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Detecting Caspase Activity in *Drosophila* Larval Imaginal Discs

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Abstract

Caspases are a highly specialized class of cell death proteases. Since they are synthesized as inactive full-length zymogens, activation – at least of effector caspases and to some extent also of initiator caspases – requires a proteolytic cleavage event, generating a large and a small subunit, two of each forming the active caspase. The proteolytic cleavage event generates neo-epitopes at both the C-terminus of the large subunit and the N-terminus of the small subunit. The cleaved Caspase-3 (CC3) antibody was raised against the neo-epitope of the large subunit and thus detects only cleaved, but not full-length, Caspase-3. Although raised against human cleaved Caspase-3, the CC3 antibody cross-reacts in other species and detects cleaved caspases, most notably DrICE and Dcp-1, in *Drosophila*. This protocol describes the procedure for use of the CC3 antibody to detect caspase activity in larval imaginal discs in *Drosophila*.

Keywords

Drosophila; Cleaved-Caspase-3; Dronc; DrICE; Dcp-1; Cell Death; Non-apoptotic Function; Imaginal Disc; Immunolabeling

1. Introduction

The genome of *Drosophila melanogaster* encodes for seven caspase genes (reviewed in [1,2]). However, only three of these are involved in the execution of apoptosis: the initiator caspase Dronc and the effector caspases DrICE and Dcp-1 [1,2]. The synthesis and activation of these three caspases is very similar to that of their human homologs [1]. Moreover, *Drosophila* is an ideal model system for studying the various functions of caspases. Overall, 50% of caspase targets during apoptosis are conserved at the protein level between flies and humans [3]. This increases to 60% when accounting for different protein targets within the same conserved pathway. Taken together with the fact that the intrinsic cell death cascade is remarkably well conserved in *Drosophila,* the fly is a very relevant model to study the roles of caspases in the initiation and execution of apoptotic cell death [2,4]. Interestingly, recent studies have also shown that the roles of caspases in the fly go

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[3]Take care to ensure the vials do not become overcrowded. Crowding in the food can not only cause larvae to wander prematurely, potentially leading to incorrect developmental timing at dissection, but can also create a stressed environment, sometimes enhancing cell death phenotypes and confounding results. Depending on the overall health of the parental lines and the expected viability of the F1 progeny, we have found allowing a 24 hour egg lay by 8 virgin females mated with 4 to 6 males in narrow vials (25mm O.D. × 99mm H) yields sufficient progeny without crowding.

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beyond traditional cell death and include a plethora of non-apoptotic functions. Caspases have been implicated in cell migration and morphogenesis [5–7], compensatory cell proliferation [8–11], cell differentiation and maturation [12,13], innate immunity [14,15], and non-apoptotic alternative cell death [16].

Initially, to detect caspase activity *in vivo,* researchers employed indirect measures such as TUNEL, acridine orange, and propidium iodide [17–19]. However, these methods highlight cells in the late stages of programmed cell death and are not specific to the intrinsic cell death pathway [20]. More recently, direct methods to detect caspase activity have been developed, which take advantage of antibodies that specifically recognize neo-epitopes that are generated after the proteolytic processing of caspases. Ideally, these antibodies do not detect uncleaved, and therefore inactive, caspases. Typically, these antibodies are raised against the neo-epitope formed at the C-terminus of the large subunit of Caspase-3. The first antibody of its kind was the CM1 antibody, which is no longer available [21]. Currently, several antibodies are commercially available that are referred to as anti-Cleaved Caspase-3 (CC3 from Cell Signaling Technology) or anti-Active Caspase-3 (Abcam). Although raised against an epitope in human Caspase-3, these antibodies also cross-react with *Drosophila* caspases [22–24] which is surprising as only eight of 13 residues within the epitope are identical between human Caspase-3 and *Drosophila* effector caspases DrICE and Dcp-1. However, follow-up studies demonstrated that only the most C-terminal three residues (ETD), which are conserved between the three caspases, are required for detection by the CC3 antibody [23]. Because these three residues constitute part of the cleavage site of the initiator caspase Dronc, the specificity of the CC3 antibody comes with the caveat that it not only detects cleaved DrICE and Dcp-1, but also a currently unknown non-apoptotic protein (or proteins) bearing a similar epitope [23]. Because exposure of these sites is dependent on the proteolytic activity of the initiator caspase Dronc, we interpret the CC3 antibody as a marker of Dronc activity rather than of DrICE activity [23]. This becomes an especially important distinction in the study of non-apoptotic functions of caspases—processes that may be dependent solely on Dronc and not involve effector caspases at all.

In this chapter, we describe a method for using the CC3 antibody in *Drosophila* larval tissues, specifically in the epithelial layers found within the developing imaginal discs. The first part describes the technique for sample preparation and fixation. Proper sample handling at this point is critical to overall success of the method. The second and third components describe the immunolabeling and visualization of the CC3 antibody, the marker for Dronc activity. With slight modifications, one could apply similar methods to a vast array of embryonic, larval, and adult tissues to aid in the study of both the apoptotic and non-apoptotic functions of Dronc.

