Chapter 19

Assessment of Neuronal Cell Death in *Caenorhabditis elegans*

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Abstract

The nematode *Caenorhabditis elegans* is a powerful experimental platform for cell biology studies. The molecular mechanisms that mediate cell death and neurodegeneration have been characterized extensively in the nematode. In addition, the availability of a wide arsenal of genetic and molecular tools and methodologies renders *C. elegans* an organism of choice for modeling human neurodegenerative diseases. Indeed, neuronal necrosis can readily be observed and examined in vivo, in the worm. In this chapter, we describe the two main approaches that are routinely used for monitoring and quantifying neuronal cell death in *C. elegans*. The first is based on direct visualization of dying cells via Nomarski differential interference contrast (DiC) microscopy, and the second on the assessment of neuronal survival by fluorescence microscopy.

Key words *Caenorhabditis elegans*, Cell death, Differential interference Contrast (DiC), Fluorescence microscopy, Nematode, Neurodegeneration, Nomarski microscopy

1 Introduction

C. elegans represents a powerful model with optimal characteristics for the investigation of cellular biology and its underlying processes. It is particularly ideal for the study of neurons and neuronal cell death. First and foremost, the organism has a transparent body that allows for direct observation in living animals without the need for invasive procedures such as skull windows. Additionally, it possesses a fixed number of cells (including neurons) of each type that are derived from a consistent and invariant developmental process. This means that each wild-type animal of the same sex has the same neuroanatomy, allowing for the reconstruction of a perfect cell lineage tree and the most complete neuronal connection maps of any model organism. Furthermore, the nematode is easily manipulated genetically and even more easily affected by RNAi, allowing

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for extensive reverse genetics experimentation. Finally, *C. elegans* has a short life span and fast reproduction, making it ideal for the observation of aging related phenotypes [1].

Cell death in *C. elegans* can be classified into two categories: apoptotic cell death, and necrotic. Apoptotic cell death generally occurs in a programmed fashion during development (exactly 131 cells die) and is mediated via *ced-3 and ced-4* [2]. Necrotic cell death, on the other hand, is generally the result of abnormality. It primarily involves the increase of intracellular Ca^{2+} due to influx from plasma membrane channels and release from storage compartments such as the ER. This increase leads to the dissolution of lysosomes via calpain proteases [3–5]. Thus, hydrolytic lysosomal enzymes like cathepsins are released to the cytosol [3, 6]. Work in our lab has also indicated a role for clathrin mediated endocytosis, intracellular transport and even autophagy in the necrotic process [7]. Neuronal cell death in *C. elegans* is primarily necrotic. A notable example of this is neuronal cell death due to deleterious Gain of Function mutations in genes of the degenerin family like *deg-1* [8] and *mec-4* [9]. Those are ion channels that, when irregularly activated, can lead to necrotic degeneration of a subset of mechanosensory neurons. A similar effect emerges from a gain-of-function mutation of an acetylcholine receptor subunit, *deg-3* [10].

C. elegans can be used to model the circumstances of neuronal cell death in more complex organisms (and particularly, humans) in order to identify the mechanisms (which are likely to be conserved) that facilitate it and test potential ways to ameliorate it. An example of this is its use as a model for excitotoxicity. The combined deletion of the glutamate reuptake transporter *glt-3* and hyperactivation of the G protein Gs subunit α can induce an excitotoxic phenotype. This allowed for studying the role of type-9 adenylyl cyclase in that process [11]. As another example, our lab has used worms to model neuronal cell death as a result of heat stroke, demonstrating that heat preconditioning can have a protective effect via the activation of HSP-16.1, which stabilizes the golgi membrane bound transporter PMR-1 and thus assists in Ca^{2+} clearance. This protective effect can also be induced in mammalian neurons [12]. Possibly one of the most prominent applications of this type is the use of *C. elegans* for the creation of "humanized" models of neurodegenerative disease (that is, worms expressing wild-type or mutant human versions of a protein) [13, 14]. This has been done with α-synuclein for the study of Parkinson's disease [15, 16] or superoxide dismutase 1 [17, 18] and TDP-43 [19] for the study of Amyotrophic Lateral Sclerosis. It has also been done with microtubule associated protein tau [20, 21], beta-amyloid peptide [22] and amyloid precursor protein [23] for the study of Alzheimer's disease.

