Detection of apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling and acridine orange in *Drosophila* embryos and adult male gonads

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In *Drosophila*, vast numbers of cells undergo apoptosis during normal development. In addition, excessive apoptosis can be induced in response to a variety of stress or injury paradigms, including DNA damage, oxidative stress, nutrient deprivation, unfolded proteins and mechanical tissue damage. Two of the most commonly used methods to label apoptotic cells in *Drosophila* are terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) for fixed tissues and acridine orange (AO) staining for live embryos or tissues. Here, we describe protocols for labeling apoptotic cells in *Drosophila* embryos and adult male gonads. Slightly modified protocols can also be applied for other *Drosophila* tissues. The AO protocol is quick, simple and allows real-time imaging of doomed cells in live tissues. However, it is difficult to combine with conventional counterstains or Ab labeling. On the other hand, this functionality is readily afforded by the TUNEL protocol, which permits the detection of apoptotic cells in fixed tissues. These staining procedures can be completed in 1–2 d.

INTRODUCTION

Apoptosis is a morphologically distinct form of active cellular suicide that serves to eliminate unwanted and potentially dangerous cells[1–8]. The defining characteristic of apoptosis is a complete change in cellular morphology. As observed by electron microscopy, the cell undergoes shrinkage, chromatin margination, membrane blebbing, nuclear condensation and segmentation, and division into apoptotic bodies that are often engulfed by neighboring cells or phagocytes[9]. A common method for examining apoptosis via DNA fragmentation is the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay. Originally described by Gavrieli, Sherman and Ben-Sasson[10], TUNEL has become one of the main methods for detecting apoptotic programmed cell death. The assay relies on the production of nicks in the DNA, which can be identified by TdT. This enzyme catalyzes a template-independent addition of dUTPs to the 3′-OH ends of double-stranded or single-stranded DNA breaks. The incorporated nucleotides form an oligomer composed of chemically modified nucleotides (e.g., digoxigenin-conjugated nucleotides) and unlabeled nucleotides in a random sequence. Labeled oligomers are subsequently detected with an immunochemical marker (e.g., an anti-digoxigenin Ab that is conjugated to a fluorophor).

Apoptosis is distinct from necrosis, which involves the loss of cell membrane integrity. However, although in recent years the TUNEL method has been improved to specifically detect DNA cleavage and chromatin condensation associated with apoptosis[11,12], there have been reports of occasional false-positive cells, including those with necrotic morphology[13]. In addition, some developmental processes involve chromatin condensation (e.g., spermatid maturation) and are also associated with a degree of transient DNA-strand fragmentation[14]. Therefore, it is important to validate TUNEL-staining results by other criteria, ideally using ultrastructural analysis for a particular cell type or tissue. For example, whole-mount immunofluorescence TUNEL staining of wild-type *Drosophila* testes reveals two types of TUNEL-positive cells: early spermatogonia (**Fig. 1a–d**) that do indeed die, and late post-meiotic elongated spermatids that undergo some degree of transient DNA fragmentation during differentiation (**Fig. 1e–h**). Co-staining with 4,6-diamidino-2-phenylindole (DAPI) reveals that the TUNEL-positive early spermatogonia degrade their DNA and die (**Fig. 1a–d**; the dying nuclei appear as a hole in the DAPI layer). By contrast, triple-immunofluorescence staining of elongating spermatids for TUNEL and differentiation markers shows that these cells are not dying, but undergo some nicking of their DNA strands when histones are replaced by protamines during spermatid maturation. A similar phenomenon has also been described in mice[14].

The TUNEL method, when used appropriately, is useful for multiple labeling[15]. However, it is relatively laborious and time consuming. Staining live tissues with the vital dye acridine orange (AO) provides a rapid alternative for visualizing apoptosis in live *Drosophila* tissues and even affords the possibility of real-time imaging using time-lapse cinematography[16]. Originally developed for the analysis of imaginal tissues[17,18], AO staining was shown to be highly selective for apoptotic cells within live *Drosophila* embryos[19] (**Fig. 2a**,**b**). Although the precise basis for this selectivity is still not fully understood, it appears that AO undergoes changes in its spectral emission properties as cellular proteins become modified during the condensation of apoptotic corpses. Importantly, AO stains both the cytoplasm and the nucleus of apoptotic cells, does not stain necrotic cells and can label apoptotic corpses inside macrophages[19]. These observations show that AO can enter and penetrate live cells readily, suggesting that altered membrane permeability is not the mechanism by which apoptotic cells are

