrane potential decreases. In healthy cells with high mitochondrial $\Delta\Psi_m$, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. On the other hand, in apoptotic

or unhealthy cells with low $\Delta\Psi_m$, JC-1 remains in the monomeric form, which shows only green fluorescence. The ratio of green to red fluorescence is dependent only on the membrane potential and not other factors such as mitochondrial size, shape, and density, which may influence single-component fluorescence signals. Flow cytometric analysis of JC-1 fluorescence is best performed using two dimensional green versus red fluorescence plots, where the ratio of green to red fluorescence allows comparative measurements of membrane potential between cell populations.

Application of JC-1 for Flow Cytometry

Flow cytometry has emerged as the technique of choice for analysis of the $\Delta\Psi_m$ in whole cells. JC-1 is excited using an argon laser at a wavelength of 488 nm. Both JC-1 aggregates and monomers exhibit green fluorescence (peak emission at 527 nm) which is measured in the FL1 channel (530 nm) on the Accuri C6 Flow Cytometer®. JC-1 aggregates show a red spectral shift (peak emission at 590 nm), and are measured in the FL2 channel (585 nm) of the C6 Flow Cytometer. Thus, healthy non-apoptotic cells will be detected in both FL1 and FL2 channels (FL1^{bright}, FL2^{bright}) and cells with altered mitochondrial function due to apoptosis or other cellular processes will remain bright in the FL1 channel, but will have reduced FL2 intensity (FL1^{bright}, FL2^{dim}).

Assay Protocol

This protocol was performed using Accuri's JC-1 Mitochondrial Potential Assay Kit (KR310).

Sample Set-up

See Table 1 for a summary of recommended samples. Several considerations need to be made when designing a JC-1 experiment.

- JC-1 fluorescence is measured in the FL1 (530 nm) and FL2 (585 nm) channels of the C6 Flow Cytometer, necessitating the use of fluorescence compensation controls for proper analysis.
- To facilitate setting FL2 minus FL1 fluorescence compensation, Accuri suggests treating cells from each experimental group with CCCP to create a strong, single positive green fluorescence control. CCCP causes quick mitochondrial membrane depolarization, greatly reducing the FL2 red fluorescence signal, thus providing a green-signal only positive control.
- An unstained control sample should also be included for each cell type and experimental condition used.

Tube	Sample Type	JC-1	FL1 (530 nm)	FL2 (585 nm)	Purpose
1	Cells	-	-	-	Unstained control, Set P1 Gate
2	Cells	+	+	Variable	Set P2 Gate
3	Cells plus CCCP (depolarized)	+	+	-	Set P3 Gate, Apply FL2-FL1 Compensation

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Cell Culture

1. Culture cells in multi-well plates or tissue culture flasks to a density optimal for apoptosis induction (typically $\leq 1 \times 10^6$ cells/mL) according to your protocol. Duplicate or triplicate wells/flasks should be prepared for each experimental group.
! CAUTION: Cells cultivated at densities $> 10^6$ /mL may begin to naturally enter apoptosis. The cell concentration and protocol for inducing apoptosis should be optimized for each experimental system.
2. Be sure to include at least one well or flask of untreated cells for each cell type and treatment condition to serve as control cells.
3. Induce apoptosis according to your protocol.
4. During the last 5 minutes of apoptosis induction, prepare the FL1-only positive fluorescence control sample by adding 1 μ L of 50 mM CCCP per 1 mL culture volume to the untreated cell samples ($C_f = 50 \mu$ M).

Preparation of JC-1 Staining Solution

(Begin preparation 30 minutes prior to the end of cell treatment.)

1. Warm fresh culture medium to 37°C.
2. Thaw an aliquot of the JC-1 Reagent at room temperature (RT). Make sure JC-1 is completely thawed and warmed to RT before diluting.
3. Mix thawed JC-1 well before dilution.
4. Prepare the JC-1 Staining Solution by diluting the reagent 1:10 in pre-warmed culture media.
5. Mix well to dissolve all particulates.

