Uncompensated Polychromatic Analysis of Mitochondrial Membrane Potential Using JC-1 and Multilaser Excitation

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The lipophilic cation JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl carbocyanine iodide) has been used for more than 20 years as a specific dye for measuring mitochondrial membrane potential ($\Delta\Psi_m$). In this unit, we revise our original protocol (that made use of a single 488 nm laser for the detection of monomers and aggregates, and where compensation was an important step) to use dual-laser excitation. Moreover, thanks to recently developed multilaser instruments and novel probes for surface and intracellular markers, JC-1 can be utilized by polychromatic flow cytometry to simultaneously detect, without any compensation between fluorescences, $\Delta\Psi_m$ along with other biological parameters, such as apoptosis and the production of reactive oxygen species. © 2015 by John Wiley & Sons, Inc.

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INTRODUCTION

The dissipation of the mitochondrial transmembrane potential ($\Delta\Psi_m$) constitutes an early and irreversible step in the cascade of events that, in several cell types, can lead to programmed cell death (apoptosis) (Galluzzi et al., 2012).

Several probes are available to measure $\Delta\Psi_m$ by flow cytometry, but some of them have a low specificity for this organelle; conflicting data in the literature about the role of $\Delta\Psi_m$ dissipation during the apoptotic process could be, at least in part, ascribed to this lack of specificity.

After excitation with a blue laser at 488 nm, the fluorescent dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl carbocyanine iodide (JC-1), a lipophilic cation existing in a monomeric form, emits in the green region. However, in mitochondria that have a high $\Delta\Psi_m$, JC-1 forms so called J-aggregates, described almost 80 years ago (Jelley, 1936), and undergoes a reversible change in fluorescence emission from green to orange. Using common flow cytometers equipped with such lasers, for several years mitochondria have been studied by detecting the two emissions of JC-1 by the normal filters present in FL1 (for monomers) and FL2 (for aggregates) (Cossarizza et al., 1993; Cossarizza et al., 1995; Polla et al., 1996; Cossarizza et al., 1997; Salvioli et al., 2000; Cossarizza et al., 2002; Lugli et al., 2007; Troiano et al., 2007; Gibellini et al., 2012; Abu et al., 2014; Marringa et al., 2014; also see older version

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of this unit at *http://onlinelibrary.wiley.com/doi/10.1002/0471142956.cy0732s41/full*). Measurements using this dye provide information on changes in $\Delta\Psi_m$ (typically, a decrease in $\Delta\Psi_m$ causes a relevant shift from orange to green fluorescence emission), as well as on total mitochondrial content (based on the intensity of the green fluorescence emission). A number of studies have since shown the superiority of JC-1 over other dyes—e.g., rhodamine 123 (R123) or 3,3′-dihexyloxadicarbocyanine iodide [$DiOC_6(3)$]—that were used for the same purpose, and demonstrated that JC-1 is also unaffected by changes in plasma membrane potential (Salvioli et al., 1997; Lugli et al., 2007; Troiano et al., 2007; also see older version of this unit at *http://onlinelibrary.wiley.com/doi/10.1002/0471142956.cy0732s41/full*).

This unit discusses a new method to detect JC-1 (see Basic Protocol 1), based upon the use of two lasers, one to excite JC-1 monomers (by the canonical 488-nm laser line), and the other to excite JC-1 aggregates (by a yellow laser emitting at 561 nm). The typical excitation by the blue laser excites JC-1 with high efficiency, but sometimes requires significant compensation between FL1 and FL2. In contrast, yellow laser allows a better resolution, and thus a clearer visualization of monomers and aggregates without compensation (Perelman et al., 2012). For this reason, we have revised our basic JC-1 protocol using the two different lasers quoted above.

Furthermore, we have recently developed another polychromatic flow cytometric assay (see Basic Protocol 2) utilizing JC-1 and other probes for the simultaneous detection of $\Delta\Psi_m$, reactive oxygen species (ROS, by CellRox DeepRed), and apoptosis (by Annexin V, detecting the exposure of phosphatidylserine on the plasma membrane). This protocol can be applied when the simultaneous analysis of multiple parameters during apoptosis is required, e.g., in investigating the role of certain proteins on cell phenotype or when testing the cytotoxicity of compounds of pharmacological interest.

CAUTION: For the protection of laboratory personnel from potential infectious agents (e.g., hepatitis and HIV), handle human samples using disposable gloves in a biological safety cabinet.

CAUTION: All probes described in this unit are potentially hazardous (see manufacturers' MSDSs), and users should wear gloves during the staining procedures.

BASIC PROTOCOL 1

BASIC DETERMINATION OF MITOCHONDRIAL MEMBRANE POTENTIAL USING JC-1: DUAL-LASER EXCITATION OF THE DYE AVOIDS COMPENSATION ISSUES

This protocol is intended for cells such as peripheral blood mononuclear cells (PBMCs) or cell lines such as RKO, HL60, MCF7, and U937. Other cell types may also be stained using minor adjustments to the steps described below.

