Chapter 9

Analysis of CDK Inhibitor Action on Mitochondria-Mediated Apoptosis

Anna Gortat

Abstract

The role of cyclin-dependent kinase inhibitors (CDKIs) is to negatively regulate cyclin-dependent kinases as a mechanism of control of cell proliferation. As such, CDKIs are being used to induce apoptosis in cancer cells to prevent their excessive reproduction. This chapter describes procedures to study apoptosis induction upon treatment with any CDKI through the evaluation of morphological and functional mitochondrial alterations, in particular, how to measure the mitochondrial membrane potential ($\Delta\Psi m$) using TMRE dye, determine the content of intracellular ATP, observe mitochondrial network morphology using HeLa cells stably expressing fluorescent reporter DsRed targeting mitochondrial matrix, observe ultrastructure of the organelle using transmission electron microscopy, and, finally, assure that mitochondrial outer membrane permeabilization takes place by assessing the subcellular localization of cyt C in HeLa cells stably expressing fluorescent cyt C-GFP.

Key words Apoptosis, ATP, CDKIs, Mitochondrial membrane potential disruption, Mitochondrial network, MOMP

1 Introduction

Cyclin-dependent kinases (CDKs) are a family of serine/threonine kinases which are essential for driving cell cycle phases in eukaryotic cells and as such are considered a potential target for anticancer treatment [1]. Cancer cells proliferate outside their normal growth restraints. Selective interruption of the cell cycle in cancer cells by interfering with CDK action triggers apoptotic cell death. Therefore, development of molecules that can specifically inhibit CDKs is a pharmacological challenge and some of them are currently undergoing clinical trials [2–4].

Apoptosis is a mechanism by which eukaryotic cells commit suicide. It permits to eliminate unwanted and defective cells through an ordered process that excludes inflammatory response induction [5]. Mitochondria play a central role in apoptosis. They control the intrinsic pathway of apoptosis [6], participate in the

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extrinsic pathway [7, 8], and additionally are implicated in nonapoptotic cell death [9–13]. When a cell starts to die, the mitochondrial outer membrane permeabilization (MOMP) occurs, and the proteins residing in the intermembrane space (IMS) are being released into the cytosol until, finally, dissipation of the mitochondrial membrane potential ($\Delta\Psi m$), shutdown of ATP synthesis, and structural collapse of mitochondria take place. This triggers caspase-dependent [14] as well as caspase-independent executionary cascade [15–17] resulting in the final dismantling of the cell.

In this section, the materials and methods used to determine the state of mitochondria are presented. We describe in detail how to measure the mitochondrial membrane potential ($\Delta\Psi m$) using TMRE dye, how to determine the content of intracellular ATP, how to observe mitochondrial network morphology using HeLa cells stably expressing fluorescent reporter DsRed targeting mitochondrial matrix, how to observe ultrastructure of the organelle using transmission electron microscopy, and, finally, how to assure that MOMP takes place by assessing the subcellular localization of cyt C in HeLa cells stably expressing fluorescent cyt C-GFP.

2 Materials

1. Culture medium adequate for the cell line of your choice complemented or not with fetal bovine serum (FBS): Here, we use high-glucose (4.5 g/l) DMEM complemented with 10 % FBS to grow HeLa cells and their derivatives.
2. Petri dishes.
3. Serological pipettes, sterile.
4. Plates 6-well, 96-well.
5. Laminar flow chamber.
6. Cell culture incubator at 37 °C and 5 % CO_2.
7. Centrifuge.
8. Conical tubes (15 and 50 ml).
9. Cover slips.
10. Hemocytometer.
11. Light microscope, e.g., Olympus.
12. Fluorescence microscope, e.g., Leica.
13. Phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.4).
14. Distilled water.
15. Dimethylsulfoxide (DMSO).
16. Ethanol absolute.

