

Live image of mammalian cell anastasis

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Introduction

The programmed cell death process known as apoptosis is an essential step both in organ regeneration and in stress response. It is a highly conserved “suicide” process in cells, which can be initiated by extrinsic or intrinsic signals both of which impinge on a group of proteases, called caspases. Activated caspases target a wide range of proteins required for dismantling cells. A few years ago our lab discovered that apoptosis, which had been considered an irreversible process, is actually reversible even at a late stage, after caspase activation. The reversibility of apoptosis, which is named *anastasis* (Greek for *rising to life*), was observed in many cultured mammalian cell lines, primary cells from diverse tissues including highly specialized cells like cardiomyocytes, and in *Drosophila*. The best way to track anastasis in cultured mammalian cells is live image. Here we present a step-by-step protocol for imaging apoptosis and recovery processes of adherent cells (like Hela cells) with mitochondria and nuclei labeled by fluorescent dyes on Zeiss LSM780.

Materials

Reagents

1. MitoView 633 (Biotium, Cat: 70055)
2. Hoechst 33342, trihydrochloride trihydrate (Molecular Probes, Cat: H-1399)
3. MEM, GlutaMAX Supplement (Life Technologies, Cat: 41090-101)
4. Heat Inactivated FBS (Sigma, Cat: F4135)
5. Pen/Strep (Life Technologies, 15140-122)
6. Trypsin-EDTA (0.25%), phenol red (Life Technologies, Cat: 25200114)
7. Commonly-used apoptotic inducers:
 - Ethanol, molecular biology grade (Fisher Scientific, Cat: BP2818)
 - DMSO (Santa Cruz Biotechnologies, Cat: sc-358801)
 - N-Acetyl-D-sphingosine (C2-ceramide, Sigma, Cat: A7191)

Equipment

1. Zeiss LSM780
2. T75 tissue culture flask (Genesee Scientific, Cat: 25-209)
3. 35mm glass-bottom culture dish (Mattek, Cat: P35G-0-20-C)
4. FoilCover (Pecan)
5. INCYTO C-chip Disposable hemocytometers (Fisher scientific, Cat: DHCN015)

Reagent setup

1. Culture medium for Hela cells.
 - 500ml MEM, GlutaMAX Supplement
 - 50ml Heat Inactivated Fetal Bovine Serum
 - 5ml Pen/Strep
 - Mix together, filtered to sterilize. Store @ 4C.
2. MitoView 633 stock solution

Reconstitute 50µg MitoView 633 in 460µl DMSO, aliquot and store @-20C for long-term storage.

3. Hoechst stock solution

Dissolve 2mg Hoechst 33342 in 1ml ddH₂O. Protect from light. Store @4C.

Procedure

Prepare cells for live imaging

1. Aspirate culture medium from T75 tissue culture flask. Add 5ml 0.25% Trypsin-EDTA solution into flask. Observe cell morphology under microscope. Aspirate Trypsin-EDTA solution when cells become round. Add 5ml culture medium into flask. Remove cells from bottom and make single cell suspension by pipetting.
2. Count cell number using hemocytometer (See appendix 1 for cell counting). Dilute 1.5×10^5 cells in 2ml culture medium. Add the 2ml cell solution in 35mm glass-bottom dish. Put the dish in 37C incubator with 5% CO₂ and 90% humidity for 18h-24h.

Note: The number of cells needed for experiments depends on cell types. At the time of inducing apoptosis, cells in the dish should be around 80% confluence. The number mentioned here is for HeLa cells.

Stain cells with selected dye to mark mitochondria and nuclei

3. Dilute MitoView 633 stock solution and Hoechst stock solution in 2ml new culture medium to make the staining solution containing 1µM MitoView 633 and 250ng/ml Hoechst right before use.
4. Aspirate the culture medium in the dish. Add staining solution and incubate cells in 37C incubator with 5% CO₂ and 90% humidity for 30min.

Note: If using cells stably-transfected with fluorescent reporters, skip step 3 and 4.

Set up Confocal setting for live image on Zeiss 780

5. Turn on Zeiss 780 following the start procedure. Log on to the computer and open Zen Black software.
6. Put the heat insert on the microscope stage. Seal the stage incubator. Turn on CO₂ on the wall.
7. In Zen Black software, select “Acquisition” tab. Under the heading ‘incubator’, check ‘Heat Unit XL’, ‘Heat Dev Humid’, ‘Heat Insert P’ and ‘CO₂’. Set all three temperature control to 37C and CO₂ concentration to 5% (Fig 1)

Note: Allow at least 30min for CO₂ concentration and temperature to be stabilized.