Typically, by using a 488-nm blue laser, it can be observed that cells with high $\Delta\Psi_m$ (that form JC-1 aggregates) emit orange fluorescence (at ~590 nm); those with low $\Delta\Psi_m$ (containing JC-1 in its monomeric form) emit green fluorescence (at ~520 nm) (Cossarizza et al., 1993). Recently it has been demonstrated that alternative excitation wavelengths can facilitate the detection of $\Delta\Psi_m$, and, most importantly, use of two wavelengths avoids the need for compensation. Indeed, the excitation wavelength 561 nm (i.e., yellow laser) is above the emission spectra of JC-1 monomers, and selectively excites J-aggregates; hence there is no need to compensate green and orange fluorescence (Perelman et al., 2012). Thus, we have adapted our original protocol (that made use of a single 488-nm laser, and where compensation was an important step) to an instrument equipped with a blue and a yellow laser (like the Attune NxT, from Life Technologies).

Materials

Experimental samples: human peripheral blood lymphocytes or monocytes, or human tumor cell lines (e.g., RKO, HL60, U937, MCF7); here we use RKO cells, which derive from a colon carcinoma and grow adherent to the plastic flask

Complete RPMI culture medium, 1 ml per sample

1 M valinomycin [dissolve valinomycin (mol. wt. 1111.32; Sigma-Aldrich) in dimethylformamide (DMF) and store in a glass container up to 6 months at 4°C] *or* 1 mM carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP; Sigma Aldrich)

2.5 mg/ml JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide): prepare by dissolving JC-1 (Life Technologies, Thermo Fisher Scientific) in dimethylformamide (DMF); store in a glass container up to 2 years at –20°C, protected from light

Phosphate-buffered saline (PBS)

3.5-ml, 55 × 12–mm plastic tubes (Sarstedt, or equivalent)

Centrifuge (Minifuge RF; Heraeus), or equivalent

Flow cytometer equipped with a 488-nm blue laser and with a 561-nm yellow laser, e.g., Attune NxT (Life Technologies)

Additional reagents and equipment for counting (*APPENDIX 3A*) and culturing (*APPENDIX 3B*) mammalian cells

Prepare cells

1. Count a sample of the experimental cells of interest (*APPENDIX 3A*).

   This protocol can be used to stain either cells growing in suspension or adherent cells after they have been released from the plate by trypsinization (APPENDIX 3B) and counted (APPENDIX 3A).

2. Collect at least 2×10^5 cells from the experimental samples in 55 × 12–mm tubes by centrifuging 5 min at 300 × *g*, room temperature. Collect the same number of cells to use for a positive control.

3. Decant and discard the medium and resuspend the cell pellet in 1 ml fresh complete RPMI culture medium.

4. For obtaining a so-called "positive control," i.e., a sample where all cells have depolarized mitochondria, prepare one sample of cells treated with valinomycin (final concentration 0.1 μM) or with carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP, final concentration 250 nM). Incubate 10 min or 45 min, respectively, at 37°C.

   Drugs such as the K^+ ionophore valinomycin or the proton translocator FCCP are able to collapse the $\Delta\Psi_m$.

   Note that to avoid problems related to intracellular drug metabolism, in some instances valinomycin is preferred over FCCP or ClCCP (and is also less expensive).

Stain with JC-1

5. Add 1 μl of 2.5 mg/ml JC-1 fluorescent probe (2.5 μg/ml final concentration) to the experimental and positive control cells and shake the cell suspension until the dye is well dispersed and gives a uniform red-violet color.

   JC-1 tends to form aggregates when added to normal aqueous medium. To avoid this, add the probe while gently vortexing.

