Chapter 11

Detection of Cell Death in *Drosophila* Tissues

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Abstract

Drosophila has served as a particularly attractive model to study cell death due to the vast array of tools for genetic manipulation under defined spatial and temporal conditions in vivo as well as in cultured cells. These genetic methods have been well supplemented by enzymatic assays and a panel of antibodies recognizing cell death markers. This chapter discusses reporters, mutants, and assays used by various laboratories to study cell death in the context of development and in response to external insults.

Key words Drosophila cell death reporters, TUNEL, Acridine orange, Immunostaining, DEVD cleavage assay

1 Introduction

Drosophila is a highly popular experimental model for studying various aspects of biological problems as they can be easily genetically manipulated and have relatively short life cycles. The *Drosophila* community over the last hundred years has built a formidable library of mutants, transgenes, transgenic reporter lines, and genetic techniques that allow study of many processes and pathways. This includes programmed cell death (PCD), which makes possible the metamorphosis from larvae to adult flies, and also plays many other important roles in development.

Similar to other organisms, cell death pathways in *Drosophila* can be activated in response to DNA damage and excess stress imposed in various subcellular compartments by extrinsic factors. While the apoptotic cascade in *Drosophila* culminates in the activation of initiator and effector caspases, the upstream components vary from canonical apoptotic genes in mammals. There are seven known caspases: Dredd [1], Dronc [2], and Strica [3] are initiator caspases; Drice [4], DCP-1 [5], DECAY [6], and DAMM [7] are effector caspases. These caspases are synthesized as inactive zymogens, but gain activity after proteolytic processing. In *Drosophila*, a family of proteins called inhibitors of apoptosis proteins (IAPs)

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play particularly prominent roles in regulating caspase activity [8]. There are three known IAPs—DIAP1 [8], DIAP2 [9, 10], and BRUCE [11–13]. There is certain degree of tissue-specificity and mutual redundancy between the caspases and IAPs and this is extensively reviewed elsewhere [14–16]. When death-inducing signals are received by cells, IAPs are inactivated by the IAP-antagonists: *hid* [17], *reaper* [18], *grim* [19], and *sickle* [20–22] (Fig. 1). Inhibition of IAPs trigger apoptosis in most somatic cells of *Drosophila*, as caspases are constantly undergoing proteolytic activation in living cells, only to be inhibited by IAPs. Specifically, the *Drosophila* initiator caspase Dronc constitutively forms a complex with the adaptor protein Dark, even without cytochrome c released from the mitochondria [23]. In living cells, the small amount of activated caspases engage in negative feedback, with the help of IAPs. In cells doomed to die, inhibition of IAPs by IAP-antagonists leads to the stable activation Dronc and Dark [24]. This leads to the activation of effector caspases such as Drice, which subsequently orchestrate apoptosis by cleaving various nuclear and cytoplasmic proteins.

A dramatic case of programmed cell death (PCD) associated with normal development occurs during metamorphosis. In this case, cell death is largely under the control of ecdysone, a steroid hormone. Spikes in ecdysone levels are seen at the interface of the major development stages: embryo hatching, larval molting, pupariation, and pupation [25, 26]. The most morphological change and correspondingly the largest ecdysone spike occurs during pupation when entire larval organs, such as the gut and salivary gland, are eliminated by PCD to give way to adult tissues. It is thus not surprising that ectopic misexpression of ecdysone and other apoptotic pathway proteins has dire developmental consequences [27, 28].

