Video Article

Live Imaging of Cell Extrusion from the Epidermis of Developing Zebrafish

George T. Eisenhoffer[1], Jody Rosenblatt[1]

[1]Department of Oncological Sciences, Huntsman Cancer Institute, University of Utah

Correspondence to: Jody Rosenblatt at jody.rosenblatt@hci.utah.edu

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Abstract

Homeostatic maintenance of epithelial tissues requires the continual removal of damaged cells without disrupting barrier function. Our studies have found that dying cells send signals to their live neighbors to form and contract a ring of actin and myosin that ejects it out from the epithelial sheet while closing any gaps that might have resulted from its exit, a process termed cell extrusion[1]. The optical clarity of developing zebrafish provides an excellent system to visualize extrusion in living epithelia. Here we describe a method to induce and image extrusion in the larval zebrafish epidermis. To visualize extrusion, we inject a red fluorescent protein labeled probe for F-actin into one-cell stage transgenic zebrafish embryos expressing green fluorescent protein in the epidermis and induce apoptosis by addition of G418 to larvae. We then use time-lapse imaging on a spinning disc confocal microscope to observe actin dynamics and epithelial cell behaviors during the process of apoptotic cell extrusion. This approach allows us to investigate the extrusion process in live epithelia and will provide an avenue to study disease states caused by the failure to eliminate apoptotic cells.

Video Link

The video component of this article can be found at http://www.jove.com/video/2689/

Protocol

Basic Workflow for the Visualization of Actin Dynamics During Cell Extrusion in the Epidermis of Developing Zebrafish

The epidermis of the developing zebrafish is comprised of two distinct layers, the surface layer (or periderm) and a basal layer of cells that contact the basement membrane[2]. The cells of the outer surface layer undergo apoptosis and are eliminated from the tissue by extrusion[3] **(Figure 1)**. To visualize this process in real time, we inject RNA encoding red fluorescent protein fused to the calponin homology domain of utrophin, an actin binding protein (RFP-UtrCH) [4,5], into one-cell stage transgenic zebrafish expressing green fluorescent protein (GFP) under the stratified epithelia promoter cytokeratin 8[6] **(Figure 2A)**. Although RFP-UtrCH is ubiquitously expressed in the animal after RNA injection, we focus on the superficial epidermal cells that express both RFP and GFP to follow actin dynamics specifically in the epidermis **(Figure 2B)**. We then treat the larvae with G418 to induce apoptotic cell extrusion and image the epidermis and actin filaments using a spinning disc confocal microscope and acquire 4D datasets.

Prior to Starting the Experiment

1. *In vitro* transcribe the RNA from a linearized DNA template using the mMessage mMachine SP6 kit (Ambion) and purify the capped RNA using the RNeasy Mini kit (Qiagen). Dilute the RNA to ~60ng/ul using sterile water, make 3-5µl aliquots and store at -80°C until ready for injection. Note: Avoid repetitive freeze/thawing to prevent degradation of the RNA.
2. Make a mold to hold the embryos during injection by pouring 40mL of 2% agarose in E3 embryo medium (E3) into a 100 x 15mm culture plate and placing a microinjection mold (Adaptive Science Tools, # I-34) in this tray. Once the agarose has solidified, remove the mold to expose the troughs in the agarose. Store the plate at 4°C until ready for use.
3. Pull capillary needles for injection using borosilicate glass with filaments (OD 1.0mm, ID 0.78, 10cm length) and a Sutter P-97 Pipette Puller at the following settings (Heat 485, Pull 50, Velocity 60, Delay/Time 90, Pressure 200). Note: Different types of glass will require optimization of the listed conditions.
4. Make up 1% Low Melt (LM) agarose in E3 and store 1mL aliquots at 42°C. Be careful as evaporation of E3 from the stocks can increase the agarose concentration.
5. Maintain adult zebrafish under standard laboratory conditions, with a regular light/dark cycle of 14 hours light and 10 hours of darkness[7]. The night before the experiment set up fish to mate by placing 3 female and 2 males in a tank separated by a divider. The next morning, change the water in the tank and pull the divider when the lights come on.

