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Necrosis in *C. elegans*

Matt Crook, Avni Upadhyay, and Wendy Hanna-Rose

Abstract

To use *Caenorhabditis elegans* to study the mechanisms for initiation and execution of necrosis, the experimentalist should be familiar with the established models of necrosis in *C. elegans* and the genetic and molecular tools available. We present a summary of two contrasting models for studying necrosis in *C. elegans* and outline the methods for scoring necrosis in each. These methods are useful for the study of necrosis under other conditions in *C. elegans* and for comparative studies both between established and new necrosis models. We also present a list of the genetic and drug tools available for perturbing pathways known to be important for initiation or execution of necrosis and describe their use in *C. elegans*. Specifically, we outline methods to inhibit autophagy, to perturb calcium homeostasis, and to disrupt lysosomal function in the *C. elegans* system.

Keywords

Cell death; Degenerins; Nicotinamide; TOR; Vitamin B3 metabolism; Mechanosensory cells; Uv1 cells; Necrosis; *C. elegans*

1 Introduction

Cell death occurs via multiple genetic programs and studies in *Caenorhabditis elegans* have been key to both the recognition of specific death programs and the elucidation of their underlying genetic control. Cell death programs can be distinguished based on the morphological and molecular changes that occur during dismantling of the cell as well as on the signals for initiation of the program. Necrosis is one such cell death program. It proceeds via a stereotypical series of morphological changes, excessive cell swelling being one of the most dramatic (reviewed in refs. 1–3). While the morphology of necrotic cells has been described in detail, we are just beginning to decipher the genetic and molecular events that underlie necrotic death, and it is becoming clear that distinct pathways for both initiation and execution of morphologically similar death programs may exist. These differences highlight the importance of multiple models for studying necrotic death and demonstrate that the *C. elegans* system will continue to be valuable to the advancement of the field of cell death.

Necrotic death in *C. elegans* is not programmed from a developmental standpoint as in some other systems, but *C. elegans* cells do respond to various insults with one or more genetically controlled necrotic-type death programs. There are several models for studying necrosis in *C. elegans* and these models are proving useful for understanding how cells die

in response to insult, a crucial first step to learning to prevent such deaths in clinical settings where necrosis plays a role in disability (e.g., after stroke or ischemia). We briefly describe two specific models for studying necrosis in *C. elegans* and then describe methods for using these systems to analyze mechanisms of necrosis.

1.1 Touch Cell Death Induced by Degenerins

Gain-of-function mutations in certain ion channel subunits cause neurons expressing these “degenerins” to necrose with predictable spatial and temporal patterns [4–6]. The best-studied degenerin gene is *mec-4*. Dominant, gain-of-function mutations in *mec-4* cause necrosis of six specific touch receptor cells (PVM, AVM, a pair of PLMs, and a pair of ALMs) (Fig. 1a, *see* wormatlas.org or ref. 7 for detailed information about cell morphology and location). Necrotic cells appear in the *mec-4(d)* mutants shortly before hatching and the corpses are absorbed by mid-L1 [8]. Genetic analysis of degenerin-induced necrosis has revealed an important role for calcium influx in induction of the necrotic program and for calcium-dependent proteases in its execution [9, 10]. Lysosomal proteases are also involved in executing necrosis downstream of calcium signaling in this model [11, 12]. Finally, efficient autophagy is required for the progression of necrosis [13, 14]. We explain how to use tools to probe each of these processes in *mec-4d*-induced touch cell necrosis.

1.2 Uv1 Cell Death Induced by Nicotinamide or Loss of PNC-1 Nicotinamidase Function

The four uterine-vulva 1 cells that lie between the uterine seam cell and the vulF cell in *C. elegans* (Fig. 2a) are sensitive to perturbations in vitamin B3 metabolism. Supplementation of wild-type animals with nicotinamide or mutation of the nicotinamidase PNC-1, which converts nicotinamide to nicotinic acid, causes these cells to necrose [15, 16]. While the morphological changes associated with uv1 and degenerin-induced necrosis are similar, studies suggest that the triggers and the signals that activate the death program in these two models are distinct, highlighting the value of further comparative analyses.