6. Incubate the samples 10 min in the dark, 37°C.

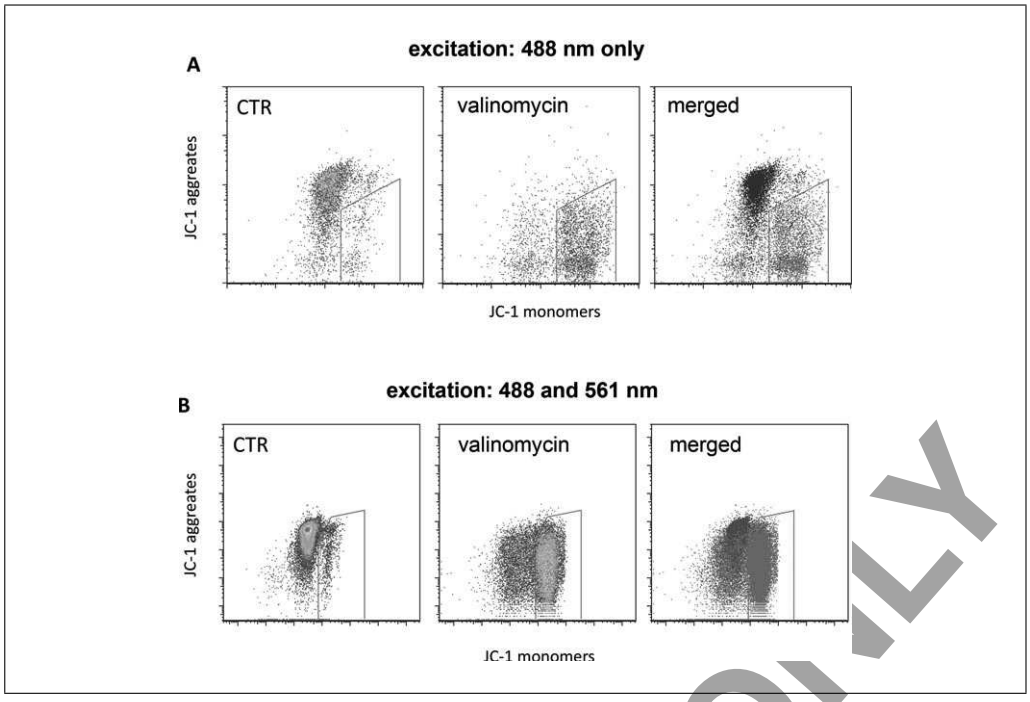

Figure 7.32.1 Changes in JC-1 fluorescence after mitochondrial membrane depolarization in RKO cells treated with valinomycin, as described in Basic Protocol 1. Samples were acquired using 488-nm laser only (**A**), or with dual-laser excitation (**B**). Control cells (CTR) were stained with 2.5 μg/ml JC-1. Note the shift to the bottom and to the right of cells with mitochondria depolarized by treatment with 100 nM valinomycin. Right panel shows the merging of the left and center panels. Green-orange compensation was ~4% and orange-green compensation was ~10%; compensation was required to better visualize monomers and aggregates.

All reagents must be at room temperature and carefully checked for pH (7.4) when used, because $\Delta\Psi_m$ is very sensitive to alterations of these conditions.

The staining procedure must be carried out away from direct intense light, and incubation must be in the dark because of the light sensitivity of JC-1.

7. Wash the cells by centrifuging 5 min at 300 × *g*, room temperature, discarding the supernatant, and resuspending the cells in 1 ml PBS for analysis on cytometer.

Set up flow cytometer

8. Detect JC-1 fluorescence of the experimental and positive control samples using a classical green band-pass filter centered at 525/50 nm for monomers detection (channel of blue laser) and a classical greenish orange band-pass filter centered at 585/42 nm (usually those for a channel collecting fluorescence signals deriving from the excitation with the blue or the yellow laser).

The most common flow cytometers are typically equipped with only a 488-nm argon or solid-state laser; no special requirements are needed to analyze $\Delta\Psi_m$. The gain of photomultipliers (PMTs) obviously depends on the cytometer used, but generally JC-1 does not require any substantial increase in PMT amplification; green-orange compensation can be ~4% and orange-green compensation ~10%. However, note that no compensation is needed if a blue and a yellow laser are used to detect monomers and aggregates, respectively.

See Figure 7.32.1 for a typical example of JC-1 staining of control (CTR) RKO cells, and of RKO cells treated with valinomycin. Detection was performed by using a single blue laser (A) or using blue and yellow lasers (B). This treatment results in a relevant change in the fluorescence distribution: cells with depolarized mitochondria can be easily identified as those going from the center of the plot to the lower right quadrant.

9. On the basis of the laser used, adjust the voltage of the respective PMTs to obtain the bivariate green versus orange distributions similar to those shown in Figure 7.32.1A and B, and then record the control sample. Use the same PMT settings for the subsequent samples.

Analyze JC-1 stained experimental samples

10. Acquire fluorescence data for experimental samples in listmode, using a log scale for the fluorescence channels.

    Cells with high $\Delta\Psi_m$ are those forming J-aggregates; thus, they show high orange fluorescence. On the other hand, cells with low $\Delta\Psi_m$ are those in which JC-1 maintains (or re-acquires) its monomeric form, and thus show green fluorescence. Once mitochondria are depolarized, JC-1 monomers redistribute in other membranous compartments with lower $\Delta\Psi$. As a consequence, the green fluorescence intensity of depolarized cells is a little bit higher than that of polarized ones simply because of the presence of a higher amount of JC-1 monomers inside the cell.

11. *Recommended for samples with heterogeneous cell populations:* Set a gate on the population of interest, then proceed with adjustment of PMTs, as well as compensation if a 488-nm laser is used. Dual-laser excitation of the dye does not require compensation.

    When the sample contains a heterogeneous cell population, it is possible to see different fluorescence patterns due to different autofluorescences and the variable content in terms of membranes and mitochondria of cell subpopulations. This is the case for peripheral blood mononuclear cells (PBMCs), lymphocytes, and monocytes, the first being smaller and having fewer mitochondria than the latter. Accordingly, the fluorescence pattern of JC-1 for such a sample shows at least two distinct peaks, one corresponding to lymphocytes, and the second, brighter in both green and orange, corresponding to monocytes. It is thus recommended to first set a gate on the population of interest, then proceed with adjustment of PMTs and compensation.

ANALYSIS OF $\Delta\Psi_M$, APOPTOSIS, AND REACTIVE OXYGEN SPECIES CONTENT BY 4-LASER POLYCHROMATIC FLOW CYTOMETRY

BASIC PROTOCOL 2

This protocol allows the analysis of $\Delta\Psi_m$ along with the detection of early apoptotic cells, and the quantification of the amount of reactive oxygen species in the cells of interest. It has been developed taking into account the possibility of simultaneously using four lasers (by using an Attune NxT from Life Technologies) and avoiding any compensation among dyes.