! CAUTION: Do not centrifuge the JC-1 reagent.

Staining Protocol: 12x75 mm Tubes

NOTE: The JC-1 concentrations and incubation times above should be used as a starting point. Experiments to titrate the JC-1 concentration and incubation time may be required to optimize assay results.

1. Use fresh culture medium warmed to 37°C.
2. After cell treatment/apoptosis induction, transfer 1 mL of each cell suspension into 12x75 mm polystyrene tubes.
3. Add 100 μ L JC-1 Staining Solution or 100 μ L culture media, according to staining condition required (see Table 1).
4. Pipette to disrupt any cell clumping.
5. Incubate samples in a CO₂ incubator at 37°C for 15-30 minutes.
6. Add 2 mL Assay Buffer (at RT) to each tube, with gentle mixing.
7. Centrifuge cells at 400 x g for 5 minutes at RT.
8. Remove the supernatant.
9. Re-suspend cells in 300-500 μ L Assay Buffer. Pipette to disrupt cell clumping.
10. Analyze samples by flow cytometry within 1.5 hour. Samples cannot be fixed.

Staining Protocol: 96-Well Plate

1. Use fresh culture medium warmed to 37°C.
2. After cell treatment/apoptosis induction, transfer 100 μ L of each cell suspension into wells of a 96-well plate (V- or U-bottom recommended).
3. Add 10 μ L JC-1 Staining Solution or 10 μ L culture media, according to staining condition required (see Table 1).
4. Pipette to disrupt any cell clumping.
5. Incubate samples in a CO₂ incubator at 37°C for 15-30 minutes.
6. Add 100 μ L Assay Buffer (at RT) to each well with gentle mixing.

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- Centrifuge cells at 400 x g for 5 minutes at RT.
- Remove the supernatant.
- Re-suspend cells in 200 μ L Assay Buffer.
- Pipette to disrupt cell clumping and transfer stained cells to tube of preference containing an additional 100 to 200 μ L Assay Buffer -OR- Analyze samples directly from the 96-well plate if using the C6 Flow Cytometer equipped with a CSampler™.
- Analyze samples by flow cytometry within 1.5 hour. Samples cannot be fixed.

Flow Cytometric Analysis

Forward and side scatter signals (FSC and SSC) and JC-1 staining patterns can vary among cell types and experimental protocols. Therefore, it is not possible to provide users with universal guidelines for setting gates. Shown here are representative examples of JC-1 analysis for a cell line (Jurkat) and for freshly isolated mouse thymocytes.

- Set FSC-H Threshold to channel 80,000 (for mammalian cell analysis).
- Create a FSC vs. SSC density plot.
 - For small cell lines and freshly isolated lymph tissues (spleen, etc.) use linear FSC (channel range 0 to 1.6×10^6) vs. linear SSC (channel range 0 to 800,000) as a starting point.
 - For larger cell lines, use log FSC vs. log SSC, then Zoom in around the main cell population after data collection.
- Create a log FL1 (X-axis) vs. log FL2 (Y-axis) density plot. (Choose either Area or Height signals.)
- Collect 50,000 to 100,000 total events from each of the three tubes listed in Table 1.
- View data for the unstained control.
- Draw a polygon gate (P1) around the cells, excluding debris, on the FSC vs. SSC plot (Figure 1a).

NOTE: Dead/dying cells tend to have a lower FSC signal than viable cells. Both populations may need to be included in the gate (Figure 1a) in order to obtain accurate red-to-green shift data in your experimental samples.

- Apply "P1 in all events" to the FL1 vs. FL2 plot (Figure 1b).