This overview of cell death in *Drosophila*, while simplistic, provides the basis for methods detailed in this chapter. Subheading 2 of this chapter discusses genetic and genotoxic methods of

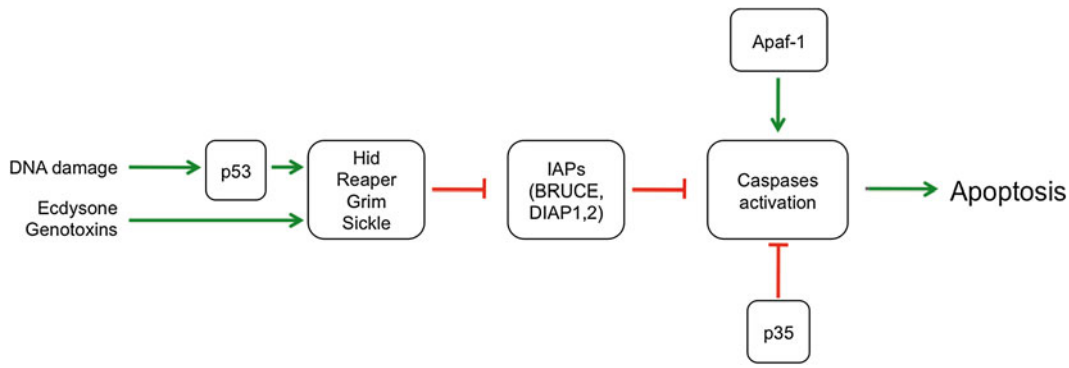

Fig. 1 A schematic showing various manipulatable elements of the cell death pathway in *Drosophila*: See text

inducing or blocking cell death. Subheading 3 discusses cell biological and biochemical assays to visualize and quantify cell death. Both sections discuss methods that can be used in larvae, adults and in cultured cells. Overall, this chapter attempts to illuminate what makes *Drosophila* a comprehensive model for studying cell death: the ability to finely regulate expression of genes with spatial and temporal control, and the variety of physiological contexts that can be simulated.

2 Materials

1. Fly stocks: Commonly used fly stocks and suggested sources are described in Table 1.
2. Media for culturing S2, S2R+, and SL2 cells: Schneider's Insect Cell Medium (Life Technologies), 10 % fetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml).
3. Phosphate buffer saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4. 10× stock can be made and stored at room temperature.

Table 1
Commonly used fly lines for modulating and observing cell death

Fly Lines	Available sources
hid, *reaper*, *grim* deletion—Df(3L)H99	BDSC, Kyoto DGGR
$dronc^{RNAi}$	VDRC, TRiP
$drice^{RNAi}$	
$dcp\text{-}1^{RNAi}$	
$dredd^{RNAi}$	
$diap1^{RNAi}$	TRiP
Gain of function	
uas-*hid*	BDSC
uas-*reaper*	BDSC, Kyoto DGGR
gmr-*hid*	
heat shock-*hid*	
Reporters	
rpr-lacZ	Laboratories of Hermann Steller, Andreas Bergmann and John Abrams
hid-lacZ	

4. Ringer's solution: 116 mM NaCl, 1.2 mM KCl, 1 mM $CaCl_2$, pH 7.4.
5. Fixative for tissue staining: 4 % paraformaldehyde, 1×PBS, made fresh (*see* **Note 1**).
6. Phosphate buffer Tween (PBT): 0.1 % Tween 20 or Triton X-100 (*see* **Note 2**), PBS.
7. Blocking buffer for immunostaining: 10 % donkey serum or 3 % BSA in PBT.
8. Acridine Orange (AO) stain: 1.25 μg/ml AO, 50 % heptane.
9. Lysis buffer for larval tissue: 50 mM Tris, 1 mM EDTA, 10 mM EGTA, 10 μM digitonin.
10. 2× reaction buffer for DEVD assay: 50 mM HEPES pH 7.4, 20 mM $MgCl_2$, 200 mM NaCl, 0.1 % NP40.
11. Fixative for cells: 10 % formaldehyde, PBS.

3 Methods

3.1 Tools for Manipulating Cell Death

This section aims to give an overview of methodologies used to either block or induce cell death. Genetic methods are useful when precise control is needed over tissue and cell type while genotoxic methods can be used to induce organism wide cell death. Chemical methods are mostly used in cell culture studies, often to corroborate results seen in vivo.

