Protocol

Visualizing Apoptosis in Embryos and the Germline of *Caenorhabditis elegans*

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Visualizing apoptosis in developing embryos or the germline of *Caenorhabditis elegans* is remarkably easy because of the transparency of the organism. The invariant pattern of cell division and programmed cell death during development makes it possible to quantify small but reproducible changes in apoptosis, which are easy to detect by light microscopy because of the refractile properties of dying cells. Although apoptotic death is easy to visualize and quantify in the germline of adult hermaphrodites, the pattern of cell death is variable, especially when triggered by stress. The most convenient method for visualization of apoptosis in vivo is light microscopy, which requires immobilizing live embryos or adult animals on slides. This protocol describes the basic methods for visualizing and analyzing apoptosis in living animals.

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MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPE: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Agar solution (4% [w/v], maintained in the liquid phase in a 55°C water bath)

C. elegans *at the appropriate stage of development, in which apoptosis has been stimulated.*

Activate apoptosis in the worms of interest by perturbing gene function (e.g., with RNAi) or by exposing to various stressors (e.g., DNA-damaging agents). (See Protocol: ***Induction of Germline Apoptosis in*** C. elegans *[Lant and Derry 2014].) Careful staging of the worms, particularly with embryos, will be needed, and typically will arise only from repeated viewings of worms/embryos at different stages.*

M9 buffer for worms <R>

Nail polish and/or petroleum jelly

Tetramisole (20 mM)

Equipment

Compound microscope with differential interference contrast (DIC) optics

Numerous transgenic strains of C. elegans *carrying fluorescent markers are available; these can be crossed into any mutant background to assess various stages of apoptosis in living animals. Thus, for high-resolution imaging*

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Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot080218

and quantification of apoptotic bodies, we recommend a compound microscope that is equipped with both DIC and fluorescence optics (e.g., Leica DMRA).

Coverslips
Eyelash glued to a toothpick
Glass slides
Image capturing software (e.g., Volocity [PerkinElmer])
Immersion oil
Incubator at 37°C
Wire (platinum)

METHOD

Preparing Slides

Note that slide mounting is necessary to assess apoptosis under DIC optical resolution, but depending on the strain, apoptotic response may be visualized using fluorescent markers with a dissecting microscope, which does not require slide mounting of the worms.

1. Add a small drop (~25 µL) of warm 4% agar to a slide. Making sure that the agar does not set, quickly lay another slide on top, perpendicular to the lower slide. Press firmly to flatten the warm agar into a thin pad (~1 mm thick). Aim to maintain a level pad so the worms remain on a level plane when viewed under the microscope.
2. Before mounting the embryos or worms, carefully remove the top slide by sliding so the thin agar pad remains on the bottom slide.

   Be careful; sometimes the agar pad will slip off or buckle as the top slide is removed.
3. If desired, dry the slide/agar pad overnight in a 37°C incubator.

   For experiments of long duration (e.g., determining corpse persistence times) the agar pad on which the worms or embryos are placed will desiccate and shrink, making it difficult to record 4D images without going out of focus. Thus, we recommend performing this step for all time-course studies.

   Proceed to Steps 4–5 (for embryos) or Steps 6–9 (for worms).

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Visualizing Apoptosis in Developing Embryos

4. Place a 2.5-µL drop of M9 buffer on a thin agar pad on a microscope slide. Pick embryos from a plate that is well fed (i.e., still has plenty of bacteria) and place them into the M9 buffer.

   When visualizing embryos, it is not necessary to use tetramisole, as embryos do not require anesthesia. Instead, we recommend mounting embryos in M9 buffer. It is important to ensure that the samples remain hydrated as they are picked and placed onto the agar pad. If the liquid evaporates, add a few microliters of M9 buffer to the embryos on the agar pad to keep them hydrated while transferring to the pad.
5. Using an eyelash glued to a toothpick, carefully drag the embryos into straight lines near one side of the M9 drop. Carefully place a coverslip over the top, sandwiching the embryos between the agar pad and coverslip. If necessary, add ~18 µL of M9 buffer under the edge of the coverslip to prevent dehydration. Seal the coverslip with nail polish or warm petroleum jelly to prevent evaporation of buffer during the observation period.

   Take care that the M9 does not evaporate during this time, which will cause embryo morphology to deteriorate and make it difficult to visualize apoptotic corpses.

   Proceed to Step 10.