2. Materials

2.1 General Supplies and Reagents

1. Many fly stocks can be obtained from the Bloomington Drosophila Stock Center (http://flystocks.bio.indiana.edu/bloomhome.htm) (See Note 1)

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2. Fly food and culture vials, incubator at 18°C or 25°C as needed, CO_2 source, humidifier, and anesthetizing pads
3. Dissecting stereoscope with independent flexible light source (ex. Fiber-Lite High Intensity Illuminator M-181-1 with dual gooseneck illuminator)
4. Nutator or rocking platform at room temperature and 4°C
5. Zeiss LSM 700 confocal microscope or similar compound microscope equipped with fluorescent lamp (ex. X-Cite Series 120Q, Lumen Dynamics), CCD camera, and image processing software such as ImageJ (http://imagej.nih.gov/ij/) [25]

2.2 Sample Preparation

1. 1x Phosphate-buffered saline (PBS): 137mM NaCl, 2.7mM KCl, 8mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.2. May be made at 10x, autoclaved, and stored at room temperature.
2. 1x Phosphate-buffered saline with Triton-X-100 (PBT): 1x PBS with 0.3% (v/v) Triton X-100. May be made at 10x, sterile filtered with a 0.22μm syringe filter and stored at room temperature.
3. 16% (w/v) Paraformaldehyde
4. Glass multi-well spot plates
5. Fine forceps (one #5 and two #55, Fine Science Tools by Dumont)
6. Microcentrifuge tubes (0.5 mL recommended)
7. Ice bucket

2.3 Immunolabeling and Visualization

1. Blocking solution: 1x PBT with 2% (v/v) Normal Donkey Serum
2. Rabbit anti-Cleaved-Caspase-3 (Asp175 Antibody, #9661, Cell Signaling Technologies) (See Note 2)
3. Appropriate fluorophore-conjugated anti-rabbit antibody
4. Vectashield Mounting Media with or without DAPI
5. 0.15mm black anodized steel insect pins and holders
6. Glass slides and coverslips
7. Clear nail polish

[1]The Bloomington *Drosophila* Stock Center has a large collection of tissue-specific and developmentally regulated GAL4 drivers, which can be combined with any number of UAS linked transgenes or RNAi constructs, which could be useful in studying the apoptotic and non-apoptotic functions of Dronc. We encourage you to consult recent original works to determine the appropriate parental lines for use in your experiments.

[2]The Cell Signaling Technologies CC3 antibody is a polyclonal antibody raised against the C-terminus of the large subunit of human Caspase-3. Some have noted varying levels of appropriate staining from lot to lot. If starting with a new lot of CC3, always compare to a lot previously determined to work in *Drosophila.* This will distinguish whether a failed procedure is due to a non-reactive antibody versus an improperly performed staining.

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8. Opaque freezer box
9. Opaque slide folder, tray, or box

3. Methods

3.1 Sample Preparation

This dissection technique is described assuming right hand dominance. Each person will likely find a variation of this method that works best for them.

1. Prepare a fresh aliquot of 4% (v/v) paraformaldehyde in 1x PBS, place on ice.
2. Using #5 forceps, transfer larva to a glass well spot plate with drops of cold 1x PBS. (See Note 4)
3. Using the #55 forceps, firmly grasp the larva in the middle with right hand, and remove the posterior half of the larva with the left. Discard the posterior half.
4. Gently holding the cuticle with the left hand forceps, grasp the most anterior part of the mouth hooks with the right forceps and push the mouth hooks through cuticle, inverting the larva and exposing the brain lobes and imaginal discs.
5. Quickly remove the salivary gland and fat body, along with any components of the larval gut, as these structures may release degradative enzymes that could compromise imaginal disc integrity.
6. Grasping the cuticle, transfer to a microcentrifuge tube with fresh PBS on ice. (See Notes 5, [6])
7. After dissecting 10 to 15 larvae, remove PBS from the tube and add 4% paraformaldehyde. Mix by pipetting until all samples are floating free from each other and the tube. Fix for 30 minutes at room temperature on a rotating platform. (See Note 7)
8. Remove fixative and wash in 1x PBT, three times for 5 minutes each at room temperature. (See Note 8)

[4]If the parental cross was set up such that dominant larval markers such as *Tubby* or a GFP reporter will positively or negatively mark the animals of interest, this is the appropriate time to sort them out. If sorting by fluorescence, keep the animals to be dissected on ice or transfer to new dishes with cold PBS immediately before dissection.

