Chapter 2

In Vivo and In Vitro Determination of Cell Death Markers in Neurons

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Abstract

Mitochondria are key regulators of cellular death. The mitochondrial membranes contain essential enzyme complexes for maintaining metabolic homeostasis and meeting the energy requirements of the cell (Tait and Green, Nat Rev Mol Cell Biol 11:621–632, 2010 and Galluzzi et al., Apoptosis 12:803–813, 2007). Thus, any perturbation of outer or inner mitochondrial membranes can lead to disruptions in the normal fluxes of key ions and metabolic proteins (i.e., ADP/ATP exchange), leading to eventual cellular death. In addition to maintaining cellular viability, mitochondria play a critical role in the initiation of programmed cell death. As initiators of the cell death process, key mitochondrial proteins [Cytochrome C (Cyt C) one of the most well-studied among them] are released from the intermembrane space during early cell death events eventually leading to caspase activation.

Release of Cyt C is a crucial step during cellular death (Tait and Green, Nat Rev Mol Cell Biol 11:621–632, 2010). Therefore, the measurement of Cyt C release can give vital information about cell death signaling. Immunolabeling against Cyt C can give an easy readout of mitochondrial integrity as well, allowing for simultaneous identification of mitochondrial viability (and/or damage) and initiation of intracellular death processes.

In this chapter, we use Cyt C as a dual marker of mitochondrial integrity and cell death and review several protocols to measure Cyt C localization into intact mitochondria and its release into the cytosol. The goal is to offer an array of assays that, combined, provide both qualitative and quantitative analysis of the relationship between mitochondrial viability and activation of an intracellular cell death process. Immunofluorescence, Western blot, and ELISA measurements of Cyt C as are discussed in detail.

Key words: Mitochondria, Cytochrome C, Immunofluorescence, Western blot, ELISA

1. Introduction

Mitochondria are essential for both life and death processes, including many metabolic pathways, energy production, calcium storage, production/regulation of reactive oxygen species (ROS),

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and regulation of apoptosis. Intact mitochondria are required for healthy cellular metabolism and for regulation of the mitochondrial transmembrane potential ($\Delta\Psi m$). Once the outer membrane of the mitochondria loses its integrity, the mitochondrial ionic and metabolic homeostasis is disrupted, leading to a series of deathly consequences for the cell (1, 2). Therefore, maintaining integrity of the mitochondrial membranes is extremely important to maintain the metabolic viability of the cell. Mitochondria can also directly trigger cell death via the release of apoptogenic proteins into the cytosol. This is accomplished by permeabilization of the mitochondrial outer membrane (MOM) – which leads to decreased ATP production, change in mitochondrial membrane conductances, increases in cytosolic calcium levels, increase in ROS production and eventually cell death. Cyt C, which normally resides within the intermembrane space (IMS) of the mitochondria, binds to the apoptotic protease-activating factor 1 (APAF1) upon cytosolic localization. This leads to oligomerization of APAF1 and formation of the so-called apoptosome (3). The apoptosome serves as a platform for dimerization and activation of initiator caspase 9, which in turn cleaves and activates executioner caspases 3 and 7, finally leading to cell death (1, 4, 5).

Neurons are highly dependent on energy provided by the mitochondria and are therefore particularly sensitive to mitochondrial dysfunction. For fully differentiated cells like neurons, cell death signals released from damaged mitochondria represent a point of no return. Therefore, when developing means to measure death markers in neurons, the release of Cyt C from the mitochondria can be a doubly useful tool to define the time window between a decrease in energy supply and commitment to death (6–8). Cyt C also represents a valuable marker for mitochondrial integrity (6): it is normally retained in intact mitochondria and morphologically it appears in a distinct punctate staining typical of healthy mitochondria. It is released into the cytosol from damaged mitochondria (which lose their characteristic punctate structure) and rapidly diffuses into the cells. At this point, it serves as a key initiator of the cell death cascade and a powerful marker to define the timing in which the cell is committed to die (1, 6, 9).

