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Measuring apoptosis at the single cell level

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Abstract

The use of live cell microscopy has made a number of contributions to the study of apoptosis. Many of the tools and techniques are available that allow us to image the key events that occur during cell death including mitochondrial outer membrane permeabilization, mitochondrial transmembrane potential changes, translocation of Bcl-2 family members, caspase activation, phosphatidylserine flip and plasma membrane rupture. We discuss these techniques here and highlight the advantages and drawbacks of using such approaches to study apoptosis.

Keywords: Apoptosis; Mitochondria; Time-lapse confocal microscopy; Cytochrome *c*; Caspase

1. Introduction

During apoptosis, many pro-death stimuli converge on the mitochondrial pathway. This leads to permeabilization of the outer mitochondrial membrane and the release of proteins that reside in the mitochondrial inter membrane space including cytochrome *c*, Omi and Smac. Diffusion of cytochrome *c* to the cytosol results in the activation of caspases, the subsequent cleavage of specific cellular substrates and death of the cell [1]. The use of live cell microscopy has led to many advances in the field of apoptosis especially with respect to studying the mitochondrial pathway.

The first descriptions of apoptosis described the morphological changes that consistently occur during this form of cell death—namely cell shrinkage, nuclear condensation, blebbing and the formation of apoptotic bodies as the cell's membrane collapses [2]. More recently, we and others have used live cell confocal and wide-field fluorescence microscopy to assess the dynamic nature of these events [3–5]. Through the use of fluorescently labeled proteins and organelle-specific dyes we can visualize, in real time, the events that occur during mitochondrial apoptosis including mitochondrial outer membrane permeabilization (MOMP), loss of mitochondrial transmembrane potential, caspase activation, plasma membrane reorganization and deterioration.

2. Single cell analysis of the events that occur during apoptosis

2.1. Visualizing cytochrome c release

When stably expressed in cells, cytochrome *c* fused to green fluorescent protein (cyt *c*-GFP) has been shown to behave similarly to endogenous cytochrome *c* [4,6]. It localizes predominantly to the mitochondria and is released when the cells are treated with a pro-apoptotic stimulus to assume a diffuse cytosolic pattern. When these cells were observed using time-lapse confocal microscopy the exact duration of cyt *c*-GFP release in single cells could be measured and was found to take approximately 5 min irrespective of stimulus and the release was complete and kinetically invariant [3,4].

Cyt *c*-GFP can be introduced into a variety of cell lines such as Hela by retroviral transduction. This allows multiple copies of cyt *c*-GFP to be expressed in each cell, which

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boosts the signal of GFP facilitating the detection of the protein by wide-field fluorescence or confocal microscopy. Often the cells must be sorted on the GFP intensity to obtain a more homogeneous population or to select clones with the correct mitochondrial localization. The extent of cyt *c*-GFP release after treatment with a pro-apoptotic stimulus that engages the mitochondrial pathway, such as actinomycin D or UV, can be measured in these cells by counting the number of cells with a diffuse GFP signal, indicating release, versus those with a punctate or mitochondrial distribution of cytochrome *c*-GFP. These cells can also be used to monitor the release of cytochrome *c* during apoptosis in real time using time-lapse microscopy (see Appendix A) and the kinetics of the release can be expressed graphically (Fig. 2, Appendix C).

The size of the GFP moiety may alter the localization or function of a protein, such as cytochrome *c*, leading to an incorrect interpretation of the behavior of the endogenous protein. In some cases, therefore, it may be desirable to use an alternative method to study, for instance, the possible size restraints of the MOMP-responsible pore. Tsien and colleagues have developed a method to visualize proteins in real time through the addition of a small tag that binds cell-permeable fluorophores [7,8]. This tag of 12 amino acids comprises a core tetracysteine motif that can bind biarsenical dyes, and it is thus named "tetracysteine" or TC tag. Two of these dyes, FlAsH and ReAsH (fluorescein/resorufin arsenical helix binder), are commercially available (Invitrogen) and are green and red emitting, respectively. Cells can stably or transiently express the tagged protein of interest, and then are stained with the dye (Appendix B). The main problem of this technique is a high level of background due to the non-specific staining of different areas around the cell. Regardless, this technique has been successfully used to monitor the release of cytochrome *c* during apoptosis in NCI-H1299 and Hela cells, and Smac, Omi, Adenylate kinase 2 and AIF in Hela cells [3,9].

