Video Article

Live Imaging of Apoptotic Cell Clearance during *Drosophila* Embryogenesis

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Abstract

The proper elimination of unwanted or aberrant cells through apoptosis and subsequent phagocytosis (apoptotic cell clearance) is crucial for normal development in all metazoan organisms. Apoptotic cell clearance is a highly dynamic process intimately associated with cell death; unengulfed apoptotic cells are barely seen *in vivo* under normal conditions. In order to understand the different steps of apoptotic cell clearance and to compare 'professional' phagocytes - macrophages and dendritic cells to 'non-professional' - tissue-resident neighboring cells, *in vivo* live imaging of the process is extremely valuable. Here we describe a protocol for studying apoptotic cell clearance in live *Drosophila* embryos. To follow the dynamics of different steps in phagocytosis we use specific markers for apoptotic cells and phagocytes. In addition, we can monitor two phagocyte systems in parallel: 'professional' macrophages and 'semi-professional' glia in the developing central nervous system (CNS). The method described here employs the *Drosophila* embryo as an excellent model for real time studies of apoptotic cell clearance.

Video Link

The video component of this article can be found at http://www.jove.com/video/50151/

Introduction

The proper elimination of unwanted or aberrant cells through apoptosis and subsequent phagocytosis is crucial for embryonic development as well as for tissue homeostasis in the adult. Phagocytosis of apoptotic cells or apoptotic cell clearance is a highly dynamic process which proceeds in four steps: (1) *recruitment* of phagocytes to the apoptotic cell ('find-me'), (2) *recognition* of the cell as a target for phagocytosis ('eat-me') and (3) *engulfment*, followed by (4) *phagosome maturation* and degradation of the apoptotic particle[1-5]. There are two types of phagocytes: 'professional' macrophages and immature dendritic cells, and the 'non-professional' tissue-resident neighboring cells, which are crucial for apoptotic cell clearance during metazoan development[6,7].

In *Drosophila* development, the elimination of superfluous cells through apoptosis occurs in three main phases, first in the mid to late embryo, then in the mid pupa, and again in the early adult. During embryogenesis apoptotic particles are removed by 'professional' phagocytes, macrophages, and by the 'non-professional' ectoderm and glia[8,9].

In our previous work we have identified a phagocytic receptor, Six-microns-under (SIMU), which is expressed exclusively in phagocytic cells during embryogenesis. Using the *simu* promoter we generated a specific marker for phagocytic cell populations (*simu-cytGFP*) which enables us to monitor macrophages, ectoderm and glia simultaneously in a live developing embryo[8]. In addition, by using specific markers for apoptotic cells in different stages of apoptosis, we are able to follow the dynamics of apoptotic cell clearance *in vivo*.

Protocol

To follow the dynamics of apoptotic cell clearance *in vivo* two populations of cells must be labeled: phagocytic cells and apoptotic cells.

To mark phagocytic cell populations we use a *Drosophila* line containing the *simu-cytGFP* marker, which labels exclusively phagocytic cells in the embryo: macrophages, glia and ectoderm[8]. One can use different markers for phagocytic cells, including lines containing a hemocyte-specific Gal4 driver (*crq-Gal4*) or glia-specific Gal4 driver (*repo-Gal4*) and a genetically encoded fluorescent reporter under UAS control (*uas-GFP*).

To monitor apoptotic cells during apoptotic cell clearance we use different apoptosis/phagocytosis markers. Annexin V (Molecular Probes) serves as an early marker for apoptotic cells [10]; Phiphilux (OncoImmunin) is a fluorogenic caspase-3 substrate, which is used as a later apoptosis marker, and LysoTracker (Molecular Probes) works as a phagosome marker. We inject these reagents using the microinjection system PicoPump PV 820.