2 Materials

2.1 Mounting Worms for Microscopy

1. Compound microscope with 100× differential interference contrast (DIC) oil immersion objective, preferably with a UV light source for fluorescence microscopy, but scoring can be accomplished without fluorescence microscopy.
2. Stereoscope for manipulating animals on culture plates.
3. 25×75×1 mm microscope slides and 22×22 mm no.1 coverslips.
4. Anesthetic.
 - a. 100 mM stock solution of tricaine in water (store aliquots at −20 °C).
 - b. 100 mM stock solution of tetramisole hydrochloride in water (store aliquots at −20 °C).
 - c. Working solution of 1 mM each tricaine and tetramisole hydrochloride in M9: 22 mM KH₂PO₄, 42 mM Na₂HPO₄, 86 mM NaCl, 1 mM MgSO₄.

5. 5.5 % agarose in water for making pads.

2.2 Strains for Scoring Necrosis

In touch cells

1. N2 wild type, available from *C. elegans* Genetics Center.
2. CB1611 *mec-4d(e1611)* X: [8], available from *C. elegans* Genetics Center.
3. ZB1656 *tdIs5* [*P_{mec-4}::GFP*] I; *mec-4d(u231)* X [10], available by request from M. Driscoll lab.

In uv1 cells

1. N2 wild type, available from *C. elegans* Genetics Center.
2. HV727 *pnc-1(pk9605)* IV: [16], available by request from W. Hanna-Rose lab.
3. HV560 *inIs179* [*P_{ida-1}::GFP*] II; *pnc-1(pk9605)* IV: available by request from W. Hanna-Rose lab.
3. Wild type or other animals carrying the *inIs179*[*P_{ida-1}::GFP*] transgene cultured on NGM plates with 25 mM nicotinamide, which induces uv1 cell death.

2.3 RNAi

1. LB broth and agar.
2. NGM agar culture plates. Add 3 g NaCl, 17 g agar, and 2.5 g bacto peptone to a 2 l flask and add ddH₂O to 975 ml. Autoclave for 50 min on liquid cycle, allow to cool to 55 °C, and then add 0.5 ml 1 % cholesterol (dissolved in 95 % ethanol and filter sterilized), 1 ml each of 1 M CaCl₂ and 1 M MgSO₄, and 25 ml of 1 M KHPO₄. Swirl to mix and pour into 10 cm plates.
3. 1 M isopropyl beta-d-1-thiogalactopyranoside (IPTG).
4. 12.5 mg/ml tetracycline.
5. 50 mg/ml ampicillin.
6. 100 mg/ml carbenicillin.
7. NGM plates with 1 mM IPTG and 25 µg/ml carbenicillin that are 2–3 days old.
8. LB agar plates with 50 µg/ml ampicillin and 12.5 µg/ml tetracycline.
9. Clones from RNAi feeding library for genes listed in Table 1.

2.4 Drug Treatments

1. Compound microscope equipped for microinjection of *C. elegans*.
2. 1 M ethylene glycol tetraacetic acid (EGTA).
3. 10 mM dantrolene in water.

4. 100 μ M Bafilomycin-A1 stock solution: Add 160 μ l of 98 % ethanol to 10 μ g bafilomycin-A1 (Sigma-Aldrich). Mix well to dissolve. Prepare aliquots. Cover the tubes with aluminum foil and parafilm the cap; store at -20°C . Avoid frequent freeze-thawing of stock solution. To prepare the 50 μ M working solution, add 10 μ l of stock solution to 10 μ l of 98 % ethanol. Keep the solution on ice and covered in foil during use.

3 Methods

General procedures for maintenance and manipulation of *C. elegans* including culturing conditions, preparing animals for microscopy, and performing genetics and microinjection are available from several excellent sources including wormbook.org [17, 18]. We recommend consultation with experienced *C. elegans* researchers for experimenters who are novices with the system.

