Application note No. 3008. Rev. 1.8

NucleoCounter® NC-3000™

Mitochondrial potential assay using the NucleoCounter® NC-3000™ system

Product description

The NucleoCounter® NC-3000™ system enables the user to perform automated cell counting and analyses of a broad range of eukaryotic cells.

Application

This protocol for the NucleoCounter® NC-3000™ system enables the user to measure the mitochondrial transmembrane potential ($\Delta\psi_m$) using the cationic dye JC-1 (5, 5, 6, 6-tetrachloro-1, 1, 3, 3-tetraethylbenzimidazol-carbocyanine iodide). Disruption of $\Delta\psi_m$ is often associated with the early stages of apoptosis.

Introduction

Cell death can occur by two distinct mechanisms, necrosis or apoptosis. Necrosis occurs when cells are exposed to harsh physical or chemical stress (e.g., hypothermia, hypoxia) while apoptosis is a tightly controlled biochemical process by which cells are eliminated and where the cell is an active participant in its own termination ("cellular suicide"). Apoptosis is one of the main types of programmed cell death which occur in multicellular organisms and is characterized by a series of events that lead to a variety of morphological and biochemical changes, including membrane blebbing, cell shrinkage, alteration of membrane asymmetry and permeability, condensation of chromatin and nucleus, DNA fragmentation, and formation of membrane bound vesicles (apoptotic bodies)

Apoptosis is both a very complex and very important process and dysregulations in the apoptosis machinery may lead to very severe diseases. A growing body of evidence suggests that resistance to apoptosis is a feature of most, if not all types of cancer. In the other hand may hyperactivity of the apoptotic processes also cause diseases such as neurodegenerative diseases as seen in Parkinson's and Alzheimer's.

Reliable detection and monitoring of apoptosis is crucial for the development of treatments for apoptosis-associated diseases and for investigating apoptotic mechanisms in general. A number of methods have been developed to study apoptosis in individual cells. One of the major problems in detecting apoptosis is that many features of apoptotic and necrotic processes overlap and therefore it may be necessary to use several independent assays before drawing definitive conclusions. Each of the available methods has its advantages and limitations making it appropriate for some applications but not others.

This application note describes a method for detection of disruption of mitochondrial membrane potential in individual cells. Loss of the mitochondrial membrane potential is known to precede apoptosis and chemical-hypoxia-induced necrosis. The lipophilic cationic dye JC-1 display potential-dependent accumulation in the mitochondria and provides a simple, fluorescent-based method for distinguishing between healthy and apoptotic cells. In healthy cells, the negative charge established by the intact mitochondrial membrane potential facilitates the accumulation of JC-1 in the mitochondrial matrix. At high concentrations JC-1 forms aggregates and become red fluorescent. In apoptotic cells the mitochondrial potential collapses and JC-1 localizes to the cytosol in its monomeric green fluorescent form.

Principle

Using image analysis, the NC-3000™ system automates detection of cells with collapsed mitochondrial membrane potential. Cells are stained with JC-1 and DAPI. Cellular JC-1 monomers and aggregates are detected as green and red fluorescence, respectively. Apoptosis and thereby also mitochondrial depolarization is revealed as a decrease in the red/green fluorescence intensity ratio. Necrotic and late apoptotic cells are detected as blue fluorescent (DAPI) cells.
After staining cells are loaded into either of two types of ChemoMetec slides: the 2-chamber NC-Slide A2™ or the 8-chamber NC-Slide A8™. Samples are analyzed using the NC-3000™ system and the amount of blue, green and red fluorescence of the individual cells is

Gydevang 43 • DK-3450 Allerod • Denmark • Phone: (+45) 48 13 10 20 • Fax: (+45) 48 13 10 21 • Support: contact@chemometec.com • Web: www.chemometec.com

quantified. The intensity of green and red fluorescence is shown in a scatter-plot displayed on PC screen. DAPI positive/non-viable cells can be defined in a displayed histogram. We recommend always including an untreated control.
The advantages of this assay are that it is simple, rapid and relatively cheap. However, the assay may not always be specific for apoptosis. Thus, cells with a decrease in red/green fluorescence intensity ratio may also represent chemical-hypoxia-induced necrotic cells. Hence, using another apoptosis assay to confirm the obtained results is recommendable.

JC-1 and DAPI staining

Materials

- Cells to be stained[1, 2]
- Phosphate buffered saline (PBS)[1, 4]
- **Solution 7** (200 µg/ml JC-1)[3]
- **Solution 8** (1 µg/ml DAPI in PBS)
- **NC-Slide A2™** or **NC-Slide A8™**

[1] provided by the user.

[2] An untreated control should be included. Preferable, use logarithmically proliferating cells as control.

[3] Avoid repeated freeze/thawing of the JC-1 stock solution. Make small aliquots after the first thaw and store at -20 °C. JC-1 is photo sensitive. Perform all staining procedures in dark.

