

Video Article

Strategies for Tracking Anastasis, A Cell Survival Phenomenon that Reverses Apoptosis

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Abstract

Anastasis (Greek for “rising to life”) refers to the recovery of dying cells. Before these cells recover, they have passed through important checkpoints of apoptosis, including mitochondrial fragmentation, release of mitochondrial cytochrome c into the cytosol, activation of caspases, chromatin condensation, DNA damage, nuclear fragmentation, plasma membrane blebbing, cell shrinkage, cell surface exposure of phosphatidylserine, and formation of apoptotic bodies. Anastasis can occur when apoptotic stimuli are removed prior to death, thereby allowing dying cells to reverse apoptosis and potentially other death mechanisms. Therefore, anastasis appears to involve physiological healing processes that could also sustain damaged cells inappropriately. The functions and mechanisms of anastasis are still unclear, hampered in part by the limited tools for detecting past events after the recovery of apparently healthy cells. Strategies to detect anastasis will enable studies of the physiological mechanisms, the hazards of undead cells in disease pathology, and potential therapeutics to modulate anastasis. Here, we describe effective strategies using live cell microscopy and a mammalian caspase biosensor for identifying and tracking anastasis in mammalian cells.

Video Link

The video component of this article can be found at <https://www.jove.com/video/51964/>

Introduction

Apoptosis (Greek for “falling to death”) is generally assumed to be a one-way process ending in cell suicide¹⁻⁷. Genetic disruption of pro-death genes results in the survival of extra cells that would otherwise die in whole animals, including cells that have already initiated the apoptosis pathway^{8,9}. Similarly, genetic manipulations allow healthy mammalian cells that artificially display “eat me” signals or that lose adhesiveness to their extracellular matrix to escape death by whole cell phagocytosis or entosis, respectively^{10,11}. However, we and others have shown that without genetic manipulation normal healthy mammalian cells and cell lines can also recover from the early stages of apoptosis¹²⁻¹⁵. Using tools to track individual cells, we have further demonstrated recovery from late stages of apoptosis^{12,13}, after cells have passed important checkpoints that typically mark the “point of no return”²⁻⁶. These checkpoints of late stage apoptosis include mitochondrial release of cytochrome c, activation of caspases, nuclear fragmentation, and formation of apoptotic bodies. We adopted a Greek compound word “anastasis”, which means “rising to life”, to describe this reversal of apoptosis at the brink of cell death²⁻⁶.

Unless the entire dying-recovery process is observed by live cell imaging, it is challenging to distinguish cells that have undergone anastasis from cells that never experienced apoptotic events. Decades of work have revealed that the morphological features of cell suicide by apoptosis are driven by evolutionarily conserved biochemical and molecular events¹⁶⁻¹⁹. These events promote self-destruction of cells to regulate developmental and homeostatic processes in unicellular and multicellular organisms by eliminating damaged or dangerous cells¹⁶⁻¹⁹. While apoptotic cells can be readily distinguished by standardized morphological, biochemical and molecular manifestations of apoptosis^{1,5,6,16,20}, currently there is no known marker specific to anastasis^{12,13}. Importantly, cells that have undergone anastasis appear to be normal healthy cells, and cells that just start reversing apoptosis appear as apoptotic dying cells^{12,13}. Thus, new tools are needed to conclude with certainty that a given surviving cell had previously experienced active apoptotic processes.

Apoptosis is generally assumed as an irreversible cascade because it is a rapid and massive destruction process. While it could take minutes to days for some cells to initiate apoptosis, once mitochondria have released apoptogenic factors such as cytochrome c into the cytosol^{21,22}, caspases can be activated within 5 minutes^{23,24}, followed by cytoplasmic and nuclear condensation within 10 min²⁵⁻²⁷, and cell death shortly thereafter²⁵⁻²⁷. Activated caspases orchestrate apoptosis by cleaving and inactivating key structural and functional components for the purpose of cellular demolition^{2,28}, such as the endonuclease inhibitor DFF45/ICAD^{29,30}. Caspases also activate pro-apoptotic factors, such as BCL-2 family member BID, which translocates to mitochondria to promote mitochondrial release of cytochrome c^{31,32}. Caspase activity also results in cell surface exposure of phosphatidylserine as an “eat me” signal for promoting engulfment of dying cells by macrophages or

neighbor cells through phagocytosis³³. Furthermore, apoptotic events render mitochondria dysfunctional, disrupting cellular bioenergetics and metabolism^{34,35,36}. Thus, recovery from such destruction seems intuitively unlikely.

Contrary to original expectations, cells can reverse the apoptotic cell death process even at a late stage. By continuously monitoring the fate of dying cells in culture, we observed the reversibility of late stage apoptosis in a range of primary cells and cell lines^{12,13}. Removal of the death stimulus allowed recovery from the overt features of apoptosis, such as mitochondrial fragmentation, chromatin condensation, DNA damage, plasma membrane blebbing, cell surface exposure of phosphatidylserine, release of mitochondrial cytochrome c, caspase activation, nuclear fragmentation, cell shrinkage, and formation of apoptotic bodies. These observations raise unanswered questions regarding the functions, consequences, and mechanisms of anastasis. To address these questions, a prerequisite is to reliably identify cells that have undergone anastasis. Here, we describe live microscopy methods and a caspase biosensor for detecting cells that have previously reversed late stage apoptosis and then survived.

Protocol

1. Preparation of Cells for Live Cell Imaging

- To facilitate the detection of morphological changes, choose adherent cells such as HeLa (human cervical carcinoma) cells that are flat on the substrate to better visualize alteration of the plasma membrane and intracellular organelles.
Note: Reversal of apoptosis has been observed in various mammalian cells^{12,13}, including primary mouse liver cells, primary mouse macrophages, primary rat cardiomyocytes, and also cell lines such as human embryonic kidney HEK-293T cells, African green monkey renal epithelial COS-7 cells, mouse cardiac muscle HL-1 cells, mouse NIH 3T3 fibroblasts, ferret (*Mustela putorius furo*) brain CRL1656 cells, human skin cancer A375 cells, human testicular cancer CRL1973 cells, human small cell lung carcinoma H446 cells, human liver cancer HepG2 cells, human breast cancer MCF7 cells, human prostate cancer PC3 cells, and human neuroblastoma SH-SY5Y cells.
- Pre-wash glass coverslips of glass bottom cell culture dishes with absolute ethanol for cleaning and sterilization.
Note 1: Use of glass bottom dishes is recommended for high quality differential interference contrast (DIC) microscopy, and high magnification confocal or epi-fluorescence microscopy (See Discussion).
Note 2: For high magnifications (40X, 60/63X or 100X objectives and high numerical apertures 1.3 or 1.4), use of cell culture dishes with thinner glass bottom (thickness 0.085-0.16 mm) provides longer working distances for imaging (See Protocol 3).
- Depending on the cell type, glass bottom culture dishes may require coating with poly-d-lysine (0.1 mg/ml), collagen (0.01% solution in 0.01 M acetic acid) and/or fibronectin (5 µg/ml) prior to seeding cells.
Note: Optimization of the type and concentration of coating agent is required for different cell lines. HeLa cells can adhere directly on glass without coating. However, some cells, such as mouse cardiac muscle HL-1 cells, cannot adhere directly to glass surfaces, but require specific culture and coating protocols³⁷.
- Seed $\sim 2-3 \times 10^5$ cells on 35 mm pre-coated glass bottom cell culture dishes and incubate at 37 degree Celsius (°C) with 5% CO₂ for a day to achieve 80% confluency.
Note 1: Optimal conditions of cell density, incubation time, and culture condition can vary, depending on the cell type. Cell confluency can affect the apoptotic response of cells (See Protocol 2). At high confluency, cells could be less sensitive to the same concentrations of apoptotic stimuli.
Note 2: Avoid over confluent cells, as high cell density can obscure morphological changes of individual cells and their organelles.