2.1 Staining with TMRE

1. Tetramethylrhodamine ethyl ester perchlorate (TMRE) stock solution: For 10 ml of stock solution, add 2.5 mg of TMRE to 10 ml of 95 % ethanol and store frozen at –20 °C. Take 20 µl of the 0.5 mM stock solution and dilute in 100 µl normal buffer (NB: 130 mM NaCl, 5.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2 \cdot 7H_2O$, 25 mM glucose, 20 mM HEPES pH 7.4) to prepare the working solution.
2. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) stock solution at 1 mM should be prepared in 99.9 % ethanol and stored at –20 °C.

2.2 Intracellular ATP Content

1. White-walled 96-well luminometer plates for cell culture.
2. Plate shaker, for mixing multiple plate.
3. Luminometer capable of reading multiwell plates, e.g., Wallac Victor 2.

2.3 Mitochondrial Network Morphology Using HeLa Cells Stably Expressing mt-DsRED

1. Paraformaldehyde (PFA).
2. Gelatin.
3. Kimwipes.

2.4 Ultrastructure of the Mitochondria Using Transmission Electron Microscopy

1. Lab-Tek chamber slides.
2. Uranyl acetate.
3. Glutaraldehyde.
4. OsO_4.
5. Glue.
6. Araldite blocks.
7. Toluidine blue.
8. Lead citrate.
9. Liquid nitrogen.
10. Ultra Cut UC-6.
11. Transmission electron microscope.
12. Triton X-100.
13. Trypsin-EDTA solution.

2.5 Observation of the cyt C Translocation Using HeLa Cells Stably Expressing cyt C-GFP

1. PFA.
2. Gelatin.
3. Kimwipes.

3 Methods

We describe here general protocols for mitochondrial network visualization using HeLa cell line or its derivatives, upon apoptosis induction by CDK inhibitors. You should adapt these protocols to a cell line and the CDK inhibitors of your choice.

3.1 Mitochondrial Transmembrane Potential ($\Delta\psi m$) Measurement by Flow Cytometry Using TMRE Dye

The majority of energy required by the cells is produced by mitochondria in the form of adenosine 5′-triphosphate (ATP). This process requires that the electron transport chain (ETC) generates an electrical potential across the mitochondrial inner membrane ($\Delta\psi m$) which is used by ATP synthase (Complex V) to fuse free phosphate with adenosine diphosphate (ADP). $\Delta\psi m$ can be detected by a series of positively charged dyes that can penetrate the mitochondrial membrane such as TMRE, TMRM, and mitotracker. TMRE (tetramethylrhodamine, ethyl ester) is a cell-permeant, positively charged, red-orange dye that accumulates promptly in active mitochondria due to their relative negative charge [18]. The lipophilic structure of this dye allows it to penetrate cell and mitochondrial lipid bilayer membrane barriers within few minutes and thus TMRE is widely used for detection of the mitochondrial permeability transition in whole cells via fluorescence analysis methods. Depolarized or inactive mitochondria with affected membrane potential fail to accumulate TMRE and thus remain unstained. Then, the dye accumulation in mitochondria may be optically detected by flow cytometry allowing for at least qualitative assessment of $\Delta\psi m$ among experimental conditions (*see* **Note 1**).

3.1.1 Staining with TMRE

1. Culture HeLa cells for a non-induced negative control as well as a positive control at 1×10^5 cells per well (2 ml/well in 6-well plate) in high-glucose (4.5 g/L) DMEM complemented with 10 % fetal bovine serum (FBS) and grow overnight at 37 °C under 5 % CO_2.
2. For the first-time flow cytometry measurement, additional two controls of untreated cell population have to be prepared to calibrate the cytometer.
3. Induce $\Delta\psi m$ depolarization by treating the cells (except the untreated controls) with 250 nM FCCP for 10 min at 37 °C (*see* **Note 2**).
4. Remove medium from cells, replace with fresh complete medium containing TMRE, and incubate for 15 min at 37 °C 5 % CO_2. One of the additional controls of untreated cells is stained with TMRE and the other one is left unstained (*see* **Notes 3** and **4**).
5. Remove the medium and detach the cells gently from the culture dish using trypsin.

