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Detecting apoptosis in *Drosophila* tissues and cells

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Abstract

In this chapter we discuss methods that can be used to study apoptotic cell death in the *Drosophila* embryo, ovary, as well as in cultured cell lines. These methods assay various aspects of the cell death process, from mitochondrial changes to caspase activation and DNA cleavage. The assays are useful for examining apoptosis in normal development and in response to developmental perturbations and external stresses. These techniques include Acridine Orange staining, TUNEL, cleaved caspase staining, caspase activity assays and assays for mitochondrial fission and permeabilization.

Keywords

Drosophila; Apoptosis; TUNEL; caspase

Introduction

Programmed cell death is an essential aspect of normal development in *Drosophila*. Cells die and are eliminated in many different tissues, beginning in gametogenesis, and extending through pupal and adult life. Much of the cell death that occurs during *Drosophila* development can be classified as apoptosis, based on both morphological and molecular criteria. These include nuclear condensation and sustained cellular integrity, along with mitochondrial changes, DNA cleavage and caspase activation. The pathways important for apoptosis are highly conserved between flies and other organisms, and most of the genes critical for the execution of apoptosis have been identified and characterized in the *Drosophila* model.

Examining the occurrence and distribution of apoptosis can be important in understanding normal development and mutant phenotypes. Apoptosis and cell clearance are rapid processes, and even small changes in the level of apoptosis can alter cell numbers

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significantly over the course of hours. Here we describe some ways in which one can detect and analyze apoptosis in *Drosophila*. We focus on methods for detecting apoptosis in the embryo and ovary, as well as in *Drosophila* tissue culture cells. These methods can also be used with slight modifications on other tissues.

1. Detecting apoptosis in the *Drosophila* embryo

Apoptosis is initiated early in *Drosophila* embryogenesis, at stage 10 (Fig. 1) [1]. The pattern of apoptosis is dynamic and occurs in many tissues in a variety of cell types. In this section we describe methods for detecting apoptotic cell death in the *Drosophila* embryo. These include Acridine Orange, TUNEL, and cleaved caspase staining.

Acridine Orange staining is a rapid assay used to visualize apoptosis in living animals, while TUNEL and staining for cleaved caspases capture static snapshots of apoptosis in fixed tissue. These latter techniques can be accompanied by antibody or Fluorescent *In Situ* Hybridization (FISH) to examine protein or gene expression in the dying cells. In our experience these techniques are robust and accurately highlight several different stages of apoptosis. Although not included here, additional techniques have been developed to detect caspase activity in living tissues, based on cleavage of a fluorescent protein expressed on a transgene [2, 3]. In addition, we describe techniques for examining changes in mitochondrial morphology and permeability, which often accompany apoptosis in embryos [4]. Many of these techniques can also be applied to imaginal tissues, with only minor modifications in the initial tissue preparation.

1.1 Acridine Orange staining of embryos

A quick way to detect apoptosis in embryos is with Acridine Orange (AO) staining [5]. This method is fast, easy and ideal for screening large numbers of embryos [6]. Dying cells retain the AO dye selectively, perhaps due to large scale alterations in pH [7]. In living cells, AO staining can be seen in lysosomes and bound to RNA and DNA. However, the staining is much brighter in dying cells [1, 8, 9]. One disadvantage to this staining method is the transient nature of the staining. Visualizing the pattern of apoptosis should be done within 15 minutes of the staining, as the dye is rapidly bleached during imaging.

1.1.1 Embryo collection

- a. Embryos can be collected on molasses or apple juice plates. Short collections are preferred, as the pattern of apoptosis varies considerably from stage to stage. Matched wild type controls are essential when assessing mutant phenotypes. Cell death begins at stage 10/11 in normal embryos, and can be assessed by AO until cuticle formation.
- b. Embryos of the desired stage are dechorionated in strainers (70um nylon cell strainers, BD Falcon), by placing them into a glass petri dish that contains 40% freshly diluted bleach or sodium hypochlorite solution (Sigma-Aldrich). Dechoronation is complete in about two minutes, when the dorsal appendages are no longer attached [10]. It is critical that the embryos are not kept in bleach for longer than necessary. As soon as the embryos are dechorionated they must be

thoroughly washed with distilled water. Any residual bleach will eliminate AO staining.