3.1.1 Genetic Tools for Blocking or Inducing Cell Death in Drosophila

The four pro-apoptotic genes, *hid*, *reaper*, *grim*, and *sickle* are clustered together in a genetic locus on the 3L chromosome [14, 29]. Various deletions of this locus have been employed to block cell death but the most commonly used strain is a third chromosome deficiency, Df(3L)H99, which deletes *hid*, *reaper*, and *grim*. Mild phenotypes have been observed when only one copy of the H99 locus remains [30–34] but homozygotic mutants abolish virtually all apoptosis [17–19, 35]. Conversely, induction of IAP-antagonists in response to DNA damage or stress (proteotoxicity, viral infection, etc.) is mediated by p53, overexpression of which can be used to induce cell death [36, 37].

Cell death can also be inhibited by deletion or RNAi knockdown of caspases such as Dronc, Drice, DCP-1, and Dredd [9], etc. On the other hand, cell death can be induced by deleting or RNAi-mediated knockdown of anti-apoptotic genes such as DIAP1 [9] using the GAL4-UAS system [38]. In this system, a promoter typically drives the expression of a transcription factor, GAL4, which in turn induces the expression of any gene downstream of a UAS element. This two-component system allows for tissue-specific control of transgene expression. A further level of temporal control can be added using a number of genetic tools. For example,

one strategy employs the GAL80 transcription factor, which inhibits its GAL4 activity. GAL80 temperature sensitive ($GAL80^{TS}$) mutants are widely used to grow animals at GAL80-permissive temperatures to the desired developmental stage before upshifting to a non-permissive temperature where GAL80 activity is inhibited and hence GAL4 activity is restored [39]. Another popular system for temporal control of gene expression is the Geneswitch system, where GAL4 is fused to a mifepristone-responsive promoter [40]. Thus GAL4 will be active only in the presence of mifepristone, which can be regulated via diet. Often GAL4-driven expression of a transgene in the entire tissue results in various side effects, for which experimental controls are difficult to design. To circumvent this issue, transgene expression can be induced in small sections of the tissue using clonal analysis. Mosaic analysis with a repressible cell marker (MARCM) utilizes site-specific recombinases under the control of inducible promoters in combination with the GAL4-GAL80 system to generate subpopulations of cells that express a given transgene [41]. Surrounding tissue that did not undergo recombination serves as a great internal control for the experiment.

In addition to the methods described above, there are several other genetic tricks that provide various degrees of control over GAL4 expression and these are discussed in detail elsewhere [42].

Transgenic lines that fuse pro-apoptotic genes such as *reaper* or *hid* directly to tissue specific promoters have also been used widely to induce apoptosis [17] and this has been the basis of many screens to identify new modulators of cell death. For example, overexpression of *hid* in the *Drosophila* eye using the GMR-promoter results in ablation of the eye (Fig. 2). This phenotype is readily visible and hence convenient to score. Our laboratory and others have utilized this system to identify many different components of the cell death machinery [9]. Table 1 lists transgenic fly lines which can be been to induce or block cell death.

In cultured S2, SL2, and S2R+ cells (Life Technologies, *see* Subheading 2, **item 2**), *hid*, *reaper*, or *grim* overexpression can induce apoptosis [43–45]. Overexpression constructs are typically under the control of an inducible promoter and are transiently transfected into cells using a lipid-based transfection reagent such as Effectene (Qiagen).

3.1.2 Genotoxic Methods to Induce Cell Death

The most common method of inducing DNA damage in flies is by ionizing irradiation. In adults, 40 Gy of gamma-ray radiation is an efficient way to induce massive apoptosis and for larvae, a lower dose (20 Gy) maybe sufficient. The animals are allowed to recover for 2–3 h prior to dissecting out desired tissues to observe for markers of apoptosis. UV irradiation at 254 nm and 40 mJ/cm^2 can also be used to induce apoptosis in embryos, adult and larval tissues [46]. Recovery time before dissection may vary from 4 to 10 h.

