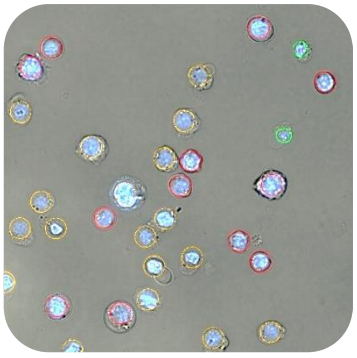
Mitochondrial Membrane Potential Studies as Early Marker of Apoptosis

Introduction



Cellular stress like DNA damage or oxidative stress can lead to the activation of the intrinsic apoptosis signaling pathway. This programmed cell death is a complex mechanism consisting of many different particularly meshing steps. In addition to their main function as ATP-supplier, the mitochondria have an important function as apoptosis regulators because one early characteristic of apoptosis is the loss of mitochondrial membrane potential (delta psi$_m$; $\Delta\Psi_m$) resulting from a disruption of the mitochondrial membrane [1,2]. This mechanism is induced by endoplasmatic reticulum (ER) stress, causing a mitochondrial Ca^{2+} and *Bcl-2 Associated X Protein (*BAX) uptake, which will be followed by the loss of $\Delta\Psi_m$ and a Cytochrome-c and ATP release – building the apoptosomes [3,4] (Fig. 1).

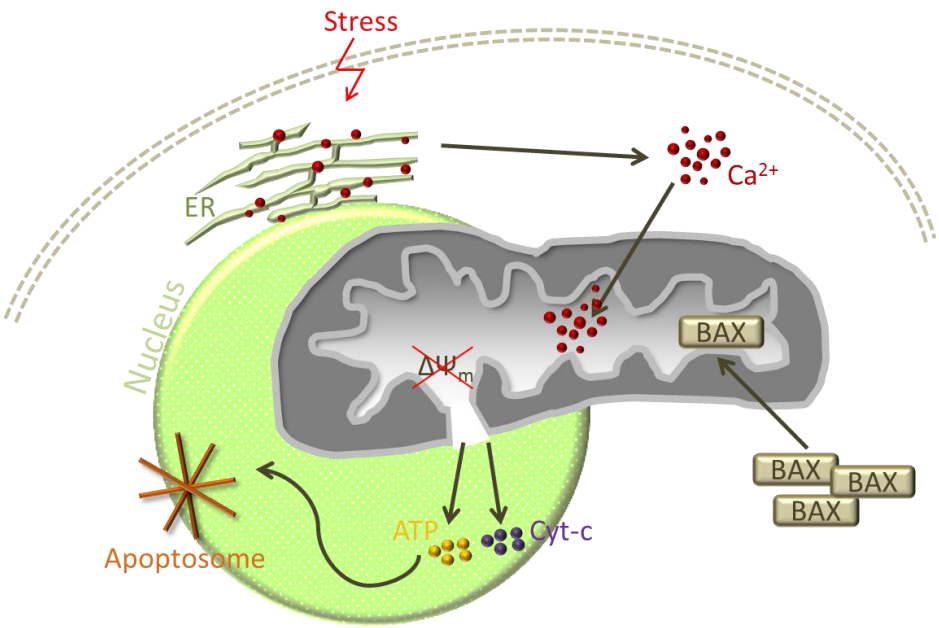


Fig. 1: Simplified scheme of intrinsic apoptosis signaling pathway.

To indicate the mitochondrial health in a suspension cell culture treated with hydrogen peroxide (H_2O_2), we used the cationic, lipophilic dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). This dye has the property to change the emitted fluorescence via accumulation and aggregation. In healthy cells JC-1 enters the negative charged mitochondrial matrix and enriches the mitochondria lumen where it builds J-aggregates after reaching a critical concentration. The J-aggregates are red fluorescent (~590 nm).
A collapse of $\Delta\Psi_m$, like in apoptotic cells, disables an accumulation of the JC-1 molecules. In those cells JC-1 remains in a monomeric, green fluorescent form (~529 nm). Therefore, early apoptotic and healthy cells are easily distinguished using the fluorescence measurements of the NyONE System (Fig. 2).