Fine analysis of the apoptotic process requires the detection of multiple cell functions at the same time, and it could be highly informative to reveal whether cells with different $\Delta\Psi_m$ also differ with respect to other parameters. This assay is recommended when studying compounds that can have differential effects on the cell populations of interest.

This protocol uses three different probes: JC-1 (for $\Delta\Psi_m$), annexin V conjugated with Pacific Blue (for detecting the exposure of phosphatidylserine on the plasma membrane, a well known phenomenon which identifies early apoptotic cells), and CellRox Deep Red (for measuring ROS production). CellRox is a cytoplasmic cell-permeable non-fluorescent (or very weakly fluorescent) reagent which, in a reduced state and upon oxidation, exhibits a strong fluorogenic signal. CellRox Deep Red can be excited by a 638-nm laser, and emits at ~665 nm. For complete information regarding the probes described here, see Internet Resources at the end of this unit.

Annexins are a family of soluble proteins (13 different isoforms) with four to eight repeats of a 75–amino acid consensus sequence relevant for Ca^{2+} binding. They are involved in membrane transport, regulation of protein kinase C, formation of ion channels,

endocytosis, exocytosis, and membrane-cytoskeleton interactions. Annexin V binds with peculiar specificity to phosphatidylserine residues, which are precociously exposed on the external leaflet of the plasma membrane during apoptosis (Lizarbe et al., 2013). Thus, when cells are annexin V positive, they have entered into an early phase of apoptosis. The annexin V–Pacific Blue conjugate is violet excitable, making it ideal for instruments with a laser at 405 nm, and for multicolor experiments that include green- or red-fluorescent dyes. The Pacific Blue-conjugated annexin V emits at ~455 nm after excitation by a violet light source.

Before starting with sample analysis, running samples stained with single fluorochromes (see steps below) is suggested to properly set up fluorescence levels. Note that also in this case there are no compensation requirements.

Materials

Cells in culture (ATCC): in suspension or adherent in 24-well tissue culture plate (as in Basic Protocol 1, we use RKO cells derived from human colon carcinoma
Complete RPMI culture medium
Phosphate-buffered saline (PBS)
CellRox Deep Red Reagent (Life Technologies)
2.5 mg/ml JC-1
(5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide); prepare by dissolving JC-1 (Life Technologies, Thermo Fisher Scientific) in dimethylformamide (DMF); store in a glass container up to 2 years at –20°C, protected from light
Annexin V binding buffer (see recipe)
?? µg/ml Pacific Blue-conjugated annexin V (Life Technologies, Thermo Fisher Scientific): store at 4°C, protected from light

3.5 ml, 55 × 12–mm plastic tubes (Sarstedt, or equivalent)
Centrifuge (Minifuge RF; Heraeus), or equivalent.
Attune NxT cytometer or equivalent cytometer equipped with four light sources for excitation at 405 nm (violet laser, for Annexin V), 488 and 561 nm (blue and yellow lasers, for JC-1), and 638 nm (red laser, for CellRox) and filters for collecting fluorescence emissions at 455/40 (for annexin V), 520/20 (for JC-1 monomers), 585/42 (JC-1 aggregates), and 660/40 (CellRox)

Additional reagents and equipment for counting (*APPENDIX 3A*) and culturing (*APPENDIX 3B*) mammalian cells and detaching adherent cells using trypsin (see *APPENDIX 3B*)

Prepare cells

1. Count a sample of the cells in culture (see *APPENDIX 3A*).

For cells in suspension

2a. Collect at least 3×10^5 cells from experimental samples by centrifuging 5 min at 300 × *g*, room temperature. Collect the same number of cells to use for a positive control.

3a. Decant and discard the medium and bring the total volume up to 1 ml with prewarmed RPMI culture medium.

For adherent cells

2b. Decant and discard the growth medium.

3b. Add 1 ml prewarmed culture medium (RPMI or similar) to the cells in the plate.

This protocol has been set up using blood cells and has been shown to work with different cell lines. However, particular attention should be given to adherent cell lines, detachment of which from the culture plate by trypsin-EDTA is required before cytofluorimetric analysis. The detachment procedure could be particularly harmful to those cells that have been damaged during the in vitro treatment, i.e., by the presence of an apoptogenic substance. In this case, the multistaining procedure described here could be performed on still-adherent cells by adding the probes directly to the culture plate.

Stain cells

4. Add the CellROX Reagent at a final concentration of 5 μM to the cells and incubate for 30 min at 37°C.

For cells in suspension

5a. To wash the staining solution from the cells, add 1 ml PBS, mix by shaking gently, and centrifuge 5 min at 300 × *g*, room temperature. Decant and discard the supernatant.

Because this protocol requires many centrifugations for the cells in suspension, the authors suggest setting the centrifugation speed as low as possible in order to avoid cellular damages due to stress. Adding 10% fetal bovine serum to PBS can decrease cell loss during washing steps.

6a. Resuspend the cells in 1 ml complete culture medium. Proceed to step 7.

For adherent cells

5b. Decant the staining solution from the cells and wash by adding 1 ml PBS, swirling, and decanting.

6b. Detach the adherent cells as follows.

i. Trypsinize cells as described in *APPENDIX 3B*.