2. Application and Removal of Apoptotic Cell Stimuli

- Shortly before use, pre-mix the apoptosis-inducing agent with 37 °C cell culture medium. Ethanol (3.6-4.5% vol/vol) served as apoptosis inducer in the present demonstration.
Note 1: Our published and unpublished data demonstrate that cells can also reverse apoptosis that is triggered by other apoptotic stimuli^{12,13}, such as dimethyl sulfoxide (DMSO, 8% vol/vol for 24 hr), staurosporine (STS, 0.5 µM for 1 hr), and taxol (1 µM for 12 hr). Optimization of dosage for triggering apoptosis is required for different kinds of cells to achieve the minimum dosage that can trigger the majority of cells in the population to undergo apoptosis.
Note 2: Pre-mixing apoptosis-inducing agents with cell culture medium is important to avoid uneven exposure of the cells to apoptotic stimuli.
- Image a group of health cells in the cell culture dish before applying the apoptosis-inducing agent to establish baseline morphologies.
- Remove the original medium from the cell culture dish, and then apply 2 ml of premixed apoptosis-inducing agent to the dish to trigger cells to undergo apoptosis. Incubate the cells at 37 °C with 5% CO₂ (or other normal culture conditions).
- Following incubation, observe the cells by light, epi-fluorescence or confocal microscopy to monitor the progression of apoptotic features.
Note 1: Cells undergoing apoptosis will display morphological, biochemical and molecular hallmarks of apoptosis that can be detected by microscopy and fluorescence-based biosensors (See Protocols 3 and 4).
Note 2: Incubation time of cells with different apoptosis inducers will vary, depending on cell type, cell conditions (such as confluency and nutrient status), and the dose of death stimulus applied. Careful titration may be required.
- When cells display hallmarks of apoptosis, remove apoptosis-inducing agent by washing the cells once with warm (37 °C, or other normal culture temperature) fresh cell culture medium.
Note 1: As apoptotic cells are more loosely attached on the culture plate, it is important to apply and remove medium gently so that cells will not be washed away.
Note 2: Live suspended cells can be recovered from the supernatant by gentle centrifugation (160 x g for 1 min) and added back to the culture dish.
- Incubate cells at 37 °C with 2 ml/35 mm dish of fresh cell culture medium in 5% CO₂. Alternatively, use conditioned medium (cell-free culture medium collected from healthy cells) to further enhance survival of apoptotic cells.
Note: Washing and incubating cells with fresh medium allow the majority of cells to reverse ethanol-induced apoptosis after ethanol exposure^{12,13}. However, repeating this washing step may be required when cells are exposed to other apoptotic stimuli (See Discussion).

3. Live Cell Microscopy

1. We recommend using an inverted confocal or epi-fluorescence microscope with environmental control (37 °C, 5% CO₂) for live cells microscopy.
Note: It is also possible to image the cells using upright microscopes with a water dipping objective. Dip the objective directly into the culture medium to image cells. However, cells can be crushed by the dipping objective during focusing.
2. Pre-warm the microscope (such as environmental control chamber, stage incubator, and objective heater) at least 2 hr before imaging.
Note: This allows microscope components to reach thermo-equilibrium, so that it can avoid the drift of focus and shift of x-y plane due to the thermal expansion and contraction of the components.
3. Place a 35 mm glass bottom dish of cultured cells (See Protocol 1) on the stage of an inverted microscope and capture cell images using a 40X or 63X plan-Apochromat objective with a numerical aperture (N.A.) 1.3 or 1.4 for imaging cells from the bottom of the dish through glass coverslips.
Note: Avoid applying over 2 ml of medium to 35 mm glass bottom dish, as the weight of the medium could cause curvature of the glass bottom, making it difficult to image a field of cells at the same focal plane.
4. Maintain cells at 37 °C or the corresponding normal temperature during the entire imaging process.
Note: The process of apoptosis, and likely the reversal of apoptosis, depends on temperature sensitive enzymatic activities. Therefore, maintaining cells at 37 °C (e.g. with a microscope stage top incubator) is important throughout the experiment. Decreased temperature could slow down the apoptotic response and the recovery response after removal of apoptotic stimulus.
5. Use a humidity device or lay a transparent foil (See Materials) on the culture dish to reduce water loss by evaporation from the medium.
Note: the foil could disrupt the polarity of light for DIC microscopy. Restore polarity by adjusting the polarizer at the light path.
6. Maintain pH in the cell culture medium (pH 6.8 -7.3) by incubating in 5% CO₂ with an environmental control chamber on the microscope.
Note: Maintenance the pH in culture medium can be also achieved by adding HEPES buffer, or by using commercial CO₂-independent medium (See Materials). Optimal conditions can vary, depending on the cell type.
7. Minimize fluorescence/laser (excitation light intensity) exposure to cells during the imaging process to avoid phototoxicity by reducing the fluorescence intensity to the lowest required to obtain high quality images of cell/subcellular structures or expressed biosensors (Details in Protocol 4).