6. Resuspend cells in fresh complete medium to a density required for flow cytometry (0.5×10^6 cells/ml) so that their final volume should be close to 0.5 ml.
7. Transfer cell suspension into a clean polypropylene tube and submit to flow cytometry measurements using settings as described below (*see* **Note 5**).

3.1.2 Flow Cytometer Settings

1. The excitation peak of TMRE is at 549 nm but using the common Argon blue line (488 nm) laser most flow cytometers yield excellent results. The emission peak of TMRE is at 574 nm and lies within the FL2 emission region.
2. A log FL2 (X-axis) versus relative cell number (Y-axis) histogram has to be created.
3. First, the untreated control cell population stained with TMRE (max $\Delta\psi m$) has to be run and the FL2 PMT voltage adjusted to allow the peak to fall within the third log decade.
4. Second, the untreated control cell population without TMRE staining (no $\Delta\psi m$) has to be run and the peak adjusted to fall within the first log decade.
5. For the final calibration step, the FCCP-depolarized positive sample stained with TMRE has to be run using the same voltage settings as above to assure that the histogram peak is visible on the X-axis. If this is not the case, PMT voltage has to be increased slightly to achieve positive control staining within the first decade of the log scale similar to the untreated control cell population without TMRE staining (*see* **Note 3**).
6. Finally, samples have to be run using the established conditions. Data can be analyzed by several software such as WinMDI FlowJo, ModFit, and Cell Quest.

3.2 Intracellular ATP Content

Apoptosis is an energy-consuming process and thus requires ATP to occur [19, 20]. For a short period of time after apoptosis induction a brief burst of ATP production can be observed [21]. However, due to inevitable shutdown of the mitochondria, intracellular concentration of ATP drops below the steady-state levels. Therefore, measurements of the intracellular ATP concentration may be considered as additional informative parameter related to mitochondrial function and metabolism [22, 23]. Here, we use ATPLite, the commercially available luminescence ATP detection assay system from Perkin Elmer. It is an ATP monitoring system based on firefly luciferase which is highly sensitive and produces a long-lived luminescence signal (half-life above 5 h). The ATPLite system permits measurement of the amount of light produced by the reaction of ATP with luciferase and d-luciferin in which, within certain limits, the emitted amount of light is proportional to the ATP concentration.

3.2.1 Determining Intracellular ATP Content

1. Culture HeLa cells at 5×10^4 per well (100 µl/well in 96-well plate) overnight. Prepare at least two wells for each predicted condition (*see* **Note 6**).
2. Series of seven 100 µl complete culture media without cells in duplicates has to be pipetted into the wells on the plate to be used for determining the ATP standard curve.
3. Treat the cells with your CDK inhibitor of choice for different times (3, 6, and 12 h) at 37 °C under 5 % CO_2. Include untreated and vehicle controls for each experiment.
4. Equilibrate reagents of the ATPLite system to room temperature for at least half an hour.
5. Prepare the ATP standard curve recommended in the manufacturer's protocol (*see* **Note 7**).
6. Reconstitute lyophilized substrate according to the manufacturer's instructions.
7. Lyse cells by adding 50 µl of mammalian cell lysis solution to 100 µl of cell suspension per well. It permits to lyse the cells and inactivate the endogenous ATPases in order to maintain the original intracellular levels of ATP. Lysis will be completed after 5 min of shaking at 700 rpm. Also, 50 µl of mammalian cell lysis solution will be added to 100 µl cell-free wells to determine the ATP standard curve.
8. Add 10 µl of each ATP standard dilution to the corresponding wells (in duplicates) and shake the plate for 5 min at 700 rpm.
9. Add 50 µl of substrate solution and let the reaction to run for 5 min in an orbital shaker at 700 rpm.
10. Incubate the plate on dark for 10 min and measure luminescence in a luminometer microplate reader (*see* **Note 8**).