1. Getting Ready

1. Heptane glue:

   Unroll double sided tape and put as much as you can in a scintillation vial, fill with heptane, seal the vial with Parafilm, and rock for 24 hr. Add heptane if the glue is too thick.
2. We allow the flies to lay on a pre-warmed grape juice agar plate + yeast paste for 2 hr, and then transfer the plate to 25 °C for 10-12 hr (depends on the desired stage) prior to microinjection and time lapse recordings of injected embryos. We collect embryos from the agar plates maintained at 25 °C, thus providing embryos from stage 15 or 16 of development.
3. Needle preparation:

   To prepare needles for injection, we use a 'Sutter instrument' needle puller and thin walled glass filaments (FHC Capillary tubing). The tip of the pooled capillary is broken to a diameter of 0.5-2 µm.
4. Coverslip with glue:

   Using a pipette tip disperse a drop of the heptane glue in one line in the middle of the coverslip. Let it dry for a few seconds. Prepare a few of these coverslips.

2. Embryo Preparation

1. Collect embryos from agar plates and transfer them to a cell strainer (SPL Life Sciences) using a clean paint brush and running water. The strainer is held over a beaker to collect waste water.
2. Wash embryos in the cell strainer using water until all the yeast paste is removed.
3. Dry out the excess water by wiping the outside of the strainer using Kimwipes.
4. Place the cell strainer in a clean Petri dish and add enough 50% bleach to cover embryos in the cell strainer. Dechorionate the embryos for 2 min occasionally (2-3 times) stirring them.
5. Rinse with water extensively until embryos loose the bleach odor.
6. Place the cell strainer into a clean Petri dish and cover the embryos with water to prevent drying up.
7. Before starting embryos setting, load 1 µl of the desired reagent into two needles (sometimes a needle can be clogged). It takes about 5 min for the liquid to reach the tip of the needle.
8. Cut a rectangular piece of grape juice agar and place it on a microscope slide. Transfer dechorionated embryos from the Petri dish to the agar piece using a clean paint brush.
9. Under a fluorescent dissection microscope select properly staged embryos expressing the GFP marker using a wet paint brush and place each embryo close to the edge of the agar piece in a row one after another (**Figure 1A**). The embryos should be placed with their ventral side up for imaging phagocytosis by glia in the Central Nervous System (CNS).
10. Set up about 10 embryos in one row.
11. Attach the embryos to a coverslip coated in the middle with a strip of the heptane glue (**Figure 1B, C**).
12. Place the coverslip in the dehydration chamber for about 5 min (this depends on the humidity of the room and the amount to be injected).
13. After drying up cover the embryos with halocarbon oil 700 (Sigma) to avoid further dehydration.
14. Place the coverslip on a microscope slide with the embryos facing up. You may put a drop of water on the slide to prevent moving of the coverslip. Embryos are now ready for injection. Injection location is typically on the lateral side in the middle of the embryo.

3. Embryo Injection

1. Attach the needle to a micromanipulator.
2. To break the needle tip put the edge of the coverslip in the field of view and adjust the needle to the same focal plane. Very carefully move the coverslip until it hits the needle tip and breaks it.
3. Focusing on the embryos, place the needle into the oil and make sure you get a liquid drop from the needle. It means the needle tip has been broken well.
4. Without touching the needle holder move the embryo into the needle and inject a drop into the embryo. Move the embryo away and proceed to the next one until all the embryos are injected.

4. Imaging

We image the embryos on an inverted confocal microscope with a 40X or 100X objective. We look for a nicely positioned embryo with the CNS in the middle, which shows strong GFP expression and a good labeling of apoptotic cells following injection.

For time lapse recordings of live embryos we usually choose 5 or 6 confocal slices (2 µm thick each). Afterwards, we make a projection of 3 slices (6 µm thickness) in order to observe whole cells.

We use markers for apoptotic cells which are stable and do not bleach easily. To avoid GFP bleaching we make recordings in intervals of 60 sec.

In some cases embryos may start rolling during the time of video recording. Therefore, we take a look once in a while at the recording in order to stop and start over if necessary.

