Chapter 7

Monitoring Mitochondrial Membranes Permeability in Live Neurons and Mitochondrial Swelling Through Electron Microscopy Analysis

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Abstract

Maintenance of mitochondrial membrane integrity is essential for mitochondrial function and neuronal viability. Apoptotic stimulus or calcium overload leads to mitochondrial permeability transition pore (mPTP) opening and induces mitochondrial *swelling*, a common feature of mitochondrial membrane permeabilization. The first phenomenon can be evaluated in cells loaded with the dye calcein-AM quenched by cobalt, and mitochondrial *swelling* can be detected by electron microscopy through the analysis of mitochondrial membrane integrity. Here, we describe a live cell imaging assay to detect mitochondrial permeability transition and the development of a detailed analysis of morphological and ultrastructural changes that mitochondria undergo during this process.

Key words Mitochondrial permeability transition, Mitochondrial swelling, Live cell imaging, Mitochondrial membrane permeabilization, Electron microscopy, Mitochondrial calcein, Cobalt, Mitochondrial ultrastructure

1 Introduction

Mitochondria are membrane-enclosed organelles present in all cellular types, including neurons, where they participate in several vital processes, such as cellular bioenergetics, metabolism, and calcium homeostasis [1, 2]. Calcium regulation is essential for the synaptic function of a neuron [3] and therefore for its viability [4]. Calcium ions influx into neurons is regulated by different cellular compartments, including the endoplasmic reticulum and mitochondria [5]. Calcium overload into mitochondria has been described as an inductor of the mitochondrial permeability transition [6], which is a phenomenon characterized by the formation of large conductance permeability transition pores (mPTP) that make the mitochondrial membrane abruptly permeable to solutes up to 1,500 Da, resulting in the loss of mitochondrial membrane potential [7]. The permeabilization of

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the mitochondrial inner membrane induces morphological changes in this organelle, including increased volume, a phenomenon known as swelling, membrane disruption, cristae disorganization, and the release of pro-apoptotic factors to the cytoplasm, to finally activate neuronal death cascades [8, 9]. For this reason, the maintenance of the structural integrity and function of mitochondria is crucial for their proper function and, therefore, for neuronal viability.

Mitochondrial swelling can be detected in vitro with a spectrophotometric assay from isolated mitochondria, as previously described [10]; however, the amount of material required to obtain high quality purification is a limiting step when the experimental model in use is a primary culture of neurons. For this reason, we describe here a direct live cell imaging technique to monitor the mPTP opening, and the development of a detailed data analysis. This assay was developed by Petronilli et al. in 1998 [11] and, later, was validated in a neuronal model [12]. The assay is based on the loading of living neurons with a calcein-AM/cobalt mix to quench the cytosolic signal of calcein, but leaving intact the mitochondrial stain. The exposure of neurons to mPTP inductors, such as the ionophore ionomycin [13], or to calcium overload results in the decay of the mitochondrial calcein fluorescence, indicating mitochondrial permeability transition. Mitochondrial swelling can be also detected through the study of certain ultrastructural parameters by transmission electron microscopy [14]. We describe here a detailed analysis to evaluate morphological changes in mitochondria exposed to permeability transition.

2 Materials

2.1 Mitochondrial Permeability Transition by Live Cell Imaging

1. Glass coverslips of 25 mm diameter (Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany).
2. 1× Neurobasal Medium (Gibco®, Life Technologies™, Carlsbad, CA).
3. B27 supplement (Gibco®, Life Technologies™).
4. DMSO (Sigma Chemicals, St. Louis, MO).
5. Stock probes: 1 mM Calcein-AM, 10 μM MitoTracker Orange CMTMRos, and 1 mM Hoechst 33342 (all from Molecular Probes®, Life Technologies™, Carlsbad, CA) in DMSO. Store at −20 °C protected from light.
6. 1 mM Cobalt (II) chloride hexahydrate ($CoCl_2 \times 6H_2O$) stock solution: To prepare 5 mL dissolve 1.1897 g in 5 mL of sterile distilled water. Store at −20 °C protected from light.
7. Tyrode buffer: 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5.6 mM glucose, pH to 7.3