3.3 Mitochondrial Network Morphology Using HeLa Cells Stably Expressing mt-DsRED

In healthy cells mitochondria form a network that undergoes constant rearrangements controlled by the balance between fusion and fission events mediated by specific proteins [24–26]. Mitochondrial fusion/fission events within the network are precisely controlled and respond to physiological and pathological conditions. It is an efficient, dynamic system to deliver energy, lipids, proteins, calcium, and metabolites between different areas of the cell [27, 28]. Also, mitochondrial DNA showed to be efficiently transferred throughout the network by matrix mixing during fusion events [29]. Mitochondria play a crucial role in apoptosis. A clear functional link of mitochondrial morphology and apoptosis exists. The rate of fission increases rapidly at the early stages of apoptosis [26] which is accompanied by cristae disorganization (*see* Fig. 1) and, in consequence, MOMP. Therefore, monitoring the mitochondrial network morphology may be an adequate determinant to establish changes in mitochondrial functioning upon CDK inhibitor treatment.

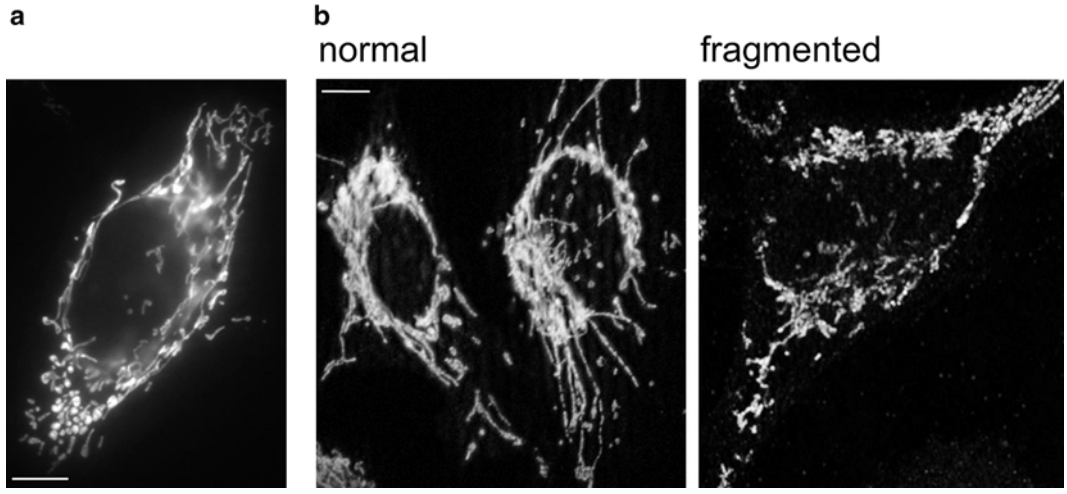

Fig. 1 Mitochondrial network is heterogenous within a cell. (**a**). Mitochondria in HeLa cells stably expressing mtDsRED form a dynamic, tubular network under physiological conditions. Mitochondrial morphology within a cell, although highly organized, appears heterogenous varying in density of clumps and tubules. (**b**). HeLa-mtDsRED were submitted (fragmented) or not (normal) to an apoptotic stimuli using 0.5 μM staurosporine (ST) for 6 h. Fragmented mitochondrial network is clearly visible upon apoptosis induction. Scale bars: 5 μm

3.3.1 Preparation of HeLa-DsRED Cells for Fluorescence

1. Place clean and sterile cover slips in a 6-well plate (you can place up to four cover slips per well).
2. Plate HeLa-DsRED cells at 1×10^5 cells (*see* **Note 9**) per well and let to attach overnight (*see* **Note 10**).
3. Treat the cells with the CDK inhibitor of your choice at different times to induce apoptosis.
4. A non-induced control as well as a positive control to fragment the mitochondrial network have to be introduced. Treat the cells with 250 nM FCCP for 10 min to depolarize mitochondria and obtain the mitochondrial network fragmentation (*see* **Note 2**).
5. Remove the medium and wash gently with PBS.
6. Fix the cover slips with 3 % PFA in PBS, incubate for 20 min at room temperature, and then wash twice with PBS to remove any remaining PFA (*see* **Note 11**).
7. Permeabilize the cells with 0.1 % Triton X-100 (in PBS) for 10 min and wash twice for 5 min with PBS to remove all the detergent.
8. Wash the cells once with PBS-gelatin 0.2 % and block with PBS-gelatin 0.2 % for 30 min at room temperature. Then, wash the cells with PBS for 5 min.
9. Drop cover slips water one by one, dry immediately using Kimwipes, and mount on 2 μl Mowiol 4-88 facing the slide.