3.1 Mounting Worms for Microscopy

1. Prepare two support slides by applying a strip of lab tape to the top and place a clean glass slide between them.
2. Melt the 5 % agarose and place a small drop on the slide using a plastic Pasteur pipette.
3. Squash the agarose drop by placing a third slide, perpendicular to the other slides, over the drop and the support slides, applying firm pressure with the thumbs over the tape, creating a round agarose pad of uniform thickness.
4. Carefully slide the top slide off of the agarose pad (*see* Note 1).
5. Add 2–5 μ l of anesthetic solution to the agarose pad.
6. Add 10–20 animals of the appropriate stage to the drop on the pad.
7. Place a coverslip over the drop, add immersion oil to the coverslip, and view with 100 $\times$ DIC objective on the compound microscope.

3.2 Scoring Necrotic Phenotypes

Necrotic cells can be visualized using DIC microscopy based on two obvious morphological characteristics: their swollen cytoplasm and their disintegrating nuclear membrane. The swollen cytoplasm expands the dying cell to several times its original size and appears empty of any organelles. The nuclear membrane maintains its original size but appears fragmented, similar to the islands in a coral atoll chain (Figs. 1b and 2e). Because necrotic cells can be cleared from the animal after a period of time, a less ambiguous and recommended method for scoring is to use transgenic strains with GFP expressed in the cells of interest (e.g., Fig. 2c). However, the choice between scoring with or without GFP will be governed by the ease of genetic crosses to put markers in the desired genetic background

¹Use a razor blade to remove the edges of the round agar pad until it is square. The square pad is easier to scan under the microscope without losing track of your position during scoring.

and the availability of a microscope with the appropriate UV light source and filters for visualizing GFP. We describe and discuss procedures for scoring using both methods.

1. Place animals on the slide for microscopy.
 - a. *mec-4d-induced touch cell necrosis for DIC*: Select first larval stage (L1) animals from a *mec-4d* strain (*see* Note 2).
 - b. *mec-4d-induced touch cell necrosis for fluorescence microscopy*: Select post-L3-stage animals from a *mec-4d* strain carrying a $P_{mec-4}::GFP$ transgene that is expressed in the touch cells (*see* Note 3).
 - c. *Uv1 cell necrosis for DIC*: Select animals that are in the second half of the fourth larval stage (L4), just after vulva eversion begins and before the uterine lumen closes (*see* Notes 4 and ⁵) from a *pnc-1* mutant strain or a wild-type strain cultured on 25 mM nicotinamide.
 - d. *Uv1 cell necrosis for fluorescence microscopy*: Select late-L4 animals, after vulva eversion has begun, or early adults, before oocytes are present, from a strain carrying a $P_{ida-1}::GFP$ transgene that is expressed in the uv1 cells.
2. Scan the slide, and for each animal at the appropriate stage, determine the presence or the absence of necrotic cells (*see* Notes 6 and ⁷) or the presence or the absence of GFP⁺ cells (*see* Notes 8 and ⁹), as appropriate.
3. Record the number of animals at the appropriate stage that were scored as well as the necrotic phenotype (*see* Notes 10 and ¹¹).

²Select animals immediately after hatching and before any darkening of the intestine because necrotic touch cells are broken down and absorbed before the end of the L1 stage. Do not score animals that are older than mid-L1. www.Wormatlas.org is helpful if additional information about staging animals is required.

³Score older animals when using the $P_{mec-4}::GFP$ transgene to avoid accidental inclusion of corpses with persistence of faint GFP signal in counts of surviving GFP⁺ cells.

⁴Staging worms this precisely is not easy to accomplish as the animals are chosen for placement on the slide. However, as the slide is being scored, it is simple to include only animals of the appropriate stage. Figure 2 has additional information about staging.

⁵Uv1 cells are not specified until early in the L4 stage. Necrotic uv1 cells do not appear until shortly after the vulva reaches the “Christmas tree” stage when the vulva begins to evert (Fig. 2b–d). The ideal time to score is just after vulva eversion begins and before the uterine lumen closes at the late-L4 stage. While the window for scoring uv1 cell necrosis is broader than that of touch cell necrosis because necrotic uv1 cells are not cleared quickly, scoring too early is still a risk. Scoring for the presence or the absence of cell corpses after L4 is possible, but the ability to count exactly how many corpses are present becomes difficult after oocytes are fertilized and deposited in the uterus.