with concentrated HCl. To prepare 0.5 L weigh 3.944 g NaCl, 0.186 g KCl, 0.132 g $CaCl_2$, 1.19 g HEPES, and 0.280 g glucose and transfer to a 0.5 L glass beaker containing 300 mL distilled water. Mix and then add 500 µL 1 M $MgCl_2$. Adjust pH with HCl and make up to 0.5 L. Make aliquots of 50 mL in Falcon tubes or glass bottles and store at −20 °C. Defrost 1 aliquot of Tyrode buffer for the assay, use it along the day and then discard. For calcium free experiments do not add $CaCl_2$ to the preparation. *See* **Note 1**.

8. Confocal microscope with appropriate laser sources (*see* below).

2.2 Mitochondrial Swelling Detection from Electron Microscopy Analysis

1. Cold artificial cerebrospinal fluid (ACSF): 124 mM NaCl, 2.69 mM KCl, 1.25 mM KH_2PO_4, 1.3 mM $MgSO_4$, 2.6 mM $NaHCO_3$, 10 mM glucose, 2.5 mM $CaCl_2$. To prepare a 10× stock solution of ACSF, weigh 72.466 g NaCl, 2.006 g KCl, 1.701 g KH_2PO_4, 1.565 g $MgSO_4$ or 3.203 g $MgSO_4 \times 7H_2O$, 21.843 g $NaHCO_3$, and 19.817 g glucose. Mix and make up to 1 L. To prepare 1× ACSF solution, dilute 100 mL of the 10× stock solution in 900 mL of distilled water. Oxygenate the solution by bubbling with an O_2/CO_2 mix for 10 min and adjust pH to 7.4 with NaOH/HCl 1 N. Add 0.368 g $CaCl_2 \times 2H_2O$ and make up to 1 L. Check again the pH and if it is necessary readjust to 7.4.
2. Cell strainer 70 µm nylon (BD Biosciences, Bedford, MA).
3. 6-well plates for cell culture.
4. Silicone tubes.
5. 0.1 M cacodylate buffer, pH 7.2 with HCl: Dissolve 2.14 g of sodium cacodylate in 80 mL of distilled water. Adjust pH to 7.2 and then make up to 100 mL with distilled water.
6. Fixative solution: 2.5 % glutaraldehyde (e.g., Sigma Chemicals): Dilute 1 mL of 25 % glutaraldehyde stock solution into 9 mL of 0.1 M cacodylate buffer, pH 7.2.
7. 1 % osmium tetroxide (OsO_4): Dilute 25 mL of a 4 % osmium tetroxide stock solution (Sigma Chemicals) with 75 mL of distilled water. Store at 4 °C.
8. 1 % uranyl acetate [UO_2 $(OCOCH_3)_2 \times 2H_2O$]: Weigh 10 mg uranyl acetate and dissolve in 1 mL distilled water.
9. Epon resin (e.g., EMbed-812, Electron Microscopy Sciences, Hatfield, PA).
10. 4 % uranyl acetate [UO_2 $(OCOCH_3)_2 \times 2H_2O$]: Weigh 0.4 g of uranyl acetate and dissolve in 10 mL pure methanol.
11. Lead citrate staining solution: mix 1.33 g $Pb(NO_3)_2$, 1.76 g $Na_3(C_6H_5O_7) \times 2H_2O$ and 30 mL distilled water in a 50 mL volumetric flask. Shake vigorously for 1 min and allow standing with intermittent shaking. After 30 min add 8.0 mL 1 N

NaOH and make up to 50 mL with distilled water. Mix until lead citrate dissolves. The final pH is 12.0.

12. Cooper grids for electron microscopy (e.g., Ted Pella Inc., Redding, CA).
13. Biosafety cabinet.
14. CO_2 incubator.
15. Vibrating microtome.
16. Ultramicrotome.
17. Transmission electron microscope.