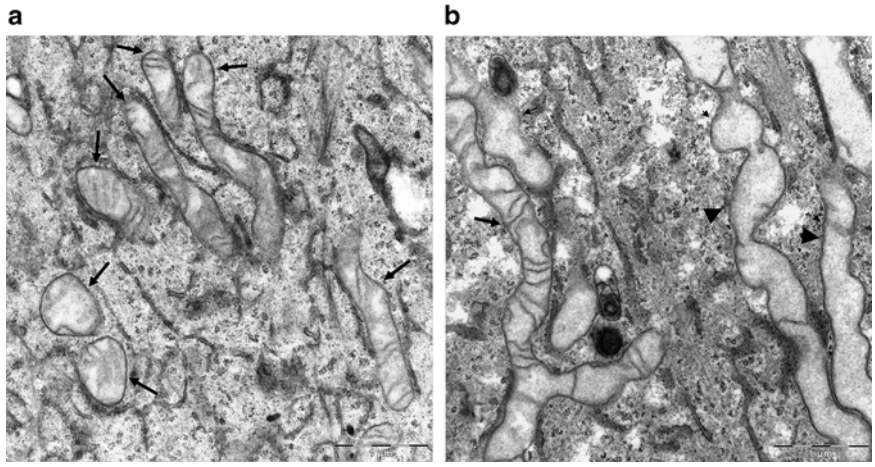

Fig. 2 Remodeling of mitochondrial ultrastructure upon apoptosis. HeLa cells were induced to apoptosis with 2 μM doxorubicin for 24 h (**b**) and submitted to transmission electron microscopy (TEM) as described. As a control, vehicle-treated cells were used (**a**). *Black arrows* in (**a**) show normal cristae organization. *Black arrow* in (**b**) shows mitochondria with partially affected, still visible cristae structure upon apoptosis induction. The cristae are not as well organized as in (**a**). *Black arrowheads* in (**b**) show mitochondria with heavily affected ultrastructure upon apoptosis induction. There are only few, very short and unorganized cristae visible. Scale bars: 1 μm

10. Dry slides overnight at room temperature, well covered from light.
11. Mitochondrial network is observed under fluorescence microscope using standard Cy3 filter.

3.4 Ultrastructure of the Mitochondria Using Transmission Electron Microscopy

Electron microscopy is a very powerful tool for the examination of mitochondrial ultrastructure in nanometer scale. It reveals that mitochondria vary in their shape; they can exist as spheres, rods, and a sausage-like structure with diameter ranging from 0.5 to 1 μm (*see* Fig. 2). During apoptosis, the ultrastructure of mitochondria undergoes a profound remodeling (*see* Fig. 2). The change in morphology is progressive and consists of losing the packed and folded appearance of the cristae. Individual crista fuses, cristae junctions open, the matrix and the intermembrane space expand, and, finally, the whole mitochondrial morphology appears vesiculated or even swollen without any cristae possible to identify [30–32] (*see* Fig. 2b).

3.4.1 Preparation of Cells for Transmission Electron Microscopy

1. Plate 3×10^4 HeLa cells per chamber in a Lab-Tek chamber slide containing four wells and treat or not with 2 μM doxorubicin or your CDK inhibitor of choice to induce apoptosis.
2. Remove the medium and fix the cells for 1 h in 3.5 % glutaraldehyde at 37 °C and then post-fix for 1 h in 2 % OsO_4 at room temperature.