In the following sections, we discuss several methods to detect retention vs. release of Cyt C from the mitochondria. We discuss using both neuronal NSC34 cells and primary cortical neurons. We originally optimized the protocols described below in NSC34 cells and used these protocols extensively in other neuronal lines like SH-SY5Y and H4 cells (6). The NSC34 cells are a hybrid neuronal line formed by the fusion of primary mouse motor neurons with neuroblastoma (10). As such, they retain many characteristics of primary neurons with the advantage of a cell line easy to maintain in culture. All the protocols have been tested and confirmed in primary cortical neurons as described below (6).

2. Materials

2.1. Cell Culture and Transfection

1. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1× penicillin/streptomycin (Pen/Strep).
2. 0.05% trypsin/0.53 mM EDTA in HBSS.
3. Lipofectamine/Plus transfection reagents (Invitrogen, Carlsbad, CA).

2.2. Cell and Spinal Cord Mitochondrial Isolation

1. Buffer A: 70 mM sucrose, 190 mM mannitol, 20 mM Hepes, pH 7.5, complete protease inhibitor cocktail (Roche, Basel, Schweiz). Note: this should be kept at 4°C for short-term use and stored at –20°C for long-term storage.
2. Buffer B: 150 mM KCl, 1 mM EGTA, 20 mM HEPES, 2 mM EDTA, pH 7.2 complete protease inhibitor cocktail. Note: this should be kept at 4°C for short-term use and stored at –20°C for long-term storage.
3. Bovine serum albumin (BSA). Note: this should be kept at 4°C.

2.3. Cell and Spinal Cord Lysis

1. Buffer A: 250 mM sucrose, 20 mM Hepes-KOH pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, complete protease inhibitor cocktail. Note: this should be kept at 4°C for short-term use and stored at –20°C for long-term storage.
2. Buffer B: 70 mM sucrose, 190 mM mannitol, 20 mM Hepes, pH 7.5, complete protease inhibitor cocktail. Note: this should be kept at 4°C for short-term use and stored at –20°C for long-term storage.
3. Digitonin extraction buffer: 0.025% digitonin, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 20 mM HEPES at pH 7.5, supplemented with complete protease inhibitor. Note: this should be kept at 4°C for short-term use and stored at –20°C for long-term storage.
4. RIPA buffer: 150 mM NaCl, 1% nonidet p40, 12 mM deoxycolic acid Na salt, 0.1% SDS, 50 mM Tris–HCl (pH 8).

2.4. ELISA

1. Cytochrome c Quantikine ELISA Kit (R&D Systems, Minneapolis, MN).

2.5. SDS–Polyacrylamide Gel Electrophoresis

1. 15% precast linear gradient polyacrylamide gel.
2. Sample buffer: 60 mM Tris–HCl pH 6.8, 25% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.1% bromophenol blue, store in aliquots at –20°C.

3. Precision plus kaleidoscope protein standard (BioRad, Hercules, CA).
4. Running buffer: 25 mM Tris base, 192 mM glycine, 0.1% SDS, pH 8.3 following dilution to 1× with water.

2.6. Western Blotting

1. Nitrocellulose membrane, 0.2 μm.
2. Blot absorbent filter paper.
3. Transfer buffer: Tris/glycine buffer.
4. Tris-buffered saline with Tween (TBS-T): Prepare 10× stock with 1.37 M NaCl, 27 mM KCl, 250 mM Tris–HCl, pH 7.4, 1% Tween-20. Dilute 100 mL with 900 mL water for use.
5. Blocking buffer: 5% (w/v) nonfat dry milk in 1× TBS-T.
6. Primary antibodies: Rabbit anti-Cytochrome C (Cell Signaling, Danvers, MA, USA) Mouse anti-map 2 (Sigma Aldrich, 1:1,000) for neuronal staining.
7. Secondary antibody: Anti-rabbit HRP conjugate (GE Healthcare, Buckinghamshire, UK), anti-mouse HRP conjugate (GE Healthcare, Buckinghamshire, UK).
8. ECL advanced Western blotting detection system (GE Healthcare, Buckinghamshire, UK).