Visualizing Germ Cell Apoptosis In Vivo

Because the germline continues to proliferate and expand throughout development, it is important to carefully stage worms used for this procedure so they are all at the same stage (e.g., 24 h post-L4). This ensures that their germlines are uniform in size.

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6. Place a drop (∼5 μL) of 20 mM tetramisole on the agar pad. Carefully pick worms with a platinum wire coated with a small amount of bacteria from the NGM plate and transfer them into the liquid.

   It is important to ensure that the worms remain hydrated as they are picked and placed onto the agar pad. If the liquid evaporates, the integrity of the animals will degrade and make it very difficult to accurately visualize apoptotic corpse morphology.

7. Once the appropriate number of worms has been added to the agar pad, carefully place a coverslip over the top, sandwiching the samples between the agar pad and coverslip. If necessary, add ∼18 μL of 20 mM tetramisole under the edge of the coverslip to prevent dehydration. Seal the coverslip with nail polish or warm petroleum jelly to prevent evaporation of tetramisole (and subsequent desiccation of the sample) during the observation period.

   Embryos develop for several hours after mounting, thereby permitting time-course analysis of apoptosis during development. In contrast, the observation of the adult germline should be limited to a few hours after mounting, as germline morphology tends to degrade after prolonged periods in tetramisole.

8. Using the 10× lens on a compound microscope, focus on the target worms.

   Often, this magnification, in conjunction with high signal labeling (such as acridine orange [AO] staining), permits the identification of the most visible/unobstructed germlines; this essentially acts as prescreening for higher magnification visualization.

9. Move to the 63× oil immersion lens and focus on nuclei in the bend region of the germline. Confirm focus on the image capturing software, as there is often a differential between the ocular lens and the camera capture.

   We find that the posterior end of the germline is often easier to visualize, as the anterior bend tends to be obstructed by the intestine.

   Proceed to Step 10.

Analyzing Apoptosis

10. Quantify corpse numbers and corpse persistence time as desired.

    Embryos will develop to hatching under the coverslip, which allows visualization of developmental apoptosis at various times over several hours. Using morphological criteria to stage the embryos, the best stages to quantify apoptosis are at the "lima bean" stage (6 h post fertilization; Fig. 1), the comma stage, the 1.5-fold stage, and the 2-fold stage (see IntroFIG7 at http://www.wormatlas.org/ver1/handbook/anatomyintro/anatomyintro.htm). Apoptosis peaks during the stages of development where embryos begin to undergo elongation—the comma and 1.5-fold stages.

    With 4D microscopy, it is easy to both quantify the number of corpses and their persistence time to determine whether abnormal numbers of corpses at specific stages of development are caused by engulfment defects. Corpses generally persist in embryos for ∼30 min before getting engulfed.

    As in embryos, apoptosis can be visualized in germlines of anesthetized adult worms for several hours. Thus, it is possible to quantify the persistence time of germline corpses to determine whether abnormal numbers are the result of engulfment defects. Germline corpses generally persist for ∼45 min before getting engulfed by the surrounding gonadal sheath cells.

    See Troubleshooting and Discussion.

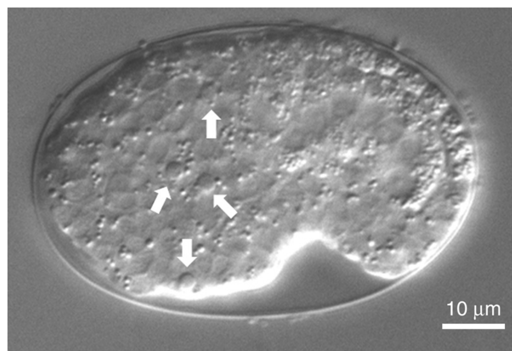

FIGURE 1. Corpses in the *C. elegans* embryo (indicated by arrows) appear as cellularized bodies or "buttons" and are spatially distinct. The above embryo is in the "bean" stage, which is ∼6 h into ex utero embryonic development. Embryo viewed by DIC using 630× oil immersion magnification.

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TROUBLESHOOTING

Problem (Step 10): Embryos are dead on viewing.
Solutions: There are a number of reasons that you may be observing dead embryos on the slide.