3. Stain the cells in the dark at 4 °C for 2 h with 2 % uranyl acetate.
4. Rinse the cells in sodium phosphate buffer (0.1 M pH 7.2), dehydrate in ethanol, and infiltrate overnight in araldite base embedding agent.
5. After polymerization, detach the embedded cultures from the chamber slide and glue to araldite blocks.
6. Cut serial semi-thin sections (1.5 μm) with an Ultracut UC-6-, mount onto slides, and stain with 1 % toluidine blue.
7. Select semi-thin sections containing cells and glue to araldite blocks.
8. Detach semi-thin sections from the glass slide by repeated freezing and thawing in liquid nitrogen.
9. Prepare ultrathin sections (0.07 μm) with the Ultracut and stain with lead citrate.
10. Submit the samples to a transmission electron microscope and obtain photomicrographs of the mitochondria.

3.5 Observation of the cyt C Translocation Using HeLa Cells Stably Expressing cyt C-GFP

Mitochondria lie at the center of the intrinsic apoptotic pathway. It is not only the bioenergetic center of the cell, but it also contains pro-death factor such as cytochrome C (cyt C) which resides in the mitochondrial intermembrane space (IMS). Release of cyt C from the IMS induces a signaling cascade that leads to demise of the cell. Thus, MOMP is the critical event that permits the release of pro-apoptotic molecules from the IMS and disrupts mitochondrial bioenergetics (*see* **Note 12**). Here, we describe how to follow translocation of the mitochondria-targeted fluorescent protein cyt C-GFP stably expressed in HeLa cells (HeLa-GFP; kind gift from C. Muñoz-Pinedo).

3.5.1 Establishing Homogeneous HeLa-GFP Stable Cell Line

First, we need to assure that the level of fluorescence intensity provided by the expression of cyt C-GFP within HeLa cells is relatively homogeneous throughout the whole population to facilitate the posterior analysis.

1. Plate 6×10^6 HeLa-GFP cells in 150 mm petri dish. 20 ml high-glucose (4.5 g/L) Dulbecco's modified Eagle medium (DMEM) complemented with 10 % fetal bovine serum (FBS) and penicillin/streptomycin mixture of antibiotics to avoid cell culture contamination is used. 1×10^6 of regular HeLa cells that do not express fluorescent reporter have to be plated in a 100 mm petri dish with 10 ml high-glucose complete DMEM. Grow the cells overnight at 37 °C under 5 % CO_2.
2. Trypsinize cells and count using hemocytometer.

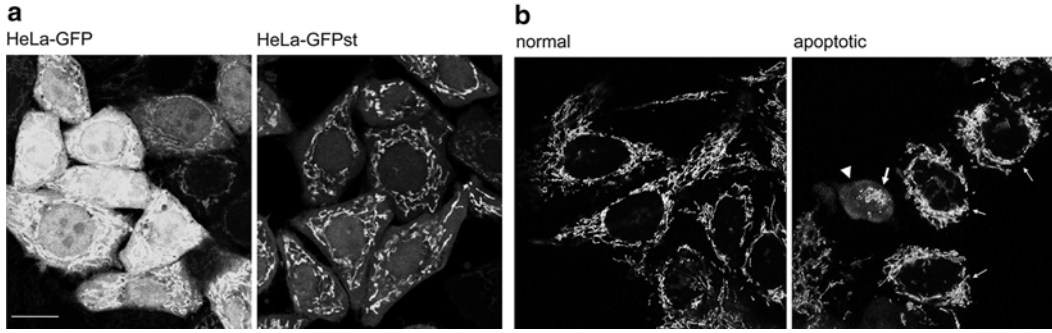

Fig. 3 Translocation of cyt C upon apoptosis induction. (**a**). HeLa-GFP cells stably expressing cyt C-GFP show heterogenous fluorescence intensity. After FACS, a homogeneous population of HeLa-GFP cells with an intermediate fluorescence intensity was selected and called HeLa-GFPst. (**b**). HeLa-GFPst were induced to apoptosis (apoptotic) or not (normal) with 2 μM doxorubicin for 24 h. *White arrowhead* shows a cell with cytosolic staining that appears upon cyt C-GFP translocation. Fragmented, collapsed mitochondria within the apoptotic cell are indicated by a *bold arrow. Thin arrows* show cells where cyt C-GFP translocation did not yet occurred. However, the cells were already affected by the apoptotic treatment as assessed by fragmentation of the mitochondrial network (compared to (**b**) normal and with ST-treated HeLa-mtDsRED cells in Fig. 1). Scale bar: 20 μm