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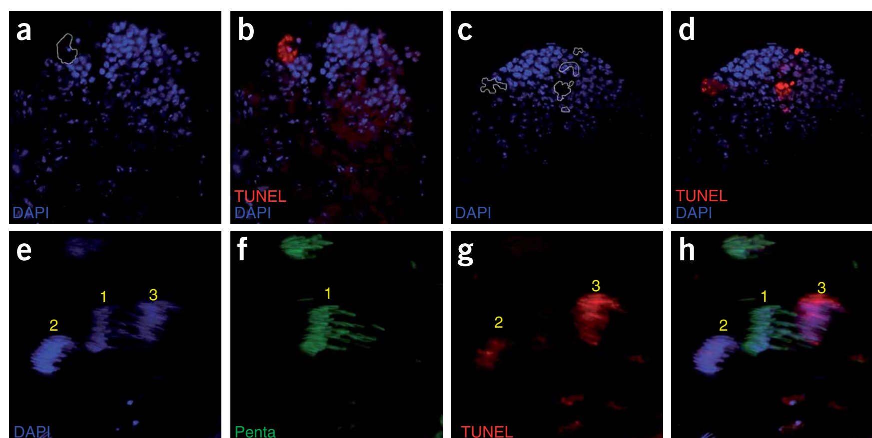

Figure 1 | TUNEL staining of *Drosophila* testes. (**a**–**d**) During pre-meiotic development, spermatogonia within the same cyst in some of the two-cell, four-cell, eight-cell and 16-cell cysts undergo synchronized death, which can be readily detected by TUNEL staining (red). The strength of the TUNEL signal is negatively correlated to the strength of the DAPI signal (blue) in the dying cells. (**e**–**h**) Triple labeling of post-meiotic elongating spermatids for DAPI (blue), TUNEL (red), which labels all 64 elongating spermatid nuclei of every cyst during the stage that correlates with histone-to-protamine transition, and hyperacetylated histone H4 (green) detected by the Penta Ab, which labels condensing nuclei prior to histone replacement with protamines[23,24]. DAPI (blue) marks all 64 nuclei within each cyst and therefore facilitates the recognition of the condensation and elongation stages of the nuclei. Notice that the TUNEL appears visible (2) in nuclei that are at a more advanced condensation stage than those in the hyperacetylated histone H4 stage (1). The TUNEL signal becomes more prominent (3) in more advanced stages. Fully matured nuclei and decondensed pronuclei in the fertilized egg are TUNEL negative (data not shown).

labeled. The ability of AO to bind to nucleic acids is well known, and enhanced nuclear staining is thought to account for its capacity to stain apoptotic cells[16,20]. However, this hypothesis is inconsistent with several other observations. In particular, AO can specifically stain advanced cystic bulges (CBs) and waste bags (WBs) in the *Drosophila* testis. As these structures only contain the extruded bulk cytoplasm — and not the nuclei of differentiating spermatids, which do not degrade their DNA — nuclear DNA cannot be the target for AO staining under these conditions (**Fig. 2c,d**)[21]. AO staining can be viewed by fluorescence or confocal microscopes using the green (522 nm) or red (568 nm) channels. The latter affords slightly 'cleaner' images due to reduced background, whereas the former offers comparatively increased sensitivity. Here we describe the detailed TUNEL-staining and AO-staining protocols used in our laboratory to analyze apoptosis in *Drosophila melanogaster* embryos and adult testes.