3 Methods

For live cell imaging, all procedures are carried out at 37 °C and under sterilized conditions unless otherwise specified.

3.1 Mitochondrial Permeability Transition by Live Cell Imaging

1. Seed neurons on glass coverslips of 25 mm diameter in a 35 mm dish or in the available format (*see* **Note 2**). Neurons are maintained in Neurobasal medium supplemented with B27 at 37 °C in an incubator with 5 % CO_2.
2. For live cell imaging, load the cells with the fluorescent probes (*see* **Note 3**). In this step work with the cells in a biosafety cabinet protected from light. Before removing the cells from the incubator, prepare the loading mix: add 1 μL of 1 mM calcein-AM stock solution, 1 μL of 1 M $CoCl_2$, and 5 μL of 10 μM MitoTracker Orange in 993 μL of Neurobasal medium to make 1 mL. Final mix concentration is 1 μM calcein-AM, 1 mM $CoCl_2$, 50 nM MitoTracker Orange (*see* **Note 4**). Hoechst dye can also be added to the loading mix to evaluate neuronal viability if necessary. *See* **Note 5**.
3. Wash cells twice with 1 mL Neurobasal medium without supplements (*see* **Note 6**). For 25 mm covers add 1 mL of loading mix to the dish and incubate neurons at 37 °C for 30 min, protected from light.
4. Remove the loaded neurons from the incubator and wash twice with 1 mL of Neurobasal medium, working in a biosafety cabinet. Change medium to Tyrode buffer and incubate cells for 10 min at 37 °C to equilibrate probes.
5. Transfer the cover to the microscope room and assemble the system as shown in Fig. 1a, b. Use little amounts of solid paraffin between both sides of the cover to fix it at the microscope system. *See* **Note 7**.
6. Set microscope parameters to start the mPTP live cell assay. For a spinning disk confocal microscope, use Texas Red filter to detect MitoTracker fluorescence, FITC for calcein-AM, and

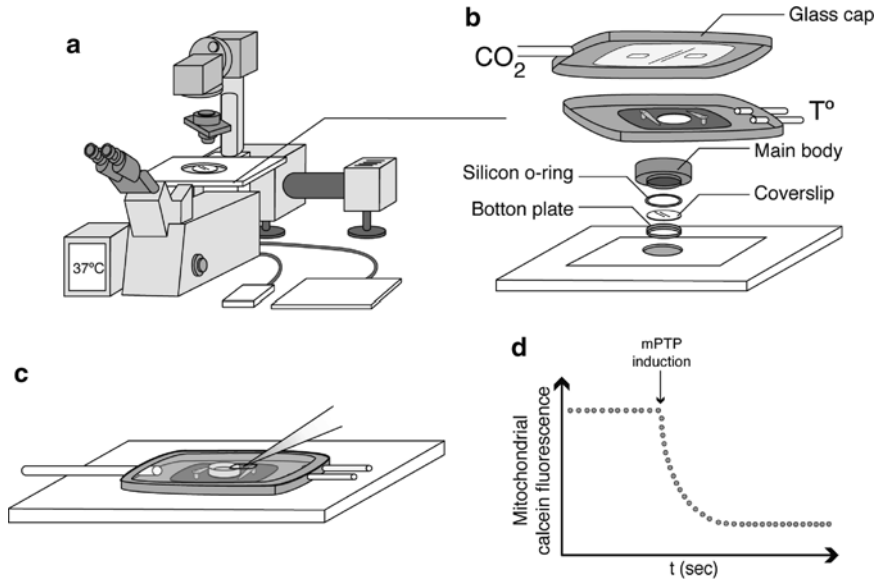

Fig. 1 Sample assembly for live cell imaging assay. (**a**) Microscope system equipped for live cell assays. (**b**) Neurons seeded on a glass coverslip mounted in the microscope chamber with CO_2 and temperature regulation. The glass cap is used to maintain the gas pressure and the temperature at 37 °C. (**c**) Closed chamber ready to use and for the stimulation of neurons. (**d**) Graphical representation of a common record obtained along the experiment after mPTP induction, i.e., with ionomycin. Mitochondrial calcein fluorescence decay is usually observed under mitochondrial permeability transition conditions

UV filter for Hoechst, or set the adequate excitation/emission wavelengths depending on the microscope used (*see* **Note 8**). Water immersion objectives are recommended for live cell imaging assays. Set temperature to 37 °C and the gas pressure to ensure a constant flux of 5 % CO_2 in the culture chamber of the microscope.