1. Injection of RNA Encoding the Fluorescently Tagged Actin Binding Protein

1. Thaw the RNA on ice. Add 0.5% Phenol Red to the RNA at a 1:1 ratio for a final RNA concentration of ~30ng/µl and load 1µl solution into a pulled capillary needle by back-filling.
2. Collect ~75 1-cell stage[8] CK:GFP embryos in E3. Pipet the collected embryos into the microinjection mold with a 5 ¾ Pasteur pipet and gently orient the embryos in the trough with FST Dumont #5 forceps. Note: Take care to work quickly as to avoid injecting into a multi-cell stage embryo, which can result in mosaic expression of the injected RNA.
3. Under a standard laboratory dissecting stereomicroscope, inject the RNA into the yolk of the embryos using a pressure-controlled microinjector (Harvard Apparatus PLI-100 Pico-Injector). A red spot (phenol red) should be visible in the embryo after injection. Inject at least 50 embryos for each experiment. (See the previous JoVE protocol for a detailed protocol on RNA injection into zebrafish embryos[9]). Note:
    1. A volume of ~2nl should be used for injection of RNA into one-cell stage embryos. To determine the amount of RNA injected, dispense a bolus of the RNA into a drop of mineral oil on a calibrated micrometer slide or under a microscope with a scale bar built into the eyepieces. The droplet of RNA should be a perfect sphere. Calculate the amount delivered by using the equation: Volume (in nl) = $4/3\pi r^3$. Adjust the volume delivered by changing the injection pressure or time on the instrument.
    2. Care should also be taken to inject the lowest amount of RNA that allows visualization of the fluorophore, as high concentrations of RNA can be toxic to the developing embryo. To determine appropriate expression level, a dose-curve experiment should be performed before running the experiment.
4. Sort the embryos to remove any unfertilized or damaged eggs and place all remaining embryos at 28.5°C.
5. After 24 hours, use a fluorescent dissecting microscope to select zebrafish embryos that have a high level of GFP (epidermis) and RFP (actin) fluorescence. Place the selected embryos at 28.5°C until ready to proceed with the drug treatment to induce apoptosis.

2. Induction of Cell Extrusion in Zebrafish Larvae using G418

We have found that exposure to the aminoglycoside antibiotic G418, or Geneticin, causes apoptosis and extrusion of epidermal cells in developing zebrafish larvae[3] by an unknown mechanism. Importantly, this treatment only works on larvae that are 4 days post fertilization and older. We find that ~ 5-25 cells can be found extruding at any given time from the fin epithelial of G418 treated larval zebrafish. As the amount of apoptotic extruding cells can vary from fish to fish, we recommend mounting multiple fish for imaging.

1. Collect and place 4 day post fertilization (dpf) zebrafish larvae exhibiting both GFP and RFP fluorescence into a 35 x 10mm culture dish containing 3 milliliters (mLs) of E3. One can treat up to 25 larvae in this size culture dish.
2. Carefully replace the media with 3mLs of E3 containing 1mg/mL of G418 and incubate larvae in the drug for 4 hours at 28.5°C. Note that G418 is light sensitive, so take care to keep the culture dish covered or in the dark.
3. Remove media and replace with pre-warmed 28.5°C E3 media containing 0.02% Tricaine and proceed to mounting the larvae.

3. Mounting Zebrafish Larvae for Imaging

1. Transfer 3 larvae to a 1.5 ml eppendorf tube and remove most of the E3 after the fish sink to bottom of tube. We have found that up to 3 animals can be properly mounted before the agarose solidifies.
2. Using a cut pipet tip, pipet 120ul of 1% LM-agarose (42°C) into the tube, mix, and then gently pipet the larvae and agarose mixture onto the coverglass in the bottom of a MatTek culture dish.
3. Using a FST pin holder with an insert pin or tungsten needle attached, quickly orient the larvae so they are straight and flat against the coverglass of the MatTek dish.

    Note: We typically use a straight pin to first orient the larvae and then an L-shaped pin to ensure they are flat against the coverglass while preventing damage to the specimens.
4. Once the agarose has solidified, gently fill the dish with E3 containing 0.02% Tricaine. Note: Alternatively, G418 can be added to the E3 in the imaging dish to continue inducing extrusion, although long-term exposure to 1mg/mL G418 will kill the larvae.