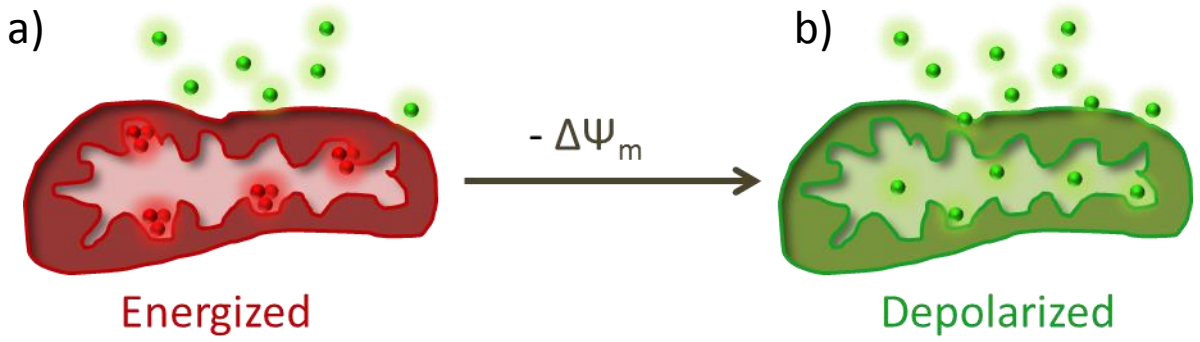


Fig. 2: Schematic illustration of JC-1 monomers (green) and J-aggregates (red) depending on the mitochondrial membrane potential.
In healthy cells mitochondria are in a energized condition with a polarized mitochondrial membrane (a). Part of the apoptotic pathway is the uncoupling of the proton gradient of mitochondria and a loss of membrane integrity. This has the consequence that the cationic JC-1 dye gets no longer actively enriched in the mitochondrial lumen and therefore it can not aggregate (b).

Aim of the study

With the NyONE and our analysis software we support many different cellular assays, including growth rate studies with the brightfield channel up to multicolor antibody analyses and toxicity studies.

One interesting field of research is the investigation of apoptosis triggers and mechanisms. With the *JC-1 Mito Potential* Operator we succeeded in distinguishing between the red and the green stained cells in a sample treated with a toxin of interest and stained with JC-1 and Hoechst. As the result of detected JC-green and JC-red cells the ratio between red and green cells could be calculated. This value is widely used as a marker for the apoptotic level of the cells.

With the present study, the efficacy of H_2O_2 as JC-1 positive control should be tested. Additionally the reproducibility and reliability of the JC-1 Mito potential operator are tested. Furthermore we examined in detail how large the analyzed area of the well should be and what cell number is needed to get representative results. Because the smaller the needed area to be measured the faster and hence cell sparing a measurement with the NyONE can be performed.

Materials

Cells/Reagents

- Nalm-6 cells (human B cell precursor leukemia)
- RPMI medium with 10 % FBS and 1 % Penicillin/Streptomycin
- Phosphate Buffered Saline (w/o Ca^{2+} & w/o Mg^{2+} (PBS^{--}))
- H_2O_2, 30 % stock-solution (e.g. Perhydrol®; MerckMillipore)
- JC-1, 200 µM stock-solution in DMSO (e.g. Santa Cruz Biotechnology)
- Hoechst 33342, 8.9 mM stock-solution in H_2O_{dest} (e.g. Santa Cruz Biotechnology)

Equipment

- Microplate with transparent bottom (e.g. Greiner Bio-One µclear 96-well plate (cat.# 655090); black plates are optimal for fluorescence imaging
- Centrifuge tubes 5 mL
- Swing out plate centrifuge
- Cell incubator
- Pipettes and tips
- NyONE microplate imaging microscope