⁶*Touch cell necrosis: mec-4d* animals have a maximum of six necrotic touch cell corpses (Fig. 1a), but on average will have two to three by DIC. In addition, the majority of easily visible necrotic cells are in the tail (Fig. 1b). Precisely counting the number of touch cells that have necrosed is challenging due to the short scoring window during which necrotic touch cells are visible before they are absorbed and disappear. This factor can result in high variance in the number of necrotic cells per animal. *Uv1 necrosis: pnc-1* mutants have a maximum of four necrotic uv1 cells (Fig. 2a) and 100 % of animals will have at least one necrotic uv1 cell. Most animals will have four necrotic uv1 cells [15].

⁷Occasionally you will see large empty vacuole-like structures lacking a disintegrating nucleus in various areas of the animal; these are most likely the result of worms being on the slide too long before scoring.

⁸One disadvantage to using $P_{ida-1}::GFP$ as a uv1 marker is its expression in the nearby HSN cell body, which, although medial to the four uv1 cells and of clearly distinct morphology (Fig. 2c), can be mistaken for a surviving uv1 cell by the novice. Alternative GFP markers more specific for the uv1 cells are available. However, markers that are expressed earlier than $P_{ida-1}::GFP$, which is only expressed after the point when the cells die, can lead to inaccuracies in scoring if an animal that is too young is accidentally included.

⁹Intervening cells can obscure both necrotic uv1 cells and GFP⁺ uv1 cells on the side of the animal opposite the objective, especially after the uterine lumen has begun to close. If the observer has difficulty scoring both sides of every animal, the side nearest the objective can be exclusively scored in each animal.

3.3 RNAi Protocol for Genes Listed in Table 1 (See Note 12)

Reagents, including drugs and genes, for perturbing known pathways involved in initiation or execution of necrosis in *C. elegans* are listed in Table 1. Allele names are provided for genes where traditional genetic experiments have been done. While either traditional genetics or RNAi could be applied with most of the genes, where no allele name is provided, RNAi has been the method applied to block gene function. RNAi can also be used to knock down the genes that are listed with alleles.

1. Streak for single colonies on an LB Amp Tet plate with an inoculum from a glycerol stock of a bacterial strain carrying the desired RNAi clone (e.g., from the *C. elegans* RNAi library (<http://www.gurdon.cam.ac.uk/~ahringerlab/pages/rnai.html>)).
2. Incubate at 37 °C overnight.
3. Pick a single colony and inoculate a 2–3 ml LB Amp broth culture.
4. Incubate at 37 °C overnight with agitation.
5. Prepare a backup glycerol stock from the overnight culture (1:2 culture:50 % glycerol).
6. Prepare plasmid DNA from the overnight culture.
7. Verify the identity of RNAi insert by sequencing with an M13 forward primer for the *C. elegans* RNAi library clones (other RNAi clones may differ).
8. Once verified, inoculate another 2–3 ml LB Amp broth culture using the glycerol stock from step 5.
9. Incubate at 37 °C overnight with agitation.
10. Add approximately 200 µl of culture to the top of NGM IPTG Carb plates. Incubate at 20 °C for 3–4 days to allow the plates to dry and the bacteria to express the

¹⁰Touch cell necrosis is reported in a number of ways:

- a. By the presence/absence of necrosis per animal, determined by observation of one or more corpses (presence) or no corpses (absence) or by observation of less than six GFP⁺ cells (presence) or exactly six GFP⁺ cells (absence).
- b. By the number of necrotic touch cells in the tail of each animal because these are the most readily visible corpses.
- c. By the total number of necrotic touch cells per animal, often reported as the number of necrotic touch cells per 100 animals. This is determined by the number of corpses observed or by subtracting the number of GFP⁺ cells observed from total number of touch cells expected, which is six.