MATERIALS

REAGENTS

- Grape/apple juice agar plates (see REAGENT SETUP)
- Bacto-agar
- PBS (see REAGENT SETUP)
- Testis buffer (TB; see REAGENT SETUP)
- Proteinase K: 200 μg ml^{-1} stock solution (Chemicon International, cat. no. 21627) ▲ CRITICAL Before use, dilute to 10 μg ml^{-1} in PBS
- 0.1% (vol/vol) Tween-20 in PBS (PBTw)
- Formaldehyde 37 wt% solution in water ! CAUTION HCOH is highly toxic, volatile and carcinogenic, can be absorbed through the skin, and is irritating or destructive to the skin, eyes, mucous membranes and upper respiratory tract; keep away from heat, sparks and open flame
- General fixative solution: 4% (vol/vol) formaldehyde solution in PBS
- 5× BSS (see REAGENT SETUP)
- BTN (see REAGENT SETUP)
- ApopTag peroxidase *in situ* apoptosis detection kit containing the following solutions and reagents: TdT enzyme, equilibration buffer, reaction buffer and stop/wash buffer (Chemicon International, cat. no. S7100) ! CAUTION The equilibration buffer, reaction buffer and TdT enzyme contain potassium cacodylate (dimethylarsinic acid) as a buffer; these components are harmful if swallowed, so avoid contact with skin and eyes, and wash areas of contact immediately
- Anti-digoxigenin-rhodamine Fab fragments (from sheep; Roche, cat. no. 1207750)
- Hoechst 33258 (bisbenzimide; see REAGENT SETUP) ! CAUTION Hoechst 33258 might be harmful by inhalation, ingestion, or skin absorption; do not breathe the dust
- Vectashield (Vector Laboratories, cat. no. H-1000)
- Vectashield with DAPI (Vector Laboratories, cat. no. H-1200) ! CAUTION DAPI is a possible carcinogen, which might be harmful by inhalation, ingestion or skin absorption, and might also cause irritation
- Bleach: 50% (vol/vol) in water ! CAUTION NaOCl is poisonous, can be explosive and might react with organic solvents; it might be fatal by inhalation, and is also harmful by ingestion and destructive to the skin
- Heptane ! CAUTION Heptane might be harmful by inhalation, ingestion or skin absorption and is extremely flammable
- Embryo fixative: 4% (vol/vol) formaldehyde solution (in PBS):heptane (1:1)
- Methanol ! CAUTION MeOH is poisonous and can cause blindness; it might be harmful by inhalation, ingestion or skin absorption
- PBT (see REAGENT SETUP)
- 1 mM AO stock solution (Sigma, cat. no. A-6014; see REAGENT SETUP) ! CAUTION AO is a mutagen and might be harmful by inhalation, ingestion or skin absorption
- Halocarbon 700 oil (Halocarbon Products)

EQUIPMENT

- Dissecting microscope
- Fluorescent or confocal microscope
- Pyrex Spot Plate, nine depressions, 85× 100 MM (Corning Incorporated) for male gonad staining
- Orbital rotator for embryo staining
- Embryo-collection cage (Genesee Scientific, cat. no. 59-101)
- 100× 15 mm polystyrene plates (Fisher Brand, cat. no. 08-757-12)
- Baskets for embryo collection (40 μm nylon cell strainer; BD Falcon, cat. no. 352340)
- Small fine-haired, artist's paintbrush
- Squirt bottle containing deionized H_2O
- Nail polish

REAGENT SETUP

Grape/apple juice agar plates Autoclave 30 g bacto-agar in 700 ml deionized H_2O for 40 min. Add 20 ml of 95% (vol/vol) ethanol to a separate vial with 0.5 g methyl paraben (*p*-hydroxymethylbenzoate), and add this solution to a container with 300 ml juice concentrate (grape, apple or other). Quickly and thoroughly mix the concentrate into the autoclaved solution and pour immediately. This 1-l mixture is enough to pour 20–30 100× 15 mm plates. Once the juice plates have cooled and hardened, store at 4 °C. Before setting up a cage, add a small

dollop of thick yeast paste to the middle of the plate (mix 1 g dry yeast and 1.3 ml H_2O to yield a paste).
PBS 137 mM NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4 and 1.76 mM KH_2PO_4 (pH 7.2)
TB 10 mM Tris-HCl (pH 6.8), 183 mM KCl, 47 mM NaCl, 1 mM EDTA and 1 mM PMSF
5× BSS 270 mM NaCl, 200 mM KCl, 37 mM $MgSO_4$, 12 mM $CaCl_2 \bullet 2H_2O$, 24 mM tricine, 1.8% (wt/vol) glucose and 8.5% (wt/vol) sucrose; adjust volume with H_2O, filter sterilize and store at 4 °C
BTN 1× BSS, 0.3% (vol/vol) Triton X-100, 5% (vol/vol) normal goat serum
Hoechst 33258 solution 4 μg ml^{-1} bisbenzimide dissolved in BTN
PBT 1× PBS, 0.1% (vol/vol) Triton X-100
1 mM stock solution of AO Dissolve 1.85 mg AO in 5 ml absolute ethanol; stock is stable for many months in a foil-wrapped container at room temperature (22–25 °C).