7. Set the time of the experiment and the interval between digital images. It is recommened to measure fluorescence changes for 15 min at least, with an image acquisition time of 10–15 s between frames. Measure a baseline for 3 min and then add the stimulus (Fig. 1c) (*see* **Note 9**). Finally, check the focus and start the experiment. It is recommended to perform positive and negative controls of the mitochondrial permeability transition pore opening to check the efficiency of the assay. *See* **Note 10**.
8. For image analysis use the NIH ImageJ software and install the "Delta F" plugin. Follow the sequential steps to analyze fluorescence changes on the images: Open the image with Image J > Image > Stacks > Z project, type the number of images corresponding to the basal line, i.e., a 3-min baseline corresponds to 180 s, if images were taken every 15 s, and then the

first 12 images correspond to the baseline; so type 1 in the start slice box and 12 in the stop slice > OK. This step creates an average image of the baseline.

9. Run "Delta F" plugin to determine fluorescence changes from the image stacks: Plugins > Stacks>T-functions > Delta F. A new image called "Delta F" is generated from this step. To obtain the value of ΔF/Fo (variation of the fluorescence between each image compared to the baseline) follow the next steps on Image J: Process > Image calculator, on Image 1 select the Delta F image created in the previous step; operation: Divide; and Image 2 is the average image created first > Yes. A new image is created called "Result of Delta F". From this image select a neurite with the polygon selection tool and perform the analysis: Images > Stacks > Plot *z*-axis Profile. A "Results" window is displayed with the mean fluorescence values corresponding to each time point. Plot the data to obtain fluorescence changes along the live cell imaging assay, as shown Fig. 1d. Repeat this step with each neurite selected until complete around 8–10 neurites per neuron.
10. Fluorescence analysis can be done with the mitochondrial calcein images to detect mPTP opening and also with those obtained from the MitoTracker loading, to detect mitochondrial membrane potential changes in response to the same stimulus (Fig. 2).

3.2 Mitochondrial Swelling Detection from Electron Microscopy Analysis

1. Prepare brain slices from rat or mouse brain [15, 16]. Cut transverse slices of 400 μm under cold ACSF using a vibrating microtome.
2. Incubate the slices in oxygenated ACSF for more than 1 h at room temperature before treatment.
3. For slices treatment, mount the slices in a 6-well plate modified as shown in Fig. 3, to keep each dish oxygenated (*see* **Note 11**). Dilute all treatments in ACSF in an appropriate volume to maintain slices immersed and avoid its contact with the air. This step is carried out at room temperature.
4. After treatment, transfer the slices one by one, with a soft brush or tweezers, to an Eppendorf tube containing 1 mL of the fixative and fix for 6 h. *See* **Note 12**.
5. After fixation, wash the slices with 1 mL of 0.1 M cacodylate buffer, pH 7.2 for 18 h at 4 °C.
6. Perform a secondary fixation with 1 % osmium tetroxide for 90 min and then wash with three washes, 5 min each in distilled water.
7. Stain samples with 1 % aqueous uranyl acetate for 60 min.
8. Dehydrate samples in acetone following these sequential steps: 1 × 50 %, 1 × 70 %, 2 × 95 %, and 3 × 100 % acetone, 20 min each.

