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Monitoring the Clearance of Apoptotic and Necrotic Cells in the Nematode *Caenorhabditis elegans*

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Abstract

The nematode *Caenorhabditis elegans* is an excellent model organism for studying the mechanisms controlling cell death, including apoptosis, a cell suicide event, and necrosis, pathological cell deaths caused by environmental insults or genetic alterations. *C. elegans* has also been established as a model for understanding how dying cells are cleared from animal bodies. In particular, the transparent nature of worm bodies and eggshells make *C. elegans* particularly amenable for live-cell microscopy. Here we describe methods for identifying apoptotic and necrotic cells in living *C. elegans* embryos, larvae, and adults and for monitoring their clearance during development. We further discuss specific methods to distinguish engulfed from unengulfed apoptotic cells, and methods to monitor cellular and molecular events occurring during phagosome maturation. These methods are based on Differential Interference Contrast (DIC) microscopy or fluorescence microscopy using GFP-based reporters.

Keywords

C. elegans; Apoptosis; Necrosis; Programmed cell death; Engulfment; Phagosome maturation; CED-1; DYN-1; PtdIns(3)P; RAB-2; RAB-5; RAB-7; HGRS-1; CTNS-1; Early endosomes; Lysosomes; Time-lapse recording; GFP; mRFP; mCherry; Differential interference contrast microscope (DIC); Deconvolution; ImageJ

1 Introduction

During an animal's development and adult life, a large number of unwanted cells are eliminated by apoptosis, a morphologically distinct type of cell death that is marked by cytoplasm shrinkage, chromatin condensation, nuclear DNA fragmentation, and the well-maintained plasma membrane integrity. Apoptotic cells are rapidly engulfed (via phagocytosis) by phagocytes (engulfing cells) within animal bodies and are degraded inside phagocytes, in membrane-bound structures referred to as “phagosomes” (Fig. 1) [1]. Apoptosis plays important roles in sculpting structures, maintaining homeostasis, and eliminating abnormal, nonfunctional, or harmful cells [2]. Efficient removal of dying cells is the necessary last step of apoptosis; in addition, it actively prevents harmful inflammatory and autoimmune responses [1, 3].

Necrosis is another type of death that is morphologically distinct from apoptosis. Necrotic cells display cell and organelle swelling, excessive intracellular membranes, and eventual

rupture of intracellular and plasma membranes (reviewed in ref. 4, 5). Necrosis is most frequently observed during cell injury, and is closely associated with stroke, neurodegeneration, heart diseases, diabetes, inflammatory diseases, and cancer [6–11]. Although historically necrosis was considered an uncontrolled cell death event caused by damage, recent discoveries made in multiple organisms demonstrated that cells possess genetic pathways that specifically trigger necrosis in response to extracellular or intracellular stimuli (reviewed in ref. 12–15). Like apoptotic cells, necrotic cells are also engulfed and degraded by phagocytes [16]. Efficient clearance of necrotic cells from animal bodies helps to resolve the wounded area; furthermore, it is also essential for reducing harmful inflammatory and autoimmune responses induced by contents of necrotic cells [16, 17].

1.1 Methods for Detecting Distinct Features of Apoptotic and Necrotic Cells in *C. elegans*

The nematode *C. elegans*, a small free-living round worm, has been established as an excellent model organism for studying the mechanisms of apoptosis and the removal of apoptotic cells due to its simple anatomy, known cell lineage, well-established genetics, and easily distinguishable apoptotic cell morphology [18, 19]. During the development of a wild-type *C. elegans* hermaphrodite, 131 somatic cells and approximately 300–500 germ cells undergo apoptosis [20–22]. In the soma, due to the fixed cell lineage, both the identity of the cells that undergo apoptosis and the timing of death are invariable in *C. elegans* [20, 21]. Apoptotic cells are rapidly engulfed and degraded by neighboring cells, many of which are sister cells of the apoptotic cells during embryogenesis [20–22]. Multiple types of cells can function as engulfing cells, including hypodermal cells, gonadal sheath cells, intestinal cells, and pharyngeal muscle cells [20–23]. One particularly useful feature of *C. elegans* is that animals at all developmental stages are transparent. Apoptotic cells are thus easily recognized within living animals under the Nomarski Differential Interference Contrast (DIC) optics as highly refractive, button-like objects that are referred to as “cell corpses” (Fig. 2a) [20–22]. DIC microscopy is thus commonly used to detect cell corpses in *C. elegans* (reviewed in ref. 19, 24). DIC microscopy, however, is unable to distinguish engulfed cell corpses from unengulfed ones because the plasma membrane of an engulfing cell is typically not visible under DIC microscope.

