Protocol

Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP Nick-End Labeling (TUNEL) for Detection of Apoptotic Cells in *Drosophila*

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A characteristic feature of apoptosis is DNA fragmentation. This fragmentation can be detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) of DNA in dying cells. Here, we present a protocol for TUNEL detection of apoptosis in *Drosophila* larval tissue, but these techniques can be adapted for other tissues and developmental stages.

MATERIALS

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

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

Reagents

DNase

Fixative (freshly prepared 4% paraformaldehyde in PBS)

Alternatively, a 20% stock can be made and stored at -20°C.

Glycerol (80% in PBS)

Alternatively, Vectashield can be used.

Hoechst 33258 (2 mg/mL stock solution)

PBTx (0.1% Triton X-100 in 1× PBS)

PBTx5 (0.5% Triton X-100 in 1× PBS)

Phosphate-buffered saline (PBS) (1×, pH 7.4; without Ca²⁺ or Mg²⁺) <R>

Sodium citrate (100 mM in PBTx [1.47 g in 50 mL of PBTx], freshly prepared)

TUNEL kit (e.g., TMR-red; Roche)

There are several commercially available kits with either fluorescent or color detection methods, and these should be used according to the manufacturer's instructions.

Equipment

Confocal or fluorescence microscope, fitted with an appropriate camera

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Dissecting microscope (e.g., Olympus)

Dissection dish

Prepare a dissection dish according to the manufacturer's instructions for the Sylgard 184 Silicone Elastomer kit (Dow Corning). The soft silicone elastomer set in the dish base is useful for preventing damage to dissection equipment.

Dissection equipment

Forceps (Dumont #5 or #55; World Precision Instruments)

Needle (26-gauge hypodermic or tungsten)

Double-sided sticky tape (optional; see Step 11)

Microcentrifuge tubes (1.5-mL)

Microscope coverslips (22 × 22 or 22 × 50 mm) and glass slides (76 × 26 mm)

Water bath (or hybridization oven, or similar) at 65°C

METHOD

1. With forceps, dissect larvae or pupae immersed in 1× PBS on a Sylgard dissection dish under a dissecting microscope at room temperature.

Dissected tissues can be processed for up to 20 min at room temperature or on ice for up to 1 h.

2. Fix tissue of interest by transferring into a 1.5-mL microcentrifuge tube containing 4% paraformaldehyde in PBS and incubate for 20 min at room temperature (22°C–25°C).
3. Wash three times with PBTx. For each wash, use 1 mL of PBTx and incubate for 5–10 min at room temperature.
4. Wash with 1 mL of PBTx5 for 5 min at room temperature.
5. Permeabilize in 100 mM sodium citrate (freshly prepared in PBTx) for 30 min at 65°C.
6. Wash three times with 1 mL each of PBTx for 5–10 min at room temperature.
7. Perform the TUNEL reaction according to the manufacturer's instructions.

For each sample, we use 100 µL of Roche TMR-red kit reagents (10 µL of the TdT enzyme and 90 µL of the labeling mix) and incubate for 3 h at 37°C.

Although TUNEL generally yields reproducible results, to avoid false positives and false negatives, appropriate controls should be included. Consider including a negative control sample that contains only labeling mix and a positive control sample that was treated with DNase. For DNase I treatment, incubate for 10 min at room temperature with DNase I at 3000 U/mL.

See Troubleshooting.

8. Remove the TUNEL solution. Wash three times with PBTx. For each wash, use 1 mL of PBTx and incubate for 5–10 min at room temperature.
9. (Optional) To detect nuclei, costain samples with 4 µg/mL Hoechst 33258 in PBS for 1 min at room temperature.
10. Wash three times with PBTx. For each wash, use 1 mL of PBTx and incubate for 5–10 min at room temperature.
11. Mount in 80% glycerol in PBS (or in Vectashield) by transferring the sample to a microscope glass slide, dissecting the tissue of interest away from other tissue if required, and then placing a microscope coverslip over tissue.

Double-sided sticky tape can be used on each end of the microscope slide to prevent the tissue from being squashed by the coverslip.

12. Examine the samples by confocal or fluorescence microscopy.

Figure 1 shows an example of a TUNEL salivary gland.

FIGURE 1. Visualization of *Drosophila* DNA fragmentation by terminal deoxynucleotidyl transfer-mediated dUTP nick-end labeling (TUNEL). Salivary glands at 14 h relative to puparium formation from (A) control larva, showing TUNEL-positive nuclei, and (C) mutant larva lacking the initiator caspase Dronc (Nc), indicating the absence of TUNEL-positive nuclei. DNA stained with Hoechst shows a similar pattern of nuclei in wild-type (B) and mutant larvae (D).

TROUBLESHOOTING

Problem (Step 7): Difficulties are experienced in labeling.

Solution: Consider using an alternative permeabilization procedure such as proteinase K treatment at Step 5 (incubate the sample at room temperature for 3–5 min in a 10 µg/mL solution of proteinase K that was prepared in PBTx) (Arama and Steller 2006; McCall et al. 2009).

RELATED TECHNIQUES

Slight modifications may be required for TUNEL in other *Drosophila* tissues. For example, TUNEL protocols for embryos include an additional postfixation step (in 4% paraformaldehyde in PBS for 20 min) following permeabilization and preceding Step 6. For additional information, see Arama and Steller (2006) and McCall et al. (2009). TUNEL can also be used for the analysis of cell death in sections from paraffin-embedded samples, necessitating the use of a color detection kit according to the manufacturer's instructions.

Because TUNEL detects DNA strand breaks, the examination of cell death after treatment with inducers of DNA damage, such as γ -irradiation, might require alternative assays, such as those employing acridine orange (see Protocol: **Use of the Vital Dye Acridine Orange to Observe Dying Cells in *Drosophila* Tissue** [Denton and Kumar 2015a]) or immunostaining for active caspase-3 (see Protocol: **Immunostaining Using an Antibody against Active Caspase-3 to Detect Apoptotic Cells in *Drosophila*** [Denton and Kumar 2015b]). This is because TUNEL can potentially detect the DNA damage induced by the treatment itself.

General advice for handling *Drosophila* in the laboratory is available in Sullivan et al. (2000).

RECIPE

Phosphate-Buffered Saline (PBS)

Reagent	Amount to add (for 1× solution)	Final concentration (1×)	Amount to add (for 10× stock)	Final concentration (10×)
NaCl	8 g	137 mM	80 g	1.37 M
KCl	0.2 g	2.7 mM	2 g	27 mM
Na ₂ HPO ₄	1.44 g	10 mM	14.4 g	100 mM
KH ₂ PO ₄	0.24 g	1.8 mM	2.4 g	18 mM
If necessary, PBS may be supplemented with the following:				
CaCl ₂ ·2H ₂ O	0.133 g	1 mM	1.33 g	10 mM
MgCl ₂ ·6H ₂ O	0.10 g	0.5 mM	1.0 g	5 mM

PBS can be made as a 1× solution or as a 10× stock. To prepare 1 L of either 1× or 10× PBS, dissolve the reagents listed above in 800 mL of H₂O. Adjust the pH to 7.4 (or 7.2, if required) with HCl, and then add H₂O to 1 L. Dispense the solution into aliquots and sterilize them by autoclaving for 20 min at 15 psi (1.05 kg/cm²) on liquid cycle or by filter sterilization. Store PBS at room temperature.

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