4. Strategies for Detecting and Tracking Anastasis during and After Apoptotic Events

1. Plasma membrane blebbing, cytoplasmic condensation, cell shrinkage and apoptotic body formation (See **Figures 1A-C, E**).
 1. Perform time-lapse live cell differential interference contrast (DIC) or phase contrast microscopy to track a group of health cells and to observe their cell morphology (See Protocol 3 for live cell microscopy, and See Discussion).
Note 1: Reduce intensity of light source for DIC/ phase contract imaging to avoid phototoxicity to the cells.
Note 2: If DIC and phase contrast microscopy are not available, use CellTracker to stain the cytosol to outline the morphology of live cells for confocal or epi-fluorescence microscopy and monitor the cell morphology.
 2. Apply cell death stimulus to trigger cells to undergo apoptosis (See Protocol 2 for application and removal of apoptotic stimuli).
 3. Observe treated cells for morphological hallmarks of apoptosis such as plasma membrane blebbing, cytoplasmic condensation, cell shrinkage and apoptotic body formation (**Figures 1A, 1B, 1C, 1E**).
 4. Wash away death stimuli, and re-supply cells with fresh medium when the cells display morphological hallmarks of apoptosis.
Note 1: Apply cell death stimulus or changing the cell culture medium on the microscope stage during time-lapse live cell imaging can be achieved by using a perfusion cell culture chamber, or can be performed directly on the cell culture dish by carefully pipetting without touching the dish, during the intervals between imaging.
Note 2: Use focus drift compensation systems to avoid out of focus of the cells due to the loss of thermo-equilibrium of the microscope system after changing the cell culture medium (See Discussion).
 5. Continuous time-lapse imaging to track the fate of cells that display hallmarks of apoptosis. Cells that reverse apoptosis can repair damage and regain normal flat morphology (**Figures 1A, 1B, 1C, 1E**).
2. Mitochondrial fragmentation, DNA/chromatin condensation, and nuclear fragmentation (See **Figures 1D, 1F, 1G**).
 1. To visualize mitochondria, stain cells with 50 nM MitoTracker red/deep red/green-fluorescent dye, and simultaneously stain the nucleus with 10 µg/ml of Hoechst 33342 blue nuclear dye in culture medium for 20 min at 37 °C with 5% CO₂.
Note 1: Reduce the concentration of dyes and duration of incubation to avoid cytotoxicity and reduce background fluorescence when needed. Optimize the staining conditions to obtain a good signal to noise ratio with the minimal amount of dye and incubation time for staining.
Note 2: Use fluorescent stains for mitochondria such as MitoTracker Red CMXRos, MitoTracker Deep Red FM, or MitoTracker Green FM, that does not leak out from mitochondria during apoptosis. This allows visualizing mitochondrial morphology during apoptosis and anastasis. See the materials and equipment table for details about these stains.
 2. Keep stained cells in the dark to avoid photobleaching.
 3. Remove excess stain by washing the cells 3 times with 37 °C phosphate-buffer saline (PBS) solution or fresh cell culture medium, with 1 min incubation in the fresh medium between each wash, and then further incubate in the fresh medium for 20 min before the final wash to allow excessive stains to exit the cells to reduce non-specific signal background for imaging.
Note: Shorten incubation times with cell medium after staining may result in high background for imaging.
 4. Perform real-time live cell confocal or epi-fluorescence microscopy to image healthy cells before apoptotic induction is applied (See Protocol 3 for live cell microscopy).
Note: Healthy cells display tubular mitochondria and round nuclei in most cell types (**Figures 1D, 1F, 1G**).
 5. Trigger cells to undergo apoptosis (See Protocol 2 for application and removal of apoptotic stimuli to the cells).
 6. Continue time-lapse live cell microscopy to observe alterations in mitochondrial and nuclear morphologies immediately after the death stimulus is applied to the cells. Observe cells to display hallmarks of apoptosis such as mitochondrial fragmentation and swelling, chromatin condensation and nuclear fragmentation, in contrast to healthy cells displaying tubular mitochondria and round nuclei.

7. When cells display hallmarks of apoptosis, wash and supply cells with fresh medium, and continue time-lapse imaging to track the fate of cells. Cells that reverse apoptosis regain normal mitochondrial and nuclear morphology (**Figures 1D, 1F, 1G**).
Note: Perform DIC microscopy in parallel when possible to obtain additional information for accessing the stages of apoptosis by observing the alterations of cell morphology in the same group of cells (See Protocol 4.1 for DIC microscopy).
3. Detection of mitochondrial release of cytochrome c using cells stably expressing a cytochrome c-GFP fusion protein (See **Figure 2**).
 1. Stain cells stably expressing cytochrome c-GFP^{23,24} with MitoTracker Red to label polarized cell mitochondria (See Steps 4.2.1 to 4.2.3 for live cell mitochondria staining).
 2. Perform real-time live cell confocal or epi-fluorescence microscopy to track healthy cells (Details in Protocol 3 for live cell imaging).
Note: The cytochrome c-GFP signal primarily localizes in the mitochondria of healthy cells (**Figures 2Ai, 2B**).
 3. Trigger cells to undergo apoptosis (See Protocol 2 for application and removal of apoptotic stimuli to the cells). Continue time-lapse microscopy to observe translocation of cytochrome c-GFP from mitochondria to the cytosol (**Figures 2Aii-iii, 2B**).
Note: Mitochondrial release of cytochrome c into the cytosol is a marker of mitochondrial outer membrane permeabilization (MOMP)⁴, a critical step in mitochondria-dependent apoptosis^{21,22}.
 4. When cells display the cytochrome c-GFP signal in the cytoplasm, remove the cell death stimulus, and supply cells with fresh medium (See Protocol 2). Continue time-lapse imaging to track the fate of cells with cytosolic GFP.
Note: Cells that reverse apoptosis regain normal cell morphology, and reduce their cytosolic cytochrome c-GFP signal presumably through degradation or translocation back to mitochondria (**Figures 2Aiv-vii, 2B**).
 5. Capture DIC images in parallel to obtain additional information for accessing the stages of apoptosis by observing alterations in cell morphology in single cells or group of cells (See Protocol 4.1 for DIC microscopy).
4. Detection of caspase activity using a caspase biosensor (See **Figure 3**)
 1. Transfect cells with the caspase biosensor NES-DEVD-YFP-NLS for 16 to 24 hr before subjecting them to an apoptotic stimulus¹³.
 2. Stain the transfected cultures with Hoechst 33342 to label cell nuclei (See Steps 4.2.1 to 4.2.3 for live cell nuclear staining).
 3. Perform real-time live cell confocal or epi-fluorescence microscopy to track healthy cells (Details in Protocol 3 for live cell imaging).
Note: The NES-DEVD-YFP-NLS biosensor signal primarily localizes in the cytosol of healthy cells due to its nuclear export signal (NES) (**Figures 3A, 3Bi and 3C**, see Discussion).
 4. Trigger cells to undergo apoptosis (See Protocol 2 for application and removal of apoptotic stimuli to the cells). Continue time-lapse microscopy to observe nuclear translocation of YFP.
Note: Activation of caspases cleaves the DEVD motif in the caspase biosensor NES-DEVD-YFP-NLS, resulting in YFP-NLS translocation from the cytosol to the nucleus (**Figures 3A, 3Bii-iv**, see Discussion).
 5. When cells display nuclear YFP, remove the apoptotic stimulus, and supply cells with fresh medium (See Protocol 2).
Note: Cells that reverse apoptosis regain normal cell morphology, but retain the nuclear YFP signal (**Figures 3Bv-x**, Cells 1 and 2, and 3D).
 6. Continue time-lapse imaging to track the fate of cells that display nuclear YFP.
Note: The nuclear YFP signal will become degraded several hours after cells have reversed apoptosis (**Figures 3B, vi-x**, Cell 1, See Results and Discussion) presumably through normal cell clearance mechanisms such as proteasome degradation.
 7. Capture DIC images in parallel to obtain additional information for accessing the stages of apoptosis by observing alterations in cell morphology in single cells or a group of cells. (See Protocol 4.1 for DIC microscopy).
5. Cell surface exposure of phosphatidylserine (See **Figure 4**)
 1. Seed cells on glass coverslips to achieve 80% cell confluency (See Protocol 1).
 2. Co-stain the cells with red-fluorescent MitoTracker and blue Hoechst 33342 stain for mitochondria and nuclei, respectively (See Steps 4.2.1 to 4.2.3 for live cell mitochondrial and nuclear staining).
 3. Trigger cells to undergo apoptosis by using an apoptosis inducer (See Protocol 2).
 4. Apply fluorescent-labeled annexin V (See Materials) to stain apoptotic cells 10 min at 37 °C before removal of the apoptosis inducer to detect exposure of phosphatidylserine on the surface of apoptotic cells.
Note 1: Healthy cells are not stained with annexin V because phosphatidylserine is normally sequestered on the inner-leaflet of the cell plasma membrane³⁸. Apoptotic cells are stained with annexin V, which binds to phosphatidylserine on cell surface (**Figures 4A and 4B**).
Note 2: Fluorescent-labeled annexin V can also be applied to stain apoptotic cells immediately after removal of the apoptosis inducer, with 10 min incubation.
 5. Remove cell death stimulus, wash the stained cells with warm PBS or fresh medium once, and then supply cells with fresh medium for 2 hr at 37 °C with 5% CO₂ (See Protocol 2).
Note 1: Cells that reversed apoptosis regain normal morphology and retain fluorescently labeled annexin V signal after regaining normal morphology (**Figures 4A, and 4B**).
Note 2: The annexin V signal decreases with time on recovered cells (See Discussion).
 6. Fix cells at chosen time points with 4% paraformaldehyde containing 8% sucrose in 1x PBS for 20 min in the dark at room temperature, and wash the fixed cells with PBS for 3 times to remove paraformaldehyde before mounting cells on glass slides coverslips with antifade mountant (See Materials) for confocal or epi-fluorescence microscopy and observe annexin V on cells after removal of apoptosis inducer.
Note 1: Sucrose in fixative preserves cell membrane structure during fixation.
Note 2: Store the paraformaldehyde solution in dark at 4°C to prevent degradation.
Note 3: Warm up paraformaldehyde solution to room temperature before applying it to cells for fixation to avoid cold shock to the cells.