The minimal amount of trypsin should be used in order to avoid both cellular damage and the presence of aggregates in the cell suspension. In fact, cell aggregates could augment background or J-aggregate fluorescence. In this case, aggregates can be eliminated from analysis by gating on singlets, which can be identified by plotting FS-area versus FS-height. In any case, when adherent cell lines are treated with apoptogenic substances, remember that apoptotic cells spontaneously detach and float in the supernatant; they should not be discarded but collected and analyzed separately or together with attached cells.

ii. Add 1 ml complete culture medium to neutralize trypsin activity.

iii. Centrifuge 5 min at 300 × *g*, room temperature, and discard the supernatant. Proceed to step 7.

7. Add 1 μl of 2.5 mg/ml JC-1 (2.5 μg/ml final concentration) to the pellet from step 6a or 6b and mix until the dye is well dispersed and gives a uniform red-violet color. Incubate the samples 10 min in the dark, room temperature.

JC-1 tends to form aggregates when added to normal aqueous medium. To avoid this, add the probe while gently vortexing.

8. Wash with 1 ml PBS as in step 5a or 5b.

9. Resuspend the cells in 195 μl annexin V binding buffer.

10. Add 5 μl of ?? μg/ml Pacific Blue–conjugated annexin V and incubate 15 min at room temperature.

Staining with annexin V is the last step of the protocol because annexin V binding to phosphatidylserine is affected by the presence of its incubation buffer. In the authors'

experience, washing or resuspending cells with PBS causes annexin V detachment from phosphatidylserine.

11. Resuspend the cells in 1 ml annexin V binding buffer.

Acquire samples on cytometer

12. First acquire blank samples and cells without CellRox, to set the level of background fluorescence for the Alexa 647 channel.

    This type of analysis requires a flow cytometer equipped with three light sources and appropriate collection filters for all the dyes (see Materials list).

13. Acquire at least 30,000 total events.

Analyze data

14. Identify cell populations on the basis of annexin V, i.e., live (Annexin V^-), apoptotic (Annexin V^+). Analyze $\Delta\Psi_m$ and ROS content in these subsets.

    Since multiple parameters are simultaneously analyzed, different techniques for data interpretation can be adopted depending on the user's interests. In this case, should the researcher be interested in detecting $\Delta\Psi_m$ and ROS production in early apoptotic or healthy cells, a gate can be designed on annexin V positive or negative cells, where the other parameters are thus analyzed (see Fig. 7.32.2).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps.

Annexin V binding buffer

0.477 g HEPES (10 mM)
1.636 g NaCl (140 mM)
0.073 g $CaCl_2$ (2.5 mM)
H_2O to 200 ml
Adjust pH to 7.4 and store up to 1 year at 4°C

Milli-Q-purified (double purified) water may also be used in this recipe.

COMMENTARY

Background Information

Mitochondria play an active role in the regulation of programmed cell death, and indeed the collapse in $\Delta\Psi_m$ can occur during the apoptotic process (Green et al., 2011). The opening of the mitochondrial permeability transition pore—a mitochondrial protein complex formed by the adenine nucleotide translocator (ANT), the voltage-dependent anion channel (VDAC), and the peripheral benzodiazepine receptor (PBR)—can induce loss of $\Delta\Psi_m$, release of apoptogenic factors, and loss of oxidative phosphorylation (Martel et al., 2014). However, whether loss of $\Delta\Psi_m$ is a cause or a consequence of the triggering of apoptosis still remains a matter of debate. Depending on the apoptotic model used, loss of $\Delta\Psi_m$ may be a late (Cossarizza et al., 1994) or an early (Zamzami et al., 1995) event. Moreover, loss of $\Delta\Psi_m$ is responsible for the release of apoptosis-inducing factor (AIF), which consequently translocates to the nucleus and promotes chromatin condensation and fragmentation (Kroemer et al., 2007). Other mechanisms initiating apoptosis (e.g., cytochrome *c* release or activation of executioner caspases) are independent of the disruption of $\Delta\Psi_m$ (Kluck et al., 1997; Bossy-Wetzel et al., 1998).

Several techniques are used to investigate the role of this organelle, including classical biochemical or molecular biology methods; flow cytometry clearly represents the most rapid and powerful tool for investigating $\Delta\Psi_m$ at the single-cell level. Many probes are available for this purpose, but some of them, e.g., R123 and $DiOC_6(3)$, are not fully adequate (Salvioli et al., 1997). As a consequence, discrepancies in the data regarding the role of $\Delta\Psi_m$ in the regulation of the apoptotic process may be also attributed to the use of inappropriate probes. A detailed analysis of other dyes is reported in *UNIT 9.14*.