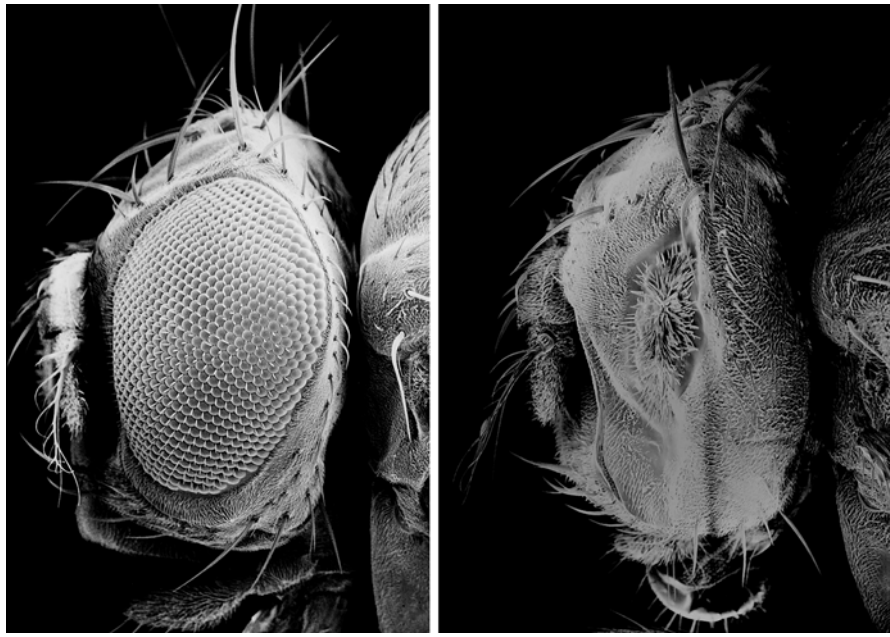

Fig. 2 High resolution image of *Drosophila* eye: Overexpression of the pro-apoptotic gene, *hid,* using the eye-specific promoter *gmr* results in eye ablation (*right*) when compared to wild type eyes (*left*)

In cultured cells cells, apoptosis can be induced by any of the following methods: 1 μM staurosporine or 10 μM ecdysone for 24 h, 200 nM actinomycin D for 3–8 h, UV-irradiation at 200 mJ/cm^2 followed by 4–12 h recovery [43, 45, 47]. When using irradiation, cells should be cultured in UV-transparent plates to allow for exposure.

3.2 Detecting Cell Death

Reflecting their roles as initiators of apoptosis in *Drosophila*, the expression pattern of *reaper*, *grim*, and *sickle* coincides with induction of apoptosis [19–21, 35]. Based on these observations, reporters have been made that can be used to monitor cell death in your favorite tissue. The first described *reaper-lacZ* reporter was generated by placing an 11-kb radiation *reaper* upstream element driving *lacZ* expression [48]. Subsequently other groups have whittled down this region to generate more fine-tuned reporters that respond to specific types of injury and stress [36, 49]. Subheading 3.2.3 describes how to examine *lacZ* expression using antibody staining. A similar reporter for *hid* has also been employed to monitor cell death in tissues [50]. Apoptotic pathways can be cell-type or tissue-type specific [51], so it maybe worth trying multiple methodologies when experimenting with a less explored tissue. Apoptotic cells can be visualized using the TUNEL labeling or by staining for specific apoptotic marker.

3.2.1 Collecting Samples of Defined Ages for Analysis

An important control to consider when designing experiments is to ensure that all animals being compared in the study, embryo, larvae, or adult, are of similar age and thus at similar developmental stages. Age is especially relevant when studying apoptosis in the developing embryo.

1. Eggs are collected in large cages on grape juice or apple juice plates smeared with yeast paste to encourage laying. Plates can be switched out in timed intervals so as to control the age of the embryo. For accurate staging, the first plate must be discarded since flies can carry embryos in their oviducts for 30 min before laying them.
2. Eggs have a protective proteinaceous covering called the chorion. Embryos must be dechorionated to allow for penetration of both staining agents and for effective lysis. Add 66 % bleach to the apple juice plates and incubate for 3 min.
3. Collect embryos in a mesh basket and thoroughly rinse with tap water for at least 3 min before transferring to a microfuge tube containing PBS (Subheading 2, **item 3**).
4. Dissect larvae and adults in PBS (Subheading 2, **item 3**) or Ringer's solution (Subheading 2, **item 4**). When dissecting several samples, store tissues in a dissection dish or microfuge tube containing PBS (Subheading 2, **item 3**).
5. For staining experiments, leave the carcass of the organism attached until just before mounting. This allows samples to sink to the bottom of tubes making washes and solution changes easier. For imaging apoptosis in S2 cells, it is best to culture them in poly-lysine coated multi-chamber slide (Nunc). The chamber allows for the entire staining process to be done on the slide and can be removed prior to mounting.