Representative Results

To study the reversal of apoptosis, tissue culture cells are first exposed to a death stimulus to trigger apoptosis. When the cells display hallmarks of apoptosis, fresh culture medium is then applied to wash away the stimulus and then incubate the dying cells to allow recovery (**Figure 1A**). Here, the key question being addressed is how far individual dying cultured cells can progress towards apoptosis and still undergo anastasis. This question can be definitively answered by continuous monitoring with biomarkers to track cell fates using the methods described below.

We first describe our strategies to detect reversal of apoptosis in tissue culture cells by using time-lapse live cell microscopy, which is required for tracking and recording the response of individual cells before, during, and after exposure to an apoptotic stimulus^{12,13}. Healthy adherent cells spread on their attached substrate (**Figure 1Bi**)^{12,13}, and displayed filamentous mitochondria and round nuclei prior to treatment (**Figures 1Ci, Di, Ei, Fi, Gi**)^{12,13}. After exposure to 3.8-4.5% ethanol in cell culture medium (vol/vol), DIC or phase contrast microscopy revealed that the cells exhibited morphological hallmarks of apoptosis, including cytoplasmic condensation, plasma membrane blebbing, and cell shrinkage (**Figures 1Bii, 1Cii-iii, and 1Eii-vi**)^{12,13}. Apoptotic body formation by the same cells was readily observed by DIC (**Figures 1Eii-vi**). Fragmentation of MitoTracker-labeled mitochondria (**Figures 1Dii-iii, 1Fii-vi**)^{12,13} as well as the condensation of Hoechst-stained nuclear DNA (**Figures 1Dii-iii, Fii-iii, Gii**)^{12,13}, and fragmentation of nuclei (**Figures 1Evi and Fvi**, white arrows), were detected simultaneously by confocal or epi-fluorescence microscopy. The cells that successfully reversed apoptosis were subsequently observed to regain normal morphology, apparently repairing their damage (**Figures 1Biii, C and Div-vi, E and Fvii-xii, and Giii**)^{12,13}. Cells under these conditions also had been shown to regain organelle motility, cell migration, and functional endocytosis based on uptake of fluorescence-emitting Quantum Dots and cell division¹³. Interestingly, some cells that reversed apoptosis displayed irregular nuclear morphology (**Figure 1Fxi**), and also abnormal cell division and formation of micronuclei (**Figures 1Giv-vii**)¹³, which is a biomarker of DNA damage, chromosome breakage and whole chromosome loss in dividing cells^{39,40}. Displaced chromosomes or chromosome fragments outside fail to be included in the daughter cell nuclei are enclosed by nuclear membrane^{39,40}. Thus, a consequence of anastasis could be the harboring of genomes that were damaged during aborted apoptosis, leading to tumorigenesis and cancer progression. The presence of micronuclei is also common in cancer cells^{40,41}.

Release of mitochondrial cytochrome *c* is a critical step to mediate or amplify caspase-dependent apoptosis²⁰⁻²². Using HeLa cells that stably express GFP-tagged cytochrome *c*, we monitored the subcellular relocation of cytochrome *c* during apoptosis and anastasis by confocal microscopy. As reported^{23,24}, cytochrome *c*-GFP localized to mitochondria prior to treatment (**Figures 2Ai and B**), but then was released into the cytosol after a death stimulus had been applied (**Figures 2Aii, 2Aiii and B**). Other characteristic morphologies such as mitochondrial fragmentation and plasma membrane blebbing, were also observed in cells that had released cytochrome *c*-GFP (**Figure 2Aiii**). These cells were reported to undergo cell death shortly after release of cytochrome *c*²³⁻²⁷. However, after removal of the death stimulus, we observed that cytosolic cytochrome *c*-GFP levels declined, mitochondria regained filamentous structures, and the plasma membrane recovered a normal appearance (**Figures 2Aiv-vii, and B**). Therefore, apoptotic cells can undergo anastasis after mitochondrial release of cytochrome *c*.

Amplification of downstream DEVD-cleaving caspases is generally assumed to be the "point of no return" in apoptosis^{2,5}. Therefore, we used a caspase biosensor NES-DEVD-YFP-NLS expressed from a plasmid¹³, making it possible to detect surviving cells that have previously experienced caspase activity (**Figure 3A**). This caspase biosensor is a polypeptide with an N-terminal nuclear exclusion signal (NES), a linker with the caspase-3/7 consensus cleavage site (DEVD)^{26,42,43}, followed by a yellow fluorescent protein (YFP) and a C-terminal nuclear localization signal (NLS). In healthy cells, this biosensor predominantly localizes to the cytosol as a function of the NES (**Figures 3Bi, Ci-iv, and D**)¹³. During apoptosis, activated caspases cleave the DEVD motif, releasing YFP-NLS, which efficiently translocates from the cytosol to the nucleus in cells that simultaneously, or subsequently, display other features of apoptosis such as DNA/chromatin condensation and cell shrinkage (**Figures 3Bii-iv, and D**)¹³. Thus, these cells retain nuclear import function following caspase activation. After removal of the apoptosis inducer, cells that undergo anastasis display morphological recovery even after caspase activation has occurred (**Figures 3Bv-x, and D**)¹³, apparently reversing late stage apoptosis. During recovery of normal morphology, the nuclear YFP signal decreases in intensity in some cells when they start to reverse apoptosis after removal of death stimulus (**Figures 3Bv-x, Cell 1**)¹³, suggesting that cells degrade the caspase-cleaved biosensor, possibly by the same mechanism used to remove other caspase-cleaved products during and after anastasis.