In *C. elegans*, a number of mutations in ion channel subunits, acetylcholine receptor subunits, and trimeric GTPases induce necrosis-like cell death that mimics the excitotoxic necrosis occurring during stroke, trauma, and neurodegenerative disorders in humans [6, 8, 15]. Necrosis-like cell deaths induced by dominant mutations of *mec-4* and *deg-3*, as well as mutations in a number of other genes, are used for studying the mechanism of excitotoxic cell death and the clearance of necrotic cells [15]. *mec-4* encodes a core subunit of a multimeric, mechanically gated Na^+ channel specifically expressed in six touch receptor neurons (AVM, PVM, ALML/R, and PLML/R) required to sense gentle mechanical stimuli along the body wall [25, 26]. Dominant, gain-of-function mutations in *mec-4* lead to hyperactive channel conductivity of Na^+ and Ca^{2+} and induce the necrosis of the six touch receptor neurons [25, 27]. *deg-3* encodes a subunit of an acetylcholine receptor ion channel [28]. A gain-of-function mutation in *deg-3* causes the necrosis of the six touch receptor neurons mentioned above and a few additional sensory and inter-neurons through hyper-activation of the acetylcholine receptor ion channel [28]. Cells undergoing necrosis in *mec-4*

and *deg-3* dominant mutants display the same distinct morphology (Fig. 2a, b). In *mec-4(dm)* mutants, during necrosis, the six dying neurons swell to many times their original size and develop cytoplasmic vacuoles and large membranous whorls, and are easily distinguishable from living cells and from apoptotic cells under DIC optics by their giant sizes (Fig. 2a) [29]. This type of cell death is independent of the CED-3 caspase pathway, and is instead triggered by the influx of Ca^{2+} into cytoplasm [27, 30]. Interestingly, despite the different death-triggering mechanism, the seven *ced* genes needed for the engulfment of apoptotic cells are also needed for the removal of necrotic cells [31], indicating that the recognition and engulfment of apoptotic and necrotic cells might share certain common mechanisms.

Besides DIC microscopy, a number of methods have been used to recognize apoptotic cells at all developmental stages in *C. elegans* based on their distinct cellular features. These include the transmission electron microscopy (TEM) for detecting cell corpses in larvae and adults, which achieves the highest resolution but is technically demanding and most time consuming, the TUNEL (terminal transferase d UTP nick end labeling) assay that detects DNA ends generated during apoptosis in embryos, and the detection of phagosomes containing dying cells in larvae and adults with SYTO dyes that stain acidic compartments, including phagosomes, inside cells (reviewed in ref. 32). Recently, several methods have been developed to detect the exposure of phosphatidylserine (PS), a membrane phospholipid kept in the inner leaflet of the plasma membrane of living cells, on the outer surface of *C. elegans* apoptotic cells using PS-binding proteins, such as MFG-E8 and annexin V, as reporters [33–35]. A chromatin-associated histone H3 reporter (HIS-72::GFP) [36], which allows us to detect the distinct condensed chromatin morphology in apoptotic cells in *C. elegans* embryos, is another cell corpse-specific marker [37]. These methods, which are based on fluorescence microscopy, are relatively easy to establish in the laboratory and take relatively short time to perform. However, they are not sufficient for monitoring the clearance status of apoptotic cells inside animal bodies because in order to accomplish this task, the engulfing cell surfaces need to be monitored.

In recent years, we have developed a series of GFP- and mRFP (or mCherry)-based reporters that label the surfaces of extending pseudopods and maturing phagosomes. These reporters offer an important strategy for distinguishing unengulfed versus engulfed but undegraded cell corpses in embryos and adult hermaphrodite gonads (Fig. 3). Furthermore, using these reporters and the DeltaVision Deconvolution Imaging System, we have established time-lapse recording protocols that enable us to quantitatively monitor the processes of engulfment as well as degradation of individual apoptotic cells in developing embryos [37–42]. These novel protocols allow us to characterize many signal transduction and membrane trafficking events occurring on the surfaces of pseudopods and phagosomes, the results of which have provided important molecular basis for understanding apoptotic cell removal [37–42]. We have listed the frequently used reporters and described the specific molecular events each reporter is suitable for monitoring (Fig. 4).