1.1.2 Staining

- a. After dechoriation and thorough washing, transfer the embryos with a brush into 12×75mm disposable glass test tubes, containing 1ml heptane and 1ml of 2.5ug/ml of Acridine Orange (see 1.7). Cap the tubes (TainerTop Closures, Sigma) and shake the tubes vigorously on an Eppendorf shaker for 5 minutes at room temperature. Vigorous shaking is required to create a good emulsion.
- b. Allow the two phases of liquid to separate, then remove the lower, aqueous phase carefully, using a glass Pasture pipette. Any larvae present in the collection will also sink through the aqueous phase, and should be removed. Add a little bit of fresh heptane. At this point, the embryos should sink to the bottom of the test tube.
- c. Using a new pipette that has been rinsed with heptane, transfer the embryos onto a glass slide while avoiding too much liquid. Rinsing the pipette with heptane will prevent embryos from sticking.
- d. Once the embryos are on the slide, soak up the excess heptane carefully using the edge of a Kimwipe, without letting the embryos dry out completely.
- e. Quickly cover the embryos with halocarbon oil followed by a coverslip. AO staining can be visualized with either FITC or RITC filters on either a fluorescent scope or a confocal.

Note: AO is a hazardous substance and precautions must be taken during use and disposal.

1.2 TUNEL

TUNEL (Terminal deoxynucleotide transferase-mediated dUTP end labeling) labels apoptotic cells in which the DNA has been cleaved [11]. During apoptosis, the degradation of DNA is executed by endonucleases including DNase II and caspase activated DNase (CAD) [12, 13]. Labeled dUTPs are added to the 3'-OH groups on single or double strand DNA breaks. This marks the nuclei of apoptotic cells in fixed embryos [6]. With newer kits that utilize fluorescently tagged dUTP, TUNEL is relatively quick and easy, and can be combined with antibody staining or FISH to further characterize dying cells (Fig. 1 & 2 respectively).

1.2.1 Embryo fixation

- a. Collect and dechorionate embryos, as described above (1.1.1). Transfer dechorionated embryos into a 12×75mm disposable glass test tube containing 1ml of heptane, and add 1ml of 4% formaldehyde fix (see 1.7). Vigorously shake the embryos for 20 minutes using an Eppendorf shaker.
- b. After fixation, the embryos will be suspended at the interface, while any contaminating larvae will sink to the bottom. Using a Pasteur pipette, transfer the

embryos into a new test tube and wash them twice with fresh heptane. Remove the heptane, but do not let the embryos dry.

- c. Devitellinize the embryos by adding equal volumes of fresh heptane and methanol to the test tube, and shaking vigorously by hand for 1 minute. Let the tube sit for a minute, allowing the devitellinized embryos to sink to the bottom. Transfer these embryos into 1.5ml microfuge tubes.
- d. Rinse the embryos twice with methanol and then twice with ethanol. Embryos can be stored in 100% ethanol at -20°C for weeks. However, TUNEL staining works best when done on freshly collected embryos.

1.2.2 TUNEL Reaction

- a. Rehydrate embryos through an ethanol series. Rinse the embryos once with ethanol, and then wash 5 minutes each with 3:1, 1:1, and 1:3 mixtures of ethanol:PBS. Finally, rinse the embryos twice with PBS-Tx (PBS with 0.1% Triton X-100).
- b. Move the embryos to small 0.5ml tubes. Do not overcrowd the tubes; embryos should fill less than half of the tube.
- c. Both TMR red and Fluorescein labeling *In situ* Cell Death Detection Kits (Roche) give strong signals. Prepare the appropriate TUNEL reaction mixture (10ul Enzyme Solution and 90ul of Label Solution per sample) immediately before use and keep the solution on ice. Remove the last PBS-Tx wash and add the TUNEL reaction mixture. Rotate the samples overnight at 4°C in the dark.
- d. Wash 8 times for 15 minutes each in PBS-Tx. Mount in Fluoromount-G (Southern Biotech).

1.3 TUNEL and antibody double labeling

- a. After rehydration (1.2.2.a), block by incubating the embryos at room temperature with constant rotation for 30 minutes in blocking solution (1% BSA diluted in PBS-Tx).
- b. Dilute desired primary antibody in blocking solution and incubate the embryos at 4°C overnight with constant rotation.
- c. Wash the embryos several times with PBS-Tx and transfer the embryos into a 0.5ml tube.
- d. Prepare secondary antibodies by diluting them into the appropriate TUNEL reaction mixture, as described above (1.2.2.c).
- e. After adding the reaction mixture with the secondary antibodies to the embryos, incubate samples in the dark at 4°C overnight with constant mixing.
- f. The following day, wash samples several times for 25 minutes with PBS-Tx and mount in desired mounting media.