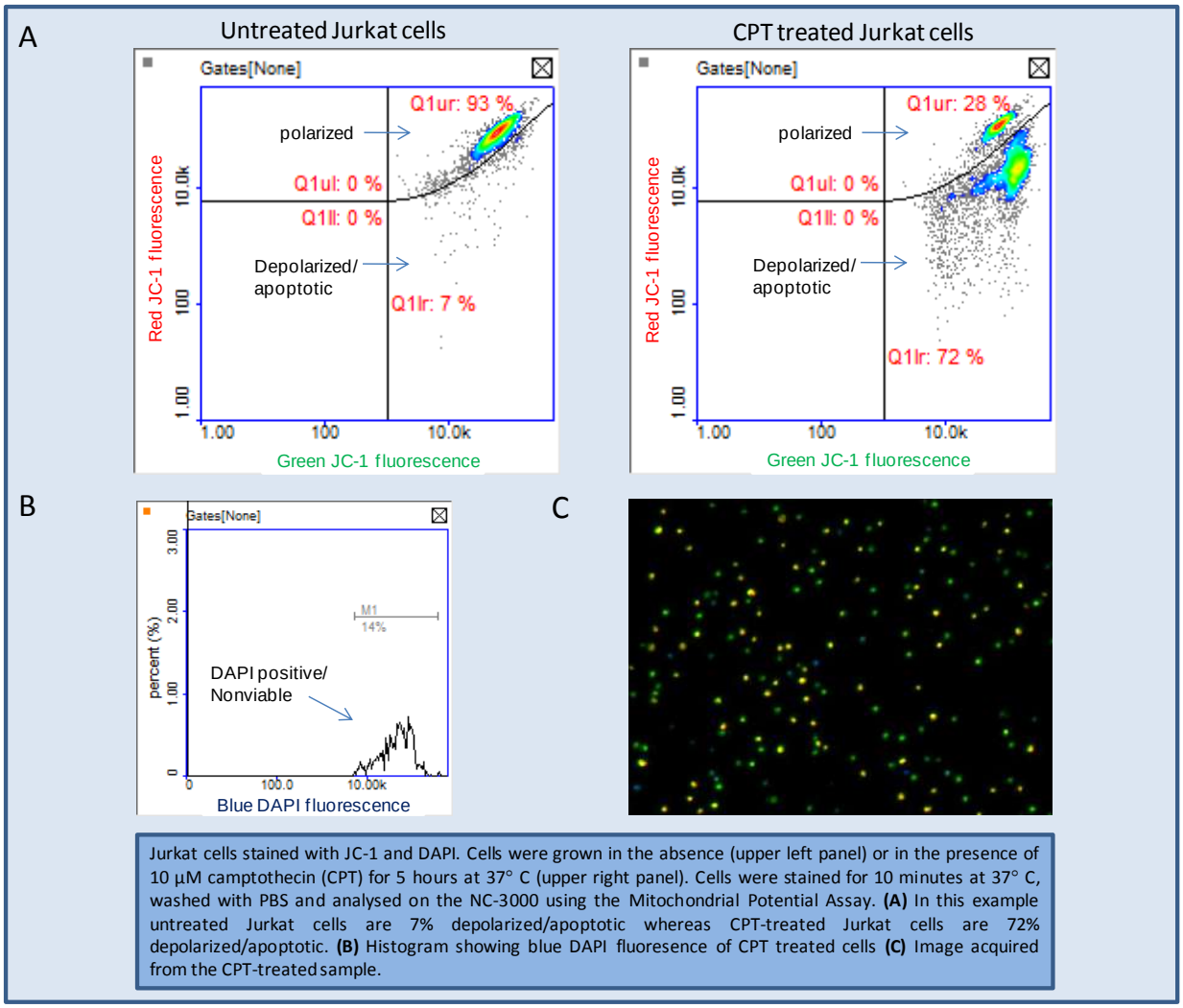
[4] Bring to room temperature before use