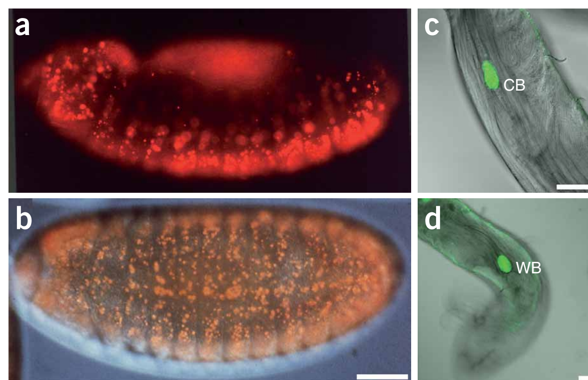

Figure 2 | AO staining of embryos (a,b) and testes (c,d). (**a**,**b**) Stage 13 embryos viewed by graphic superimposition of confocal optical sections (**a**, lateral view) or fluorescence microscopy (**b**, double exposure of Nomarski and red channel, ventral view). Anterior is left. Although the distribution of dying cells changes notably during embryonic development, the pattern of cell death for any given stage is remarkably reproducible[19]. (**c**,**d**) Live testes viewed by confocal optical sections (superimposition of Nomarski and green channel). AO specifically stained both the CB (**c**) and the WB (**d**)[21]. Scale bars, 50 μm.

PROCEDURE

Tissue collection

1| For details of how to dissect adult testes for TUNEL or AO staining, see **Box 1** and **Figure 3**. To collect embryos, set up cages of healthy well-fed flies; typically, a single cage with 500–1,000 flies (0.5–1 g) and a male:female ratio of 1:1 should be set up for each line being examined. However, if staged embryos are being collected over short time periods (<1 h) or the line does not lay many eggs, the number of flies might be increased to ∼5,000 (5 g). Alternatively, several cages can be used. Flies kept under a consistent light-regulated circadian rhythm are best for collecting eggs, and will lay them in bursts around their perceived dawn and dusk.
▲ CRITICAL STEP In addition to the genotype being tested, set up cages to collect positive and negative control embryos. As the pattern of cell death is dynamic and reproducible[19], staining specific stages in wild-type strains can serve as a positive control. However, most programmed cell death that normally occurs during *Drosophila* embryogenesis is blocked in embryos homozygous for H99, which is a small deletion that includes the cell-death activator genes *reaper*, *hid* and *grim*[15]. Staining H99 embryos provides an excellent negative control.

2| To examine all embryonic stages, allow the flies to lay eggs on grape/apple juice agar plates smeared with yeast paste for 21 h at 25 °C, without changing the collection plates. Collect and fix a volume of 50–100 μl embryos for each staining. Cell death normally occurs from stage 11 (6–7 h after egg laying) until the end of embryogenesis. Embryos can also be collected for a short period of time (0–3 h) and then aged to the desired stage. For example, to obtain late stage 10 and stage 11 embryos only, allow the flies to lay eggs for 2 h, then remove the plate from the cage and continue the incubation of the embryos at 25 °C for an additional 5 h; all embryos should be 5–7 h old.

3| Gently dislodge the embryos from the plate by adding H_2O and brushing the surface of the plate with a small artist's paintbrush. Transfer the embryos into a small embryo basket and thoroughly wash off the yeast with H_2O.

4| To facilitate efficient penetration of the TdT or AO, the embryonic chorion membrane must be removed (dechorionation). To dechorionate the embryos, place the embryo baskets in a glass Petri dish partially filled with a 50% bleach solution (the level should be just below the rim of the basket). Use a Pasteur pipette to continually rinse the embryos with the bleach solution.
▲ CRITICAL STEP Because the potency of the bleach varies, monitor the dechorionation process under a dissecting microscope. When the dorsal appendages have dissolved on 80% of the embryos (2–3 min), immediately proceed to the next step. This will prevent damage to the embryos from overexposure to bleach.

5| Remove the bleach by washing thoroughly using a squirt bottle filled with deionized water.