2.7. Immunofluorescence

1. Fixative solution: Prepare a fresh solution of 2% paraformaldehyde/2% sucrose in PBS.
2. Ice-cold methanol.
3. PBS 1×: 37 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4, store at room temperature.
4. Permeabilization solution: 0.5% (v/v) Triton X-100 in PBS.
5. Blocking solution: PBS/5% FBS.
6. Primary fluorescent-conjugated mouse-anti-Cytochrome C (1:50, Alexa Fluor 546, Invitrogen), mouse anti-map 2 (Sigma Aldrich, 1:200–1:500) for neuronal staining.
7. Secondary antibody: Fluorescent conjugated anti-mouse (i.e., AlexaFluor from Invitrogen).
8. Mounting media: ProLong AntiFade, with or without DAPI (Invitrogen).

2.8. Cortical Neuron Isolation

1. Neurobasal media (Invitrogen) 4°C.
2. 10% penicillin streptomycin solution (Fisher).
3. 1× primiocen (InvivoGen).
4. 2% B27 (Invitrogen).
5. 5% FBS (Cellgro).
6. 0.3 mM l-glutamine (CellGro).

3. Methods

Cyt C is a highly soluble protein, physiologically associated with the inner mitochondrial membrane, where it acts as a component of the electron transport chain (11). Cyt C also plays a crucial role in cell death mechanisms and, in particular, it is released from mitochondria in response to toxic stimuli (12, 13). Cyt C release from mitochondria is, therefore, a good marker of cell death, especially as associated with mitochondrial toxicity (4–6). Ideally, one would like to define timing and mode of Cyt C release from damaged mitochondria both qualitatively and quantitatively (5). We have described methods for a qualitative immunofluorescence analysis of Cyt C localization and mitochondrial morphology as well as semiquantitative and quantitative analyses of the amount of Cyt C either retained or released from mitochondria by using Western blot and ELISA techniques. Our approach has been to use, whenever possible, all three techniques for a comprehensive analysis of the relationship between mitochondria morphology and activation of the cell death pathway (6).

Each of the above methods has its own values: (a) immunofluorescence staining followed by confocal analysis of Cyt C localization allows for visualization of mitochondrial morphology and initiation of cell death cascade, (b) Western blot allows for a semiquantitative evaluation of Cyt C retention in damaged mitochondria, and (c) ELISA allows a definite quantitative estimate of Cyt C in the cytosol.

When staining cells for Cyt C, there are three possible outcomes depending on the viability of the mitochondria and eventually of the cell: (a) in healthy mitochondria, Cyt C has a punctate staining (see Fig. 1a), which nicely correlates with the typical punctate-like structure of healthy mitochondria and displays a similar staining to a classical mitochondrial markers (i.e., MitoTracker) (see Fig. 1a); (b) in damaged, dysfunctional mitochondria Cyt C appears fuzzy staining still within the immediate outer membrane; and (c) finally, in cells with fully damaged mitochondria, Cyt C staining is diffused across the cytosol. Only at this late point, there is activation of executioner caspases in the cytosol (4, 5). Upon toxic insults, there is a gradual change in staining pattern: it progressively becomes fuzzy, losing the defined punctate structure, until it completely diffuses across the cell (see Fig. 1b).

If a semiquantitative approach is desired, mitochondrial and cytosolic fractions can both be analyzed by Western blot analysis for Cyt C content (14). An advantage of this system is that Cyt C can be semiquantitatively measured from in vitro isolated mitochondria and: (1) treated with toxic insult of choice or (2) it can be measured by purifying mitochondria from cells and/or tissues