Anastasis can also be detected without live cell microscopy. This can be achieved by using fluorescently labeled annexin V^{38,44}, which binds and marks externalized phosphatidylserine (PS) at an early step in apoptosis (**Figure 4A**). For example, as expected, healthy and untreated neonatal rat primary cardiac ventricular myocytes cannot be labeled with annexin V (**Figure 4B**)¹³, as phosphatidylserine is restricted to the inner leaflet of the plasma membrane of healthy cells³⁸. In contrast, ethanol-induced apoptotic dying cells expose phosphatidylserine on the cell surface (**Figure 4B**)¹³, and therefore, can be labeled with the fluorescent annexin V^{38,44}. Noticeably, annexin V-labeled cells can regain normal morphology after removal of the death stimuli, suggesting that primary cardiomyocytes have reversed apoptosis (**Figure 4B**)¹³. Others have previously applied a similar strategy to suggest that recovery from apoptosis could occur *in vivo*. In an *in vivo* model of transient ischemic injury, caspase-dependent annexin V labeling of cardiomyocytes in rabbits and mice apparently resulted in the internalization of annexin V by surviving cells after transient ischemia⁴⁵, suggesting that anastasis could occur in live animals. In addition, two other studies used cell sorting to identify a fraction of annexin V-labeled mouse BCL₁.3B3 B lymphoma cells (exposed to anti-immunoglobulin antibodies that induce apoptosis in BCL₁.3B3) and mouse mammary carcinoma MOD cells expressing temperature-sensitive p53 (that causes apoptosis after incubated below permissive temperature) continued to proliferate after they were returned to normal culture conditions^{14,15}. Collectively, these studies suggest that annexin V is useful for determining the fate of cells that have reversed apoptosis without live-cell microscopy imaging. However, there are caveats with this strategy, as annexin V fluorescence is not permanent (**Figure 4B**)¹³, remaining detectable for only a few hr after removal of the death stimulus, so that these methods cannot provide long term tracking of cells that reversed apoptosis.

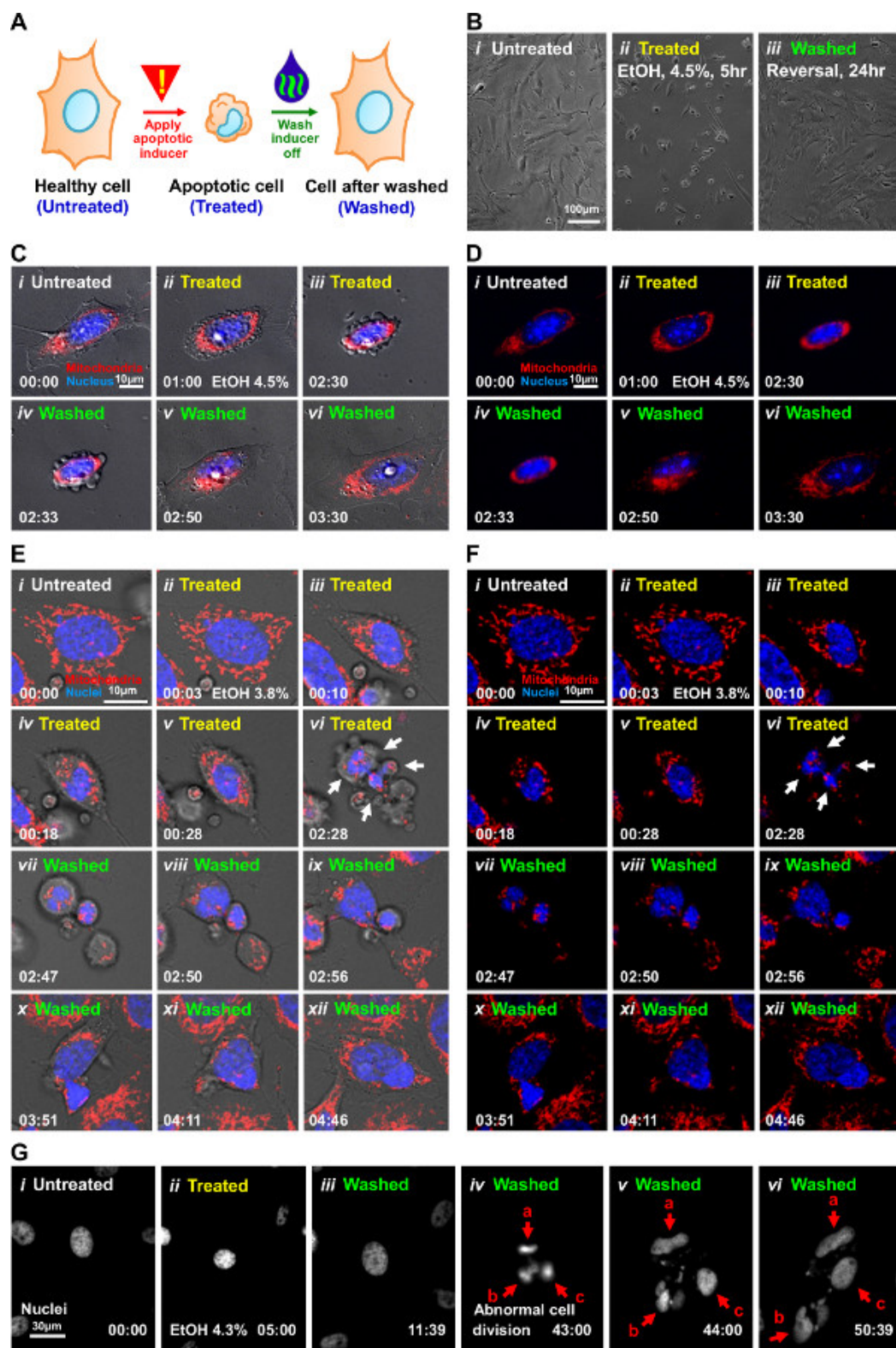


Figure 1: Tracking reversal of apoptosis by live cell imaging. (Reprinted with permission from Tang et al., MBoC 23, 2240-2252¹³, for Figures 1A-D, and G). (A) Approach to induce apoptosis and subsequently allow cultured cells to recover after washing away the apoptosis inducer. (B) Time-lapse live cell phase contrast microscopy of healthy primary mouse liver cells (untreated), the same group of cells that were treated with 4.5% ethanol in culture medium (vol/vol) for 5 hr (treated), and then washed and further incubated with fresh culture medium for 24 hr (washed). Scale bar, 100 µm. (C) Continuous time-lapse live cell epi-fluorescence microscopy of the same primary mouse liver cell before ethanol treatment (i), at the indicated times after treatment with 4.5% ethanol in cell culture medium for 2.5 hr (ii and iii), and after washing and incubating with fresh medium to allow recovery for 1 hr (iv - vi). Merged images of MitoTracker-stained mitochondria (red) and the Hoechst-stained nucleus (blue) were visualized by epi-fluorescence microscopy, and cell morphology by DIC microscopy. Scale bar, 10 µm. (D) Merged

fluorescence images only (without DIC) from panel **C** reveal organelle morphologies. **(E)** Continuous time-lapse live cell confocal microscopy of the human small cell lung carcinoma H446 cells before ethanol treatment (i), after treatment with 3.8% ethanol in cell culture medium for 2 hr 28 min (ii-vi), and after washing and incubating with fresh medium to allow recovery (vii-xii). Merged images of MitoTracker-stained mitochondria (red) and the Hoechst-stained nuclei (blue) were visualized by confocal microscopy, and cell morphology by DIC microscopy. White arrows indicate a fragmented nucleus in apoptotic bodies (vi). Scale bar, 10 μ m. **(F)** Merged fluorescence images only (without DIC) from panel **E** reveal organelle morphologies. **(G)** Continuous time-lapse live cell epi-fluorescence microscopy for monochrome Hoechst-stained nuclear imaging of a single HeLa cell that undergoes imprecise cell division. Before ethanol treatment (i), treatment with 4.3% ethanol in cell culture medium for 5 hr (ii) and after ethanol removal (washed, iii-vi). Panels iv reveals abnormal cell divisions. Red arrows indicate the major nuclei in the divided cells (iv- vi). Scale bar, 30 μ m. Times are indicated as hr:min. [Please click here to view a larger version of this figure.](#)