BOX 1 | DISSECTING ADULT TESTES

1. Dissect the male gonads (testes) from newly eclosed males in a drop of fresh TB (see REAGENT SETUP) on a glass microscope slide. Testes from older males can also be used, although they display decreased morphology. Place an anesthetized male next to the drop, hold near the top of the abdomen with a pair of fine forceps in one hand, grasp the external genitalia with another pair of forceps and pull into the TB drop. Keep the testes attached to the male genital tract to minimize loss of sample during solution removal (large samples are visualized better and have less chance of being drawn into the pipette).
2. Proceed according to **Box 2** for TUNEL staining or **Box 3** for AO staining.

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Fixation

6| Fix/treat embryos according to either option A for TUNEL staining or option B for AO staining. AO is a vital dye and therefore the embryos are stained without fixation. For the equivalent procedures for adult testes, see **Box 2** for TUNEL staining and **Box 3** for AO staining.

(A) TUNEL staining

(i) Use a small artist's paintbrush to transfer the embryos to a 1.5-ml tube containing embryo fixative. Fix at room temperature for 20 min with harsh agitation ≥150 rpm at 180° relative to the orbital rotator.
▲ CRITICAL STEP Do not over-fix as a long fixation time results in decreased devitellinization efficiency afterwards. The vitelline envelope is largely impermeable. The heptane in the fix solution knocks holes in the membrane.

(ii) After fixation, the embryos will lie at the interface between the lower and upper layers. Use a yellow pipette tip and

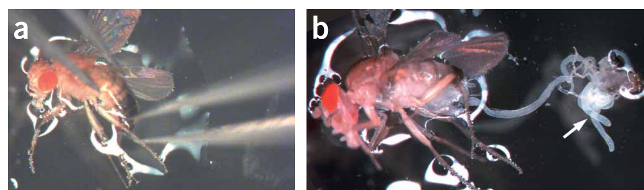

Figure 3 | Dissecting male gonads. (**a**) An anesthetized male was placed next to a drop of TB and held near the top of the abdomen with a pair of fine forceps (upper left); another pair of forceps was used to grasp the external genitalia (lower right). (**b**) The external genitalia were pulled into the TB drop. The white arrow points to the exposed testes.

BOX 2 | TUNEL STAINING OF ADULT TESTES

1. Collect the dissected samples in a Pyrex spot plate standing on ice and containing 400 μl general fixative solution. After dissection, incubate in the fixative at room temperature for 20 min more.
2. Rinse the samples in 400 μl PBS and wash twice in 400 μl of 1× BSS (10 min per wash). To minimize the loss of sample during solution removal, use a Pasteur pipette, and carefully and slowly remove the solutions under a dissecting microscope.
3. Treat with 400 μl proteinase K (10 μg ml^{-1} in PBS) at room temperature for 3–5 min. Proteinase K makes cleaved DNA accessible to the terminal transferase.

▲ CRITICAL STEP If you plan to double stain with another Ab, consider reducing or eliminating the proteinase K digestion time and proceed to Step 7. Proteinase K is an endolytic protease that cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic or hydrophobic amino acids. In our experience with multiple labeling, certain Ab signals are reduced notably following proteinase K treatment.

? TROUBLESHOOTING

4. Wash twice in 400 μl PBTw (5 min per wash).
5. Re-fix in 400 μl of 4% (vol/vol) formaldehyde solution in PBS at room temperature for 20 min. Over-fixation often results in low signal, whereas under-fixation often results in high background.
6. Wash in 400 μl PBTw five times (5 min per wash).
7. Wash in 50–100 μl equilibration buffer (ApopTag kit) at room temperature for 1 h.
8. Mix reagents in a ratio of 70% (vol/vol) reaction buffer (ApopTag kit) to 30% (vol/vol) TdT enzyme (ApopTag kit). Incubate samples in 50–100 μl TdT reaction mix at 37 °C for 3 h.

▲ CRITICAL STEP A negative control can also be performed without active TdT (using H_2O instead). Include the proteinase K digestion to control for nonspecific incorporation of nucleotide or nonspecific binding of enzyme conjugate.

■ PAUSE POINT Alternatively, incubate overnight at 37 °C. If using a Pyrex spot plate, seal it with Parafilm and incubate inside a humidified chamber to prevent the samples from drying out.