1.2 The Strategy to Determine Whether an Apoptotic Cell Is Engulfed

Previously, the only method to distinguish whether an apoptotic cell was engulfed or not was transmission electron microscopy, which required facility and expertise in electron microscopy, and was hard to be applied to real-time recording [23, 43, 44]. We established a strategy for determining whether a dying cell is engulfed using fluorescence microscopy [39, 41]. This strategy utilizes a GFP::RAB-7 reporter, which is expressed in engulfing but not dying cells from the *P_{ced-1} gfp::rab-7* transgene and is evenly distributed in the cytoplasm of engulfing cells [39]. In this background, an unengulfed cell corpse is not visible by GFP yet can be detected by its distinct DIC morphology (Fig. 3a). Once a cell corpse is engulfed, GFP::RAB-7 is recruited to the surface of the nascent phagosome and remains associated with the phagosome until its complete degradation (Fig. 3a, b) [39]. Even in a mutant in which the enrichment of GFP::RAB-7 to phagosomal surface is blocked, a phagosome that contains a cell corpse would appear as a dark sphere in a background of GFP⁺ cytoplasm of the host cell (Fig. 3a) [39, 41]. In wild-type 1.5-fold stage embryos, 91 % of cell corpses detected under DIC optics were labeled with a high level of GFP::RAB-7 [39], indicating that they were internalized inside phagosomes. Likewise, in the gonads of wild-type hermaphrodites, almost all of the germ cell corpses distinguished under DIC optics (mean = 4.7) were inside phagosomes labeled with GFP::RAB-7 on their surfaces (Fig. 3c, d) [41]. In *unc-108(n3263)* mutant animals, which bear a point mutation in the small GTPase RAB-2 and are defective in the degradation of cell corpses [37], 96 % of the germ cell corpses (mean = 14.1) were inside bright GFP::RAB-7(+) circles, indicating that they reside in phagosomes and that the recruitment of RAB-7 to phagosomes was normal (Fig. 3d) [41]. In contrast, in mutants with strong defects in the engulfment such as *ced-1(e1735)* or *ced-5(n1812)*, most (89 % and 88 %, respectively) of the large numbers (mean = 62.5 and 38.8, respectively) of DIC(+) germ cell corpses were neither surrounded by GFP::RAB-7 circles nor inside dark GFP(−) holes, indicating that they were not engulfed by gonadal sheath cells (Fig. 3c, d) [41]. These results have validated the effectiveness of using GFP::RAB-7 as a reporter to determine whether a persistent cell corpse is engulfed [41].

1.3 Time-Lapse Recording of the Engulfment and Degradation of Apoptotic Cells

1.3.1 Pseudopod Marker—CED-1 is a single-pass transmembrane protein expressed in engulfing cells and acts on cell surfaces as a phagocytic receptor for neighboring apoptotic cells [23]. CED-1 recognizes the cell-surface features of cell corpses, and clusters on the phagocytic cups and then transiently on nascent phagosomes (Fig. 4a) [23, 39, 41]. This feature enables a CED-1::GFP reporter to label the extending pseudopods throughout the entire engulfment process (Fig. 4a, d). In addition, CED-1::GFP is particularly useful for detecting partially engulfed cell corpses in engulfment-defective mutants (except the *ced-7* mutants), because the blockage or delay of pseudopod extension around cell corpses do not affect the ability of CED-1 to recognize cell corpses and cluster on phagocytic cups [23, 33]. As a consequence, in these mutants, CED-1::GFP is observed as bright, distinct partial circles around cell corpses, which represent not-enclosed phagocytic cups [23, 33].

In wild-type *C. elegans* embryos, the clustering of CED-1::GFP around a cell corpse is detectable throughout the entire engulfment process (~5 min) and the first 9 min of phagosome maturation, which lasts 50–70 min in total (Fig. 4a) [39]. As a result, at any

given moment, only a small portion of cell corpses are labeled by CED-1::GFP in animals that display normal engulfment activity.

1.3.2 Phagosomal Markers—We have developed a number of GFP- or mRFP-tagged markers that have the ability to associate with phagosomal surfaces. These include DYN-1, the large GTPase that mediates the signal from CED-1 to initiate phagosome maturation, RAB-5, RAB-2, and RAB-7, three small RAB GTPases that act as membrane tethering factors to facilitate the fusion between distinct intracellular organelles with phagosomes, 2×FYVE::GFP, a reporter for phosphatidylinositol-3-phosphate (PI3P), a phosphoinositide species that is specifically enriched on the surface of endosomes and phagosomes and that acts to initiate phagosome maturation, and HGRS-1 and CTNS-1, specific markers for early endosomes and lysosomes, respectively, which are recruited to phagosomal surfaces and fuse to phagosomal membranes (Fig. 4d and the reference therein). Each of these markers allows the time-lapse monitoring of a specific subcellular event that promotes phagosome maturation. Most of these markers are enriched on the surfaces of phagosomes transiently with different lengths of duration (Fig. 4c). To follow a phagosome throughout the maturation process, GFP::RAB-7 is the most suitable marker because it starts to be enriched on phagosomal surfaces within 5 min of nascent phagosome formation, and remains on the phagosomal surface until the complete degradation of the cell corpse inside (Fig. 4b). We found that in wild-type embryos, the disk-like DIC morphology of a cell corpse appears when engulfment starts, and disappears ~30 min after the initiation of cell-corpse degradation (N. L. and Z. Z., unpublished observation). Comparing to the DIC morphology, GFP::RAB-7 thus is a much reliable marker for following the entire removal process of engulfed cell corpses.

1.3.3 The Choice of Apoptotic and Engulfing Cells to Monitor During Time-Lapse Recording—Among the 113 cells that undergo apoptosis during embryogenesis [21], we choose to monitor the clearance of three apoptotic cells referred to as C1, C2, and C3 (Fig. 3b). These three cells are located at the ventral surface of an embryo, in approximately the same or adjacent focal planes, and are engulfed at approximately the same time, between 320 and 330 min post-first cleavage (the first embryonic cell division) [38]. C1, C2, and C3 are each engulfed by a different ventral hypodermal cell, ABplapppa, ABpraapppa, and ABplaapppp, respectively, while these hypodermal cells extend their cell bodies to the ventral midline (Fig. 3b) [38]. These temporal and spatial features make it easy to identify C1, C2, C3 and their engulfing cells; furthermore, they allow the recording of the clearance of all three cell corpses in the same time-lapse series.