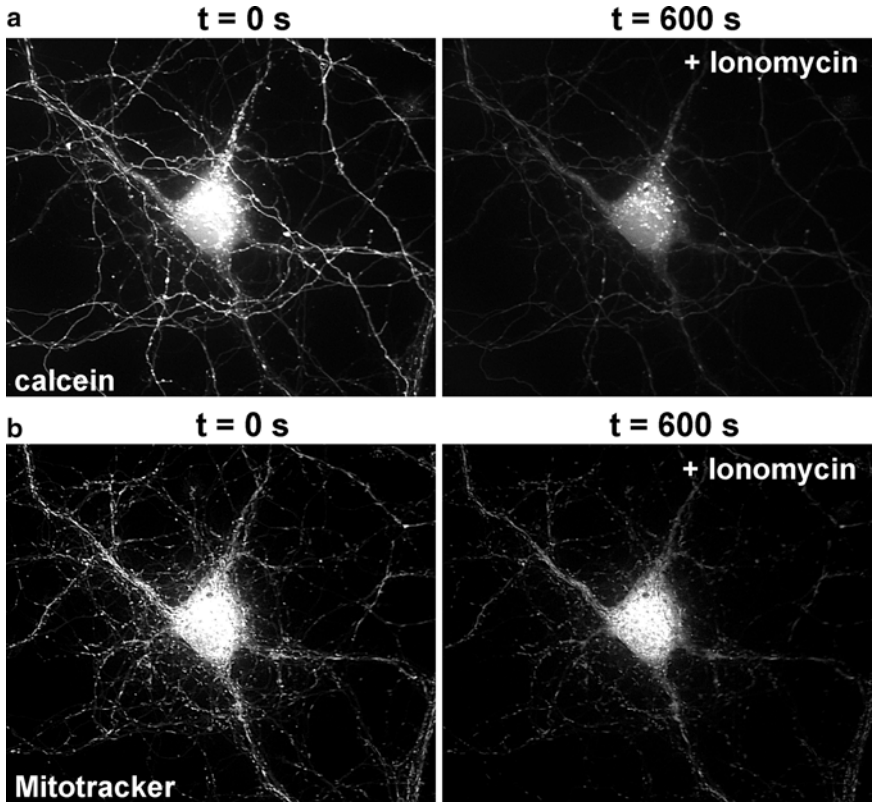

Fig. 2 Images obtained from the mPTP live cell imaging assay. Neurons were loaded with the loading Mix, containing calcein-AM, cobalt, and MitoTracker Orange. (**a**) Images show mitochondrial calcein fluorescence dissipation on neurons exposed to 0.5 μM ionomycin which indicates mitochondrial permeability transition. Images show fluorescence changes at the beginning ($t=0$) and at the end ($t=600$) of the experiment. (**b**) Images show the mitochondrial membrane potential loss in the same neuron shown in (**a**) under ionomycin stimulus

9. Place samples in 1:1 Epon:acetone overnight and embed them in 100 % Epon resin into a mold. Place mold containing samples in 60 °C oven for 24 h to polymerize.
10. Cut ultrathin sections (60–70 nm) with an ultramicrotome and collect them on 300-mesh copper electron microscopy grids (Fig. 3). Stain with 4 % uranyl acetate for 2 min and lead citrate for 5 min [17].
11. Examine the samples in a transmission electron microscope. For morphological analysis of mitochondria is recommended to capture digital images with a 16.500–20.000× magnification.
12. For mitochondrial ultrastructural analysis take 40–50 digital images per sample and evaluate membrane integrity of each mitochondrion by manually detecting structural abnormalities. Mitochondria with an overall intact structure are considered healthy, but loss of membrane continuity is considered an index of disruption. The same approach is applicable to cristae integrity analysis (Fig. 4).