1.4 TUNEL with RNA Fluorescent *In Situ* Hybridization (FISH)

Combined RNA FISH and TUNEL can be useful in examining cell death gene transcription in dying cells (Fig. 2). It can also be useful to identify the cell types that are dying, examining expression of cell identity markers, or to look at the expression of various genes in dying cells. Transcript analysis is often more robust than protein expression analysis in dying cells [14]. RNA expression is visualized with labeled anti-sense RNA probes. The signal of the probe is then amplified via fluorochrome-conjugated tyramide reaction [15]. This protocol provides our modifications and optimizations of FISH [16, 17].

1.4.1 RNA Probe preparation—Various methods can be used to synthesize antisense RNA probes from template DNA. DIG and biotin RNA labeling kits are available by Roche Applied Science (for more details see reference [17]). In our experience DIG labeled probes work best when visualizing expression in embryos.

1.4.2 Pre-hybridization

- a. Embryos are collected, aged and fixed as described above (1.2.1), and left in 100% ethanol overnight.
- b. Rehydrate embryos by performing the following washes: once in ethanol, once in 1:1 ethanol:PBT, and twice in PBT-20 (PBS with 0.1% Tween20).

*Note: RNase-free solutions should be used throughout the entire *in situ protocol*.

- c. Fix the embryos for 20 minutes in 4% formaldehyde (diluted in PBT-20) with constant mixing /rotating. This will be the second time the embryos are being fixed. After fixation, wash the embryos four times with PBT-20 for 2 minutes each.
- d. Add a working solution of proteinase K (3ug/ml see 1.7) to the samples and incubate at room temperature for 15 minutes. Then, move the embryos onto ice for 45 minutes. This incubation time may vary for different tissues.

*Note: This proteinase K step is critical. Under-digestion can prevent even probe distribution, while over-digestion can disrupt tissue integrity. Prevent agitation of the embryos while they are being treated with proteinase K.

- e. Wash the embryos four times quickly with PBT-20, and post-fix the embryos again for 20 minutes in 4% formaldehyde followed by three standard washes of 5 minutes each with PBT-20.
- f. Rinse the embryos with 1:1 mixture of PBT-20:pre-hybridization buffer (see 1.7) and wash once with 100% pre-hybridization buffer. At this stage the embryos can be stored in -20°C for weeks. Large batches of embryos can be processed up to this point, after which they can be separated for using different probes.

1.4.3 Hybridization

- a. Block the embryos in hybridization buffer. Heat the hybridization buffer at 80°C for 3 minutes and cool on ice for at least 5 minutes. Add enough denatured buffer

to the samples so that they remain properly submerged. Incubate at 56°C for 2 hours.

- b. The probe should be diluted in hybridization buffer with a concentration of 50-100ng in a total volume of 500ul. Denature the probe by heating at 80°C for 3 minutes and cooling on ice for at least 5 minutes. Remove the buffer from the embryos and add the hybridization buffer with the probe (probe solution). Incubate embryos overnight or 12 to 16 hours at 56°C.
- c. Following hybridization, stringent washes are important to remove excessive probe and reduce non-specific background. All washing solutions should be pre-heated to 56°C. Remove probe solution* and wash the embryos twice with pre-warmed pre-hybridization buffer. Then incubate the embryos at 56°C for 15 minutes.

*Note: Ideally fresh probe solution should be used each time. However, it is possible to store the hybridization buffer with probe at -20°C and use it more than once. Before each use, denature the probe solution, as described above.

- d. Wash the embryos for 15 minutes each at 56°C with the following mixtures of pre-hybridization buffer with PBT-20: 3:1, 1:1 and 1:3. Then wash in PBT-20 for 3 times for 5 minutes each at room temperature.

1.4.4 Developing the signal

- a. Block embryos for 30 minutes with constant mixing in PBTB (1XPBS + 0.1% Tween-20 + 1% milk powder).
- b. Incubate the embryos at 4°C rotating overnight with HRP conjugated anti-Dig antibody (1:1000)(Roche Diagnostics 11207733910).
- c. Wash the embryos for 10 minutes each: six times with PBTB, once with PBT and twice with PBS for 5 minutes each.
- d. Tyramide amplification is used to boost the signal. Prepare the tyramide conjugate with the amplification buffer provided in the tyramide kit (Tyramide Signal Amplification (TSA) Plus Cyanine 3/Fluorescein System, Perkin Elmer Life Sciences) at 1/50 dilution and keep on ice. After the last PBS wash, transfer embryos into 0.5ml tubes, and add tyramide solution to cover the embryos. Incubate at room temperature for 2 hours in the dark with constant rotation or mixing.
- e. After the tyramide reaction, it is very important to wash the embryos well. Wash the embryos 8 times for 10 minutes each, rotating in the dark.
- f. Once the samples are washed thoroughly, they can be directly stained for TUNEL, using the TMR red or Fluorescein *In situ* Cell Death Detection Kit, as described above (1.2.2.c) (Fig.2).