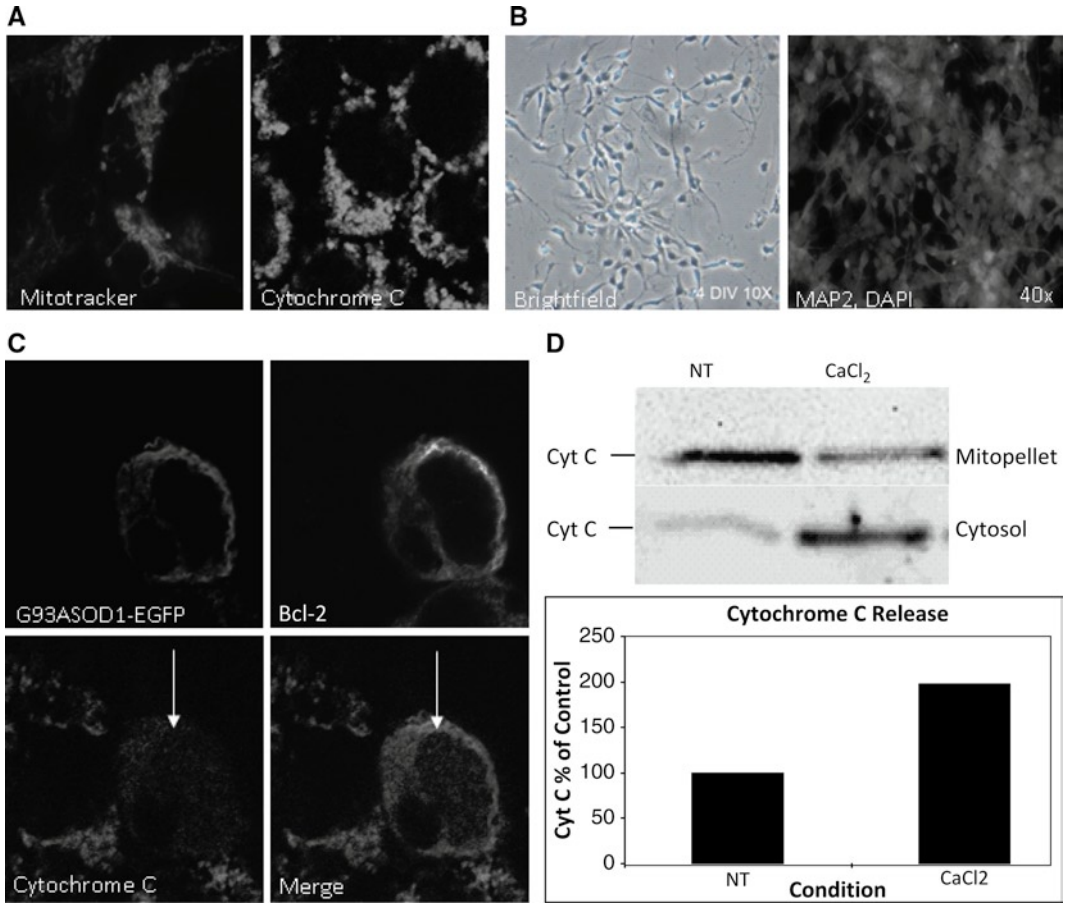


Fig. 1. Determining Cytochrome C (Cyt C) release in cell lines. (**a**) Nontreated NSC34 cells were stained with MitoTracker (*left panel*) or probed for Cyt C (*right panel*) for observation of intact, healthy mitochondria. Comparison of Cyt C with known mitochondrial stain gives same intact, punctate expression pattern across the cell. (**b**) NSC34 cells were transfected with a toxic protein (amyotrophic lateral sclerosis-linked mutant SOD1-EGFP) to induce release of Cyt C. In the transfected (*cell with arrow*), Cyt C displays a diffuse staining across the cell, indicating release. Conversely, nontransfected cells retain punctate staining, indicating intact mitochondria and no release into the cytosol. (**c**) Cortical neurons were stained for neuronal marker MAP2 and nuclear marker DAPI. (**d**) Mitopellet and cytosolic fractions of nontransfected (NT) and positive control ($CaCl_2$) NSC34 cells were run on Western blot and probed for Cyt C to determine amount retained in mitochondria and released into cytosol. Cytosolic fraction was also analyzed by ELISA; representative blot shown in *lower panel*.

previously exposed to toxic insults. This method follows a classical Western blot readout and measures the intensity of the Cyt C band in the "mitopellet" fraction (see Fig. 1d, upper panel); a potential caveat with Western blot analysis is that cytoplasmic proteins can be too diffuse to detect. However, ELISA allows for a quantitative analysis of cytoplasmic proteins and provides a more sensitive output than the Western blot (see Fig. 1d, lower panel). The Cyt C ELISA assay is a colorimetric assay – higher Cyt C cytosolic concentrations result in an intense coloration of the cytosolic sample with increased absorbance at 450 nm (6).