4. Live Imaging of Actin Dynamics and Individual Epithelial Cell Behaviors During Cell Extrusion using a Spinning Disc Confocal Microscope

We have found that the entire process of apoptotic cell extrusion from the epidermis of developing zebrafish larvae takes approximately 20 minutes[3]. Here we demonstrate how to setup a time-lapse imaging experiment to collect a series of z planes through a single layer of the zebrafish epidermis to visualize the extrusion process. We have found that typical widefield fluorescent microscopes cause significant photobleaching of the actin filaments and do not allow for timelapse imaging. Therefore, to maximize our resolution and prevent photobleaching, we use a spinning disc confocal microscope. Below we describe how to set up the experiment using the Andor IQ software with a Nikon microscope, although the principles discussed can be applied to similar microscope set-ups.

1. First, turn on the Argon (for excitation of GFP at 488nm) and HeNe (for excitation of RFP at 561nm) lasers, microscope, camera and epifluorescent lamp.
2. Within the Andor IQ software (version 1.10.1), create a time series protocol containing the wavelengths you want to image, the frequency of the intervals between image captures and the number times the capture should be repeated.
3. Gently place the MatTek dish containing your specimen on the microscope stage and use a 20x objective with transmitted light to focus on the larvae.
4. Move the 40X water immersion objective (N.A. 0.8) into the correct position. Using this objective, we can film approximately 20-25 cells per field, which allows us to follow cellular behaviors during extrusion while also resolving sub-cellular actin dynamics. Note: The same mounting

and imaging strategies can be applied to upright microscopes, although a 40X water immersion objective with a long working distance must be used.

5. Carefully place a drop of water on the top of the 40x objective and quickly place the MatTek dish on top. Note: Zeiss Immersol immersion solution can also be used if evaporation of the water is a problem.
6. Identify the specimen using phase or DIC illumination and then use 488nm epifluorescence to focus specifically on the GFP positive epidermis.
7. Switch to confocal scanning mode. Adjust the laser intensity and exposure time for each of the channels (488nm and 561nm) accordingly. Take care to balance the laser power and time of exposure for optimal detection of your signal and to prevent any photobleaching or toxicity.
8. To identify extruding cells, use epifluorescence to visualize the GFP labeled epidermis and identify a group of cells in a classic rosette pattern, consisting of a collection of cells surrounding a small round cell in the middle. Using confocal scanning mode, there should also be an accumulation of the RFP-labeled actin visible as a ring around the small round cell in the middle, a hallmark of cell extrusion. Once you have identified an extruding cell, quickly set up the microscope to acquire 4D (X-Y-Z-Time) confocal datasets.
9. Set the upper and lower points within the z plane to visualize a single layer of the epidermis, including the extruding cell, and select the step size. For time-lapse imaging, set the intervals for image capture and number of repetitions. For example, we typically acquire a z-series spanning 5-10µm, with a 1 µm step size, every 1-2 minutes for 30 minutes.
10. To view the data, we typically make 3-D projections of the z series and save the data set as a movie file that is compatible with Quick Time (Apple). In this manner, we can visualize contraction of the actin ring and epithelial behaviors that occur around the dying cell over time.

5. Representative Results

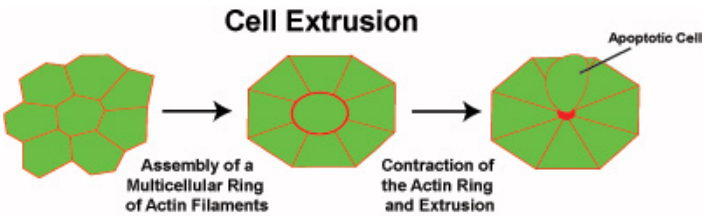

Figure 1. Schematic of apoptotic cell extrusion. Actin filaments are shown in red, GFP positive epidermal cells are green.