2 Materials

2.1 Nematode Strains

Strain selection is experiment dependent. The strains used in the examples here are the following:

1. For Nomarski DiC microscopy.
   (a) N2 Bristol: The standard WT strain of *C. elegans*.
   (b) *mec-4(u231)* X: Referred to as *mec-4(d)*. Expresses a dominant negative form of *mec-4* in 6 touch receptor neurons: ALML and ALMR, AVM, PVM, and PLML and PLMR.
2. For fluorescent microscopy.
   (a) SK4005: zdIs5 I (WT;*Is*[*mec-4::GFP* + *lin-15*(+)]). Referred to as $P_{mec\text{-}4}$GFP. Expresses GFP in the 6 touch receptor neurons affected by *mec-4(d)*.
   (b) zdIs5 I; *mec-4(u231)* X: Referred to as *mec-4(d)* *x* $P_{mec\text{-}4}$GFP. Cross of the two abovementioned worms. Expresses both GFP and the dominant negative form of *mec-4* in the target neurons.

2.2 Equipment and Reagents for General Worm Manipulation

1. Dissecting microscope (e.g., Nikon SMZ 745).
2. Platinum wire pick: this is used to routinely transfer worms from one plate to the next to ensure they remain well fed.
3. M9 buffer: M9 is the primary liquid solvent for *C. elegans* applications. To prepare 1 L of M9, mix 3 g of KH_2PO_4, 6 g of Na_2HPO_4, and 5 g of NaCl in 1 L of distilled water. Autoclave and after cooling add 1 mL of 1 M $MgSO_4$.

2.3 Nematode Food

(All procedures explained here need to be performed under sterile conditions.)

1. Normal Growth medium. (for experiments not involving RNAi).
   (a) Making the plates: To prepare 1 L of NGM, add 3 g of NaCl, 2.5 g of Bacto peptone, 17 g of agar, and 0.2 g of streptomycin to about 900 mL of distilled water in a large conical flask. Stir using a magnetic stir bar for 5 min and then autoclave the mixture. Simultaneously autoclave 100–200 mL of water at a separate small bottle. Subsequently, let them cool down until the large flask is at about 55 °C. Then, while continuously stiring with a magnetic stir bar, add 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, 25 mL of 1 M KPO_4 (pH = 6), 1 mL from a 5 g/mL cholesterol in 100% ethanol solution and 1 mL from a 10 g/ml nystatin in 70% ethanol solution. Use the extra sterilized water to bring the final volume to 1 L, and distribute the solution into 60 × 15 mm petri plates (7–10 mL per plate).

(b) Preparing the bacteria: Inocculate a single colony of the OP50 *E. coli* strain in 50 μL of liquid Luria–Bertani (LB) medium and let it grow for approximately 6 h at 37° in a shaking incubator.

(c) Final step: Seed about 200 μL per plate of the resulting liquid bacterial culture on the NGM plates and let it grow overnight at room temperature. It should form a dense bacterial lawn on the seeded area.

2. RNAi medium.

(a) Making the plates: Same as the NGM recipe above with one change: instead of adding streptomycin powder before sterilizing the mixture in the autoclave, add ampicilin after cooling it, to a final concentration of 100 μg/mL.

(b) Preparing the bacteria: For each HT115 *E. coli* strain carrying a relevant RNAi expressing vector (including the empty control vector, pL4440) innoculate a single colony in 5 mL of LB containing 100 μg/mL ampicilin and 10 μg/mL tetracyclin. Let it grow for 14–16 h in a shaking incubator. Then incubate a portion of this liquid culture (at a ratio of 50 μL per mL of LB) in an appropriate (depending on the number of needed plates) volume of liquid LB containing only 100 μg/mL ampicilin. Let it grow for 3–4 h in a shaking incubator.

(c) Final step: Seed about 200 μL per plate of the final liquid bacterial culture on the RNAi plates and let it grow overnight at room temperature. It should form a dense bacterial lawn on the seeded area. It is recommended to use these plates within 48 h for optimal RNAi effectiveness (also *see* **Notes 1** and **2**).