¹¹Uv1 necrosis can be reported in a number of ways:

- a. By the total number of necrotic uv1 cells per animal (or per side of the animal nearest the objective—see Note 8). This number is determined by either the number of corpses observed or subtracting the number of GFP⁺ cells observed from the total number of cells expected, which is four (two per side).
- b. As the proportion of uv1 cells that survive out of the total number of possible uv1 cells.
- c. By the presence/absence of necrotic uv1 cells per animal or per side of the animal nearest the objective.

¹²For a comprehensive guide to RNAi in *C. elegans*, see the Reverse Genetics chapter from www.Wormbook.org (http://www.wormbook.org/chapters/www_introreversegenetics/introreversegenetics.html). We carry out RNAi by feeding, both for ease of use and because of the availability of the *C. elegans* RNAi feeding library (<http://www.gurdon.cam.ac.uk/~ahringerlab/pages/rnai.html>).

double-stranded RNA. Five plates per RNAi treatment usually provides enough worms of the right stage to score.

11. Add animals to the prepared RNAi plates (*see* Note 13).
 - a. For *mec-4d*-induced necrosis, pick five to ten L2 larvae to each RNAi plate.
 - b. For *uvl* necrosis, pick five L4 larvae to each RNAi plate (*see* Note 14).
12. Incubate RNAi plates at 20 °C until the progenies are ready to score as described (*see* Note 15).

3.4 Drug Treatments to Perturb Calcium Homeostasis (Table 1)

1. Add EGTA, a Ca²⁺ chelator, or dantrolene, a chemical that inhibits Ca²⁺ release from the ER (*see* Note 16), to NGM agar before pouring the culture plates to a final concentration of 50 mM and 10 µM, respectively.
2. Spot plates with *E. coli* OP50 and incubate at 20 °C for 3–4 days.
3. For *uvl* necrosis, pick five L4 larvae to each plate. For *mec-4d*-induced touch cell necrosis, pick five to ten L2 larvae to each plate (*see* Note 14). Five plates per treatment usually provides enough worms of the right stage to score (*see* Note 17).
4. Incubate plates at 20 °C until progenies are at the appropriate age to score, which will take approximately 3–4 days, depending on strain and treatment.

3.5 Drug Treatment to Disrupt Lysosome Function (Table 1)

1. Inject the body cavity of gravid hermaphrodites with a working solution of Bafilomycin-A1 (*see* Note 18).
2. As a control inject a matched set of animals with 98 % ethanol.
3. Transfer injected animals to an NGM plate with OP50 food (*see* Note 19).
4. Culture the animals in the dark at 20 °C overnight.
5. Transfer surviving animals to fresh NGM plates with OP50.
6. Score the progeny of injected animals at the appropriate stage as described above.

¹³Washing worms three times in M9 before placement on the RNAi plates may reduce some of the variability of the RNAi effect caused by carryover of OP50 from stock plates.

¹⁴It is important to take the time to transfer the younger larvae when planning to score touch cell necrosis because gene expression must be knocked down before the embryos form. When planning to score *uvl* necrosis, it is not necessary to select such young animals and using older larva can avoid potential problems that gene knockdown may cause in late-stage larval development.

¹⁵Incubate approximately 3–4 days, depending on strain and RNAi clone.

¹⁶Solubility of dantrolene in water at this concentration can be problematic. Mix the stock solution thoroughly by vortexing to ensure that the final concentration is as close as possible to the desired concentration.

¹⁷Altering Ca²⁺ availability affects ovulation in *C. elegans*. As a result, animals on EGTA or dantrolene may become sterile or produce fewer progeny than normal; therefore the number of plates per treatment may need to be increased.

¹⁸We recommend microinjection of this drug. Because Bafilomycin-A1 is not soluble in water, it is difficult to add to culture plates as with the drugs to manipulate calcium. Moreover, the drug is expensive and larger quantities are needed when used as a supplement in culture plates as compared to when administered by injection. Acute exposure to Bafilomycin-A1 via soaking in drug solution is a possible substitute for injection but this treatment is associated with higher lethality.

¹⁹Animals are transferred to new plates to eliminate the progeny that were embryos in the uterus during microinjection and, thus, were not exposed to drug.