3.2.2 TUNEL Assay

TUNEL is an abbreviation for Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and is based on the detection of fragmented DNA ends, which are characteristic of apoptotic cells. A deoxynucleotidyl transferase (TdT) is used to add modified nucleotides to double- and single-stranded DNA breaks. The modified nucleotides, such as digoxigenin-dUTP, can then be detected using specific antibodies. Several commercial kits provide complete panels of reagents with different dUTP. This protocol describes staining of tissues or cells using Apoptag (Millipore).

1. Incubate sample in fixative (Subheading 2, **item 5**) for 20 min (*see* **Note 1**).
2. Wash fixed samples in PBT (Subheading 2, **item 6**) for 5 min thrice. This step permeabilizes samples using the detergent, to allow for reagent penetration.

3. Equilibrate samples in the buffer provided at room temperature.
4. Incubate in appropriate volume (till tissue is submerged) of the TdT reaction mixture (2:1 mixture of reaction buffer and TdT enzyme) for 1 h at 37 °C.
5. Stop the reaction by the adding the stop buffer (diluted 34:1 with distilled water). Agitate for 15 s and incubate at room temperature for 10 min.
6. Wash three times with PBT, 5 min each.
7. Incubated with a fluor-conjugated anti-digoxigenin antibody according to manufacturer's instructions for 2 h at room temperature.
8. If desired, counterstain with DAPI (300 nM final concentration) for 5 min at room temperature toward the end of the previous incubation (*see* **Note 3**).
9. Wash three times with PBT, 10 min each.
10. Mount samples on glass slide in a suitable medium such as Vectashield (Life Technologies) or 50 % glycerol (sterile) and visualize using a fluorescent or confocal microscope (Fig. 3).

3.2.3 Immunostaining of Apoptotic Cells

While TUNEL assays for a specific aspect of apoptosis, i.e., DNA damage, there are other markers of apoptosis that can be stained for. Table 2 lists a subset of antibodies that are commercially available or generated by various laboratories to detect pro-apoptotic proteins. The choice of apoptotic marker is dictated by how apoptosis was induced and in which cell type. Unlike mammalian cells, cytochrome *c* release is not necessary for apoptosis in *Drosophila* so anti-cytochrome *c* antibodies are generally not used

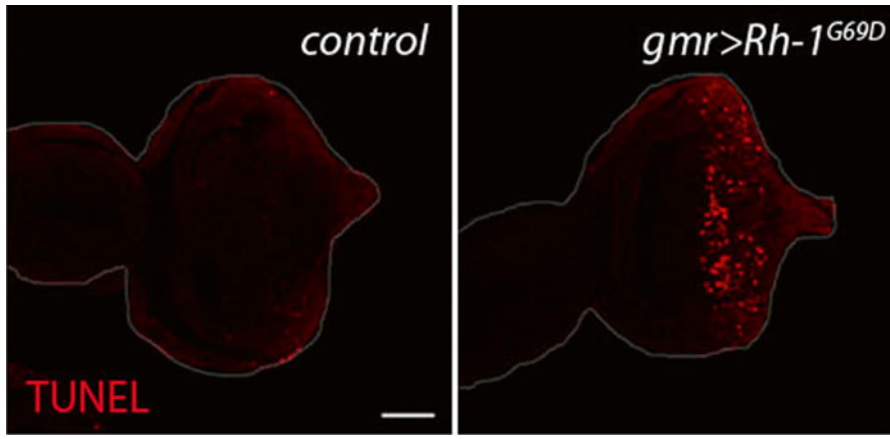

Fig. 3 TUNEL staining of third instar larval eye imaginal discs: Discs expressing mutant Rh-1^{G69D} that imposes stress in the endoplasmic reticulum show increased TUNEL staining (*right*) in comparison to the wild type control (*left*)

[52]. If using a *lacZ* reporter line, then an anti-βgal antibody can be used to visualize *rpr* or *hid* transcriptional activity. Staining for apoptotic markers can also be performed in combination with TUNEL (*see* **Note 4**).