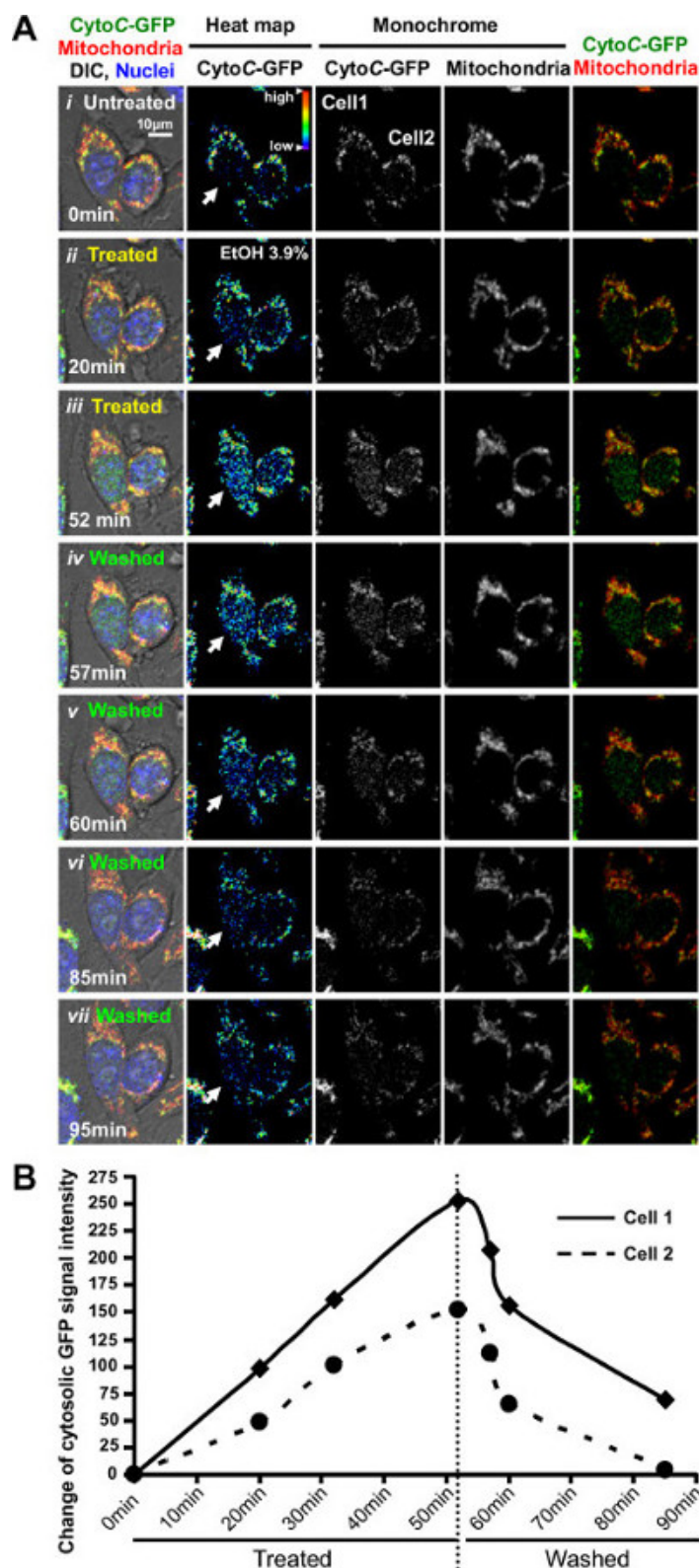


Figure 2: Detecting reversal of apoptosis after mitochondrial release of cytochrome c (A) Continuous time-lapse live cell confocal microscopy of HeLa cells stably expressing cytochrome c-GFP (CytoC-GFP) before (i), during (ii-iii) and after (iii-vii) exposure to 3.9% ethanol in cell culture medium. Merged confocal images of CytoC-GFP (green), MitoTracker-stained mitochondria (red), and Hoechst-stained nuclei (blue) are combined with DIC images (left panel). Unmerged versions are shown separately for CytoC-GFP, presented as a heat map of the signal intensity (left middle panel), monochrome image of CytoC-GFP (middle panel), and monochrome image of mitochondria (right middle panel). Merged images of CytoC-GFP and mitochondria (right panel). **(B)** The change of the cytosolic cytochrome c-GFP signal intensity of the cells, Cell 1 and Cell 2, as indicated in the monochrome CytoC-GFP image at Panel Ai. Times are indicated as hr:min. [Please click here to view a larger version of this figure.](#)