3. Centrifuge the cells for 5 min at $700 \times g$ at room temperature and resuspend the pellets in room temperature PBS to a density of 1×10^6 cells per ml.
4. Submit the cells to fluorescence-activated cell sorter (FACS) using blue Argon series laser for emission and the FL1 detector (*see* **Note 13**).
5. Calibrate FACS using as a negative control regular HeLa cells that do not express fluorescent reporter, and then run a small sample of HeLa-GFP cells to adjust the FL1 PMT voltage by allowing the peak of GFP fluorescence to fall within the third log decade.
6. Then, recover HeLa-GFP cells with an intermediate fluorescence intensity of cyt C-GFP (between the second and the third log decades) (*see* **Note 14**).
7. Plate 1×10^5 of the recovered cells per well in 6-well plate and grow overnight.
8. Inverted fluorescence microscope can be used to confirm that the cells within the wells show a homogeneous intensity of fluorescence.
9. After this procedure we refer to these cells as HeLa-GFPst (*see* Fig. 3a).

3.5.2 Observation of cyt C Translocation Using Fluorescence Microscopy

1. Place clean and sterile cover slips in a 6-well plate. Plate 1×10^5 of Hela-GFPst cells per well and left to attach overnight.
2. Treat the cells with 2 μM doxorubicin for 24 h or the CDK inhibitor of interest to induce apoptosis. Include a non-induced control.

3. Remove medium from wells and wash them gently with PBS.
4. Fix cover slips with 3 % PFA, incubate for 20 min at room temperature, and then wash twice with PBS to remove any remaining PFA (*see* **Note 15**).
5. Permeabilize cells with 0.1 % Triton X-100 (in PBS) for 10 min and then wash twice with PBS to remove all the remaining detergent.
6. Wash cells with PBS-gelatin 0.2 % and block with PBS-gelatin 0.2 % for 30 min at room temperature. Then, wash with PBS for 5 min.
7. Drop cover slips into water, dry immediately using Kimwipes, and mount on 2 μl Mowiol 4-88 facing the slide.
8. Dry slides overnight at room temperature, well covered from light.
9. Observe the samples under fluorescence microscope using standard FITC filter.

4 Notes

1. We strongly recommend to use TMRE at concentrations below 50 nM. At low concentrations, it does not form aggregates (nonquenching conditions) in cell membranes and interacts with membrane proteins minimally; thus the transmembrane distribution of TMRE is directly related to the membrane potential in accordance to the Nernst equation [18, 33]. To avoid dye aggregation, TMRE concentrations ranging between 0.5 and 30 nM are used in our setups. We recommend to determine empirically the best concentration of TMRE to be used for each cell line and treatment conditions. It is always desirable to use the lowest possible dye concentration to avoid any variation in its degree of binding to mitochondria which may result in greater dye accumulation than predicted. In practice, at nonquenching concentrations TMRE does not inhibit the electron transport chain (ETC) [34] which makes it a reliable dye for $\Delta\psi m$ studies. Also, at high concentrations TMRE may stain endoplasmic reticulum in addition to mitochondria and that would interfere with the interpretation of results.
2. We recommend to use mitochondrial oxidative phosphorylation (OxPhos) inhibitor FCCP to depolarize $\Delta\psi m$. FCCP is a protonophore which uncouples the ETC from OxPhos system by inducing reversal of the ATPase and thus depolarizes mitochondria by increasing their permeability to protons. Collapse of $\Delta\psi m$ can be achieved by addition of 250 nM FCCP 10 min prior to measurement. However, in the absence of FCCP any inducer of apoptosis may be used, e.g., 0.5 μM staurosporine (ST), for 6 h to collapse $\Delta\psi m$.