2.4 Imaging

1. DiC capable microscope (e.g., Zeiss Axio Imager Z2 Epifluorescence/DIC Microscope).
2. Fluorescent microscope (e.g., Thermofisher Scientific EVOS FV Auto 2 Imaging System).
3. Microscope slides and coverslips.
4. 2% Agarose pads: To make these, mix 1 g of agarose into 50 mL of distilled water and heat until it is completely dissolved (the solution looks perfectly clear). While the solution is still hot, put a droplet (~1 cm in diameter) onto a microscope slide. Immediately put a second slide on top and press so that the droplet is spread and flattened. Let it cool completely for about 2 min and carefully remove the second slide.

5. Eyelash hair pick. Used to transfer worms from plates onto the agarose pads before observation to minimize stress/damage to the animals.
6. Anesthetic: 20 mM Tetramisole hydrochloride solution in M9 which paralyzes worms by acting as an agonist to acetylcholine receptors (also *see* **Note 3**).

3 Methods

3.1 Sample Preperation (for Either Microscopy Method)

1. Prepare age synchronised populations of worms for each experimental condition/sample. This can be done either at the L4 stage, when worms are easily discernible by their highly visible vulva, or at the egg stage, via hypochlorite treatment of gravid adults or egg laying.
2. Place a 20 μL droplet of anesthetic (tetramisole or sodium azide) on top of an agarose pad. Gently transfer worms into the droplet using the eyelash hair pick.
3. Gently place a coverslip on top of the droplet and pad.
4. During observation, ignore any worms that have been damaged due to mishandling (exhibiting vulval rupture). Ensure that the worms do not dry out during observation.

3.2 Locating Neuronal Cell Corpses Via Nomarski Differential Interference Contrast (DiC) Microscopy

1. Setup your microscope for DiC observation. We recommend 20–40× magnification.
2. Count number of cell corpses, which are visible as enlarged vacuooles with somewhat irregular shape (Fig. 1) (*see* **Note 4**).
3. Calculate the % of neurodegeneration per worm via dividing the number of vaccuoles by the maximum number of potentially affected neurons.

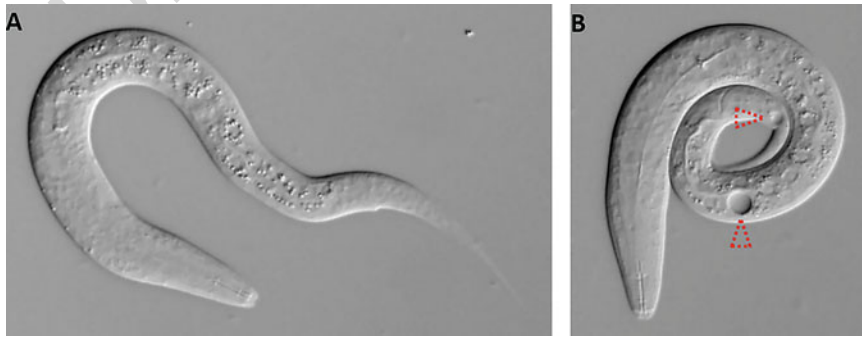

Fig. 1 (**a**) N2 worm at late L1 stage. No vacuoles are present. (**b**) *mec-4(d)* worm at the same age. The arrowheads point to two necrotic vacuoles: one at the tail, indicating the death of a PLM neuron, and one posterior to the vulva, indicating the death of the PVM neuron