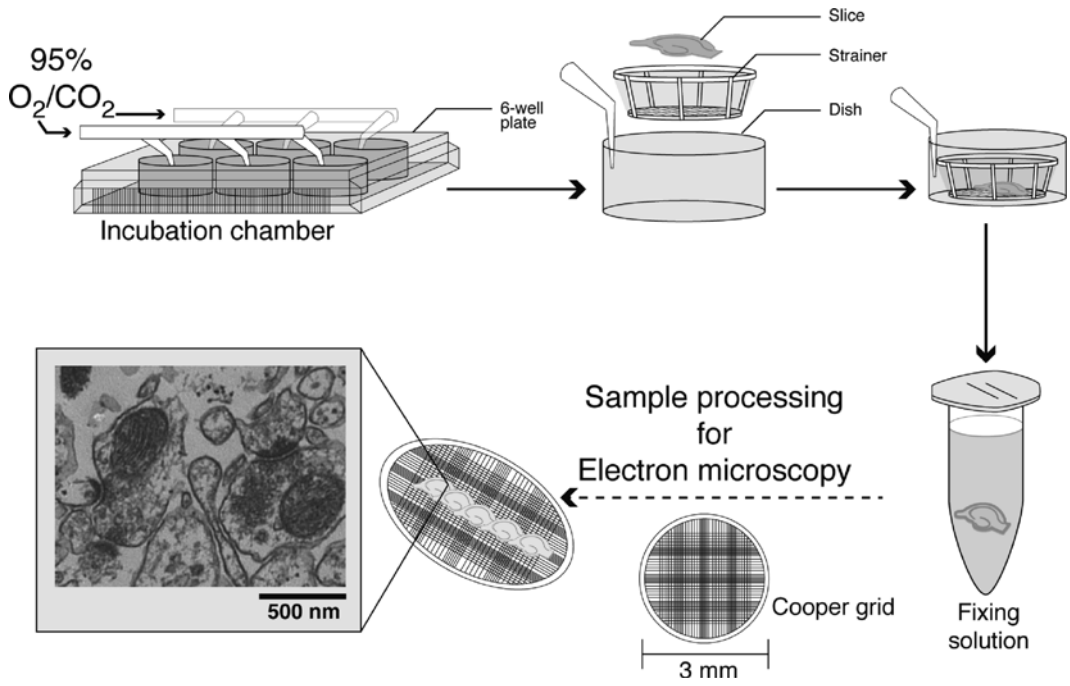

Fig. 3 Slice preparation and treatment for electron microscopy. Incubation chamber is used to treat slices separately. Each well contains a cell strainer to keep the slices protected from bubbles generated by the oxygenation system. After the incubation time, the slices are immersed in fixing solution, processed for electron microscopy and sequentially collected on copper grids for electron microscopy analysis. A representative image obtained with the microscope is shown at the end of the flowchart

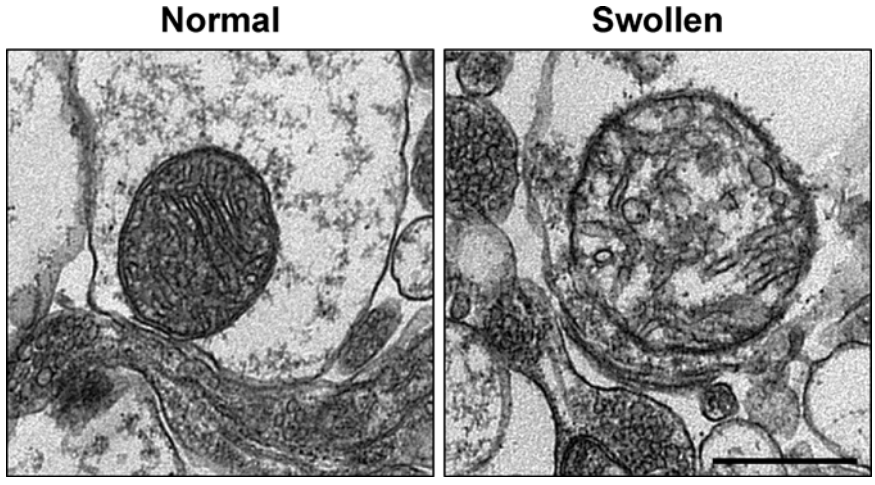

Fig. 4 Mitochondrial swelling detection by electron microscopy. Images show normal mitochondria with intact membrane and cristae (*left picture*) and a swollen mitochondrion (*right picture*) which is larger than normal and also displays membrane disruption and cristae disorganization, the common features of mitochondrial swelling by permeability transition. Bar = 500 nm