1.5 Staining for cleaved caspase

The caspase proteases are the major downstream effectors of apoptotic death. The Dcp-1 caspase in *Drosophila*, a homologue of mammalian caspase-3, is important for

developmental apoptosis. Full length Dcp-1 does not have proteolytic activity, but when Dcp-1 is cleaved, it becomes active [18]. A recently developed antibody to cleaved Dcp-1 (Cell Signaling Technology) provides a way to visualize caspase activation in dying cells (Fig.1). It should be noted that in the past, an antibody to cleaved-Caspase-3 (raised against cleaved human Caspase-3) was commonly used as a general apoptotic marker in *Drosophila*. However, lot to lot variation in this antibody limits its usefulness. Both the cleaved Dcp-1 antibody and the cleaved Caspase-3 antibody clearly recognize apoptosis-related epitopes, as staining is eliminated in embryos lacking cell death. However, the exact protein recognized is unclear [19]. For more details, see below (2.3).

- a. Collect, dechorionate, fix and rehydrate embryos as described above (1.2). Rinse the embryos twice with PBS-Tx.
- b. Block by incubating the embryos at room temperature with constant rotation for 30 minutes in 1% BSA diluted in PBS-Tx.
- c. Dilute cleaved Dcp-1 antibody at 1:100 in blocking solution and incubate the embryos at 4°C overnight with constant rotation.
- d. Wash the embryos several times with PBS-Tx over a 30 minute period.
- e. Add the appropriate secondary antibody and incubate the samples in the dark at 4°C overnight, with constant mixing.
- f. The following day, wash samples several times for 30 minutes with PBS-Tx and mount in Fluoromount-G. Allow some time for Fluoromount-G to solidify by placing the slides in the dark at room temperature.

1.6 In vivo analysis of mitochondrial dynamics

Mitochondrial fission and fusion are altered in apoptotic cells, and changes in mitochondrial dynamics can influence the apoptotic sensitivity of cells (reviewed in [20]). Mitochondrial morphology can be assessed with a ubiquitously expressed mitochondrial localized GFP, SpSqEYFP-mito (mito-GFP) [21], in which EYFP is fused to a mitochondrial targeting sequence from Complex VIII of cytochrome C oxidase (Fig. 3). Alternatively, an antibody to the alpha subunit of ATP synthase 5A (Mitosciences MS507, available from AbCam) can be used to label mitochondria [22].

- a. Collect embryos (or discs), fix and block, as described above (1.5).
- b. Anti-GFP (Invitrogen, A-11122, 1:50) antibody can be used to visualize mito-GFP. Block and dilute the antibody in PBS with 0.3% Triton X-100 with 5% NGS (Fig. 3).
- c. Alternatively, dilute ATP synthase antibody 1:500 in PBS with 0.3% Triton X-100 with 5% NGS, and incubate overnight at 4°C.
- d. After washing as described above (1.5), block and incubate with Alexa-tagged secondary antibodies (1:200).
- e. Mitochondrial length can be quantified using the protocol of Song et al, adapted for Nikon confocal microscope [23]. In brief, the maximum projection of images from

wing discs is skeletonized. All objects in the examined field (60×4) become a single pixel wide and the number of pixels forms a single skeleton that is equal to its length. The lengths of the skeletons can then be measured for each field using Nikon Elements software.

1.7 Solutions

Acridine Orange—A 2.5ug/ml working solution of Acridine Orange diluted in PBS is made from a 10mg/ml Acridine Orange stock solution (Molecular Probes/Life Technologies). The working solution can be kept at room temperature in the dark for months, and the concentrated stock can be kept at 4°C. Acridine Orange should be handled with gloves.

Embryo Fixative—4% formaldehyde diluted in PBS from 16% EM grade stock (Polysciences). This diluted fixative can be stored for 1 month at 4°C.

Proteinase K—A stock solution of 20mg/ml Proteinase K (Sigma Aldrich) is made and kept frozen. The working solution of 3ug/ml is made in PBT-20 (1XPBS + 0.1% Tween-20).

Pre-hybridization buffer—50% formaldehyde + 25% 20X SSC diluted in RNase free water

Hybridization buffer—50% Formaldehyde + 5X SSC + 100ug/ml heparin + 100ug/ml DNA herring sperm + 0.1% Tween-20 diluted in RNase free water. Solution should be filtered through a 0.2um filter and can be stored in -20°C for months.