1. Incubate sample in fixative (Subheading 2, **item 5**) for 20 min (*see* **Note 1**).
2. Wash fixed samples in PBT (Subheading 2, **item 6**) for 5 min thrice (*see* **Note 2**). This step permeabilizes samples using the detergent, to allow for reagent penetration.
3. Incubate samples in blocking buffer (Subheading 2, **item 7**) for an hour at room temperature. Remove blocking buffer and rinse with PBT three times. Remove as much PBT as possible before proceeding to next step.
4. Add primary antibody solution (Table 2) diluted appropriately in PBT and incubate either for 2 h at room temperature or overnight at 4 °C (*see* **Note 5**).
5. Wash samples three times with PBT, 5 min each.
6. Incubate with fluor-conjugated secondary antibody (Alexa Fluor, Life Technologies) diluted 1:1000 in PBT for 1 h at room temperature. If combining with TUNEL staining, include anti-digoxigenin antibody in the secondary antibody solution.
7. Counterstain and mount as described in TUNEL staining.

Table 2
Antibodies for cell death markers

Antibody	Source
cleaved-dcp-1	Cell Signaling Technology
cleaved caspase 3	Cell Signaling Technology
hid	Santa Cruz, Hermann Steller, Hyung Don Ryoo
DIAP1	AbCam, Bruce Hay, Hyung Don Ryoo
Dronc	Sharad Kumar
Drice	Sharad Kumar
ATP5a (mitochondrial marker)	AbCam
Cytochrome C	Zymed
anti-βgal	Sigma

3.2.4 Acridine Orange Staining

Acridine Orange (AO) is a cell-permeable organic compound that is retained selectively by dying cells because of their internal pH imbalance [53]. Since AO is used primarily in live tissues, options for counterstaining samples with other markers are limited. AO staining is popularly used for screening large numbers of embryos [35] but can also be used in other tissues such as imaginal discs [54]. While the staining protocol for AO is brief, so is the time available to observe the tissue after staining.

1. For embryos: Add sufficient amount of AO stain (Subheading 2, **item 8**) to dechorionated embryos and shake vigorously. Incubate for 5 min at room temperature. Mount the embryos from the interphase on to a glass slide and visualize in the green or red channel within 15 min.
2. For tissues: Incubate freshly dissected tissues (not fixed, *see* **Note 6**) in 0.5–10 μg/ml AO for 5 min at room temperature. The concentration of AO varies depending on tissue type and can be optimized. Mount tissues on glass slide and visualize as above.

3.3 Biochemical Assays

3.3.1 Colorimetric Assay for Caspase Activity

A direct approach for quantifying cell death activity is by measuring *hid* and *reaper* levels by either western blotting (Table 2) or RT-PCR (Table 3). A broader method is measuring caspase activity. One method to assess caspase activity is by western blotting for cleaved caspase (anti-Dronc, -Drice) using antibodies described in Table 2. A more precise assessment of cell death can be obtained by measuring caspase activity using synthetic substrates. Several synthetic substrates have been developed and their catalysis can be measured colorimetrically or by fluorescence. DEVD-pNA is a peptide caspase substrate whose sequence is similar to the caspase cleavage site in PARP. Cells or tissues prepared as described in Subheading 3.2.1 can be used in the assay as follows.

1. Add appropriate amount of lysis buffer (Subheading 2, **item 9**) to samples and vortex intermittently for 10 min to enable complete lysis. Larger tissues or whole organisms can be homogenized in the lysis buffer using a pestle.