[5]Do not attempt to fully dissect the imaginal discs from the brain lobes or cuticle at this point. The discs are very small and very delicate even after fixation. By leaving them attached to the bulkier structures during the staining process, it will be much easier to transfer, fix, wash, and stain the discs without damaging them. The excess structures will be removed later. (See 3.3.2)

[6]Someone who is proficient in this technique can dissect on average one larva every 30 to 45 seconds. However, a novice may find that it takes significantly longer. Exposed discs may be transferred to fresh PBS in a microcentrifuge tube and kept on ice until fixation, but they should never be left for more than one hour. When beginning, dissect fewer animals at a time to reduce the time discs spend exposed in PBS before fixation.

[7]Do not over fix samples. This protocol does not have a separate antigen retrieval step and excessive fixation can mask epitopes. Do not exceed 60 minutes of fixation at room temperature or 1.5 hours on ice. Best results will come from the standard 30 minutes of fixation at room temperature.

[8]Exposed discs may be stored in PBT at 4°C overnight without any decrease in quality. Discs stored up to a week have been used successfully, but there can be noticeable decreases in quality of the samples.

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3.2 Immunolabeling of Samples

1. Preincubate samples in fresh blocking solution for 15 minutes at room temperature on a rotating platform. (See Note 9)
2. Incubate with anti-Cleaved-Caspase-3 antibody (1:100-1:500) diluted in fresh blocking solution, overnight at 4°C on a rotating platform. (See Notes 10, 11)
3. Wash in PBT three times for 5 minutes each at room temperature.
4. Incubate with a fluorophore conjugated anti-rabbit antibody for 6 hours at 4°C on a rotating platform. (See Note 12)
5. Wash in 1x PBT twice for 15 minutes each, followed by one wash in 1x PBS for 15 minutes, all at room temperature on a rotating platform.
6. Incubate in Vectashield Mounting Media for at least one hour at 4°C on a rotating platform. (See Note 13)

3.3 Mounting of Samples and Visualization of Cleaved-Caspase-3 antibody

1. Using #55 forceps carefully transfer samples to a glass slide with a drop of Vectashield. (See Note 14)
2. Using the dissecting needles (insect pins in appropriate holders), separate the imaginal disc of interest from the surrounding tissue. For example, if removing the eye-antennal disc, sever the connection between the antennal tissue and the mouth hooks. Then carefully separate the eye tissue from the brain lobes and sever the connection at the optic stalk.
3. Remove the cuticle and other debris from the slide and arrange discs for imaging.
4. Carefully apply a coverslip over the samples and seal on at least two sides with clear nail polish. (See Note 15)

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[9]All incubations from this point forward should be carried out in the dark to prevent degradation of the antibody or photobleaching of the sample. This is best achieved by keeping samples in a light-blocking freezer box. If this is not available, another option is to wrap sample racks in aluminum foil.

[10]Primary antibody may be reused for subsequent samples. Users will even notice an improvement in quality of CC3 staining with a reduction in background after the first one or two trials.

[11]It may be useful to include one or two additional antibodies at the same time to provide context to any CC3 positive cells—for example, co-staining 3rd instar eye imaginal discs with the pan-neuronal marker ELAV would distinguish the developing photoreceptor neurons in the posterior from the as of yet undifferentiated proliferating cells in the anterior. Take care to make sure that each antibody was raised in a different species which will prevent cross-reaction during the secondary incubation (ex. rabbit anti-CC3 and rat anti-ELAV labeled with donkey anti-rabbit FITC and donkey anti-rat CY3 work very well together)

[12]We routinely carry out our secondary incubations for 2.5 hours at room temperature, but this does increase background fluorescence. When trying a new antibody (primary or secondary) or when in need of publication quality images, carry out the secondary incubation at 4°C.

[13]If using Vectashield with DAPI, incubate for at least 2.5 hours. DAPI staining will only improve with longer incubation prior to mounting. Samples may even be stored in Vectashield for a few days at 4°C in order to allow mounting and imaging to occur on the same day (See Note 16)

[14]It is important to use sufficient Vectashield so that samples do not dry out on the slide during dissection; however, too much can also cause problems. If the droplet is too deep, structures will be free floating and it will be difficult to hold them in place to make clean cuts. Also, if there is too much Vectashield on the slide when placing the coverslip, samples will often move around and overlap, and any excess fluid outside the edges of the coverslip will prevent the nail polish from creating a secure seal.

[15]This step is most essential if slides will be imaged using oil immersion. If imaging with dry objectives, slides can be imaged and then sealed prior to storage. If there is too much Vectashield, the nail polish may pucker up or completely peel off within 24 hours. Repeat sealing process as necessary.

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5. View and image discs with a confocal microscope or compound microscope with fluorescent light source.

6. Store slides not currently being imaged in an opaque slide folder, tray, or after sufficient drying time in a slide box. (See Note 16)

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[16] We have found that when mounted properly, slides may be stored at 4°C overnight or at −20°C for at least a week without significant reduction in quality of CC3 signal. However, other antibodies or fluorophores may not be as stable. Best results will come from slides imaged on the same day they are mounted.

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