? TROUBLESHOOTING

9. Prepare stop buffer by adding 1 volume of stop/wash buffer (ApopTag kit) to 34 volumes of dH_2O. Remove the TdT reaction mix and incubate the samples in 400 μl stop buffer at 37 °C for 3–4 h.
10. Wash in 400 μl PBTw three times (5 min per wash).
11. Block in 400 μl BTN solution at room temperature for ≥1 h.
12. Apply 400 μl anti-digoxigenin (diluted 1:200 in BTN) to the samples and incubate in the dark at room temperature for 2 h.

■ PAUSE POINT Alternatively, incubate overnight at 4 °C. If you plan to double stain with another primary Ab, add the appropriate concentration to the anti-digoxigenin mix.

13. Wash four times in 400 μl of 1× BSS (20 min per wash; see REAGENT SETUP). If no secondary antibodies are to be used, proceed to Step 16.
14. Incubate with secondary Ab (diluted in 400 μl of 1× BSS) in the dark (for fluorescent-conjugated antibodies) at room temperature for ≥1 h. If more than one primary Ab was used, add the appropriate secondary antibodies to the same mix.
15. Wash twice in 400 μl of 1× BSS (10 min per wash).
16. To prepare the testes for mounting, use fine forceps to transfer them into a drop of 1× BSS on a glass microscope slide and to separate them from the male genital tract. For mounting, we use Vectashield or any glycerol-based mounting medium. Put a drop (20 μl) of the mounting medium on a glass microscope slide and use fine forceps to transfer the testes into the drop. Burst existing bubbles with a needle or aspirate them with a yellow tip. Hold the coverslip at 45 °C relative to the glass microscope slide, slowly lower it until it touches the mounting medium, then cover the testes completely and seal with nail polish. If you plan to also visualize the nuclei, the DAPI-containing Vectashield mounting medium is highly efficient.

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BOX 3 | AO STAINING

1. Incubate testes in a drop of 0.6 μg ml^{-1} solution of AO in TB on a glass microscope slide for 5 min.
▲ CRITICAL STEP The AO solution must be fresh; immediately before use, dilute 1.6 μl of 1 mM AO stock solution (see REAGENT SETUP) in 1 ml TB.
2. Wash the testes briefly by transferring them into a drop of TB on a glass microscope slide using fine forceps.
3. Mount in a drop (20 μl) of TB on a new glass microscope slide. Hold the coverslip at 45 °C relative to the glass microscope slide, slowly lower it until it touches the TB, then cover the testes completely and examine immediately. The testis cells will continue to metabolize for ≥15 min in TB.
4. Examine quickly by fluorescence/confocal microscopy. The red (568 nm) channel provides cleaner images with less background, but the green (522 nm) channel offers better sensitivity.
▲ CRITICAL STEP As this staining is performed on live tissue, and because of the tendency of the AO fluorophor to photobleach after several seconds, the tissue must be examined and photographed immediately.

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angle the tube to carefully remove the bottom formaldehyde phase and most of the upper heptane layer without removing the embryos.

(iii) Add 1 ml methanol and vortex vigorously for 20 s to remove the vitelline membrane.
▲ CRITICAL STEP It is essential that the tubes are either vortexed or shaken vigorously by hand. The vitelline membrane will burst when the tissue swells. Devitellinized embryos (usually 50%; this figure can be increased by reducing the amount of embryos in a tube) should sink to the bottom of the tube within a few minutes.

(iv) Remove most of the fluid, including that containing the embryos that did not sink, which will have retained their vitelline membrane and will not stain. Wash three times with 1 ml methanol. For these and all subsequent washes, the wash solution is removed each time after allowing the embryos to sink to the bottom. Use a Pasteur pipette or a 1-ml blue tip to remove the wash; the embryos are not transferred to a clean tube.
■ PAUSE POINT Store at –20 °C in methanol if staining is to be performed at a later date. Also, it is claimed that embryos that have spent several days in cold storage stain better.

(v) Rehydrate the embryos by washing in 1 ml solution containing 75%, 50%, and 25% (vol/vol) methanol in PBS (see REAGENT SETUP) for 5 min per wash. Finally, rinse in 1 ml PBS.