Procedure

1. Prepare 2 x 2.5 mL of a cell suspension, to reach a cell concentration of up to 3 x 10^5 cells/mL
2. Add 100 µM H_2O_2 (27.78 µL) to one sample and add the appropriate volume of H_2O_{dest} to the other sample
4. Incubate cells 1 h at 37 °C. Invert centrifugation tube 3-4 times during incubation or incubate on a slowly rotating device (30 rpm)
5. Remove the H_2O_2 and centrifuge cells 10 min at 400 x g
6. After cell centrifugation aspirate supernatant
7. Resuspend cells carefully in 2.5 mL warm medium
8. Add 6.25 µL JC-1 stock-solution (final concentration 0.5 µM) and 0.8 µL Hoechst 33342 stock-solution (final concentration 2.85 µM)
9. Protect cells from light during the following steps.
10. Incubate cells 30 min at 37 °C in the dark (with inverting or rotating as in step 4.)
11. Remove the dyes, centrifuge cells again 10 min at 400 x g and discard supernatant
12. Resuspend cells carefully in 2.5 mL warm medium
13. Prepare mixtures with different ratios of H_2O_2 treated vs. H_2O_2 untreated cells
    - 0 % treated cells + 100 % untreated cells
    - 25 % treated cells + 75 % untreated cells
    - 50 % treated cells + 50 % untreated cells
    - 75 % treated cells + 25 % untreated cells
    - 100 % treated cells + 0 % untreated cells
14. Add 100 µL of each mixture per well to the microplate (3 replicates per mixture)
15. Centrifuge the plate for one minute at 30 x g in the swing-out rotor
16. Remove the plate carefully out of the centrifuge and place it into the sample holder of the NyONE imager

Results

With the performed experiments we were able to demonstrate that all Nalm-6 cells have lost the mitochondrial membrane potential after the indicated H_2O_2 incubation. This is apparent from the fact that no red JC-1 fluorescence is detected in wells containing 100 % H_2O_2-treated cells (Fig. 3 a-c).

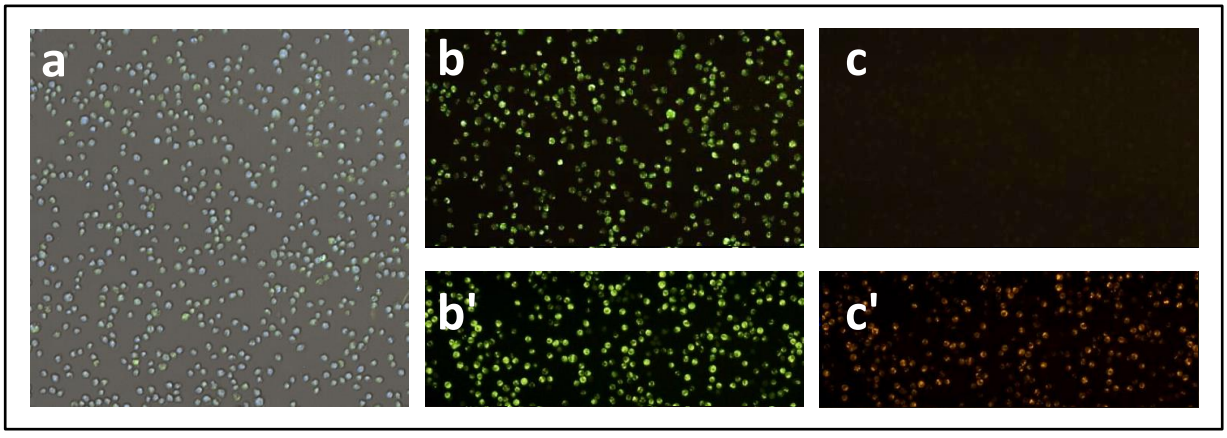


Fig. 3 a-c: Influence of H_2O_2 treatment on Nalm-6 cells
a) Overlay of brightfield, Hoechst, JC-1 green and JC-1 red (10x). **b)** Green emission (530/43 nm). All cells include the green, monomeric form of JC-1. **b')** In comparison, another sample without H_2O_2 treatment. **c)** Red emission channel (607/70 nm). As a result of H_2O_2 incubation, no J-aggregates are detectable, suggesting that mitochondrial membrane is depolarized. **c')** In comparison, another sample without H_2O_2 treatment. All cells include J-aggregates.