2. Detecting apoptosis in the *Drosophila* ovary

During *Drosophila* oogenesis, programmed cell death and engulfment of the germline occur in response to environmental and developmental signals. Multiple cell death mechanisms including apoptosis, autophagic cell death, and necrosis contribute to the elimination of the germline at distinct stages throughout oogenesis [24-26]. During mid-oogenesis, the germline which includes the nurse cells (NCs) and oocyte (O), undergoes programmed cell death in response to poor environmental conditions such as starvation, and the germline debris is subsequently engulfed by the surrounding layer of epithelial follicle cells (FCs) (Fig. 4A-D) [26-28]. In contrast, the NCs undergo a developmental programmed cell death during the late stages of oogenesis once they have fulfilled their role of supporting the developing oocyte (Fig. 4E-H'). While the mechanisms of programmed cell death during oogenesis are complex, apoptosis plays an essential role during starvation induced programmed cell death in mid-oogenesis and a minor role during the developmental programmed cell death of the nurse cells during late oogenesis [26, 29]. As in the embryo, apoptotic cell death in the ovary is characterized by the activation of caspases and DNA cleavage. Because of the large size of many cells in the ovary, cell death can also be detected by the condensation of DNA, reduced cellular volume, and blebbing of the plasma membrane [30, 31]. The objective of this section is to discuss methods to detect apoptotic events in the *Drosophila* ovary, including DAPI staining, cleaved Dcp-1 antibody staining, and TUNEL.

2.1 Ovary dissection

2.1.1 Preparation of flies for dissection—Prior to dissection, select 10 females and 10 males between 3-10 days old and transfer them to a fresh food vial containing a dollop of yeast paste. Transfer the flies to vials containing fresh yeast paste 1-2 more times such that they are on yeast paste for a total of 48 hours. Well-conditioned females will have swollen abdomens containing well-developed ovaries. If dying mid-stage egg chambers are preferred, transfer well-conditioned flies to apple juice agar starvation vials for 16-24 hours.

2.1.2 Dissection—This technique has been previously described [32-34].

- a. Anesthetize flies on CO₂ pad, and sort females from males.
- b. Using forceps (Dumont #5, Fine Science Tools), hold the female fly between the abdomen and thorax, and submerge in a glass well containing Grace's Insect media (Fisher).
- c. With a second pair of forceps, pull gently on the terminal end of the female to remove the ovaries.
- d. Using glass Pasteur pipette, transfer dissected ovaries to another glass well containing Grace's media, and discard the carcass.
- e. Once all 10 females have been dissected, use forceps to remove debris and gently tease the ovaries.
- f. Using a glass Pasteur pipette, transfer the ovary tissue in Grace's media to a 1.5ml Eppendorf tube. Keep all the tissue within the narrow section of the pipette. Allow ovaries to settle on bottom of the tube. Proceed to fixation as quickly as possible (<20 minutes after dissecting) in order to preserve cellular structures.

2.2 DAPI staining

DAPI (4',6-diamidino-2-phenylindole dichloride) is a DNA specific stain that emits blue fluorescence when excited by UV light, allowing for the visualization of DNA. DAPI staining is a simple procedure that can be used in conjunction with other fluorescent probes. For example, DAPI can be combined with an antibody against a membrane protein to clearly distinguish FCs from NCs [27]. In the *Drosophila* ovary, DAPI staining is used as a tool to visualize egg chambers throughout oogenesis and identify those that are dying during mid-oogenesis. In a healthy egg chamber, DAPI labels the polyploid NC nuclei and the surrounding layer of smaller FC nuclei (Fig. 4A). In a very early dying egg chamber, the NC nuclei begin to condense, as evidenced by brighter DAPI staining (Fig. 4B-C). As the egg chambers progress through apoptosis, the fragmentation of the NC nuclei is visible (Fig. 4C-D). Overall, DAPI staining is a quick and easy protocol that allows for the visualization of apoptotic events that occur during oogenesis.

- a. Using a plastic fine-tipped transfer pipette, remove Grace's media from the Eppendorf tube without removing any ovary tissue.
- b. Add 500ul of Grace's fix (see 2.5) and 250ul of heptane to ovary tissue (total volume = 750ul). Rotate for 20 minutes at room temperature (RT).