1.4 A Strategy to Identify Necrotic Touch Neurons with the Aid of Both a Touch Neuron Reporter and the Distinct DIC Morphology

To facilitate the characterization of necrotic-cell removal during development, we have developed P_{mec-7} *mCherry*, an mCherry reporter specifically expressed in touch neurons under the control of the *mec-7* promoter, a touch neuron-specific promoter (Fig. 2c) [45]. A combination of the distinct DIC morphology displayed by cells undergoing necrosis (Fig. 2a) and the presence and disappearance of mCherry signal allows us to reliably determine the time points when necrosis occurs and when a necrotic cell is removed (Fig. 2c).

2 Materials

The materials and methods described here are specific for the detection of apoptotic and necrotic cells in *C. elegans*. For general materials and methods for raising and handling *C. elegans*, please see ref. 46. For general introduction of using DIC microscopy in *C. elegans*, please see ref. 47.

2.1 General

1. 4 % agarose solution, prepared by heating 2 g agarose in 50 mL autoclaved deionized water until agarose is completely melted. After usage, the solidified solution can be stored at room temperature and melted in a microwave oven again.
2. M9 Buffer (1 L): 3 g KH_2PO_4 , 6 g Na_2HPO_4 , 5 g NaCl, 1 mL 1 M MgSO_4 dissolved in 850 mL H_2O , add H_2O to 1 L, autoclave.
3. 30 mM sodium azide (NaN_3) in M9 buffer.
4. Microscope slides, coverslips (22×22 mm), Pasteur pipette and bulb, high vacuum grease (Dow Corning), DeltaVision immersion oil $N = 1.514$ (Applied Precision), handmade worm pick, which is a platinum wire mounted on a Pasteur pipette.

2.2 Equipment and Software

1. Nikon SMZ645 Stereomicroscope or any stereomicroscope from other manufacturers for handling of *C. elegans*.
2. An Olympus IX70-DeltaVision microscope (Applied Precision) equipped with 20 $\times$, 63 $\times$, and 100 $\times$ Uplan Apo objectives, fluorescence light source and excitation and emission filter sets, DIC microscopy accessories, motorized stage (X, Y, and Z axis), a Coolsnap HQ2 digital camera (Photometrics), and the SoftWoRx 4.0 software (for the deconvolution and processing of images) (Applied Precision). For fluorescence imaging, two sets of fluorescence filters, both from Chroma Inc., are used, including the GFP filter (excitation wavelength 475/28 nm; emission wavelength 525/50 nm) for the GFP signal and the mCherry filter (excitation wavelength 575/25 nm; emission wavelength 632/60 nm) for the mCherry signal.
3. A temperature control chamber mounted over the DeltaVision microscope that maintains the temperature of the stage at 20 °C. Alternatively, the DeltaVision microscope can be kept in a room where the temperature is maintained at 20 °C.
4. A PC computer for image processing and analysis.
5. The ImageJ software (downloaded from (<http://rsb.info.nih.gov/ij/index.html>)) for quantitative image analyses.

2.3 *C. elegans* Strains

- a. The CED-1: :GFP marker

Strain ZH231, which carries an integrated reporter gene $P_{ced-1}::gfp$.
Genotype: *enIs7* [$P_{ced-1}::gfp$] [33].

b. The GFP: *rab-7* marker

Strain name: ZH1112. Genotype: *unc-76(e911); enEx478 [pUNC-76 (+) and P_{ced-1} gfp: *rab-7*] [39]. This strain carries the reporter construct P_{ced-1} gfp: *rab-7* as well as pUNC-76(+), a plasmid containing the wild-type *unc-76* gene, in the same transgenic array. Transgenic animals are normal for locomotion, whereas non-transgenic animals are Unc (Uncoordinated). To cross the transgenic array to the strains of your interest, follow standard genetic operation [46].*

3 Methods

3.1 Using DIC Microscopy to Score the Number of Apoptotic Cell Corpses

3.1.1 Scoring at Different Developmental Stages—Apoptotic cell corpses can be recognized as small, reflective, disk-like objects in living animals using DIC microscopy (Fig. 2a). To access the clearance of apoptotic cells, which is a dynamic process, it is critical to score the number of cell corpses at defined developmental stages and within the defined regions of an animal for meaningful comparison of results obtained from different genetic backgrounds.

A commonly used assay to quantify the phenotypes of the mutants defective in cell-corpse removal is to count the number of persistent cell corpses in the head (the area between the anterior end of the worm and the anterior boundary of the intestine) of a newly hatched L1 larva (Fig. 2a). In the head of newly hatched L1 larvae (hatched within 1 h) with normal cell-corpse removal activities, no cell corpses are observed (Fig. 2b); on the other hand, mutations that block cell-corpse removal result in the persistent presence of cell corpses, most of which are generated during mid-embryogenesis, in the head (Fig. 2a) [19, 48].