(vi) Treat with 1 ml proteinase K (10 μg ml^{-1} in PBS) at room temperature for 3–5 min. Proteinase K makes cleaved DNA accessible to the terminal transferase.
▲ CRITICAL STEP If you plan to double stain with another Ab, consider reducing or eliminating the proteinase K digestion time and proceed to Step 6Ax. Proteinase K is an endolytic protease that cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic or hydrophobic amino acids. In our experience with multiple labeling, certain Ab signals are reduced considerably following proteinase K treatment.
? TROUBLESHOOTING

(vii) Wash twice with 1 ml PBTw (5 min per wash).

(viii) Re-fix in 1 ml of 4% (vol/vol) formaldehyde solution in PBS at room temperature for 20 min. Over-fixation often results in low signal, whereas under-fixation often results in high background.

(ix) Wash in 1 ml PBTw five times (5 min per wash).

(x) Wash in 50–100 μl equilibration buffer (ApopTag kit) at room temperature for 1 h.

(xi) Mix reagents in a ratio of 70% (vol/vol) reaction buffer (ApopTag kit) to 30% (vol/vol) TdT enzyme (ApopTag kit). Incubate samples in 50–100 μl TdT reaction mix at 37 °C for 3 h.
▲ CRITICAL STEP A negative control can also be performed without active TdT (use water instead). Include the proteinase K digestion to control for nonspecific incorporation of nucleotide or nonspecific binding of enzyme conjugate.
? TROUBLESHOOTING
■ PAUSE POINT Alternatively, the embryos can be incubated overnight at 37 °C.

(xii) Prepare stop buffer by adding 1 volume of stop/wash buffer (ApopTag kit) to 34 volumes of dH_2O. Remove the TdT reaction mix and incubate the samples in 1 ml stop buffer at 37 °C for 3–4 h.

(xiii) Wash in 1 ml PBTw three times (5 min per wash).

(xiv) Block in 1 ml BTN (see REAGENT SETUP) solution at room temperature for ≥1 h.

(xv) Apply 0.1–1 ml anti-digoxigenin (diluted 1:200 in BTN) to the samples and incubate in the dark at room temperature for 2 h.
■ PAUSE POINT Alternatively, the embryos can be incubated overnight at 4 °C. If you plan to double stain with another primary Ab, add the appropriate concentration to the anti-digoxigenin mix.

(xvi) Wash four times in 1 ml of 1× BSS (20 min per wash; see REAGENT SETUP). If no secondary antibodies are to be used, proceed to Step 6Axix. If you plan to stain the nuclei, use Hoechst 33258 after this step (**Box 4**).

(xvii) Incubate with secondary Ab (diluted in 0.1–1 ml of 1× BSS) in the dark (for fluorescent-conjugated antibodies) at room temperature for ≥1 h. If more than one primary Ab was used, add the appropriate secondary antibodies to the same mix.

(xviii) Wash twice in 1 ml 1× BSS (10 min per wash). If you plan to stain the nuclei, use Hoechst 33258 after this step (**Box 4**).

(xix) Remove as much of the BSS solution as possible and add 40 μl mounting medium to the embryos in the microfuge tube; we use Vectashield or any glycerol-based mounting medium. Use a Pasteur pipette or a yellow tip cut in the middle to transfer the embryos (in ∼20–30 μl mounting medium) onto a glass microscope slide. Burst existing bubbles with a needle or aspirate them with a yellow tip. Hold the coverslip at 45 °C relative to the glass microscope slide, slowly lower it until it touches the mounting medium, then cover the embryos completely and seal with nail polish.

BOX 4 | HOECHST 33258 STAINING OF NUCLEI

1. Rinse the embryos twice in 1 ml BTN (30 s per wash).
2. Stain the embryos with 1 ml of 4 μg ml^{-1} Hoechst in BTN for 5 min.
3. Rinse twice in 1 ml of 1× BSS (30 s per wash).
4. Mount the embryos as described in Step 6Axix.

(B) AO staining

(i) Use a small artist's paintbrush to transfer the embryos to a 1.5-ml tube containing 0.5 ml heptane and 0.5 ml of 13.5 μM AO in PBS (dilute 1 volume of 1 mM AO stock solution in 73 volumes of PBS; see REAGENT SETUP).
▲ CRITICAL STEP This is a vital staining procedure and it is mandatory that every care is taken to preserve tissue viability. Any trace of detergents (such as Triton-X), bleach, prolonged tissue storage or fixatives will ruin the staining.