- c. Using a fine-tipped transfer pipette, remove Grace's fix and heptane.
- d. Rinse with PBS-Tx (PBS with 0.1% Triton X-100) twice.
- e. Wash with PBS-Tx three times for 10 minutes/wash on a rotator (a total wash time of 30 minutes).
- f. After final wash, remove PBS-Tx from ovaries.
- g. Add 1-2 drops of VectaShield Mounting Media with DAPI (Vector Laboratories) and store overnight at 4°C.
- h. Using a glass Pasteur pipette, transfer the ovary tissue in VectaShield Mounting Media with DAPI to a glass slide. Using tungsten needles (Carolina Biologicals), break apart ovarioles and spread evenly across glass slide. Then, carefully place cover slip on top and seal with nail polish. Slides can be stored at 4°C.
- i. Visualize on fluorescence or confocal microscope with UV settings.

2.3 Cleaved Dcp-1 Antibody staining

Dcp-1 is an effector caspase in *Drosophila* that is required for NC death during mid-oogenesis [35]. Until recently, there have been limited antibodies to visualize Dcp-1 activity in dying cells. Here we present an immunofluorescence technique to visualize cleaved Dcp-1 in mid-stage dying egg chambers, using a new antibody against cleaved Dcp-1 (cDcp-1) from Cell Signaling Technology (CST). Importantly, western blot analysis performed by CST has found that the antibody recognizes cleaved Dcp-1. However, the blot also contained another cell death specific band that was very prominent. Therefore, we cannot assume that the immunofluorescence that we see is Dcp-1, but we do know that it is death specific and Dcp-1-dependent in the ovary (AKT, unpublished). Using this technique we have found that healthy egg chambers do not contain any caspase activity (Fig. 4A). However, in very early dying egg chambers we see localization of epitopes recognized by the cDcp-1 antibody at the outer membrane of the NCs. We also see the formation of vesicle structures in the FCs indicating the NC material is being engulfed by the FCs very early in the death process (Fig. 4B). As the egg chambers progress through death, we see continued localization to the outer membrane of the NCs and to vesicles within the follicle cells (Fig. 4C-D). We have found this antibody to be a useful marker for dying egg chambers and for engulfment.

- a. Using a plastic fine-tipped transfer pipette, remove Grace's media from the Eppendorf tube without removing any ovary tissue.
- b. Add 500ul of Grace's fix and 250ul of heptane to ovary tissue (total volume = 750ul). Rotate for 20 minutes at room temperature (RT).
- c. Using a fine-tipped transfer pipette, remove Grace's fix and heptane.
- d. Rinse with PBS-Tx twice.
- e. Wash with PBS-Tx three times for 20 minutes/wash on a rotator (a total wash time of 60 minutes). After last wash, remove PBS-Tx from tube.
- f. Block for 1 hour at RT with PBANG (see 2.5) on rotator.

- g. Remove PBANG from tube.
- h. Dilute primary antibody, anti-Cleaved Dcp-1 (Cell Signaling Technology, Catalog #9578) 1:100 in PBANG, using a total volume >400ul. Incubate overnight at 4°C on a rotator.
- i. After overnight incubation, remove PBANG/primary antibody solution and rinse twice with PBS-Tx.
- j. Wash for 2 hours with PBS-Tx + 0.5% BSA, changing wash at least four times.
- k. Dilute secondary antibody, goat-anti-rabbit Cy3 (Jackson ImmunoResearch) 1:200 in PBANG, using a total volume of >400ul.
- l. Incubate ovaries in secondary antibody for 1 hour at RT on rotator. From this point forward, protect samples from light.
- m. Remove secondary antibody and rinse two times with PBS-Tx.
- n. Wash for 2 hours with PBS-Tx + 0.5% BSA, changing wash at least four times
- o. Rinse once with PBS.
- p. Add 1-2 drops of Vectashield Mounting Media with DAPI, store overnight at 4°C.
- q. Using a glass Pasteur pipette, transfer the ovary tissue in VectaShield Mounting Media with DAPI to a glass slide. Using tungsten needles, break apart ovarioles and spread evenly across glass slide. Then, carefully place cover slip on top and seal with nail polish. Slides can be stored at 4°C.
- r. Visualize on fluorescence microscope with UV and rhodamine filters, or confocal microscope with laser settings for DAPI and Cy3.

2.4 TUNEL staining

DNA fragmentation is a critical event during apoptosis that leads to the destruction of a cell. DNA fragmentation occurs during cell death in mid-oogenesis in response to starvation, as evidenced by visible DNA fragments when DAPI staining is performed (Fig. 4A-D). Additionally, it has been demonstrated that dying mid-stage egg chambers are TUNEL positive [36]. Although apoptotic cell death is only a minor contributor to the elimination of the nurse cells during developmental programmed cell death [29], DNA fragmentation has been observed with TUNEL staining during late oogenesis [6, 36-38]. In particular, TUNEL positive NC nuclei are present in wild-type stage 12 and 13 egg chambers (Fig. 4F'-G'). A variety of TUNEL kits are available and have been described in the literature for use on the *Drosophila* ovary. The general workflow of TUNEL staining includes fixation, permeabilization, equilibration, a labeling reaction, a stop reaction, and staining with DAPI. Most recently, we have had success with the DeadEnd Fluorometric TUNEL System (Promega). This kit was also used successfully for TUNEL staining of the germarium and mid-stage egg chambers [39]. Compared to previous published techniques [33], we found that permeabilization of the tissue with proteinase-K is too harsh and that the washes with PBS-Tx are sufficient. Furthermore, we found that this kit yields consistent results in less time. We have outlined the procedure that we used below.