The assay described above, which relies on the persistent presence of embryonic cell corpses for at least 6 h, is suitable for detecting strong but not weak removal defects. In mutants in which cell-corpse removal is slowed down but not blocked, often only one to two cell corpses are detectable at young L1 stage in the head [41]. A more sensitive and comprehensive assay for inefficient removal is to score the number of cell corpses in the entire embryo and at different stages. The stages that we score at are: bean, comma, 1.5-fold, twofold, threefold, and early and late fourfold stages. Embryos at these stages, which correspond to ~320, ~380, ~420, ~460, ~520 to ~605, and ~700 to ~790 min after the first cleavage (the first cytokinesis), respectively, are easily recognizable using DIC microscopy by their distinct body morphology [21, 38, 41].

Germ cells that undergo apoptosis during germ line development or are induced to die by DNA damaging agents can be scored in the adult hermaphrodite gonad using DIC microscopy [22, 41, 42, 49]. Again, to obtain reproducible results, it is critical to score in animals of defined age. The most commonly used samples are adult hermaphrodites that are aged 48 h post the mid-L4 larval stage.

3.1.2 Mounting Animals on an Agar Pad

1. Melt the 4 % agarose solution by heating it in a microwave oven.

2. Dispense a drop of agarose solution on a glass microscope slide and flatten the drop immediately with another glass slide. Wait until agarose solidifies, then gently separate the two slides by sliding one against the other. An agarose pad provides support to the coverslip so that the living specimens are not over-squashed.
3. Cut the round agarose pad into an approximately 12 × 12 mm square with the edge of a glass slide. Place 3 µL of 30 mM NaN₃ in M9 buffer at the center of the pad (*see* Note 1).
4. Under the Nikon SMZ 645 Stereomicroscope, transfer animals at the stage of choice with a worm pick from a plate to the drop of 30 mM NaN₃ in M9 buffer, gently disperse eggs with a worm pick.
5. Gently place a coverslip over the drop of liquid. Remove any solution outside the coverslip with tissue paper.

3.1.3 Observation Under the DIC Microscope

1. Align the DIC light path carefully for optimal DIC effect according to the manufacturer's instruction (www.applied-precision.com).
2. Under the 63× or 100× objective, identify cell corpses and score the number. As *C. elegans* is transparent under the light microscopy, by focusing from the bottom to the top of the animal, cell corpses in the z-axis of the entire desired region can be scored.
3. Alternatively, instead of scoring directly from the eyepiece, serial z-section DIC images could be captured (see below for z-sectioning) and the number of cell corpses could be scored later by replaying the serial images on the computer. This method speeds up the image capturing process and avoids the long-term effect of NaN₃ in altering the DIC appearance of cell corpses (*see* Note 1).

3.2 Distinguish Necrotic Touch Neurons with the Aid of P_{mec-7} mCherry

In *mec-4(dm)* mutants, the necrosis of four of the six touch neurons occurs during embryogenesis and the other two deaths occur during L1 larval stage [29]. Under DIC optics, necrotic cells can be distinguished from living cells and apoptotic cells by their giant sizes and swelling morphology (Fig. 2a, b). To reliably evaluate the defect in removing necrotic cells in certain mutant backgrounds, it is also essential to count the number of necrotic corpses at appropriate developmental stages. In *mec-4(dm)* mutant worms, most necrotic cells are removed by the L3 larval stage [29, 31]. In mutants defective in the engulfment of necrotic cells, such as *ced-1* or *ced-7* mutants, the majority of necrotic cells last in the body until L3 or L4 larval stages [31]. In order to follow the clearance of necrotic touch neurons in different mutant backgrounds, we use a combination of P_{mec-7} mCherry, which labels the touch neurons in both the live and necrotic status, and DIC microscopy,

¹NaN₃ anesthetizes and immobilizes animals. Larvae and adults are immobilized within a few min after incubation with the 30 mM NaN₃ solution. It takes NaN₃ a much longer time to penetrate eggshells. For scoring embryos younger than the twofold stage, it is not necessary to use NaN₃, since vigorous body movement of embryos does not start until that stage. Note that after 1 h incubation in the NaN₃ solution, the DIC morphology of larvae and adults starts to become abnormal, whereas that of embryos are not affected.

which distinguishes the distinct morphology of necrotic neurons, to distinguish touch neurons throughout larval development (Fig. 2c).

1. Staging worms: Newly hatched L1 larvae are defined as L1 larvae hatched within a 1 h period. Newly hatched L1 larvae are transferred to new plates. Young L2, L3, and L4 larvae are larvae that have been staged for 15, 24, and 33 h after hatching at 20 °C.
2. Using the protocols described above, mount larvae on slides. Under 100× magnification, using the mCherry filter set to distinguish the fluorescent signal (excitation filter 575 nm, emission filter 632 nm) and identify touch neurons.
3. Capture serial z-section images of both DIC and the area of a worm that contains the touch neuron of your interest at 1.0 μm/section optic interval. Adjust the exposure time to the optimal.
4. Deconvolve the mCherry z-section images using the Softworx 4.0 software.