(ii) Vigorously shake at room temperature for 5 min, either by hand or with harsh agitation ≥150 rpm at 180 °C relative to the orbital rotator.
▲ CRITICAL STEP It is important to generate a fine emulsion of the heptane and aqueous phase.

(iii) Allow phases to separate, remove embryos from the interface using a Pasteur pipette and place in a ∼40 μl drop of Halocarbon 700 oil on a glass microscope slide. This inert oil allows oxygen to pass through, while keeping the embryos moist. The oil prevents the embryos from drying out during microscopy imaging. Place a coverslip over the embryos and seal with nail polish.

(iv) Examine quickly by fluorescence/confocal microscopy. The red (568 nm) channel provides cleaner images with less background, but the green (522 nm) channel offers better sensitivity.
▲ CRITICAL STEP As this staining is performed on live tissue, and because of the tendency of the AO fluorophor to photobleach after several seconds, the tissue must be examined and photographed immediately.

● TIMING

Approximate timeline for staining embryos

Embryo collection and washing: Steps 1–5, 1 h to overnight
Fixation for TUNEL: Steps 6Ai–6Aiv, 40 min, with the option to store embryos from a few days to several weeks
Rehydration, proteinase treatment and re-fixing: Steps 6Av–6Aix, ∼2 h
Labeling: Steps 6Ax–6Axii, 4 h (or 1 h and then incubate overnight), followed by 3–4 h of washing
Primary Ab treatment and washes: Steps 6Axiii–6Axvi, 3 h and 20 min (or 1 h and 20 min and then incubate overnight), followed by 1.3 h washing
Secondary Ab incubation, washing and mounting: Steps 6Axvii–6Axix, 2 h
AO treatment: Step 6B, 20–30 min

Approximate timeline for staining testes

Dissection of testes: 1 h depending on dissection time (**Box 1**)
Fixation for TUNEL: 50 min (**Box 1**)
Proteinase treatment and re-fixing: ∼1.3 h (**Box 2**)
Labeling and post-labeling washes: 4 h (or 1 h and then incubate overnight), followed by 3–4 h of washing (**Box 2**)
Primary Ab treatment and washes: 3 h and 20 min (or 1 h and 20 min and then incubate overnight), followed by 1.3 h washing (**Box 2**)
Secondary Ab incubation, washing and mounting: ∼2 h (**Box 4**)
AO treatment: 15 min (**Box 3**)
Hoechst 33258 staining of nuclei: 10 min (**Box 4**)

? TROUBLESHOOTING

See **Table 1**.

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TABLE 1 | Troubleshooting table.

Step	Problem	Solution
6Avi, Box 2, Step 3	In double labeling, low immunoreactivity of additional immunochemical marker due to susceptibility of the antigen to proteinase K treatment.	Consider reducing proteinase K digestion time or eliminating it completely. If omitting the treatment with proteinase K reduces the TUNEL signal, another pretreatment for exposure of the DNA should be tested. For example, instead of proteinase K, pretreat with detergent; 0.5% Triton X-100 can be applied for 10 min.
6Axi, Box 2 Step 8	If a high percentage of the nuclei are stained with, but not without, TdT, there appears to be artifactual staining of the DNA only.	Use less of the TdT reagent. Instead of 1:3 dilution, use dilutions in the range of 1:5 to 1:16. Maintain the reagent volume by adding water.

ANTICIPATED RESULTS

The TUNEL and AO assays are both highly reproducible in embryos and male gonads. It is recommended that each experiment is performed using negative and positive control samples, as indicated in the PROCEDURE. Although the TUNEL enzymatic-labeling method is time-consuming, it is sensitive and specific. Recently, additional methods for the detection of apoptotic cells in *Drosophila* have become available, such as whole-mount staining of *Drosophila* tissues with the CM1 Ab (anti-cleaved caspase-3; Cell Signaling Technology, cat. no. 9661), which cross-reacts with cleaved effector caspase in the fly and/or detection of caspase-3-like activity (DEVDase) in tissue lysates[22]. The ApopTag kit for TUNEL labeling has been traditionally used; however, other kits might also be used (for example, the *In Situ* Death Detection Kit from Roche, cat. no. 12156792910, has been recently demonstrated to work successfully with embryos). Examples of expected results for wild-type embryos and testes are shown in **Figures 1,2**. Images that demonstrate the dissection of male gonads are shown in **Box 1** and **Figure 3**.

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