- One possibility (if you are employing RNAi, or observing a mutant strain or products of a cross) is that the eggs may be dead before plating as part of a mutation phenotype. In some cases, a mutant phenotype may result in embryonic lethality after much developmental apoptosis has occurred, for example in the case of large chromosomal deficiencies (Sugimoto et al. 2001). For such studies, it is important to obtain embryos at very early stages of development (2- to 8-cell stage) by cutting open gravid hermaphrodites to follow their lineages to precisely score apoptotic events. We also refer the reader to the less labor-intensive method of scoring surviving nuclei in the pharynx if the animals survive to hatching, where many extra cells are located when apoptosis is suppressed (Schwartz 2007).
- A second possible cause may be transfer time from plate to slide. Often if this is not done rapidly (and the embryos desiccate on the pick) you will be unable to observe developmental apoptosis. Finally, embryos will cease dividing and eventually die if they are in an anoxic environment. Thus, mounting embryos with minimal amounts of bacteria and ensuring that there are plenty of air bubbles in the agar pad help prevent hypoxic conditions under the coverslip.

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Problem (Step 10): Air bubbles are present (in the agar pad or tetramisole) that obscure worm physiology.
Solution: Before the agar setting, if air bubbles have formed, use the edge of a coverslip to puncture the bubbles. Similarly, with the application of tetramisole, if air bubbles are noticeable on the pad, use the edge of a coverslip to puncture them before putting worms in the anesthetic. Note that air bubbles can be beneficial in preventing hypoxic conditions, when making long duration (i.e., time course) observations. In these cases, make sure to manipulate the worms in the tetramisole so that they do not lie directly on the bubbles (causing imaging issues).

Problem (Step 10): Worms are dead/severely damaged on viewing.
Solution: There are a number of reasons that this may occur. (Making sure the worms you mount are not already dead or damaged on the plate is a good habit!) In the transfer between plate and slide (and tetramisole), do not leave the worm on the pick in excess of 30 sec, as desiccation will begin and can result in death by the time the worm is mounted. After the worms have been placed on the slide, do not be tempted to apply pressure on the coverslip to “secure” its hold. This will cause the worms to burst and prevent you from accurately quantifying apoptosis. The capillary force between the coverslip and the tetramisole should keep it in place (unless there is too much solution on the slide—in which case the coverslip may slide off).

Problem (Step 10): Germlines appear damaged or obstructed.
Solution: Damaged germlines can be the result of a specific mutation or RNAi treatment, or damage can be incurred by desiccation from prolonged time on the pick. As mentioned above, damaged germlines or unexpected apoptotic phenotypes (particularly in terms of engulfment rates) can result when worms are dying on the slide. As in embryos, the hypoxic conditions imposed by slide mounting can affect germline quality in adult worms.

Cases of germline obstruction from intestinal “blocking” are common, and are more prominent in the anterior of the worm, particularly in older animals which tend to retain their eggs. We recommend mounting more than 50 worms per condition and only quantifying corpses in animals with clearly visible germlines in all focal planes. In our experience, the posterior germline is consistently less obstructed by the intestine and therefore better for quantifying apoptosis.

Problem (Step 10): Germline corpses are persisting in excess of “standard” engulfment time.

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Solution: You may have discovered a gene that regulates corpse engulfment, or there is insufficient oxygen for the worms to remain viable. If you suspect that defective engulfment is not linked to the particular condition or gene that you are studying, the persistence may be caused by lack of oxygen in the slide. Avoid hypoxic conditions as referred to above. Bacterial consumption of oxygen is usually not a problem, because in most cases, it is not necessary to have adults under coverslips for more than a few hours.

Problem (Step 10): Despite the induction of DNA damage (via genetic or genotoxic means), the apoptotic effect appears too small to quantify.

Solution: An alternative method would be to quantify germline apoptosis in an engulfment-defective background, such as the *ced-1* mutant. By preventing engulfment, corpses will persist (giving the impression of increased corpse numbers), which will shift the distribution of corpses to a normal curve (see, e.g., Ross et al. 2011).

DISCUSSION

The methods described here are the most simple, linear preparative means to assess apoptosis. Corpse counting and quantification of the cellularized "buttons" (Fig. 2) in the germline requires practice. For quantification, it is critical to have experience with discriminating apoptotic corpse morphology. Thus, it is recommended to practice double-blind quantification of apoptotic corpses in various mutant strains that have high and low levels of apoptosis to gain expertise. Attuning your eyes to these morphological features, and differentiating early from late, is simply a matter of patience and repetition.