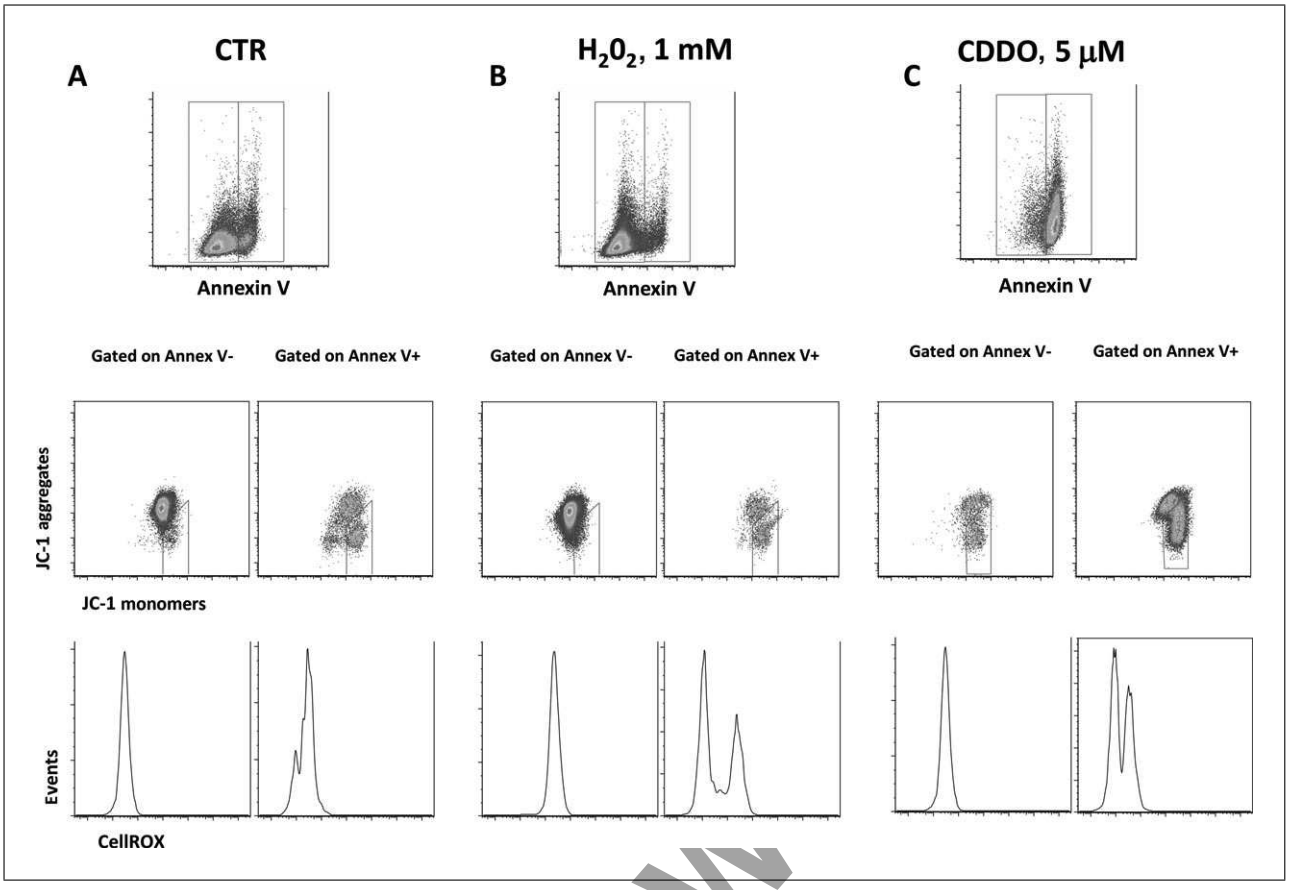

Figure 7.32.2 Multilaser, uncompensated analysis of apoptosis, mitochondrial membrane potential, and production of reactive oxygen species. RKO cells were cultured in the absence (**A**) or presence (**B**) of H_2O_2 (1 hr) and (**C**) 5 μM CDDO (24 hr). Cell were stained as described in Basic Protocol 2. Viable and apoptotic cells were identified by positivity for annexin V; $\Delta\Psi_m$ was analyzed by JC-1, ROS production by CellRox Deep Red.

We have demonstrated that JC-1 is an excellent potentiometric probe, having the peculiar ability to change color reversibly depending on the $\Delta\Psi_m$. This property is due to the reversible formation of JC-1 aggregates upon polarization of mitochondrial membrane, which causes a shift in emitted light from ~530 nm (emission of monomers) to ~590 nm (emission of J-aggregates). In living cells, the color of the dye changes reversibly from green to orange as the mitochondrial membrane becomes more polarized (Reers et al., 1991). Aggregate formation begins at potential values on the order of 80 to 100 mV, and reaches the zenith at ~200 mV.

When 488 nm was the sole available laser line, researchers had to cope with compensation, which had to be set up considering the spillover of the two fluorescences, and required not only the preparation of "biological negative controls" (i.e., samples of cells treated with a depolarizing agent to see the area where cells with a low $\Delta\Psi_m$ tended to go), but also a certain experience on the part of the operator. In any case, excitation with 488-nm laser was quite efficient and allowed, and is currently allowing, a significant number of studies. Modern flow cytometers have more excitation sources than in the past. The main advantage of a second excitation source for JC-1 aggregates is well evidenced by fact that compensation is no longer needed, since yellow laser does not excite JC-1 monomers (Perelman et al., 2012).

JC-1 staining can be combined with multiple probes in a polychromatic flow cytometric assay to detect changes in $\Delta\Psi_m$ together with other parameters during apoptosis; Basic Protocol 2 can be useful and informative, because several cell functional subsets with different characteristics can be simultaneously identified in a given population. This makes it possible not only to discriminate cell death, but also to investigate whether similar compounds exert differential effects in the same cell type. This type of analysis, combined with high-throughput technologies, could be adopted for the screening of the toxicity of a variety of compounds, in order to obtain multiple information about the investigated molecules.