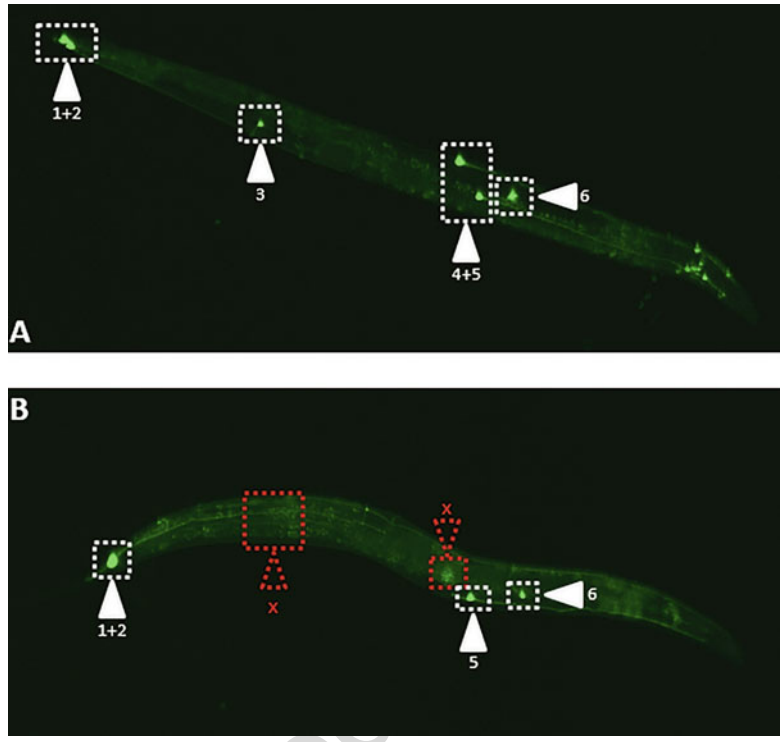

Fig. 2 (**a**) $P_{mec\text{-}4}$GFP worm, D1 adult. The arrowheads show all 6 neurons potentially affected by *mec-4(d)* are visible: PLML and PLMR (1 + 2), PVM (3), ALML and ALMR (4 + 5) and AVM (6). (**b**) *mec-4(d)* x$P_{mec\text{-}4}$GFP worm at the same age. The empty arrowheads show the positions of a missing neuron (PVM) and a blurry dying neuron (one of the ALM pair)

3.3 Identifying Loss of Fluorescently Tagged Neuronal Cells Via Fluorescent Microscopy

1. Setup your microscope for observation under the appropriate fluorescence channel. Low (4–10×) magnification is generally recommended to assess for the survival of neuronal cell bodies. Imaging at higher magnifications (40× or more) can provide better detail, especially on axonal condition, but will generally require Z-stack deconvolution since, due to the thinness of the neuron and the anatomy of the worm (bilateral symmetry), all neurons of a group are unlikely to be equally in focus at the same level.
2. Living neuronal cells should appear as bright and sharp spots. Blurry spots are likely in the process of dying and should not be counted (Fig. 2) (*see* **Note 5**).
3. Calculate the % of neuronal survival per worm by dividing the number of living neurons by the maximum number of potentially affected neurons.

4 Notes

1. For experiments that utilize RNAi, it is necessary to remember that most neurons of the worm are not affected by normal RNAi feeding. It will be necessary to transgenically express the RNA transmembrane transporter, SID-1, in the desired target neurons.
2. An additional point for RNAi experiments, for models that exhibit early neuronal cell death, like *mec-4(d)*, is that efficient silencing may not be achieveable at normal condiditions due to the narrow timeframe. Performing the experiments at 15° instead of the normal 20° to prolong organismal development might improve RNAi effectiveness.
3. In neuronal cell death experiments on models where acetylcholine signaling is relevant to the phenotype, tetramisole cannot be used as an anesthetic since it directly interferes with that system. In that case we recommend the use of 10 mM sodium azide (NaN_3, solution in M9), an inhibitor of oxidative phosphorylation.
4. Corpses of necrotic cells may not persist for a long time in the worm, as they can be removed by phagocytosis. This means that, depending on the model, this technique may only be effective within a narrow time window. For instance, in the *mec-4(d)* worms used as an example here the corpses can only be observed until the L2 stage of development. A way to partially circumvent this issue is to use a worm background in which corpse removal is inhibited due to a mutation, such as *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-8*, or *ced-10* mutants [24].
5. It is possible that, at low magnifications, the projections of two neuronal cell bodies on the camera lens can be close enough that it is hard to distinguish them, creating the illusion that there is only one neuron. That can be resolved by also counting the axons, and/or switching to a higher magnification and examining all z-stack levels, since, as mentioned before, all neurons of a group are unlikely to be equally in focus at the same level.
6. Unless the study is focused around the effects of starvation on neuronal cell death, the experimental worms should be well fed. Due to the existence of transgenerational effects in *C. elegans* [25, 26] it is a good idea to extend this rule to the prior three (at least) generations.

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