3.3 Time-Lapse Recording to Monitor Engulfment and Degradation of Apoptotic Cells in Real Time in Embryos

The DeltaVision Deconvolution Microscope is a white-light microscope that relies on specially designed computer deconvolution algorithm to achieve high resolution [50]. Comparing to conventional confocal microscope, the DeltaVision results in less photo-bleaching of images and less photo-damage to living specimens, and offers comparable, under some conditions even superior, resolution and sensitivity. Here we describe a specific protocol for image capture and time-lapse recording that we developed using the DeltaVision. For step-by-step operation of the DeltaVision microscope and the SoftWoRx software, see the manufacturer's instruction (www.appliedprecision.com).

3.3.1 Mounting Embryos on a Microscope Slide

1. Follow the description of Subheading 3.1.1 to prepare an agarose pad on a microscope slide. Spot 3 μL M9 buffer in the center of the pad, transfer eggs to the pad, disperse eggs in M9 buffer (*see* Note 2).
2. Gently squeeze a thin line of high vacuum grease around agarose pad and cover the pad gently with a coverslip. Avoid air bubbles. Vacuum grease prevents the drying of the agarose pad and allows air exchange. No more than 50 eggs should be loaded onto one slide, and eggs should be sufficiently dispersed in M9 solution (*see* Note 3).

3.3.2 Identifying Cell Corpses C1, C2, C3 and Their Engulfing Cells—C1, C2, and C3 are located at the ventral surface of an embryo, in approximately the same or adjacent focal planes, and are engulfed at approximately the same time, between 320 and 330 min post-first cleavage (Fig. 3b). C1, C2, and C3 are each engulfed by a different ventral hypodermal cell (Fig. 3b) [38]. After identifying the embryo at the right stage and

²Anesthetization is not necessary since at the particular embryonic stages for recording, there is minimal embryonic body movement. NaN₃ stops embryonic development and should be avoided.

focus on the ventral surface, one can easily identify C1, C2, and C3 and their engulfing cells by following the above temporal and spatial features. The clearance process of all three cell corpses can be captured in the same time-lapse series, using a z-stack containing 8–12 serial z-sections (at 0.5 μm /section) at every time point.

1. Place the prepared slide on the microscope stage, start microscope operation. During this time period, align the DIC light path for optimal DIC effect. Open the SoftWoRx program.
2. Using the GFP channel, identify embryos that carry the transgenic array. Under the 100 $\times$ objective, identify transgenic embryos whose ventral side faces the objective and which are at ~ 320 min post-first cleavage or younger (Fig. 3b) (*see* Note 4). Once an appropriate embryo is identified, its exact location on the slide should be recorded using the “point marking” function of the SoftWoRx program.

3.3.3 Time-Lapse Recording

1. Set up microscope parameters. Use the 100 $\times$ objective. For capturing DIC images, exposure time is usually set at 0.1 s. The exposure time is 0.1 s or shorter for each channel and each z-section (*see* Note 3). If the signal is weak, 2×2 binning is recommended (*see* Note 3).
2. Set up the recording program. Serial z-sectioning is performed from the ventral surface of an embryo towards the center. The setting of 8–12 z-sections at 0.5 μm /section is sufficient to include C1, C2, C3 in one z-section series (cell corpses are of 2.5–3 μm in diameter). An image size of 374×374 pixels is sufficient for capturing the entire embryo if 2×2 binning is performed (*see* Note 3). For recording the engulfment process, which lasts ~ 5 min in a wild-type strain, 10–20 time points at a 1-min interval is sufficient if recording starts at a time between 310 and 320 min post-first cleavage. For the degradation process, which lasts ~ 50 –70 min in wild-type embryos but could last much longer in degradation-defective mutants [39], we record for 100–120 min at a 2-min interval. After embryos reach

³How to ensure that embryos develop normally during time-lapse recording?

- a. To ensure normal embryonic development in a chamber with limited oxygen supply, load no more than 50 eggs onto the glass slide, disperse eggs thoroughly in the M9 solution, and carry over as little bacteria as possible.
- b. To avoid photo-damage of embryonic development and photo-bleach of fluorescence signals, use a highly sensitive CCD camera so that the light exposure time could be minimized, and restrain or avoid direct observation of fluorescent light under the eyepiece. Instead, “snapshots” with the camera should be used for finding and setting the focal plane to begin the recording. For weak fluorescence signals, use the “ 2×2 binning” function to keep the exposure time minimal. In addition, include only the necessary number of z-sections at each time point. As a rule of thumb, for recording of two channels, the exposure time of each channel should be kept below 0.2 s per z-section.
- c. Several signs can help us identify the photo-damage of embryonic development. Data obtained from those embryos whose development is arrested due to photo-damage are not useful. We rely on a few embryonic morphology changes to determine whether the development is proceeding in the normal time course. For example, the period from the bean- to the comma-stage, lasts 60–70 min. During this period, an embryo rotates 90°. In addition, a period from the comma stage to the 1.5-fold stage lasts ~ 40 min. A significant elongation of any of these time intervals is a sign of developmental arrest.