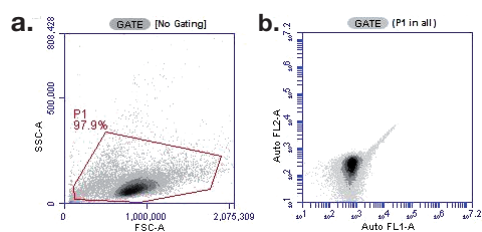


Figure 1.

- Light scatter gate with P1 drawn,
- FL1 vs. FL2 with Gate (P1 in all) applied for unstained, untreated cells (Tube 1).

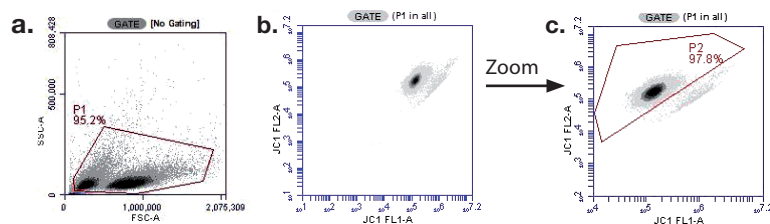


Figure 2.

- Light scatter gate and
- FL1 vs. FL2 for cells with JC-1 and without CCCP (Tube 2).
- Zoom of FL1 vs. FL2 plot with P2 gate drawn.

- View staining of cells with JC-1 using sample Tube 2 (Table 1). Adjust P1 if needed (Figure 2a).
- The FL1 vs. FL2 plot should now contain a large, double positive population (Figure 2b).
- Adjust the FL1 axis view with Zoom or Plot Spec Tools (Figure 2b). Draw a polygonal gate, P2, around the main population (Figure 2c).
- View JC-1 staining of sample Tube 3 (Figure 3a). The percent of cells in the P2 gate for the CCCP control will be the "background" value to be subtracted from the percent P2 events in all test samples of the applicable treatment group.

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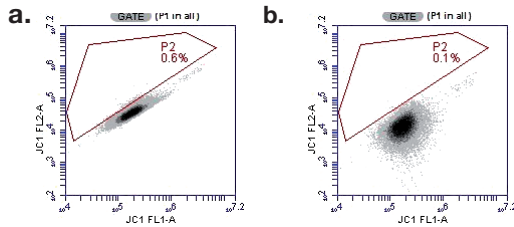


Figure 3.

(a) JC1-FL1 vs. JC1-FL2 plot for cells with JC-1 and CCCP (Tube 3)
 (b) same plot after 12% FL2 minus FL1 compensation has been applied.

12. Two methods are available to reduce background if an unacceptable percentage of cells from Tube 3 fall inside P2. With the high resolution of the Accuri C6 Flow Cytometer, we have found that using the CFlow[®] Zoom Tool to precisely adjust the P2 gate to exclude background is effective without the need for fluorescence compensation. Alternatively, fluorescence compensation can be used to further resolve the FL2+ and FL2- populations (Figure 3b). We have obtained equivalent results using either method (Figure 4).

Method 1: CFlow Zoom Tool

- Use the Zoom Tool to magnify the region of the JC1-FL1 vs. JC1-FL2 plot where the FL2- and FL2+ populations seem to overlap. Zoom can be performed more than once if necessary.
- Adjust the perimeter of P2 to more precisely exclude background from the CCCP control sample (Tube 3).
- Upon achieving an acceptable background level, zoom out as needed and proceed to Step 13.