Table 3
Primers for amplification of pro-apoptotic genes [56]

Gene	Primer sequence
reaper	AGTCACAGTGGAGATTCCTGG
	TGCGATATTTGCCGGACTTTC
hid	ACGGCCATCCGAATCCGAAC
	TGCTGCTGCCGGAAGAAGAAGTT

2. Clear lysate by centrifuging at 10,000 × *g* for 10 min at 4 °C. Lysates can be stored in −70 °C for several weeks with minimal loss in caspase activity.
3. Mix lysate with equal volume of 2× reaction buffer (Subheading 2, **item 10**). Add 10 μM (final) Z-DEVD-pNA or Ac-DEVD-pNA (SCBT) and incubate for 37 °C for 1 h.
4. Measure absorbance at 400 nM (or other wavelength depending on substrate). Samples maybe diluted to obtain readings in a linear range if necessary.

3.3.2 Viability Assays in S2 Cells

An alternative to staining for cell death in cultured cells is to assess viability by using an exclusion dye such as trypan blue or an inclusion dye such as crystal violet. It is important to start with equal number of cells per sample so as to be able to compare values directly. Crystal violet staining is more suitable for high throughput experiments since it can be read using a plate reader. Crystal violet stains all cells adherent to the plate so it relies on the assumption that apoptotic cells will be washed away. Cells cultured in 6-well dishes and transfected or treated with appropriate apoptotic inducers are assayed as follows.

1. Aspirate culture media, add 250 μl fixative (Subheading 2, **item 11**), and incubate for 15 min at room temperature.
2. Rinse three times with distilled water.
3. Incubate with 250 μl 0.5 % crystal violet solution for 30 min at room temperature on a shaker.
4. Rinsing with tap water ten times to remove excess dye. Leave plates inverted on a paper towel to drain water completely.
5. Elute with 500 μl of 10 % acetic acid for 10 min at room temperature on a shaker.
6. Diluted eluate in water 100-fold before measuring absorbance at 595 nm.

Trypan blue staining is more suitable for kinetic monitoring of samples. It is based on membrane impermeability of the blue dye thus negatively marking live cells. A suspension of S2 cells in PBS is mixed with trypan blue (Sigma) solution such that the final concentration of trypan blue is 0.04 %. After incubating for 5 min at room temperature, the number of alive versus dead cells can be counted using a hemocytometer or a cell counter (Bio-Rad). Other cell viability assays are reviewed in [55].

4 Notes

1. The concentration of paraformaldehyde (or formaldehyde) in the fixative can be adjusted depending on the samples being stained. Although 4 % paraformaldehyde is standard, there are antibody or TUNEL labeling protocols that use as low as 1 % of fixative.
2. The precise condition for antibody labeling may vary from tissues to tissues. For example, larval imaginal disc epithelium is an accessible tissue, and the PBT solution contains typically 0.2 % of either Tween-20 or Triton X-100. However, embryos and adult eyes and brains are less permeable to antibodies, and require a higher concentration of detergent (we suggest 0.3 %). If the quality of the antibody labeling is unsatisfactory, one might want to vary the concentration of detergent.
3. Samples can be counterstained with a cytoskeletal marker such as fluor-conjugated Phalloidin (Life Technologies) to observe cellular and nuclear morphology. Early apoptotic nuclei have a condensed appearance. Mitochondrial markers show shattered-glass-like patterns in apoptotic cells. Staining for pro-apoptotic proteins such as *hid* show their localization to the mitochondria and appear as a punctate pattern.
4. 4: This technique can also be performed on samples already incubated with TdT (but before anti-digoxigenin staining).
5. The primary antibody incubation step has scope for optimization in antibody dilution ratios, incubation times and temperatures. Typically, primary antibody solutions can be reused by storing at 4 °C in 0.01 % sodium azide for up to a month.
6. It is important to remember that acridine orange or trypan blue labeling should not be performed with fixed tissues. These assays work only when tissues contain live cells. By contrast, TUNEL or antibody labeling assays require prior fixation of tissues.

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