Furthermore, we could demonstrate that the JC-1 measurements with the NyONE and the evaluating of the images with the *JC-1 Mito Potential* operator are exceedingly reproducible. Results with a low standard deviation between different experiments under same conditions could be achieved (Fig. 4).

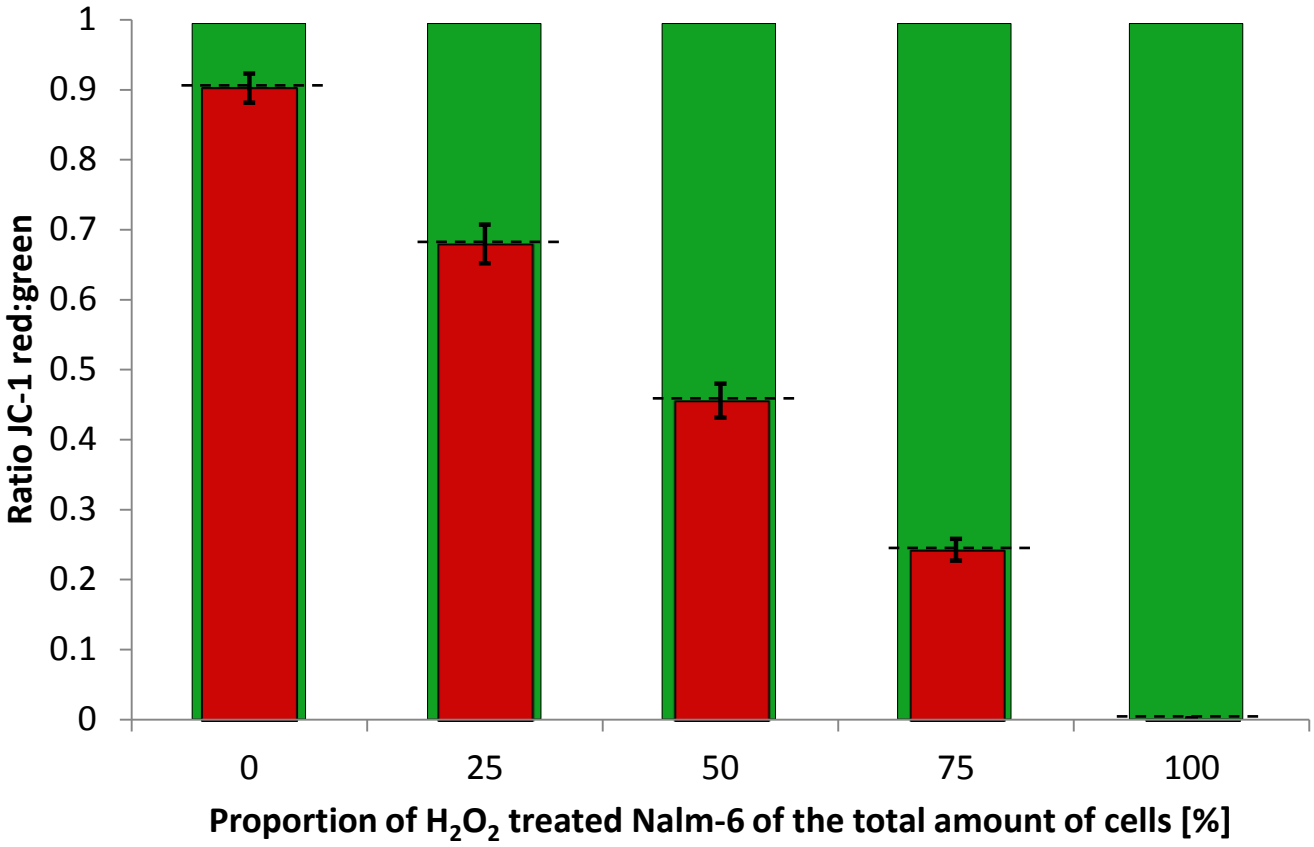


Fig. 4: JC-1 staining of H_2O_2 treated Nalm-6 cells
Cells were treated 1h with 100 µM H_2O_2 and stained 30' with 0.5 µM JC-1. These cells were mixed in different ratios with untreated JC-1 stained cells (N=3; n=3). After image analysis with the *JC-1 Mito Potential* operator the ratio between red and green detected cells was calculated (dotted lines on the bars). The red bars show the part of the J-aggregates containing cells and the green bars show the part of JC-1 green stained cells. The JC-1 concentration and the incubation time were set in a way that JC-green is always detectable whereas apoptosis induction only affects (decrease) the formation of red J-aggregates.

Another aim of these experiments was to investigate the needed minimum size of the evaluated area and how a variance of the number of cells affects the reproducibility of the results. For this purpose, cells were treated and mixed as described above. Three different experiments with three different cell concentrations were performed ($2.3x10^4$, $1.7x10^4$, $0.7x10^4$ cells per well). Each experiment was measured once with 9 subwells and then again with 1 subwell in the center of the well. With 9 subwells 32.4 % and with 1 subwell 3.6 % of the total area of a well of a 96-well microplate were detected. The comparison of individual measurements showed that no significant differences in the red:green ratios could be detected among the different cell concentrations used. Only a tendency is recognized that for the highest cell number measurings of 9 subwells, a slightly higher ratio exists contrary to the 1-subwell measurings, whereas the lowest cell concentration (measurements with 1 subwell) indicates a slightly increased red:green ratio compared to the measurements of 9 subwells (Fig. 5).