- a. After dissecting the ovaries, use a plastic fine-tipped transfer pipette to remove Grace's media from the Eppendorf tube without removing any ovary tissue.
- b. Add 500ul of Grace's fix and 250ul of dH₂O to ovary tissue (total volume = 750ul). Rotate for 15 minutes at room temperature (RT).
- c. Wash with PBS-Tx three times for 10 minutes/wash on a rotator (a total wash time of 30 minutes).
- d. Wash twice for 5 minutes each in 500ul of equilibration buffer.
- e. To each sample, add 90ul of equilibration buffer, 10ul of nucleotide mix, and 2ul TdT enzyme. From this point forward, protect samples from light.
- f. Incubate in water bath for 3 hours at 37°C, protected from light.
- g. Dilute 20XSSC solution 1:10 in dH₂O to make 2XSSC solution.
- h. After 3 hour incubation, add >300ul of 2X SSC to sample for 1 minute to stop the reaction.
- i. Wash with >300ul of 2X SSC solution for 15 minutes at RT on rotator.
- j. Wash three times with PBS-Tx for 10 minutes (total wash time= 30 minutes).
- k. Add 1-2 drops of Vectashield Mounting Media with DAPI, store overnight at 4°C.
- l. Using a glass Pasteur pipette, transfer the ovary tissue in VectaShield Mounting Media with DAPI to a glass slide. Using tungsten needles, break apart ovarioles and spread evenly across glass slide. Then, carefully place cover slip on top and seal with nail polish. Slides can be stored at 4°C.
- m. Visualize on fluorescence or confocal microscope with UV and FITC settings.

2.5 Solutions

Grace's fix—375ul of Grace's media and 125ul of 16% paraformaldehyde (Electron Microscopy Sciences), opened fresh <1 week.

PBS-Tx—1X PBS with 0.1% Triton-X100

PBANG—PBS-Tx + 0.5% BSA (Fisher) + 5% Normal Goat Serum (Invitrogen)

3. In vitro cell death detection assays in *Drosophila*

Drosophila S2 cells have proven useful for assessing the role of broadly expressed genes in regulating cell death. Both overexpression and knock down strategies are easily employed in S2 cells (and other *Drosophila* cell lines). It should be noted that S2 cells are highly phagocytic, and dying cells are rapidly engulfed by their neighbors. Thus it is important that cell death be assayed at short time points after induction. Here we present several assays that are specifically useful for the analysis of cell death in cultured cells.

3.1 Quantitative assay for assessment of Caspase activity in *Drosophila* cells

Caspase activity can be detected in S2 cell lysates using a substrate conjugated to fluorescent aminomethyl coumarin. The non-cleaved peptide-coumarin is non-fluorescent, however when it is cleaved by active caspases it becomes fluorescent. This assay is modified from Zimmerman et al. [40].

- a. S2 cells are grown at 25°C in Schneider's medium. After apoptosis induction approximately 5×10^6 cells are lysed in 150ul NP-40 lysis buffer. Protein concentration is assayed in cell lysates by diluting 1:10 using the BCA protein assay kit (Pierce) according to the manufacturer's instructions [41, 42]. Incubate 20ug of cellular protein in a 50ul volume of 20uM DEVD-7-amino-4-methylcoumarin (A1086 Sigma, also available as a kit) in 20mM PIPES (pH 7.2), 100mM NaCl, 1mM EDTA, 0.1% CHAPS, 10% sucrose, and 10mM dithiothreitol, in a final volume of 50ul.
- b. After 1 hour incubation at room temperature, the cleaved substrate is detected in a fluorescence spectrophotometer. The excitation and detection wavelengths are set to 365 nm and 460 nm, respectively. Values should be normalized to the negative control.
- c. Reliable controls for this assay include omitting the lysate (negative control), inducing cell death with Actinomycin D or UV or adding active Caspase 3 (Calbiochem) (positive controls) [4], and omitting the substrate (negative control). Additionally, caspase inhibitors (e.g. 1ul of a 10mM stock of the broad-spectrum Caspase inhibitor zVAD-fmk (Calbiochem) should block detectable caspase activity.
- d. The percent apoptotic cells of each lysed cell population should be assessed for morphological changes to further confirm occurrence of apoptosis [42].