2.2. Loss of mitochondrial transmembrane potential

Apart from its role in apoptosis, cytochrome *c* also plays an integral role in the electron transport chain that produces ATP and provides energy to the cell. The electron transport chain is comprised of a number of protein complexes (I–V) that are localized to the inner mitochondrial membrane. As electrons are transported from one complex to the next protons are produced and pumped from the mitochondrial matrix to the intermitochondrial space. This creates an electrochemical potential across the membrane known as the mitochondrial transmembrane potential ($\Delta\psi_m$). Upon MOMP and cytochrome *c* release the $\Delta\psi_m$ is lost [10].

This loss of $\Delta\psi_m$ can be measured in single cells by measuring the loss of TMRE or TMRM (Tetramethyl rhodamine ethyl-or methyl-ester) over time. These fluorescent dyes bind to mitochondria with high $\Delta\psi_m$ and the dye is released from the mitochondria when $\Delta\psi_m$ dissipates.

Loss of TMRE has been observed to occur at the same time as the release of cytochrome *c*-GFP and is often used as a good approximation of the occurrence of MOMP (Fig. 1). However, careful observation of TMRE loss versus cytochrome *c* release in cells undergoing apoptosis has demonstrated that the two events are not simultaneous [11]. Loss of $\Delta\psi_m$ actually occurs slightly later than release of cytochrome *c*. Furthermore it is possible to observe loss of $\Delta\psi_m$ without cytochrome *c* release. The electron transport chain uncouplers FCCP or CCP immediately induce loss of TMRE when added to cells but do not cause cytochrome *c* release. Also when caspases are inhibited $\Delta\psi_m$ is decreased but is restored over time, while cytochrome *c* release is normal [11] .

There are other dyes available that also measure $\Delta\psi_m$ each with their own advantages and drawbacks [12]. Rhodamine 123 is similar to TMRE but quenches at high concentrations and is also quite toxic to most cells. CMXROS has the advantage of being fixable. JC1 is a dual color dye that is green when it is cytosolic but shifts to red fluorescence when it binds to the mitochondria. These dyes do not rapidly efflux, however, when $\Delta\psi_m$ dissipates and therefore are less useful for single cell imaging (Table 1).

2.3. Translocation of Bcl-2 family proteins

MOMP is primarily regulated by the Bcl-2 family of proteins. Some of these proteins are constitutively located on mitochondria or other intracellular membranes. Others, however, translocate to mitochondria during apoptosis. A GFP-Bax fusion protein has been used to study Bax trans-

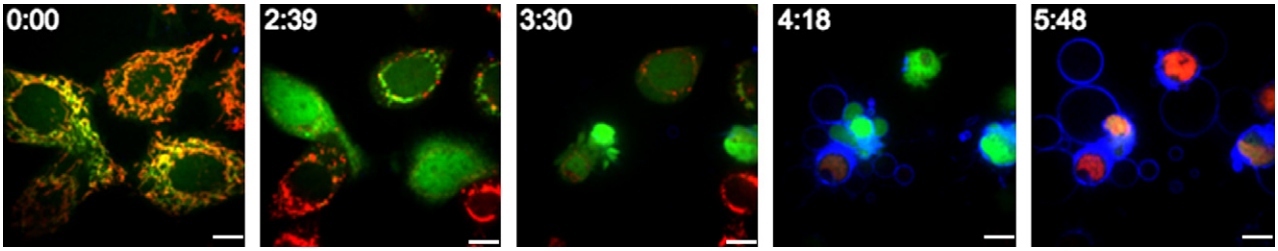

Fig. 1. Time-lapse analysis of the morphological changes that occur during apoptosis. Hela cyt *c*-GFP cells were stained with TMRE (50 nM, red), Annexin V-APC (1% (v/v), blue) and PI (0.4 μg/ml, pink) and treated with actinomycin D (1 μM). The time after exposure to actinomycin D is at the top left of each panel. Scale bars (10 μm) are at the bottom right of each panel.