3. Working solution (100 nM) of TMRE from a stock solution (0.5 mM) is prepared. It is used only on the day of preparation.
4. TMRE working solution is diluted to a final concentration of 30 nM directly in the fresh complete medium.
5. After staining with TMRE, samples should be stored protected from light until they can be analyzed on the flow cytometer. However, we strongly recommend to perform the measurements as soon as possible and as late as within 30 min after TMRE equilibration. Temperature can affect TMRE staining; therefore we recommend to avoid storing the samples on ice prior to analysis.
6. For plating the cells we use white opaque 96-well plates. White plates, in contrast to the black ones, reflect light and will maximize light output signal; therefore we strongly recommend their use.
7. For ATP standard curve we recommend to prepare standards of 1×10^{-5} M, 1×10^{-6} M, 1×10^{-7} M, 1×10^{-8} M, 1×10^{-9} M, 1×10^{-10} M, and blank. Values of intracellular ATP concentrations should fall within this range of ATP concentrations.
8. It is very important to handle the plate in reduced illumination conditions prior to measurement for avoiding the plate phosphorescence which may result in higher background levels.
9. The major advantage of using stable cell line expressing a fluorescent reporter is that changes in mitochondrial network morphology can be visualized in living cells. Also, if required, DsRED fluorescence is stable under fixation conditions, thus enabling a wide range of flexibility for a variety of applications.
10. For tracking the mitochondrial network morphology, we use HeLa cells stably expressing an mtDsRED fluorescent reporter (kind gift from M. Rojo). mtDsRED bears mitochondrial targeting sequence fused to 5′ end of the DsRED molecule, thus targeting the DsRED fluorescent reporter to the mitochondrial matrix. This permits to observe morphology of the mitochondrial network in extreme conditions such as apoptosis without diffusion of the fluorescent signal as occurs when using proteins residing in the mitochondrial IMS, e.g., cyt C and SMAC. Proteins residing in the IMS get translocated to the cytosol upon apoptosis induction whereas mtDsRED staining remains in the mitochondrial matrix, thus making the fragmentation processes observable (*see* Fig. 1b, normal and fragmented).
11. PFA fixation is a method of choice for membrane-bound and cytoskeletal structure observation and for that reason we use it here to visualize mitochondrial network. Prepare a 12 % PFA stock solution in PBS. It is difficult to dissolve. Weight the exact amount of PFA and add it to warm PBS. Stir for a couple

of hours in a water bath. Do not exceed 60 °C. You can add a few drops of 10 N NaOH to facilitate the dissolution. Once prepared, store frozen at −20 °C for up to 1 year. Prepare a 3 % working solution by diluting with PBS just before use and throw away the excess. Check each batch of PFA before use.

12. Until now, we were determining apoptosis by measuring mitochondrial membrane depolarization and cellular ATP concentration and observing mitochondrial network morphology. In most cases, all these can be used as surrogate for measuring MOMP which is the determinant of commitment to apoptosis but additional techniques must be used to assure that MOMP actually took place. One has to keep in mind the restriction of each of the methods described previously. The drop in $\Delta\psi m$ is not always equivalent to MOMP. Some protonophores, i.e., FCCP used as a positive control in our measurements, depolarize mitochondria in the absence of MOMP. The same is true for the mitochondrial network fragmentation. Although mitochondrial fragmentation is indeed associated with apoptosis, excessive mitochondrial fragmentation can occur in a variety of conditions independently of apoptosis processes, such as that occurring upon exposure to carbonyl cyanide m-chlorophenyl hydrazone (CCCP), uncoupling agent that disrupts the electrochemical potential of the mitochondrial inner membrane. To unambiguously establish the occurrence of MOMP a translocation of the IMS residing factors such as cyt C must be assessed (*see* Fig. 3b).
13. The green fluorescent protein (GFP) absorbs light with an excitation maximum of 395 nm and fluoresces with an emission maxima of 510 nm. However, it may be excited at 488 nm by a standard blue Argon series laser and the emission peak can be detected in the FL1 detector.
14. The key for an efficient recovery of cells during FACS is the optimal number of cells and their density. We recommend to prepare 5 ml of cell suspension at the density of 1×10^6 cells per ml.
15. PFA fixation will affect the GFP fluorescence only slightly, in contrast to methanol or ethanol fixation which can completely abolish the GFP signal.

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