3.2 In vitro Cyt c release assays

Cyt c release in apoptotic S2 cells can be detected by a fluorescence-activated cell sorting (FACS) assay [43]. In this assay, the plasma membrane of S2 cells is permeabilized with digitonin without affecting the permeability of the mitochondrial membrane. Released Cyt c exits the permeabilized cell membrane and only remaining mitochondrial Cyt c is stained with an anti-Cyt c antibody. Thus, Cyt c staining is high when the mitochondria remain intact and is reduced when the mitochondria are permeabilized.

- a. Harvest cells (1×10^5) and permeabilize with digitonin (Fluka Biochemica 50ug/ml in PBS with 10mM KCl) for 5 min on ice. Permeabilization of the plasma membrane in over 95% of the cells should be verified in an aliquot (5%) of the cells, by Trypan blue exclusion assay (Life Technologies).
- b. Mitochondrial Cyt c is detected by immunohistochemistry. Fix cells in 4% paraformaldehyde in PBS, followed by permeabilization with 0.5% Triton X-100 in phosphate-buffered saline. Washes and incubation with antibody are done in 0.1% Tween in PBS. Cells are stained with anti-Cyt c primary antibody (Zymed, clone

7H8.2C12 1:200) followed by Alexa 488-conjugated donkey anti-mouse secondary (Molecular Probes 1:200).

- c. Analyze staining levels by FACS. Cells in the low fluorescence peak have undergone mitochondrial permeabilization. Controls containing no digitonin should result in the absence of the low fluorescence peak.

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Figure 1. Nuclear and cytoplasmic markers of apoptosis in embryonic development

Embryos are immunostained with cleaved Dcp-1 (1:100 red) and TUNEL (Fluorescein-green), as described. Note that TUNEL labels nuclear changes while cDcp1 is largely cytoplasmic (arrow). Some cells are labeled with both TUNEL and cDcp-1, but a surprising number are single labeled. Engulfed corpses retain both cDcp1 and TUNEL labeling (circle). The yolk auto fluoresces (in green and yellow) at later stages of development.

Figure 2. FISH with TUNEL to visualize reaper transcription in dying cells

Transcription of the cell death gene reaper is detected in doomed and dying cells of the ventral nerve cord in stage 14 embryos. FISH (green) detects reaper transcripts in the cytoplasm of many cells at this stage. Labeling with TUNEL (red) reveals reaper expressing cells that have cleaved DNA (white arrow), as well as reaper expressing cells that are not TUNEL positive (yellow arrow). These cells may be at early stages of apoptosis. Not all dying cells express reaper (pink arrow). (A) is a lower magnification image of a whole embryo (maximum intensity projection), while (B-B'') are higher magnification images of the indicated region, with a more limited number of optical sections.

Figure 3. Mito-GFP permits easy visualization of mitochondrial length

Wing discs from larvae carrying SpSqEYFP-mito (mito-GFP) were stained with anti-GFP and DAPI. In control, extended mitochondria are visible (A'). When the mitofusin MARF is knocked down in the disc, mitochondrial fusion is blocked and the mitochondria are much shorter.

Figure 4. Visualization of apoptosis during *Drosophila* oogenesis

A-D) Wild-type mid-stage egg chambers from starved flies are stained with DAPI to mark the DNA (blue) and cleaved Dcp-1 antibody (red). A) Each egg chamber has three cell types: the oocyte (O), the nurse cells (NC), and the follicle cells (FC). A healthy mid-stage egg chamber does not contain any cleaved Dcp-1 staining. B-D) Progressively dying mid-stage egg chambers stain positively for cleaved Dcp-1 on the outer membrane of the NCs. Staining is apparent in very early dying egg chambers B), and in vesicles within the FCs (yellow arrowhead). Egg chambers display characteristics of apoptotic cell death including fragmentation and condensation of the NC nuclei (white arrowhead). E-G, E'-G') Wild-type stage 11-14 egg chambers from well conditioned flies are stained with DAPI (blue) and TUNEL (green) (red arrowhead) to mark fragmented DNA. E-E') Stage 11 egg chamber does not contain any TUNEL positive NC nuclei. F-F') Stage 12 egg chamber contains NC nuclei with positive TUNEL staining. G-G') Stage 13 egg chamber contains TUNEL positive NC nuclei. H-H') Stage 14 egg chamber has no NC nuclei.