Representative Results

Representative frames from a movie in which apoptotic cells are labeled with Annexin V, and glia, ectoderm and macrophages are labeled with *simu-cytGFP* are shown in **Figure 2**. Each frame is a projection of 3 slices 2 µm each.

The embryo of stage 15 is properly positioned showing the embryonic CNS in the middle with well labeled glia (g). Macrophages (m), which are mostly outside the CNS, show strong cytoplasmic GFP expression. Ectodermal cells are also labeled with cytoplasmic GFP (e). Many Annexin V positive cells are seen inside and outside the CNS. To follow the engulfment event is not easy. We highlighted one event with a white rectangle where a glial cell is engulfing an apoptotic particle. Note the probing behavior of the glial cell: touching the apoptotic particle a few times without engulfing it and then ending up with engulfment of the Annexin V labeled apoptotic cell (**Figure 2**).

Additional markers for different apoptotic stages could be used with the same procedure.

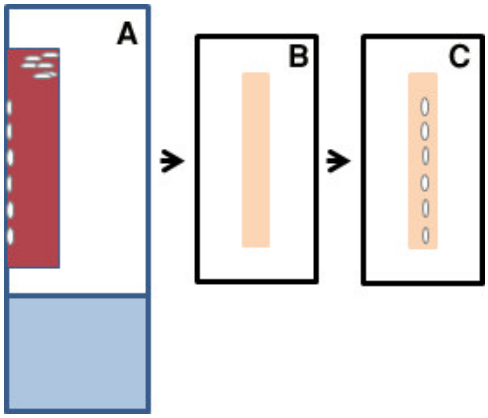

Figure 1. Schematic representation of embryo preparation for microinjections. A. A microscopic slide containing a piece of agar on which embryos are transferred after dechorionation. About 20 embryos are placed in line, close to the edge of the agar piece, with their ventral side up, for imaging the CNS. **B.** A coverslip containing a strip of heptane glue in the middle. **C.** The coverslip from B with embryos attached to the heptane glue strip.