13. To evaluate mitochondrial swelling, measure morphological parameters, such as area, diameter, and perimeter [18] with ImageJ software. Set scale parameters in ImageJ: Analyze > Set scale > introduce distance in pixels and unit of length. *See* **Note 13**.
14. Set the parameters to be measured in ImageJ: Analyze > Set measurements > click in, Area, Perimeter and Feret's diameter.
15. Measure mitochondrial morphology with the polygon selection tool of ImageJ by surrounding each mitochondrion and press Ctrl + M on the keyboard to display the Results window. Plot the results in a column graph and as a scatter plot graph to analyze the whole population of mitochondria. *See* **Note 14**.

4 Notes

1. To maintain the osmolarity of the Tyrode buffer under calcium free conditions, add 3.997 g NaCl to the preparation instead 3.944 g, to change the concentration from 135 to 136.8 mM NaCl.
2. Glass coverslips of other diameters may be used depending on the chamber that is going to be mounted onto the microscope stage for the live cell assay. Some microscopes have chamber adaptors for 12, 25, or 40 mm diameter coverslip. If the microscope does not have chamber adaptors for different coverslip size, glass bottom dishes can also be used to seed cells.
3. Try to load just one cover per assay to avoid fluorescence decay during the time waiting between each experiment.
4. Other MitoTracker stains could be used to evaluate the mitochondrial membrane potential during the mPTP live cell assay, as MitoTracker Red CMXRos, but higher concentration of this probe is needed (between 200 and 1 μM). It is also important to check the bleaching of the probe depending on the microscope available.
5. Add 1 μL of 1 mM Hoechst 33342 dye to 1 mL of loading mix. It is recommended to measure Hoechst stain only at the beginning and at the end of the experiment and not along it to avoid probes bleaching.
6. If the cells were previously treated before the live imaging experiment, it is necessary to wash them to eliminate the stimulus. Also it is important to wash the cells to deplete them from B27, since it could interfere with the stimulus that is going to be used for the live cell assay.
7. Put the paraffin only at the edges of the cover to maintain an adequate cellular field to observe at the microscope. Some systems are magnetically sealed and do not need paraffin.

8. Hoechst λex/em = 350/461 nm. The ex/em peaks of calcein after hydrolysis are 494/517 nm. MitoTracker Orange λex/em = 554/576 nm.
9. For live cell imaging, the stimulus can be added manually with a micropipette or through a peristaltic pump.
10. Use 0.5 μM of the ionophore ionomycin, as a positive control of mitochondrial permeability transition. A loss of mitochondrial calcein is appreciated when this stimulus is added. As a negative control, incubate neurons for 30 min at 37 °C with 20 μM cyclosporin A (CsA) to inhibit mPTP opening before the stimulus. A control of probe bleaching is also needed. To evaluate this issue, measure the fluorescence of the probe for the duration of the experiment by simply adding vehicle.
11. An oxygenated dish can be manually made by introducing silicone tubes adapted to a white pipette tip. Fix the tip to the inside wall of the dish until the middle of the well and connect the tube to the oxygen. Be sure to keep a constant oxygen flux along the experiment, avoiding bubbles directly on the slices. Mount the slices into a cell strainer (usually used for organotypic cultures) inside each dish. Under these conditions the slices can be maintained between 6 and 8 h without affecting cell viability (*see* Fig. 3).
12. Prepare a fresh fixing solution every time you need it and only the amount that is necessary for each experiment. Do not freeze.
13. To determine distance in pixels, measure the length of the scale bar using the straight line selections tool of ImageJ and then open the set scale window and introduce the following parameters: *distance in pixels* is automatically determined by the software when the scale bar is measured with the straight line tool; the *known distance* corresponds to the length of the scale bar measured from the image; and the *unit length* also depends on the scale bar unit usually in μm. Click on "Global", and the final scale should be in pixel/μm.
14. The scatter plot graphs allow a deep analysis of the data, because they can be divided by percentiles to determine which population is affected by a determined treatment, as previously described (*see* [18]).

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