8. In the tissue culture hood, insert the dish into the

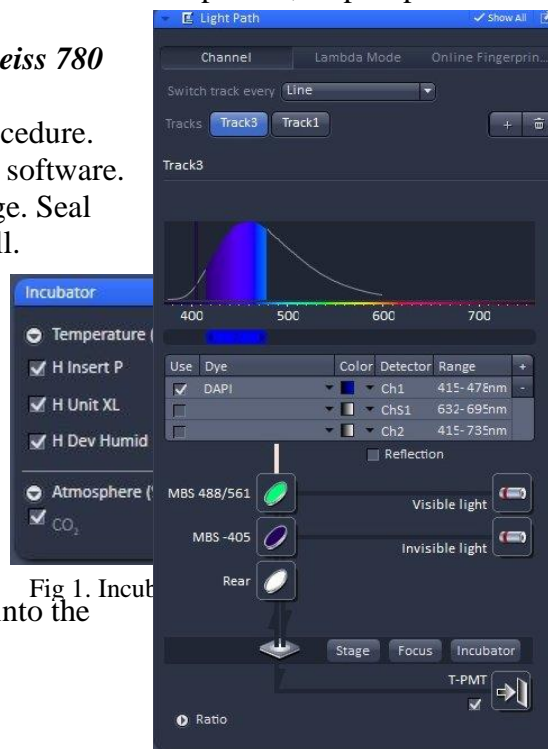


Fig 1. Incubator

Fig 2. To take DIC image, check T-PMT in DAPI channel.

- dish holder firmly. Remove the lid, and place FoilCover over the dish to prevent evaporation during imaging. Bring the assembled dish unit to microscope room.
9. Put the dish unit on heat insert. Switch to 20X objective. Select 'Locate' tab in software, focus on cells in DAPI channel through eyepieces.
 10. Switch to 'brightfield' and turn on transmitted light to set up DIC (See appendix 2 for how to set up DIC on Zeiss LSM780).
 11. In Zen Black software, select 'acquisition' tab. Set up channels needed for imaging according to the excitation/emission wavelength of the fluorescent dyes or proteins used in the experiments. DIC image can be taken with 405nm, 488nm or 561nm laser. Check the T-PMT in the light path if DIC is needed (Fig 2).
 12. Click 'Live' to adjust laser power, pinhole, and master gain. Set the laser power as low as possible to minimize phototoxicity. Generally, the power of 405nm laser and 488nm laser should not exceed 0.6% and the power of 633nm laser should not exceed 1%. For MitoView 633 and Hoechst staining, 0.2% 405nm laser and 0.6% 633nm laser are enough to get bright signals. An example of setting is shown in Fig 3.
 13. Under configuration, check 'positions' and pull up the position list. Select at least 4 positions and adjust the focus for each position. Do not include the position you used to adjust the laser settings.
 14. 'Focus Devices and strategy' window: select 'definite focus' as autofocus mode. Check 'Autofocus every n Timepoint' and 'Autofocus every n Position'. Set the frequency of autofocus you want.
 15. Check 'time series'. In the 'Time series' window: Set the interval to 10min. Set the number of cycles you want to take.
 16. 'Autosave': Check 'autosave'. Choose the folder where you want to save the files and name your files.

Live imaging

17. After setting up all parameters, click 'start experiment'. Take 2 or 3 images of untreated cells.
18. Dilute apoptotic inducer and MitoView 633 in 2ml culture medium right before use. During the 10min interval, open the stage incubator, gently remove the CO₂ pad and FoilCover. Aspirate culture medium in the dish. Add the medium containing apoptotic inducer and MitoView 633 in to the dish slowly. Put FoilCover and CO₂ pad back. Seal the incubator.

Note: When removing and putting back FoilCover, keep the orientation of FoilCover the same to maintain the DIC quality.

19. In next captured image, check the focus. Adjust the focus if necessary.
20. After desired time of treatment, open the stage incubator. Gently remove the CO₂ pad and FoilCover. Aspirate medium in the dish. Add 2ml new medium containing 1 μ M MitoView 633 in to the dish slowly. Put FoilCover and CO₂ pad back. Seal the incubator.

Critical: Apoptotic cells are loosely attached to the bottom of the dish, so it is very important to do this step gently to keep most cells in situ after medium change. When adding new medium, set the outflow speed of pipet to slow.

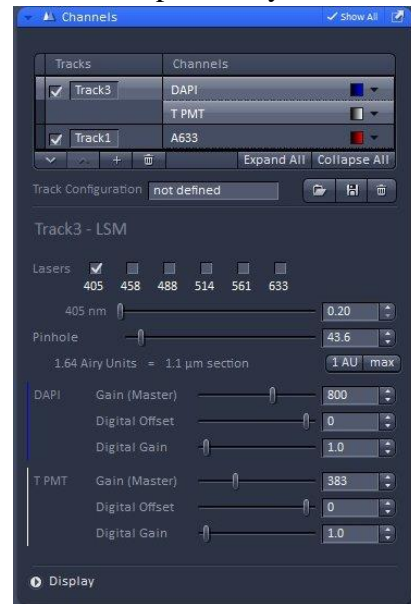


Fig 3. An example of channel setting.