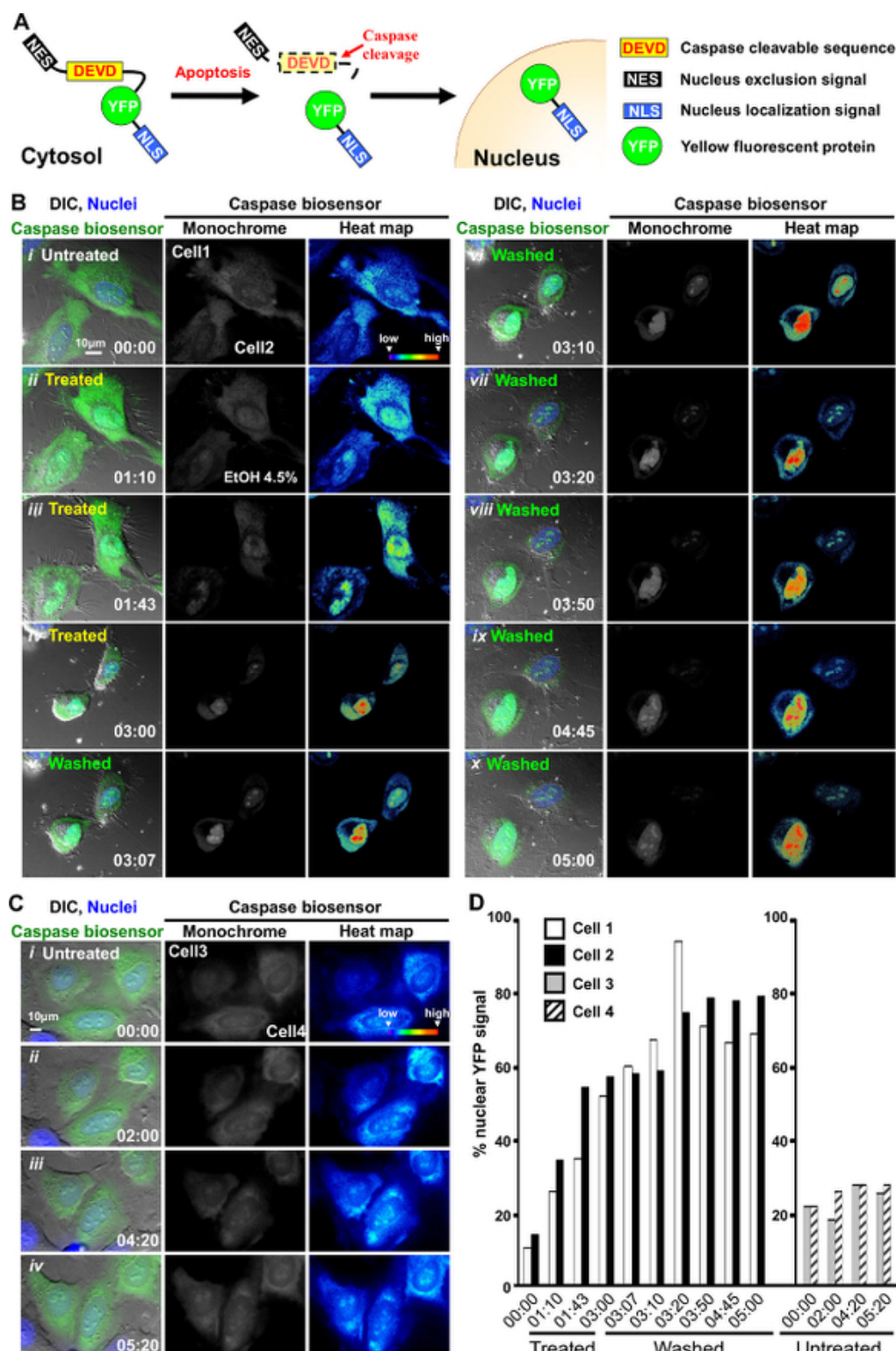


Figure 3: Detecting reversal of apoptosis after caspase activation (Reprinted with permission from Tang et al., *MBoc* 23, 2240-2252¹³, for Figures 3A-D). **(A)** Diagram of the caspase biosensor fusion protein composed of a nuclear export signal (NES), the DEVD caspase cleavage site, yellow fluorescent protein (YFP), and the nuclear localization signal (NLS). **(B)** Continuous time-lapse live cell confocal microscopy of HeLa cells expressing the caspase biosensor NES-DEVD-YFP-NLS before (i), during (ii-iv) and after (v-x) exposure to 4.3% ethanol in cell culture medium. Merged confocal images of the caspase biosensor (green), Hoechst-stained nuclei (blue) and DIC images (left panel), a monochrome images of YFP signal (middle panel), and a heat map indicating the YFP signal intensity (right panel). **(C)** Untreated controls analyzed as in panel B. **(D)** Quantified fluorescence intensities for the activated nuclear caspase biosensor in individual cells (numbered 1 and 2 in panel Bi) before, during, and after exposure of ethanol, and in untreated controls (numbered 3 and 4 in panel Ci) were calculated as the percent of YFP present in the nucleus (nuclear/total cell YFP signal intensity x 100%). Times are indicated as hr:min. Scale bar, 10 μ m. [Please click here to view a larger version of this figure.](#)

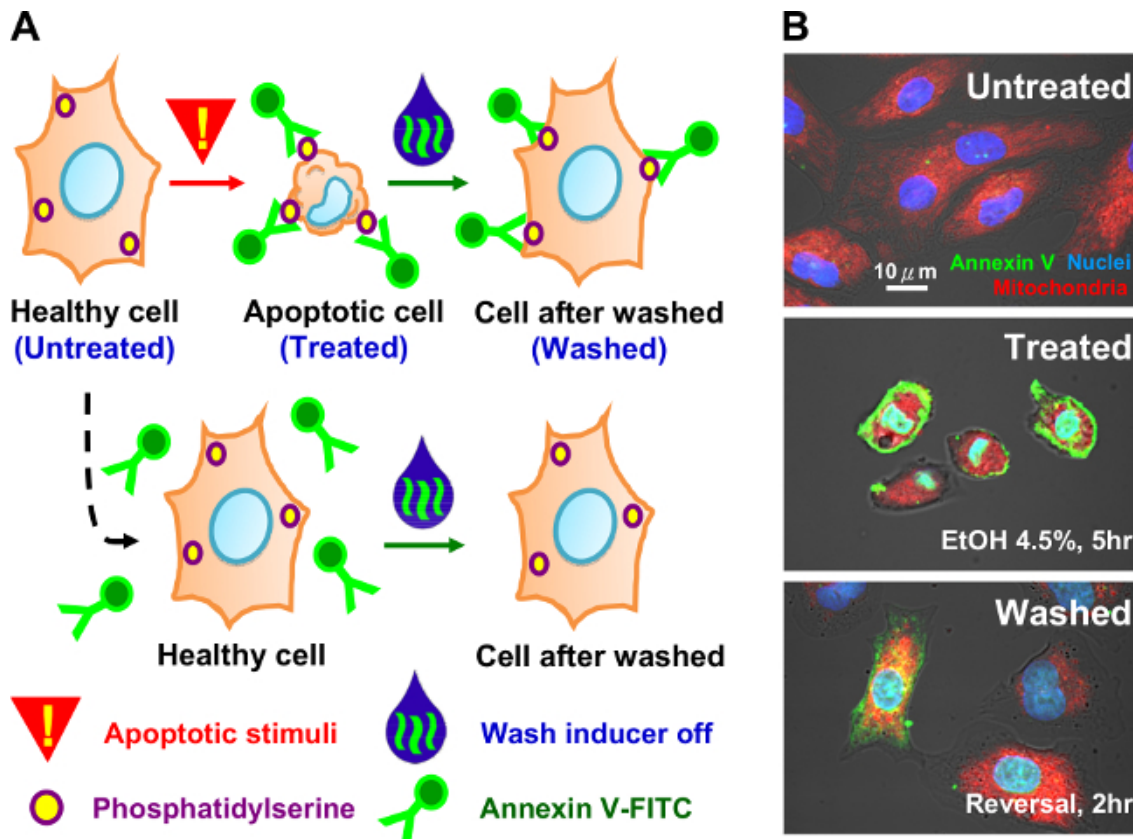


Figure 4: Detecting reversal of apoptosis by fluorescent-labeled annexin V (Reprinted with permission from Tang et al., *MBoc* 23, 2240-2252¹³, for Figures 4A-B). (A) Diagram of the annexin V-FITC anastasis assay used in panel B. (B) Neonatal rat primary cardiac ventricular myocytes were exposed to 4.5% ethanol in cell culture medium for 5 hr, and then incubated with annexin V-FITC for 10 min before removal of the ethanol medium. Cells were either fixed immediately (treated), or fixed after refeeding with fresh medium for an additional 2 hr recovery (washed). Untreated cells that were exposed annexin V-FITC serve as control (Untreated). MitoTracker-stained mitochondria (red), Hoechst-stained nuclei (blue) and annexin V-FITC (green) were visualized by confocal microscopy, and cell morphology was visualized by DIC microscopy. Scale bar, 10 μ m. [Please click here to view a larger version of this figure.](#)