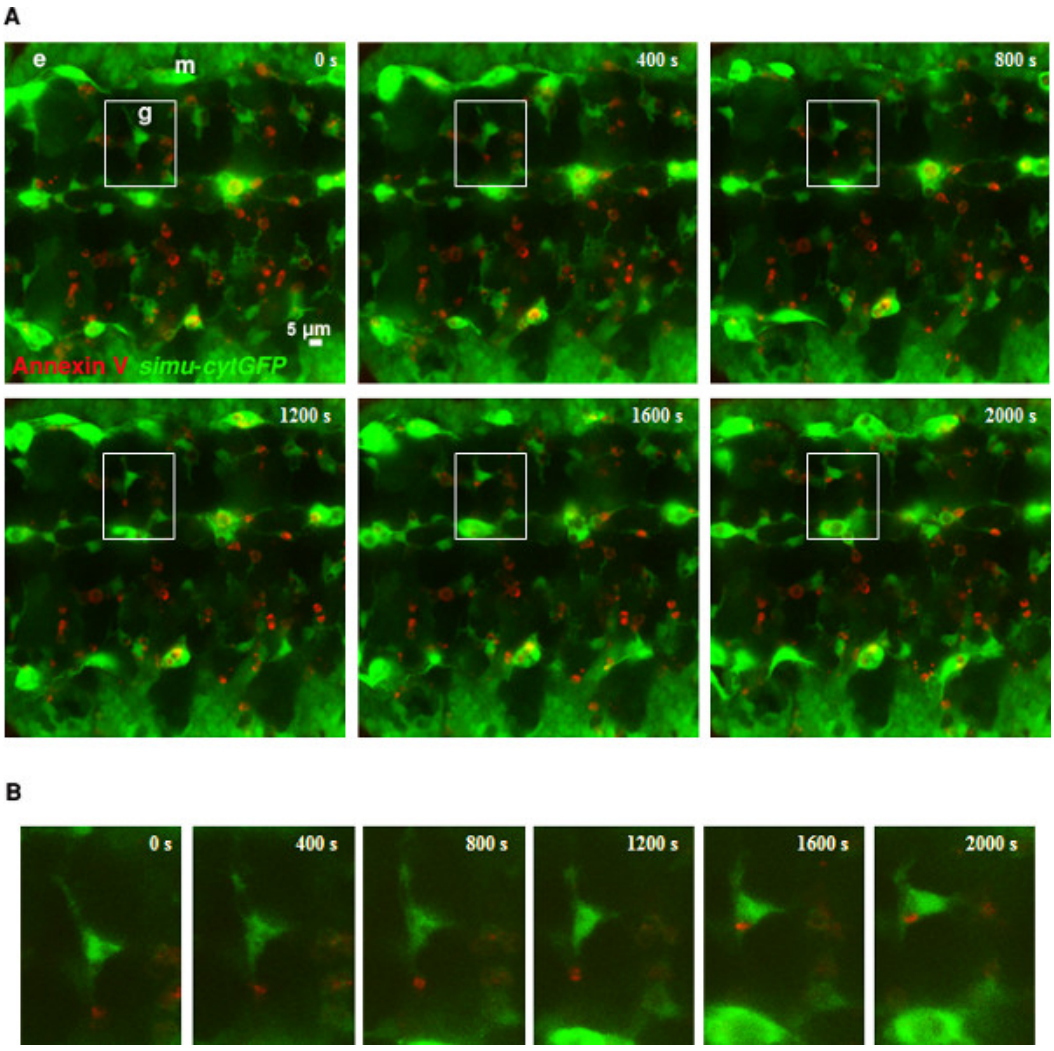

Figure 2. Dynamic analysis of apoptotic cell clearance. Time-lapse recordings of apoptotic cell clearance in a stage 15 embryo. Glia (g), macrophages (m) and ectoderm (e) are labeled with *simu-cytGFP* (green). Apoptotic cells are marked with the fluorescent Annexin V (red). **A.** Selected frames from a representative movie are shown. A glial cell engulfing an apoptotic cell is depicted by a rectangle. **B.** Close up views of the marked rectangle.

Discussion

Apoptotic cell clearance is a critical last stage of apoptosis, which is highly dynamic. Therefore real time studies of the process are of utmost importance. Here we describe a protocol that enables monitoring of apoptotic cell clearance in living developing *Drosophila* embryos. In this procedure, two populations of cells must be marked using specific markers: apoptotic cells and phagocytes. The *simu-cytGFP* reporter is suitable for monitoring phagocytic macrophages, glia and ectoderm simultaneously in the same embryo, *w*hich makes it possible to follow different phagocytic cell populations at the same time. This is a great advantage for studying 'professional' and 'non-professional' clearance concurrently, comparing specific aspects of the process such as rate, specificity and degradation ability. A disadvantage of this approach is that *simu-cytGFP* marker is expressed extremely strongly in macrophages and much weaker in glia, which could be tricky to adjust in the same movie. However, one can image apoptotic cell clearance by macrophages separately from this process in the CNS, by driving GFP expression with specific Gal4s (*crq-Gal4* for macrophages and *repo-Gal4* for glia).

Different markers for apoptotic cells reflecting specific molecular and morphological alterations are of great value for understanding the dynamics and reversibility of apoptosis and cell clearance. Moreover, real time recordings of apoptotic cell clearance using these markers add additional information about mutant phenotypes, which could not be achieved by using conventional immunohistochemistry techniques in fixed embryos.

The most difficult step of the described protocol, when imaging the developing CNS, is the extremely precise positioning of the embryos before injection. Even a slight move of the coverslip during attachment of the prepared embryos to the glue could change their position, which would not be appropriate for imaging the CNS. The embryo attaching step should be done with maximum attention.

Development of additional markers for specific stages in apoptosis and phagocytosis would be extremely desirable for finer dissection and possible manipulation of apoptotic cell clearance. The protocol presented here serves as a good basis for future development.

Disclosures

The authors declare that they have no competing financial interests.

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