⁴Two distinct features that can help identify embryos at this stage are as follows: (1) the ventral surface slightly invaginates on both sides, and (2) the three soon-to-be engulfing cells are located at the lateral sides, in a wedge shape, with the tip of each cell less than halfway extended towards the ventral midline (Fig. 3b).

~460 min post-first cleavage, rapid body movement starts, which interferes with image recording.

3. Using the “point marking” and “point visiting” functions of the software to record multiple embryos in the same program. Using the parameters described above, at least three embryos can be recorded in the same program in a time interval of 2 min.
4. Keep observing images from time to time. Adjust the starting focal plane during the interval of recording if any change of focal plane occurs. Abort recording if an embryo slows down or stops its development due to photo-damage (*see* Note 3).
5. After recording is completed, deconvolve images using SoftWoRx.
6. Open deconvolved files with softWoRx, open DeltaVision files using ImageJ for quantitative analysis (*see* below). Save tiff or jpg images of your choice for processing using Adobe Photoshop.

3.3.4 Measuring Signal Intensity on Phagosomal Surfaces—The dynamic changes of the signal intensity of CED-1 and RAB-7 indicate the progress of engulfment and phagosome maturation; in addition, alteration of the dynamic pattern of these signals in mutant backgrounds suggest specific defects in phagosome formation and maturation [39, 41]. The signal intensity of CED-1 and RAB-7 on phagosomal surfaces is quantified by measuring the fluorescence intensity of CED-1: :GFP and GFP: :RAB-7, respectively. The absolute fluorescence signal intensity, however, varies from embryos to embryos due to the different expression levels of the transgene. Thus, we use the relative signal intensity represented by the ratio of the intensity on phagosomal surface to that in an adjacent area inside the cytosol to indicate the enrichment of CED-1 or RAB-7 on phagosomal surfaces. We use the software ImageJ to quantify fluorescence signal intensity.

1. Open a DeltaVision image file in the ImageJ program. Increase the magnification of the image until the boundary of phagosome can be clearly distinguished.
2. Use the freehand selection tool to define a donut-like and closed area with one continuous line that surrounds the surface of a phagosome.
3. Select the Measure tool from the Analyze menu to display the modal value of the fluorescence signal intensity measured in this area (*see* Note 5).
4. Use the freehand selection tool to select an area in the engulfing cell cytosol adjacent to the phagosome. Repeat **step 3** to obtain the modal value.
5. Calculate the ratio of the values obtained from the phagosomal surface and that obtained from the cytosol.
6. Plot the ratio over time.

⁵The modal and mean values are usually very similar. An abnormally bright pixel on phagosome surface, however, is ignored in modal value, whereas it is counted and increases mean value. Therefore, the modal value is more resistant to signal noise and reflects the signal intensity more accurately.

3.3.5 Measuring the Volume of a Phagosome over Time—During phagosome maturation, the volume of a phagosome decreases as the content is gradually digested, and is a reliable index that reflects the progression of the degradation of apoptotic cells [39, 41].

1. Open image series in ImageJ. Among a z-stack of serial optical sections, identify the middle section of a phagosome in the z-axis, which represents the equator plane.
2. Set up the $\mu\text{m}/\text{pixel}$ scale (*see* Note 6) by selecting Set Scales in the Analyze menu and entering the scale for each pixel. As a reference, images obtained from the DeltaVision using the 100 $\times$ objective and subject to 2×2 binning have a scale of 0.133 $\mu\text{m}/\text{pixel}$.
3. Increase the magnification of the image until the boundary of a phagosome can be clearly distinguished. Use the freehand selection tool to draw a continuous line along the phagosome surface. Always draw along the path that has the brightest signal.
4. Select the Measure tool from Analyze menu to display the area (A) of the selected shape (the phagosome) in μm^2 .
5. Regarding a phagosome as a sphere, calculate the radius (r) of the phagosome using the formula $A = \pi r^2$. Calculate the volume of the phagosome (V) using the formula $V = 4/3 \pi r^3$.
6. Plot the phagosome volume over time.

3.4 Distinguishing Defects in Engulfment from Defects in Phagosome Maturation Using GFP::RAB-7

The principle of this assay has already been explained in Subheading 1.2. This assay can be conducted in embryos at specific developmental stages and in the gonads of adult hermaphrodites at specific stages.

1. Using the protocols described above, mount embryos or adult hermaphrodites on slides and identify embryos at the stage of your choice. 30 mM NaN_3 is used to immobilize adult worms.
2. To analyze embryos, capture serial z-section images of an entire embryo at $40 \times 0.5 \mu\text{m/s}$ optic interval (*see* Note 7). Other parameters for image recording are the same as described above except a time course for recording is not necessary. Score the number of GFP::RAB-7(+) rings and the number of GFP::RAB-7(−) dark spheres inside engulfing cells using deconvolved serial z-section images. The total of these two numbers represent the number of phagosomes that contain engulfed cell corpses. Also score the number of cell corpses that display the distinct DIC morphology but are neither labeled with a GFP::RAB-7 ring nor appear as a dark

⁶The $\mu\text{m}/\text{pixel}$ scale designates the size of each pixel in the image, which can be obtained from the program with which the image is captured.