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Fig. 1. Scoring touch cell necrosis. **(a)** Schematic diagram indicating approximate positions of the six touch cells in the animal. The *shaded Xs* are left/right pairs of cells and may not be visible in the same focal plane, particularly in the mid-region of the body. **(b)** *mec-4d(e1611)* L1 larva with two necrotic cells (*arrows*) in the tail

Fig. 2.

Staging animals for scoring uv1 necrosis. (a) Schematic diagram indicating approximate positions of the four uv1 cells in the animal. There are two uv1 cells on each side, flanking the vulva. Only two uv1 cells are visible in a single focal plane. (b–d) A temporal series of wild-type, mid to late L4 hermaphrodites, showing the morphology of the vulva. (b) The “Christmas tree” stage: uv1 cells begin to die shortly after this stage and animals should be scored when slightly older. (c) A slightly older animal. The first subtle signs of eversion are evident. The two large medial cells (arrows) are moving towards one another and will eventually make contact, as in the older animal in (d). The protrusions into the vulval lumen in the ventral vulva (arrowheads) are beginning to “droop” ventrally and this feature becomes more evident in (d). Asterisks mark the open uterine lumen in all animals. (c' and c'') Fluorescent images in two different focal planes from the same animal as in (c). This animal carries the *P_{ida-1}::GFP* transgene. Arrows indicate the two uv1 cells visible in the same focal plane. The uv1 cells appear to have a convex surface on the ventral side and a concave surface on the dorsal side. The HSN cell (arrowhead) is in a slightly different focal plane and has a distinct morphology with a fl at ventral surface. (e) *pnc-1(pk9605)* mutant with a necrotic uv1 cell (arrow)

Table 1

Genes and drugs for manipulating processes that affect initiation and/or execution of necrosis in various systems

<i>Autophagy</i>	
Loss of function of these genes will block autophagy and rescue <i>mec-4d</i> -induced touch cell necrosis [13, 14], but not uv1 necrosis	
<i>unc-51(e369)</i>	Atg1p ortholog, S/T kinase
<i>bec-1(ok700)</i>	Atg6/Vps30/Beclin1 ortholog
<i>lgg-1</i>	Atg8p ortholog, member of preautophagosome complex
<i>Ca²⁺</i>	
Loss of function of these genes or application of these drugs will dampen an increase in cytosolic Ca ²⁺ and rescue <i>mec-4d</i> -induced touch cell necrosis [10], but not uv1 necrosis	
<i>crt-1(bz29)</i>	ER Ca ²⁺ -binding protein/chaperone
<i>cnx-1</i>	ER Ca ²⁺ -binding protein
50 mM EGTA	Ca ²⁺ chelator
10 μ M dantrolene	Blocks ER Ca ²⁺ release
<i>TOR-mediated nutrient sensing</i>	
Loss of function of these genes will block TOR-mediated nutrient sensing and exacerbate <i>mec-4d</i> -induced touch cell necrosis [14]. This may be via a negative effect of Tor on autophagy	
CeTor/ <i>let-363</i>	Tor1/2p PIK ortholog
<i>Lysosomal proteases</i>	
Loss of function of these genes or application of this drug will disrupt the function of lysosomal proteases and will rescue <i>mec-4d</i> -induced touch cell necrosis [11] and uv1 necrosis [15]	
50 μ M Bafilomycin-A1	Inhibits processing of cathepsin D, a lysosomal protease [19]
<i>vha-2, vha-10, vha-12</i>	Subunits of V-ATPase that cause cytosolic acidification and activation of lysosomal proteases [11]
<i>asp-3, asp-4</i>	Aspartyl protease, <i>asp-3</i> and <i>asp-4</i> act in parallel and downstream of <i>clp-1</i> and <i>tra-3</i> [9]
<i>clp-1</i>	Calpain homologue, acts in parallel with <i>tra-3</i>
<i>tra-3</i>	Atypical calpain regulatory protease

Allele names are provided for genes where traditional genetic experiments have been done. Where no allele name is provided, RNAi is recommended as a method to block gene function. Additional information about any *C. elegans* gene is available at Wormbase.org