In the example shown in Figure 7.32.2, RKO cells (human colon carcinoma) were treated for 24 hr with 5 μM 2-cyano-3,12-dioxo-oleana-1, 9(11)-dien-28-oic acid, methyl ester (CDDO), a Nrf2 activator that inhibits cell proliferation and induces differentiation and apoptosis in several cancer cells. Note that in panel A most control cells (CTR) or most cells treated with H_2O_2 are viable and have a high $\Delta\Psi_m$ if compared to cells treated with CDDO. In panel C, apoptotic elements can be easily identified on the basis of the positivity to annexin V. In all populations, $\Delta\Psi_m$ can be easily analyzed: apoptotic cells display a loss of $\Delta\Psi_m$, while living cells display well polarized mitochondria. Note that in the treated sample viable cells are characterized by a decrease in J-aggregate median fluorescence and thus display a $\Delta\Psi_m$ that is lower than that present in untreated cells (see middle plots of panels A and C).

Critical Parameters and Troubleshooting

The use of JC-1 with probes emitting in the deep red is in principle possible, e.g., despite the fact that JC-1 can still emit at wavelengths >670 nm.

Anticipated Results

Changes in $\Delta\Psi_m$ can occur under a variety of physiopathological conditions. Studies on apoptosis are now complicated by the observation that many types of cell death exist, each of them with peculiar intracellular targets. During apoptosis, mitochondria can be the first actors, or they can be involved in subsequent steps. Thus, careful (and repeated!) time-course analyses are required to establish whether depolarization of mitochondria occurs because of, or is the cause of, apoptosis. Similar considerations can be applied if one considers a variety of other parameters that can be analyzed along with $\Delta\Psi_m$.

Time Considerations

In general, the staining protocol with only JC-1 (Basic Protocol 1) is very fast and simple, and requires <1 hr. It is suggested to acquire the samples immediately. The time for analysis is usually short. The first step is to set the quadrants (for JC-1) in the control sample, check the correct position of the quadrants with the valinomycin-treated sample (all the fluorescence in the lower right quadrant for JC-1, a significant decrease for the other probes), and finally analyze the other files. Once the setting of the flow cytometer is accomplished, acquisition take a few minutes, depending on the number of tubes that have to be analyzed and of the speed of the instrument. Clearly, using an acoustic flow cytometer able to acquire up to 35,000 events per second (like the aforementioned Attune NxT) facilitates high throughput.

Basic Protocol 2 (uncompensated, polychromatic analysis of apoptosis) takes more time. Sample preparation requires up to 1.5 hr. In this protocol, data analysis is obviously longer because of the number of parameters that are under investigation and the numerous functional subsets of cells potentially identifiable by such an approach. However, software for automatic data analysis of hundreds of samples (e.g., FlowJo; TreeStar) and mathematical methods have been developed to simplify the comprehension of polychromatic flow cytometry data (see (Petrausch et al., 2006; Lugli et al., 2010; Roederer et al., 2011). In particular, the use of Principal Component Analysis can be quite advantageous when huge data sets are generated (derived from multiple time points and compound concentrations) and when the simultaneous analysis of multiple parameters from a multidimensional space is needed (Lugli et al., 2007a, b; Lugli et al., 2007a, b; Troiano et al., 2007).

Literature Cited

Abu, N., Akhtar, M.N., Yeap, S.K., Lim, K.L., Ho, W.Y., Zulfadli, A.J., Omar, A.R., Sulaiman, M.R., Abdullah, M.P., and Alitheen, N.B. 2014. Flavokawain a induces apoptosis in MCF-7 and MDA-MB231 and inhibits the metastatic process in vitro. *PLoS One 6*;9:e105244.

Bossy-Wetzel, E., Newmeyer, D.D., and Green, D.R. 1998. Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J.* 17:37-49.

Cossarizza, A., Baccarani-Contri, M., Kalashnikova, G., and Franceschi, C. 1993. A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5′, 6,6′tetrachloro1,1′,3,3′tetraethylbenzimidazol-carbocyanine iodide (JC-1). *Biochem. Biophys. Res. Commun.* 197:40-45.

Cossarizza, A., Mussini, C., Mongiardo, N., Borghi, V., Sabbatini, A., De Rienzo, B., and Franceschi, C. 1997. Mitochondria alterations and dramatic tendency to undergo apoptosis in peripheral blood lymphocytes during acute HIV syndrome. *AIDS* 11:19-26.

Cossarizza, A., Kalashnikova, G., Grassilli, E., Chiappelli, F., Salvioli, S., Capri, M., Barbieri, D., Troiano, L., Monti, D., and Franceschi, C. 1994. Mitochondrial modifications during rat

thymocyte apoptosis: A study at the single cell level. *Exp. Cell Res.* 214:323-330.

Cossarizza, A., Franceschi, C., Monti, D., Salvioli, S., Bellesia, E., Rivabene, R., Biondo, L., Rainaldi, G., Tinari, A., and Malorni, W. 1995. Protective effect of N-acetylcysteine in tumor necrosis factor-alpha-induced apoptosis in U937 cells: The role of mitochondria. *Exp. Cell Res.* 220:232-240.