Method 2: Set Compensation

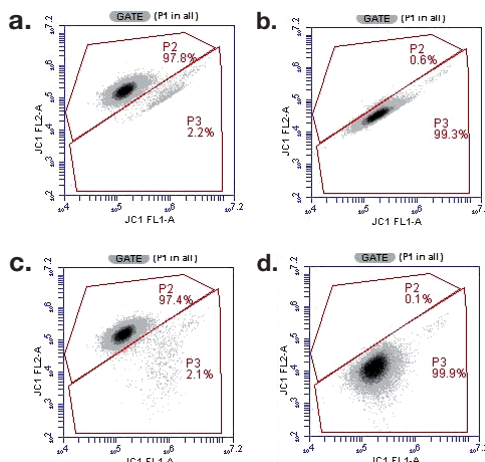
- Open the Compensation Settings dialog box.
- Click on the “FL1” button under the words “Correct FL2 by subtracting a percentage of”.
- A value between 11% and 12% is a good starting point on the C6 Flow Cytometer.
- Click the “Preview” button.
- If an acceptable background for Tube 3 now falls inside P2 (Figure 3b), review the data for Tube 2 (Figure 4c).
- Apply these correction values to all samples (Compensation Settings dialog box).

13. While viewing the data for Tube 3, draw a polygon P3 around the major population of cells, and then adjust the lower edge of P2 to meet (but not overlap) the upper edge of P3 (Figure 4).

14. Set an appropriate Run Limit in the CFlow Collect Tab (from 10,000 to 50,000 cells in P1) and collect the data for all remaining samples.

Figure 4.

JC1-FL1 vs. JC1-FL2 before (a and b) and after (c and d) 12% FL2 minus FL1 correction has been applied to cells with JC-1 and without CCCP sample (Tube 2) and cells with JC-1 and CCCP sample (Tube 3). P3 is drawn around the major population of the cells with JC-1 and CCCP sample (Tube 3), and the lower border of P2 is adjusted to meet the upper border of P3.



No (0%) FL2 minus FL1 compensation:

97.8% (cells with JC-1 and without CCCP) – 0.6%
 (cells with JC-1 and CCCP) = 97.2%

12% FL2 minus FL1 compensation:

97.4% (cells with JC-1 and without CCCP) – 0.1%
 (cells with JC-1 and CCCP) = 97.6%

15. The appropriate P1, P2 and P3 regions within each experimental group may need to be adjusted, as light scatter and JC-1 staining characteristics may change with treatment, BUT:
16. Once the appropriate P1, P2, and P3 gates have been determined using the untreated, apoptotic and CCCP control samples, the location must be maintained for all samples within a given cell type/treatment group for that experiment on a given day.
17. In addition to cell lines, the JC-1 assay protocol also works well on fresh tissue isolates (Figure 5).

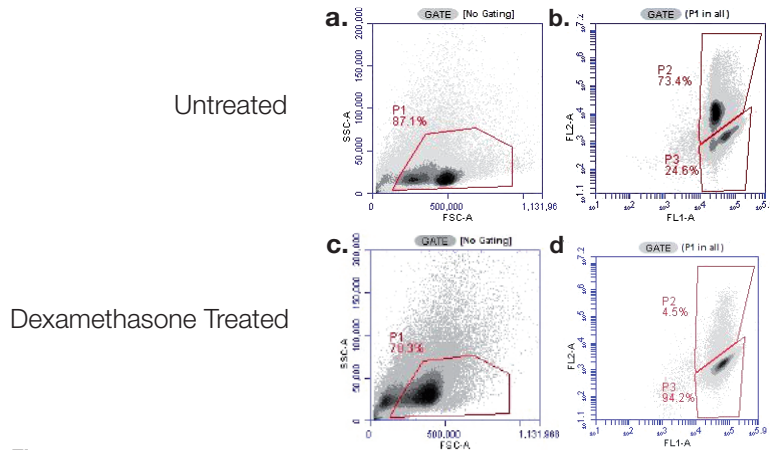


Figure 5.

Effect of dexamethasone on mitochondrial membrane potential in primary thymocytes. Mouse thymocytes were isolated from 24-week old C57B6 mice and mechanically disrupted. Cell suspensions were incubated for 24 hours in a 37°C, 5% CO₂ incubator either with or without 10 μM dexamethasone. Cells were stained according to the kit directions. The majority (>73%) of untreated mouse thymocytes maintained normal ΔΨ_m with bright JC-1 staining detected in both FL1 and FL2 (FL1^{bright}, FL2^{bright}; upper right plot), while >94% of cells exposed to dexamethasone remained bright in the FL1 channel but had reduced FL2 intensity (FL1^{bright}, FL2^{dim}; lower right plot), indicative of compromised ΔΨ_m.