Assessing corpse numbers throughout the stacks (Z-plane) of the germline is critical to accurately obtaining corpse numbers, which occasionally show only small variation between strains/conditions. It is important to ensure that the animals, whether embryos or adults, are carefully staged so data are reproducible. A steady hand is also required when moving embryos, so make sure to monitor your caffeine intake before the experiment! In addition, we highly recommend performing double-blind apoptosis counts to prevent observer bias. By quantifying corpse persistence times it is possible to determine whether abnormal corpse numbers are caused by defects in the execution or engulfment phases of the apoptotic program. Whenever possible, we also recommend confirming results using multiple alleles, transgene rescue (when working with mutant strains), and RNAi. This is particularly important for analyzing germline apoptosis in strains/conditions where the differences in average corpse number are subtle. We therefore recommend using engulfment defective mutants such as *ced-1* or *ced-5* to increase the sensitivity of detecting subtle changes in apoptosis.

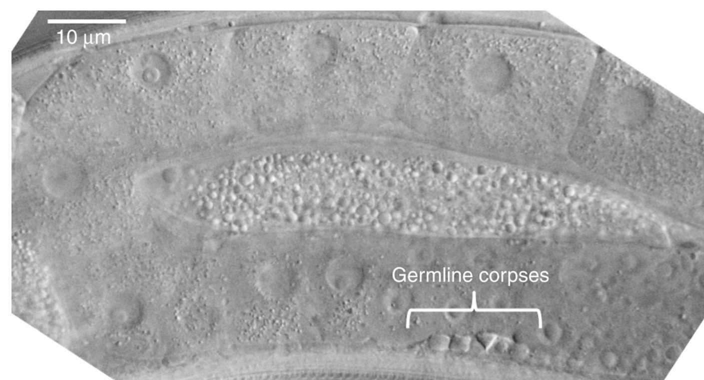

FIGURE 2. *C. elegans* germline indicating corpses as a result of irradiation (60 Gy). Corpses appear, as in the embryo, as cellularized bodies or "buttons" in (and just before) the bend of the pachytene region of the germline. Germline viewed under 630× oil immersion magnification.

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Data Analysis

Proper statistical methods are necessary for comparing apoptotic corpse numbers. This is particularly important when comparing corpse numbers in undamaged germlines with those that have been subject to stress (Fig. 2). In our experience, physiological apoptosis tends to follow a Poisson distribution in wild type and most mutant animals, whereas after stress, corpse numbers follow a normal (bell curve) distribution. Comparing these types of data distributions cannot be achieved with a Student's *t*-test, for example. Rather, we recommend the use of statistical methods such as the Mann–Whitney U test, which does not require different data sets to follow the same distribution. For a good refresher on the proper use of error bars and statistics in biology, we recommend the reviews by Cumming et al. (2007) and Fay and Gerow (2013).

It is important to consider the potential impact of proliferative changes when analyzing corpse numbers in the germlines of various mutant strains. Because the somatic lineages of *C. elegans* follow invariant patterns of cell division and cell death, analysis of apoptotic events in developing embryos is very precise. In the germline, cell deaths are more stochastic and can be influenced by changes in proliferation. To ensure that decreased numbers of corpses observed in the germline are not simply the result of reduced proliferation and therefore fewer cells capable of undergoing apoptosis, it is important to compare corpse numbers with the total number of germ cells. This can be achieved using fluorescent reporters such as mCherry-tagged histone H2B (i.e., strain OD141) that mark all nuclei in the germline. Alternatively, germlines can be fixed and stained with DAPI and the nuclei quantified. In the case of somatic apoptosis, the invariant lineage of *C. elegans* makes it possible to visualize extra (undead) cells in vivo without the use of fluorescent markers, but this usually takes a very patient and seasoned pro.

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RELATED TECHNIQUES

Visualizing germline apoptosis can be enhanced through the use of strains that express fluorescent apoptosis markers. Many markers of apoptosis routinely used in cultured cells, such as terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL), AO staining, and immunohistochemistry to a growing list of apoptotic proteins, can also be applied to *C. elegans*.

RECIPE

M9 Buffer for Worms

1. Dissolve 3 g of KH_2PO_4, 6 g of Na_2HPO_4, and 5 g of NaCl in 1 L of H_2O.
2. Autoclave for 20 min.
3. Add 1 mL of 1 M $MgSO_4$.
4. Store at room temperature. After 2 wk, check for visible contamination before use.

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