Protocol

1. For each sample, suspend cells in 1 ml medium, PBS or other appropriate buffer at approximately $1x10^6$ cells/ml.
2. Add 12.5 µl of **Solution 7** (final concentration: 2.5 µg/ml) to the cell sample and incubate 10 minutes at 37° C.
    a. Duration of the staining may depends on the specific cell type. If necessary incubation time can be extended to 30 minutes.
3. Centrifuge stained cells at 400 *g* for 5 min. at room temperature and remove the supernatant completely without disturbing the cell pellet.
4. Resuspend cell pellet in 1-2 ml PBS by pipetting, centrifuge at 400 *g* for 5 min. at room temperature and remove the supernatant completely without disturbing the cell pellet.
    a. Careful washing of the sample is crucial for achieving valid results
5. Wash again. Resuspend cell pellet in 1-2 ml PBS by pipetting, centrifuge at 400 *g* for 5 min. at room temperature and remove the supernatant completely without disturbing the cell pellet.
6. Resuspend cell pellet by pipetting in 0.25 ml **Solution 8** and analyze immediately
7. Engage NucleoCounter® NC-3000™ by starting the accompanying software.
8. Depending on the number of samples a 2-chamber slide (**NC-Slide A2™**) or an 8-chamber slide (**NC-Slide A8™**) can be used.
    a. **NC-Slide A2™**: Load ~30 µl of each of the cell suspensions into the chambers of the slide. Place the loaded slide on the tray of the NucleoCounter® NC-3000™ and select "**Mitochondrial Potential Assay**", sample unit **NC-Slide A2** and press RUN.
    b. **NC-Slide A8™**: Load ~10 µl of each of the cell suspensions into the chambers of the slide. Place the loaded slide on the tray of the NucleoCounter® NC-3000™ and select "**Mitochondrial Potential Assay**", sample unit **NC-Slide A8** and press RUN.

Cellular blue, green and red fluorescence is quantified and cells with collapsed mitochondrial potential exhibit a decrease in red/green fluorescence intensity ratio. The green, red and blue fluorescence will be displayed in scatter plots and histograms. Gates in the scatter-plot can be used to demarcate depolarized/apoptotic cells (see figure below). DAPI positive/non-viable can be defined in the displayed histogram.

Gydevang 43 • DK-3450 Allerod • Denmark • Phone: (+45) 48 13 10 20 • Fax: (+45) 48 13 10 21 • Support: contact@chemometec.com • Web: www.chemometec.com



Jurkat cells stained with JC-1 and DAPI. Cells were grown in the absence (upper left panel) or in the presence of 10 µM camptothecin (CPT) for 5 hours at 37° C (upper right panel). Cells were stained for 10 minutes at 37° C, washed with PBS and analysed on the NC-3000 using the Mitochondrial Potential Assay. **(A)** In this example untreated Jurkat cells are 7% depolarized/apoptotic whereas CPT-treated Jurkat cells are 72% depolarized/apoptotic. **(B)** Histogram showing blue DAPI fluoresence of CPT treated cells **(C)** Image acquired from the CPT-treated sample.

Troubleshooting

Inappropriate loading of the NC-Slides:

Due to variations in chamber volumes the exact amount needed to fill the chamber may vary. Make sure that the chamber is completely filled and that no excess liquid spreads into other chambers or onto the top of the cover slip. Furthermore, avoid introduction of air bubbles into the chambers. Insufficient filling and air bubbles may cause cell movement compromising the quality of the image analysis.

Inadequate washing of samples:

It is important to do two washes with PBS. During washing make sure that the supernatant is completely removed in order to get rid of extracellular JC-1 aggregates. The presence of extracellular JC-1 aggregates will compromise the results since they are included in the cell count.

Gydevang 43 • DK-3450 Allerod • Denmark • Phone: (+45) 48 13 10 20 • Fax: (+45) 48 13 10 21 • Support: contact@chemometec.com • Web: www.chemometec.com

Handling and storage

For handling and storage of ChemoMetec instruments and reagents and NC-Slides refer to the corresponding product documentation. For other reagents refer to the material data sheet from the manufacturer of the reagents and chemicals.

Warnings and precautions

For safe handling and disposal of the ChemoMetec reagents and NC-Slides refer to the corresponding product documentation and the NucleoCounter® NC-3000™ user's guide. For other reagents refer to the safety data sheet from the manufacturer of the reagents and chemicals required for this protocol. Wear suitable eye protection and protective clothes and gloves when handling biologically active materials.

Limitations

The NucleoCounter® NC-3000™ system is FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USE. The results presented by the NucleoCounter® NC-3000™ system depend on correct use of the reagents, NC-Slide and the NucleoCounter® NC-3000™ instrument and might depend on the type of cells being analyzed. Refer to the NucleoCounter® NC-3000™ user's guide for instructions and limitations.

Liability disclaimer

This application note is for RESEARCH PURPOSES ONLY. It is not intended for food, drug, household, or cosmetic use. Its use must be supervised by a technically qualified individual experienced in handling potentially hazardous chemicals. The above information is correct to the best of our knowledge. Users should make independent decisions regarding completeness of the information based on all sources available. ChemoMetec A/S shall not be held liable for any damage resulting from handling or contact with the above product.

Product disclaimer

ChemoMetec A/S reserves the right to introduce changes in the product to incorporate new technology. This application note is subject to change without notice.

Copyright

Gydevang 43 • DK-3450 Allerod • Denmark • Phone: (+45) 48 13 10 20 • Fax: (+45) 48 13 10 21 • Support: contact@chemometec.com • Web: www.chemometec.com