21. In next captured image, check the focus. Adjust the focus if necessary. Image recovery process for 4-20h.

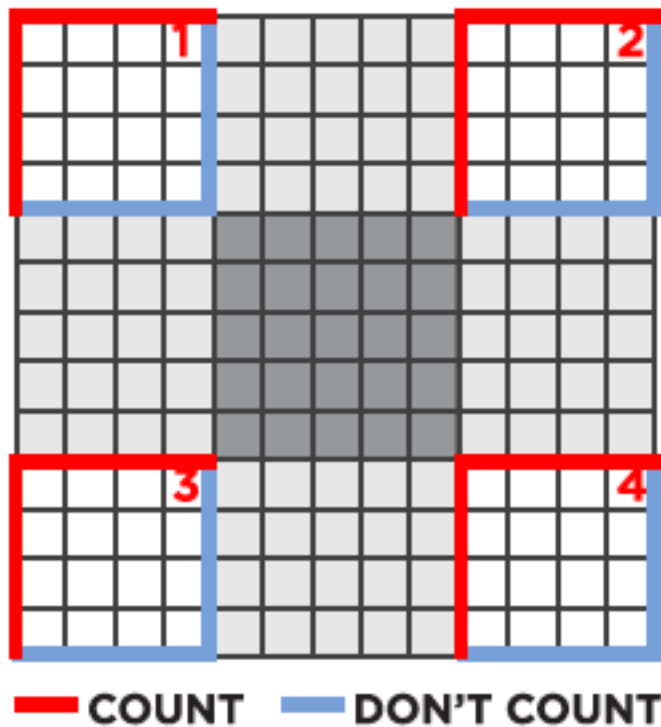
Anticipated results

A representative movie of mammalian cell anastasis should contain the following: 1) images of untreated cells showing flat cells spreading on the bottom of the dish with elongated mitochondria (with 20X objective, mitochondria morphology may hardly be seen) and large nuclei; 2) During apoptosis induction phase, apoptotic markers like membrane blebbing, nucleus shrinkage and condensation, mitochondria fragmentation can be seen; 3) During recovery phase, cells gradually regain attachment to the matrix and normal morphology.

MitoView 633 has cytotoxicity in long-term incubation, so is not good for imaging long-time recovery. Cells stably-transfected with IMS-DsRed reporter can be used as a substitute.

Appendix 1: Cell counting using hemocytometer

1. For 1 flask (T75) of HeLa cells, after trypsinization and removal of trypsin, pipette the cells with 5ml medium to make single cell suspension. Then transfer 1ml cell suspension to a new tube, add 1ml medium to make 1:1 dilution, mix well.
2. Pipet 10 μ L of diluted cell suspension into the hemocytometer through one of the inlets.
3. Count cells in the four corner squares on the hemocytometer (1–4). To avoid overcounting cells that are half-in and half-out of the squares, consistently count cells that are touching only two of the four boundaries of any square. Record the counts of the four squares. Use this representation of a hemocytometer to help you:



Note: If there are too many cells to count, dilute the cells. Counts are not accurate if there are less than 100,000 cells/mL (which corresponds to 10 cells per square).

4. Calculate using the formula below to get the cell concentration of the diluted cell suspension

$$\text{Cells/mL} = [(\text{Total cell counts in 4 squares})/4] \times 10^4$$

Then the cell concentration in the original 5ml cell suspension is

$$\text{Cells/mL} = [(\text{Total cell counts in 4 squares})/4] \times 2 \times 10^4$$

(Modified from online protocol)

Appendix 2: Set up DIC on Zeiss LSM780.

1. Focus sample using 20X objective.
2. Move the condenser to the lowest position (towards the sample).
3. Open the aperture diaphragm.
4. Close the luminous-field diaphragm until the edges of octagon in the view can be seen.
5. Focus the condenser until the edges appear sharp.
6. Center the image of the luminous-field diaphragm with the centering screws.
7. Open the luminous-field diaphragm until the edges are just outside the visible region through eyepieces.
8. Open the Zen Black software. In the 'Ocular' window under 'Locate' tab, select the correct Aperture filter. For 20X and 40X objective, use DIC II.
9. In the 'acquisition' tab, check the quality of DIC image under 'Live' mode. FoilCover is orientated. If used, rotate the FoilCover to get the best DIC image. And during the whole experiment, keep the same orientation.

(modified from *DIC Imaging using Laser Scanning Microscopes on Inverted Stands* from Zeiss)