Cossarizza, A., Pinti, M., Moretti, L., Bricalli, D., Bianchi, R., Troiano, L., Fernandez, M.G., Balli, F., Brambilla, P., Mussini, C., and Viganò, A. 2002. Mitochondrial functionality and mitochondrial DNA content in lymphocytes of vertically infected human immunodeficiency virus-positive children with highly active antiretroviral therapy-related lipodystrophy. *J. Infect. Dis.* 185:299-305.

Galluzzi, L., Kepp, O., and Kroemer, G. 2012. Mitochondria: Master regulators of danger signalling. *Nat. Rev. Mol. Cell Biol.* 13:780-788.

Gibellini, L., De Biasi, S., Pinti, M., Nasi, M., Riccio, M., Carnevale, G., Cavallini, G.M., Sala de Oyanguren, F.J., O'Connor J.E, Mussini, C., De Pol, A., and Cossarizza, A. 2012. The protease inhibitor atazanavir triggers autophagy and mitophagy in human preadipocytes. *AIDS* 26:2017-2026.

Green, D.R., Galluzzi, L., and Kroemer, G. 2011. Mitochondria and the autophagy-inflammation-cell death axis in organismal aging. *Science* 333:1109-1112.

Jelley, E.E. 1936. Spectral absorption and fluorescence of dyes in the molecular state. *Nature* 138:1009-1010.

Kluck, R.M., Bossy-Wetzel, E., Green, D.R., and Newmeyer, D.D. 1997. The release of cytochrome c from mitochondria: A primary site for Bcl-2 regulation of apoptosis. *Science* 275:1132-1136.

Kroemer, G., Galluzzi L., and Brenner, C. 2007. Mitochondrial membrane permeabilization in cell death. *Physiol. Rev.* 87:99-163.

Lizarbe, M.A., Barrasa, J.I., Olmo, N., Gavilanes, F., and Turnay, J. 2013. Annexin-phospholipid interactions. Functional implications. *Int. J. Mol. Sci.* 14:2652-2683.

Lugli, E., Pinti, M., Nasi, M., Troiano, L., Ferraresi, R., Mussi, C., Salvioli, G., Patsekin, V., Robinson, J.P., Durante, C., Cocchi, M., and Cossarizza, A. 2007. Subject classification obtained by cluster analysis and principal component analysis applied to flow cytometric data. *Cytometry A* 71:334-344.

Lugli, E., Roederer, M., and Cossarizza, A. 2010. Data analysis in flow cytometry: The future just started. *Cytometry A* 77:705-713.

Marringa, W.J., Krueger, M.J., Burritt, N.L., and Burritt, J.B. 2014. Honey bee hemocyte profiling by flow cytometry. *PLoS One.* 9:e108486.

Martel, C., Wang, Z., and Brenner, C. 2014. VDAC phosphorylation, a lipid sensor influencing the cell fate. *Mitochondrion*.

Perelman, A., Wachtel, C., Cohen, M., Haupt, S., Shapiro, H., and Tzur, A. 2012. JC-1: Alternative excitation wavelengths facilitate mitochondrial membrane potential cytometry. *Cell Death Dis.* 3:e430.

Petrausch, U., Haley, D., Miller, W., Floyd, K., Urba, W.J, and Walker, E. 2006. Polychromatic flow cytometry: A rapid method for the reduction and analysis of complex multiparameter data. *Cytometry A* 69:1162-1173.

Polla, B.S., Kantengwa, S., François, D., Salvioli, S., Franceschi, C., Marsac, C., and Cossarizza A. 1996. Mitochondria are selective targets for the protective effects of heat shock against oxidative injury. *Proc. Natl. Acad. Sci. U.S.A.* 93:6458-6643.

Reers, M., Smith, T.W., and Chen, L.B. 1991. J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. *Biochemistry* 18:4480-4486.

Roederer, M., Nozzi, J.L., and Nason, M.C. 2011. SPICE: Exploration and analysis of post-cytometric complex multivariate datasets. *Cytometry A* 79:167-174.

Salvioli, S., Ardizzoni, A., Franceschi, C., and Cossarizza, A. 1997. JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: Implications for studies on mitochondrial functionality during apoptosis. *FEBS Lett* 411:77-82.

Salvioli, S., Dobrucki, J., Moretti, L., Troiano, L., Fernandez, M.G., Pinti, M., Pedrazzi, J., Franceschi, C., and Cossarizza, A. 2000. Mitochondrial heterogeneity during staurosporine-induced apoptosis in HL60 cells: Analysis at the single cell and single organelle level. *Cytometry* 40:189-197.

Troiano, L., Ferraresi, R., Lugli, E., Nemes, E., Roat, E., Nasi, M., Pinti, M., and Cossarizza, A. 2007. Multiparametric analysis of cells with different mitochondrial membrane potential during apoptosis by polychromatic flow cytometry. *Nat. Protoc.* 2:2719-2727.

Zamzami, N., Marchetti, P., Castedo, M., Zanin, C., Vayssiere, J.L., Petit, P.X., and Kroemer, G. 1995. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J. Exp. Med.* 181:1661-1672.

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