Oftentimes, the nature of the experimental systems used and the technical difficulties associated with them limit the analysis to one technique over the other. For instance, when using Cyt C as a cell death marker, one relies on cell culture systems. Toxic proteins are often used to induce cell toxicity and transient transfection and/or expression systems are commonly used tools to assay toxicity of proteins in neuronal cells. Experimental limitations typical of transiently transfected cellular models are linked to the fact that, in these systems, the cell population is not completely homogeneous and, at any given time, only a percentage of cells express the toxic protein(s), and it is therefore poised to undergo cell death. In this case, a qualitative – single cell – analysis of Cyt C localization by immunofluorescence may be the only possible experimental approach. Moreover, at least in transiently transfected cells, the time window to observe changes in mitochondrial function and subsequent Cyt C distribution is usually short (6–72 h) making it difficult to evaluate cumulative toxic effects or end point phenomena. Defining the precise time course of toxicity is also crucial and may also represent a limiting factor in choosing more quantitative analysis than immunofluorescence/confocal microscopy analysis of Cyt C localizations.

While single cell analysis of Cyt C localization following a toxic stimulus, represents a good starting point to define Cyt C as a marker of cell (and mitochondrial) toxicity, more quantitative measures of its translocation from the mitochondria to the cytosol are needed to define (a) its role in triggering cell death, (b) the threshold of Cyt C release needed to get to the point-of-no return into the death pathway, and (c) to quantify the extent of toxicity of the toxic insult used in the experiments studied. Therefore, upon optimization of the experimental conditions used (e.g., high transfection efficiency of a toxic protein, correct dose of toxin), it is recommended to combine the immunofluorescence data with Western blot and ELISA analyses of Cyt C content in isolated mitochondria and cytosol, respectively.

The ELISA method described above is ultimately the most accurate and quantitative assay to establish toxicity and the extent of cell death in any given sample.

3.1. Isolating Mitochondria from NSC34 Cells (see Materials in Subheading 2.2)

Protocol adapted from Pallotti and Lenaz (15, 16).

Before Starting, Make Up Solutions

1. 1 mL BSA + 4 mL buffer A.
2. 2 mL solution (1) + 2 mL buffer B.
3. 2 mL buffer A + 2 mL buffer B.

1. Use ~6 confluent dishes of NSC34 cells.
2. Collect cells, spin at 600 × *g*, 4°C, 10 min at 4°C (see Note 1).

3. Remove supernatant, resuspend pellet in 4 mL cold PBS (from now on, keep on ice).
4. Spin resuspended pellet down at 600 × *g*, 10 min at 4°C.
5. Remove supernatant, resuspend pellet gently in 800 µL solution (2); once completely resuspended transfer solution to 2-mL glass homogenization tube.
6. Homogenize resuspended pellet at 4°C.
7. Transfer homogenized solution to a clean Eppendorf tube.
8. Spin down homogenate at 1,000 × *g*, 10 min at 4°C.
9. Discard pellet, transfer supernatant into new, clean Eppendorf tube.
10. Centrifuge at 10,000 × *g* for 10 min at 4°C.
11. Gently pipette off supernatant.
12. Resuspend pellet in 100 µL solution (3).
13. Centrifuge at 10,000 × *g* for 10 min at 4°C.
14. Resuspend pellet in 100 µL buffer A.
15. Centrifuge at 100,000 × *g* for 1 h at 4°C.
16. Resulting pellet is mitochondrial fraction, supernatant is cytosolic fraction.

3.2. Isolating Mitochondria from Spinal Cord (Use Materials in Subheading 2.2)

Protocol adapted from Fieni et al. (17).

Before Obtaining Spinal Cord, Prepare Solutions

1. 1 mg/mL trypsin + 2 mg/mL collagenase in PBS w/out Ca^{2+} or Mg^{2+}.
2. Pre-incubate Solution to 37°C.
3. 3 mL BSA + 22 mL buffer A.
4. 2 mL solution (1) + 2 mL buffer B.
5. 2 mL buffer A + 2 mL buffer B.