Co-staining with JC-1, Reagents Detectable in FL3 and FL4

It is possible to co-label cells with JC-1 and antibodies for cell surface markers or other viability markers, as long as the fluorescence emission of the additional label is spectrally resolved from JC-1.

Compatible dyes include, but may not be limited to:

- The viability dye 7-amino actinomycin D (7-AAD; detectable in FL3 of the C6 Flow Cytometer),
- Fluorochromes: PE-Cy7 (FL3), PerCP-Cy5.5 (FL3), APC (FL4) and Alexa 647 (FL4).

When planning to include additional markers, single-color control samples need to be included for each marker to allow correct fluorescence compensation. See Table 2 for suggested samples.

Table 2. Sample for JC-1 Staining plus FL3 and FL4 Fluorochromes						
Tube	Sample Type	JC-1	FL1 (530 nm)	FL2 (585 nm)	FL3 (670 nm)	FL4 (675 nm)
1	Cells	-	-	-	-	-
2	Cells	+	+	variable	-	-
3	Cells + CCCP (depolarized)	+	+	-	-	-
4	Cells + CCCP (depolarized)	+	+	-	+	-
5	Cells + CCCP (depolarized)	+	+	-	-	+
6	Cells + CCCP (depolarized)	+	+	-	+	+
7	Cells + CCCP (depolarized)	+	+	variable	+	+

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Antibodies or washable dyes:

1. Add with the JC-1 staining solution in Step 3 of the JC-1 Staining Protocol.
2. All incubations and washes should be performed according to the remaining steps of the JC-1 protocol.
3. Analyze samples within 1.5 hour by flow cytometry. Samples cannot be fixed.

Annexin-V:

1. Perform Steps 1-8 of the JC-1 Staining Protocol.
2. Resuspend in calcium-containing Annexin binding buffer warmed to 37°C and stain according to the manufacturer's recommendations.
3. Analyze samples immediately by flow cytometry. Samples cannot be fixed.

7-AAD:

1. Perform steps 1-9 of the JC-1 Staining Protocol.
2. Add 7-AAD cell viability stain to appropriate samples and stain according to manufacturer's recommendations.
3. Analyze samples immediately by flow cytometry. Samples cannot be fixed.

Trouble-Shooting

Problem	Possible Causes	Recommended Solutions
No staining	Samples have been fixed with a fixative such as paraformaldehyde.	Assays should be performed without any fixation.
Poor staining	<ol style="list-style-type: none"> 1. JC-1 staining solution has been centrifuged. 2. Stained cells have been exposed to strong light. 	<ol style="list-style-type: none"> 1. Do not centrifuge JC-1 staining solution as this will precipitate the reagent. 2. Analyze the stained cells immediately after washing.
Control cells without treatment show low ratio of red to green signal	Control cells are not healthy.	Use only healthy cells.
Staining is too strong	JC-1 staining solution is too concentrated for this cell type.	Dilute JC-1 staining solution.

References

1. Green, D.R. and Reed, J.C. Mitochondria and apoptosis. *Science* 281, 1309-1312 (1998).
2. Petit, P.X., Lecoeur, H., Zorn, E., et al. Alterations in mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis. *J. Cell Biol.* 130 (1), 157-167 (1995).
3. Salvioli, S., Ardizzoni, A., Franceschi, C., et al. JC-1, but not DiOC₆(3) or rhodamine 123, is a reliable fluorescent probe to assess $\Delta\Psi_m$ changes in intact cells: Implications for studies on mitochondrial functionality during apoptosis. *FEBS Lett.* 411, 77-82 (1997).

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