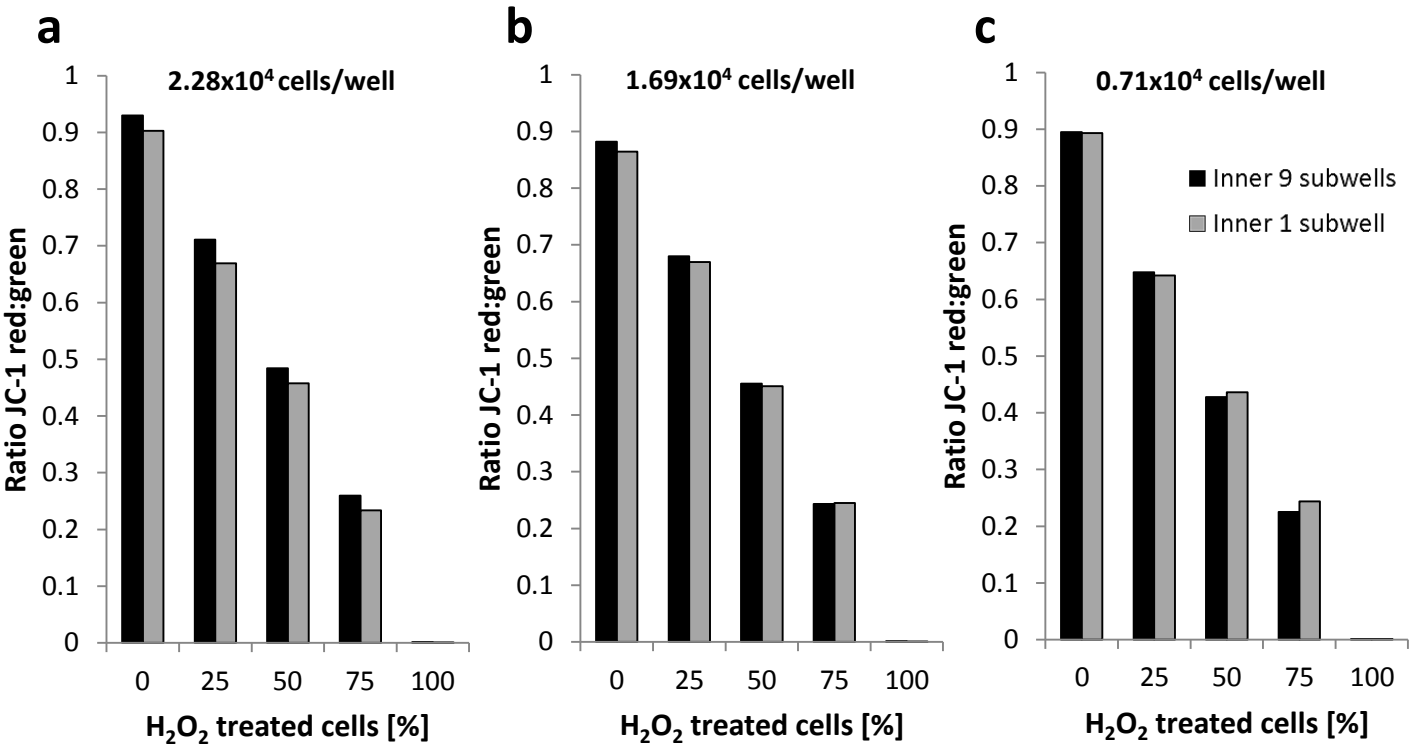


Fig. 5 (a-c): Comparison between 9 and 1 subwells and different cell numbers/well.
Shown is the effect of different numbers of cells on the reproducibility of the measurement method. **a)** 2.3x10^4, **b)** 1.7x10^4, **c)** 0.7x10^4 cells per well. The y-axis indicates the red: green ratio and the x-axis indicates the proportion of H_2O_2-treated cells to the total cell solution. The black bars show the measurement of 9 subwells/well of a 96-well microplate and the grey bars show the measurement of 1 subwell/well of a 96-well microplate (N = 3, n = 3).

Conclusions

We have developed a protocol that shows on human Nalm-6 cells that H_2O_2 is suitable as a positive control for JC-1 experiments. This result allows to avoid the use of highly toxic protonophores, which act as proton gradient uncoupler, such as Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) or Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP).

A great advantage in the establishment of the JC-1 protocol was that in contrast to other methods (e.g. flow cytometry) the images of the cells are archived and can visually be inspected and reprocessed if necessary.

The carried out experiments show that, due to the mixing ratios of treated to untreated cells, the expected results can be obtained very accurately using the *JC-1 Mito potential* operator of SynenTec. This is ensured by the complex image processing algorithms and the individually and extensively modifiable image analysis parameters.

The results demonstrate the robustness and reproducibility of this application. Even varying cell numbers or different measuring ranges do not influence the results significantly. Thus it can be said that the advantages of image cytometry, combined with automated measurements of microplates and our high image resolution is very suitable for apoptosis and mitochondrial membrane potential studies. However, according to different cell lines and toxins of interest the staining protocol should be optimized to ensure that every cell can be detected.

References

[1] Liu, X., Kim, C.N., Yang, J., Jemmerson, R. and Wang, X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell, 86, 147-157.

[2] Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T. and Tanaka, N. (2000) Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science, 288, 1053-1058.

[3] Riedl, S.J. and Salvesen, G.S. (2007) The apoptosome: signalling platform of cell death. Nat Rev Mol Cell Biol, 8, 405-413.

[4] Saleh, A., Srinivasula, S.M., Acharya, S., Fishel, R. and Alnemri, E.S. (1999) Cytochrome c and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation. J. Biol. Chem., 274, 17941-17945.

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