1. Once spinal cord is obtained, cut into small pieces and let it sit in 10-mL beaker in 2 mL of solution (1) at 37°C for 15 min.
2. After 15 min, add 2 mL of solution (2) to cord, place in homogenization tube.
3. Homogenize cord with Potter homogenizer (Teflon/glass; 4°C, 600 × *g*).
4. Transfer cord homogenate to ultracentrifuge tube. Spin at 800 × *g* for 10 min at 4°C.
5. Resuspend the first nuclear pellet in 4 mL solution (2) and rehomogenize. Spin both supernatants at 800 × *g*, 10 min, 4°C (see Note 2).

6. Combine the two supernatants obtained (8 mL), rehomogenize the bigger pellet in one of the tubes after resuspending in 8 mL solution (2).
7. Spin everything (two tubes, 8 mL) at 800 × *g*, 10 min, 4°C.
8. Combine the supernatants (16 mL), get rid of the pellets, and spin at 10,000 × *g* for 20 min, 4°C.
9. Resuspend this pellet in 2 mL solution (3). Spin at 10,000 × *g* for 20 min, 4°C.
10. Resuspend pellet in 2 mL solution (4), spin at 100,000 × *g* for 1 h, 4°C. Resulting pellet is mitochondrial fraction, supernatant is cytosolic fraction.

3.3. Preparing the Samples to Assay CytC Release in ELISA and WB, from Mouse Spinal Cord Extracts or Transiently Transfected Cells (Use Materials in Subheading 2.3)

1. Homogenize mouse spinal cords and/or lysates of NSC34 cells in 800 µL of buffer A, and spin at 750 × *g* at 4°C for 10 min.
2. Rehomogenize the pellet in 400 µL of buffer A, spin at 750 × *g* at 4°C for an additional 10 min.
3. Combine the supernatants from these two homogenization steps in one solution; spin combined supernatant at 750 × *g* at 4°C for 10 min. Repeat spin with resulting supernatant.
4. Spin supernatant from step 3 once at 10,000 × *g* at 4°C for 15 min.
5. Resuspend each pellet in 80 µL of buffer B.
6. Incubate 25 µL of mitochondria with 20 µL of buffer B. Spin samples at 100,000 × *g* for 1 h at 4°C.
7. Analyze the resulting supernatant for Cyt C by ELISA following the manufacturer's instruction (see Notes 3 and 4), whereas the resulting mitopellet will be resuspended in buffer B and analyzed by WB.
8. Mitopellet for WB analysis of Cyt C release obtained as follows: collect 6–7 confluent Petri dishes (10 mm) of NSC34 cells.
9. After 24 h, detach and wash cells with 1 mL of PBS. Spin down at 500 × *g* and lyse in 100 mL of digitonin extraction buffer supplemented with complete protease inhibitor.
10. Spin down lysates at 15,000 × *g* and lyse the resulting pellet containing mitochondria in 100 mL of RIPA buffer.
11. Prepare 20 µg of proteins for WB by adding 5× sample buffer and boil for 5 min.

3.4. SDS–PAGE and WB

1. Assemble the gel unit using 15% polyacrylamide gels, add running buffer and connect to a power supply (see Note 5).
2. Load the samples, and run the gels at 100 V.

3. The samples that have been separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) are transferred to supported nitrocellulose membranes electrophoretically.
4. Assemble the transfer unit to allow protein transfer from the gel to the nitrocellulose membrane, add transfer buffer, connect the unit to a power supply and apply 100 V for 1 h.
5. Once the transfer is complete the cassette is taken out of the tank and carefully disassembled.
6. The nitrocellulose is then incubated in blocking buffer for 1 h at room temperature on a rocking platform.
7. The blocking buffer is discarded and the Cyt C primary antibody is added on the membrane (1:1,000 dilution in blocking buffer) for 1 h at room temperature on a rocking platform.
8. The primary antibody is removed and the membrane is washed three times for 10 min each, in TBS-T.
9. The anti-rabbit secondary antibody is freshly prepared for each experiment in a 1:10,000 dilution in blocking buffer and added to the membrane for 1 h at room temperature on a rocking platform.
10. The secondary antibody is removed and the membrane is washed three times for 10 min each, in TBS-T.
11. During the final wash, 2 mL aliquots of each portion of the ECL reagent are warmed separately to room temperature and the remaining steps are done in a dark room under safe light conditions. Once the final wash is removed from the blot, the ECL reagents are mixed together and then immediately added to the blot, which is then rotated by hand for 1 min to ensure even coverage; chemiluminescence has been evaluated using a ChemiDoc acquiring system.