⁷The average thickness of an embryo is 20 μm (Z. Z., unpublished observation).

sphere inside an engulfing cell. This number represents the number of unengulfed cell corpses.

3. For analyzing adult gonads, the same method is used except that $40 \times 1.0 \mu\text{m/s}$ intervals are used to capture a z-stack to accommodate the depth of an adult hermaphrodite.

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Fig. 1.

A diagram illustrating the process that removes apoptotic and necrotic cells. *C. elegans* necrotic cells are much bigger than apoptotic cells. For the sake of illustration, the “dying cell” being engulfed is drawn to resemble an apoptotic cell. Note that necrotic cells do not undergo any shrinkage process before being engulfed

Fig. 2.

Using Differential Interference Contrast (DIC) microscopy to detect apoptotic and necrotic cells. (a) and (b) DIC images of L1 larvae. Scale bars: 10 μ m. (a) Head (top) and tail (bottom) of an L1 larva of the *ced-1(e1735); mec-4(dm1611)* genotype showing apoptotic cells (arrowheads) and one necrotic cell (arrow). (b) One L1 larva of the *deg-3(u662)* genotype showing three necrotic cells (arrows). Two are in the head region and the other is in the tail. (c) Monitoring necrotic cells during larval development with the aid of mCherry. DIC (a–d, i–l), mCherry (m–p), and merged (e–h) images of the tails of wild-type (a–h) and *mec-4(dm1611)* (i–p) larvae at different stages (larval developmental stages as labeled) expressing P_{mec-7} mCherry. Arrows indicate live touch cells. In the tail of a larva, usually only one of the two touch neurons is visible within one focal plane. (b, f) showed an

exception, in which both touch neurons (*arrows*) are visible within one focal plane. *Arrowheads* indicate necrotic touch cells. Dorsal is to the top. Scale bars: 6 μ m

Fig. 3.

Strategy of using a phagosomal marker to distinguish engulfed from unengulfed somatic and germ cell corpses. (a) Diagram illustrating GFP::RAB-7 ($P_{ced-1} gfp::rab-7$) as a reporter for distinguishing phagosomes and monitoring the degradation of a cell corpse and the recruitment of RAB-7 to phagosomal surfaces. Adapted from ref. 41. (b) Fluorescence (a) and DIC (b) images of a wild-type embryo expressing $P_{ced-1} gfp::rab-7$ at ~330 min-postfirst embryonic division. Phagosomes C1, C2, and C3 are labeled with enriched GFP::RAB-7 (arrows). The boundary of three ventral hypodermal cells (identities labeled) that have engulfed apoptotic cells C1, C2, and C3 are traced with lines. Anterior is to the top. Ventral faces readers. Scale bars: 10 μ m. Adapted from ref. 40. (c) Diagrams of an adult *C. elegans* hermaphrodite (left) and a cross section of the distal gonad arm (right) illustrating the strategy using GFP::RAB-7 expressed in gonadal sheath cells under the control of P_{ced-1} to distinguish engulfed from unengulfed germ cell corpses. The dashed line indicates a microscopic focal plane, under which the images in (d) were captured. GFP::RAB-7 is evenly distributed in sheath cell cytoplasm and is recruited to phagosomal surfaces. A phagosome containing a cell corpse would either be labeled by GFP::RAB-7, or, in certain mutants, remain a GFP(-) dark hole surrounded by the GFP(+) cytoplasm of the sheath cell. An unengulfed cell corpse would not be visualized by GFP::RAB-7 yet would be visible by its DIC cell corpse morphology. Adapted from ref. 41. (d) DIC (a-d) and GFP (e-h) images of adult hermaphrodite gonads in wild-type and different mutants expressing GFP::RAB-7 in gonadal sheath cells. Worms were staged at 48 h after L4 stages. Arrows indicate GFP::RAB-7(+) phagosomes. Open arrowheads indicate unengulfed cell corpses. Dorsal is to the top. Scale bars, 20 μ m. Adapted from ref. 41

Fig. 4. Time-lapse recording of the engulfment and degradation of apoptotic cells in *C. elegans* embryos (**a**) and (**b**). Time-lapse images of the engulfment (**a**) and phagosome maturation (**b**) processes in wild-type embryos. Scale bars: 2 μ m. “0 min” is the time point that engulfment is just complete. *Filled arrows* indicate extending pseudopods, *open arrows* indicate phagosomes, and an *arrowhead* indicates the engulfing cell prior to engulfment. (**c**) The temporal order of the phagosome localization of multiple engulfment and phagosome maturation factors and the incorporation of endosomes and lysosomes. Data represent mean durations of GFP-tagged reporters localized on pseudopods or phagosomes, obtained from time-lapse imaging experiments. “0 min” represents the time point when engulfment is complete. The transition from *light* to *dark color* indicates the gradual increase of signal

intensity. **(d)** A list of GFP-tagged reporters for monitoring apoptotic-cell removal. Numbers in superscript correspond to the numbered references