Discussion

Anastasis refers to the phenomenon where cells that have activated cell death pathway subsequently reverse the dying process and survive. Here, we have demonstrated that live cell imaging can be used to confirm that the same individual cells in fact can reverse apoptotic cell death process at a late stage, and then continue surviving and reproducing. Our protocols describe several optimized cell type-specific treatment conditions to induce apoptosis and allow a large proportion of the cells to undergo the reversal of apoptosis monitored with several biomarkers for verification. Regarding technical issues, glass bottom dishes were used to culture cells for imaging^{12,13}, because of the highly transparent thin glass between cells and the microscope objective, which allows long working distances for high quality confocal or epi-fluorescence microscopy in high magnification, facilitates observation of classic apoptosis including mitochondrial fragmentation, release of cytochrome c, and nuclear condensation and fragmentation. Glass also does not distort the polarity of light, to allow high quality DIC microscopy for monitoring of membrane blebbing, cell shrinkage of cells, and apoptotic body formation during apoptosis. DIC microscopy has advantages over phase contrast microscopy to observe these morphological hallmarks of apoptosis, as DIC enhances the contrast of unstained and transparent cell samples, improving detection of cell morphologies. If DIC and phase contrast microscopy are not available, CellTracker can be used to stain the cytosol to outline the morphology of live cells for confocal or epi-fluorescence microscopy and monitor the cell morphology.

The application of appropriate doses of death stimuli and the strategies of the removal of the stimuli are critical steps for detection of anastasis. We have chosen to use low concentrations of ethanol as a death stimulus for the experiments described here because a large percent of treated cells can uniformly undergo apoptosis and can recover from this, potentially milder, death stimulus. Nevertheless, these approaches can also be applied successfully with other death stimuli, as we have reported^{12,13}. However, some death stimuli are inherently more challenging than others, such as staurosporine (STS), a commonly used apoptosis-inducing agent. Perhaps because it binds its substrates with high affinity⁴⁶⁻⁴⁸, repeated washes are essential but still may not efficiently remove STS from the cells. In this case, using lower drug concentrations and shorter drug incubation time that can still activate apoptotic dying process could be helpful to allow more cells to reverse apoptosis. Re-feeding cells with conditioned cell culture medium can also enhance reversal of apoptosis better than the fresh cell culture medium. Apoptotic cells are usually loosely attached to the culture dish surface particularly on glass surfaces, hence it is important to wash away the apoptosis inducers gently to avoid detaching cells. Coating glass dishes with poly-D-lysine, collagen and/or fibronectin could increase cell adherence for allowing certain types of cells, such as cardiomyocytes³⁷, to attach properly on the glass surface of cell culture dishes, particularly after exposure of cells to a death stimulus.

The process of apoptosis, and likely the reversal of apoptosis, depends on enzymatic activities that can be influenced by temperature. Therefore, maintaining cells at 37 °C or at their normal culture condition is important throughout the live imaging process to ensure reproducibility of experimental results. Pre-warm microscope before starting live cell imaging is also a critical step that allows the microscope components to reach thermo-equilibrium. This can avoid the drift of focus and shift of x-y plane due to the thermal expansion and contraction of the components during live cell imaging. Applying or removing the death stimulus by changing the medium a culture dish with either a pipet or by perfusion in the pre-warmed microscopy system during time-lapse imaging can still cause drift of focus due to changes in temperature or volume of the medium. Z-stack acquisition can help to image cells at the correct focal plane, but will increase the risk of phototoxicity due to additional exposure of cells to fluorescent lasers. Alternatively, use of focus drift compensation systems, such as an infrared laser-based automatic focus correction system, can keep cells at the same focal plane during time-lapse live cell imaging by maintaining the distance between the cells/glass and the objective without additional exposure of the cells to short wavelength, phototoxic fluorescence.

Mitochondrial release of apoptogenic proteins such as cytochrome *c*, and activation of effector caspases, such as the downstream/effector caspase-3 and caspase-7, have been generally considered to be the "point of no return" in the apoptotic cell death process^{2,5,6}. Western blots have been used to detect the cleavage (activation) of caspase-3 and its substrates such as poly(ADP)-ribose polymerase-1 (PARP) and DFF45/ICAD in the whole cell population during apoptotic induction and after removal of apoptotic stimuli, and revealed that cleaved caspase-3, ICAD, and PARP showed up in cells during exposure to death stimuli, but disappeared after the cells were re-fed with fresh culture medium¹³. However, these methods reveal the general condition in cell population, but do not distinguish the individual cells that will ultimately die from those that will reverse apoptosis and then survive. Biochemical fractionation to analyze release of mitochondrial cytochrome *c* has similar caveats. Therefore, to track the fate of individual cells after cytochrome *c* release and caspase-activation, two of the most recognized hallmarks of apoptosis^{2,5,6}, GFP-tagged cytochrome *c* (CytoC-GFP) and a caspase biosensor, such as the one used here NES-DEVD-YFP-NLS, combined with live cell microscopy circumvent these problems by directly observing morphological recovery and the translocation of the CytoC-GFP biosensor in the same cells. These results indicate the reversibility of apoptosis after mitochondrial cytochrome *c* release and caspase activation.

The current challenge for studying anastasis is the lack of labeling techniques to detect cells that have undergone anastasis at any point throughout their lifespan, *in vivo* or *in vitro*. Stable expression of the caspase biosensor NES-DEVD-YFP-NLS also permits detection of caspase activity if caspases are activated again in the future, but does not permanently record caspase activity as the activated biosensor will be degraded without sustained caspase activity¹³. Other previously reported caspase biosensors, such as the FRET-based SCAT biosensors (e.g. ECFP-[DEVD]-Venus)^{26,49} as well as fluorescent reporters Apoliner (CD8-RFP-[DQVD]-GFP)⁵⁰ and CPV (e.g. CD8-[DEVD]-Venus)^{51,52}, can be used to detect caspase activity in whole animals and in cultured cells, but have similar limitations. Similarly, CytoC-GFP cannot be used to track cells that reversed apoptosis in long term, as it is released from mitochondria into the cytosol during apoptosis and is subsequently degraded. Annexin V labeling has been used to track cells that reversed apoptosis, but the signal intensity also declines with time, so is not useful for long term tracking of anastasis^{13,45}. Continuous long-term *in vivo* imaging could be used to track the fate of the same cells after exposure of death stimuli. However, long-term *in vivo* imaging is still challenging to perform in most live animals. Therefore additional approaches are needed to extend anastasis research to whole animal studies, and for screening drugs that could promote or inhibit anastasis using tissue culture cells

In the future, newer protocols and techniques that track long-term cell fate are needed to develop and test the physiological, pathological and therapeutic implications of anastasis. We proposed that anastasis could be a general cell survival mechanism to rescue injured cells that are difficult to replace, such as mature neurons and cardiac cells¹³. However, anastasis of apoptotic cancer cells after chemotherapeutic treatments could result in recovery of dangerous cells leading to recurrence of resistant tumors^{12,13}. Anastasis could also be an important mechanism of tumorigenesis, as some cells display oncogenic transformation after they reversed apoptosis¹³. Therefore, enhancing anastasis could be a new therapeutic strategy for inhibiting neurodegeneration and heart failure, while suppressing anastasis as a new way for preventing or treating cancers. Developing biosensors to track reversal of apoptosis in disease model systems will advance the understanding of the cell survival mechanisms of anastasis, and potential therapeutics to intractable diseases by modulating anastasis.

Disclosures

The authors have nothing to disclose.

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