3.5. Immunofluorescence Analysis of Cyt C Release

If Transfecting a Toxic Protein

1. Plate NSC34 cells in two-chamber slides with 500,000 cells/chamber.
2. 24 h after plating transfect cells with 200 ng of plasmid; lipofectamine PLUS is recommended (follow manufacturer's protocol).

If not Transfecting, Begin Here

3. 24 h after transfection fix cells for 20 min with a solution of 2% paraformaldehyde/2% sucrose at room temperature.
4. Remove fixing solution and treat cells with ice-cold methanol, for 10 min, at room temperature.
5. Wash slides once in PBS, 5 min at room temperature, gently shaking.

6. Add blocking buffer (PBS/5% FBS) for 1 h at room temperature, rotating.
7. Incubate slides were then incubated with fluorescent-conjugated mouse anti-Cyt C (1:50, AlexaFluor 546) for 1 h in blocking buffer.
8. Rinse slides three times in PBS.
9. Mount coverslips on slides using ProLong Antifade, dry for 2 h, nailpolish and analyze (see Notes 6 and 7).

3.6. Cortical Neuron Isolation

Isolate cortical neurons from nontransgenic E15 mice.

1. Dissect embryos and place cortices in dissection solution.
2. Spin cortices at 80 × *g* for 5 min and placed in trypsin/EDTA-HBSS for 20 min at 37°C.
3. Spin sample three times at 80 × *g* for 5 min to remove trypsin.
4. Triturate sample with a fire-polished Pasteur pipette until the suspension is homogeneous before an additional spin at 80 × *g* for 5 min.
5. Discard supernatant, resuspend cells, and if transfecting or running IF, count them before plating.
6. Once plated, cortical neurons can be assayed for Cyt C as described.

4. Notes

1. For Cyt C measurements by Western blot and/or ELISA, after collecting the cells all steps should be performed on ice or at 4°C. Mitochondria are highly temperature sensitive and Cyt C retention into mitochondria and its detection are also negatively affected by prolonged incubation at room temperature.
2. When isolating spinal cord mitochondria, the spin at 800 × *g* should be repeated at least three times. This spin is performed to clean the final mitochondrial pellet as much as possible of nuclei, debris, etc. Therefore, performing this spin several times results in a "cleaner" final pellet.
3. When running ELISA the first time, run three dilutions of your sample with a tenfold change in dilution factor against the standard curve. This will roughly give you an idea of what protein concentration your samples are and whether they will fit within the linear range of the standard curve.
4. Avoid bubbles as much as possible when running ELISA – bubbles/foam can affect the readings.

5. These instructions assume the use of a BioRad Mini Protean System gels but they are easily adaptable to other formats.
6. When analyzing Cyt C release by immunofluorescence, it is best to visualize the cells under the microscope when cells are not directly on top of each other. Therefore, plating conditions should be optimized in order not to have an overconfluent filed of cells. This is particularly important for dividing neuronal cells (e.g., NSC-34). For primary neurons, it is crucial to disperse the cells at plating to avoid overlapping. If cells are overconfluent, the Cyt C staining in the juxtaposing cell may interfere with the signal derived from the cell that is being visualized.
7. When staining for Cyt C release in immunofluorescence, use a secondary antibody/cellular marker (i.e., DAPI staining of nuclei) to mark the cells – when Cyt C becomes diffuse, it can be very difficult to distinguish cellular outlines by mitochondrial staining alone.

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