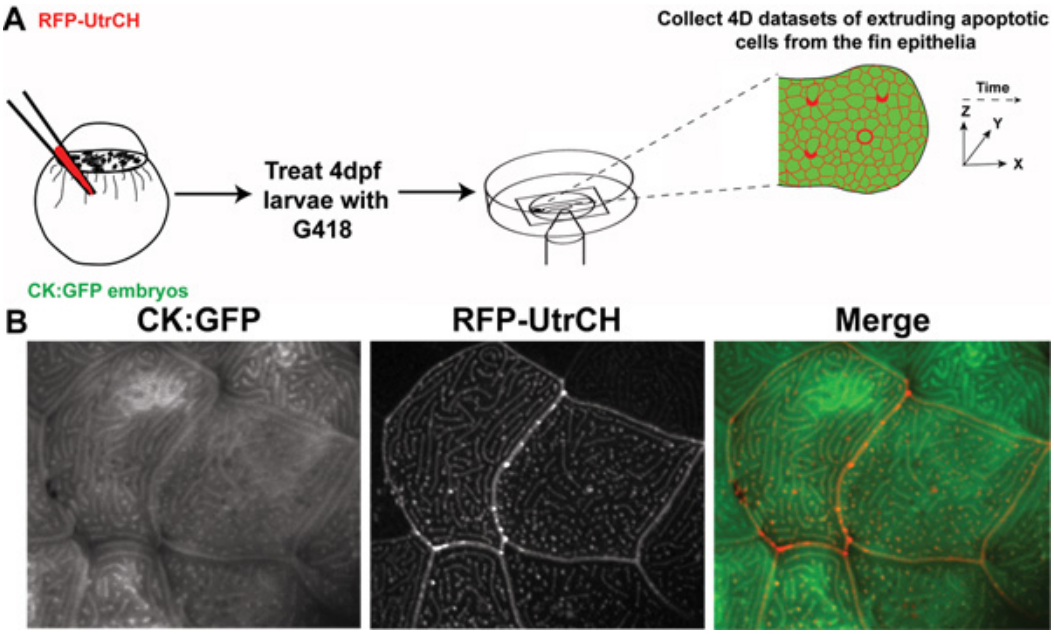

Figure 2. Experimental Workflow. A. Schematic of the workflow for the experiment. **B**. Expression of RFP-UtrCH in the epidermis of a 4dpf CK:GFP zebrafish.

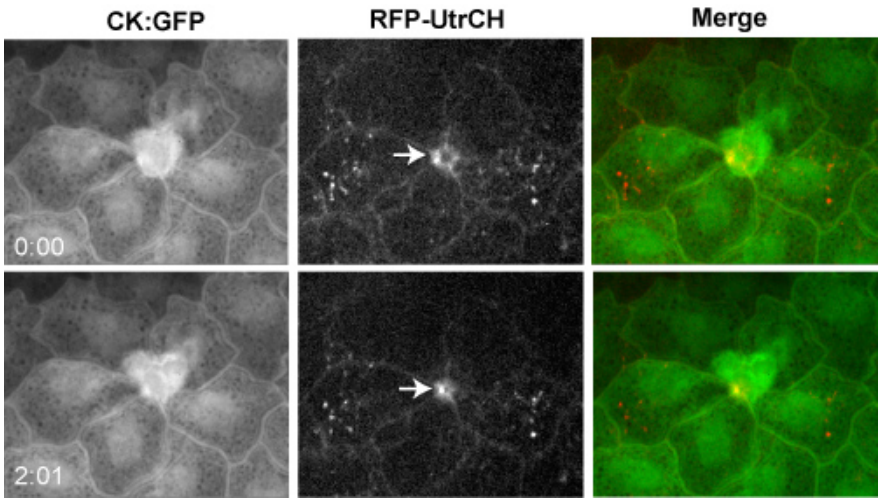

Figure 3. Still frames (z-projections) from a time-lapse movie that follows live actin dynamics during the process of epithelial cell extrusion. Arrows denote the ring of actin formed by neighboring cells which contracts and closes over the course of 2 minutes.

Discussion

The protocol described here demonstrates a simple and straightforward method to visualize the process of extrusion in a live epithelial tissue. This type of experiment allows us to examine subtleties of actin dynamics not previously appreciated in fixed tissue analyses, and therefore, complements common immunofluorescent methods. We can use this protocol in combination with chemical inhibitors or genetic mutants to better analyze the extrusion process and study disease states associated with the failure to remove and clear apoptotic cells, which we expect will lead to inflammatory responses or the accumulation of damaged cells, as seen in cancer. For example, our laboratory has previously shown that chemical inhibitors can be used in combination with G418 to alter the targeting of the actin filaments along the basolateral surface of the cell, which effects the direction a cell extrudes[3]. One limitation to the current method is that due to the stochastic and unpredictable nature of cell death, our datasets tend to focus on the later stages of extrusion. To study the formation of the multicellular actin ring and initiation of contraction, future experiments will apply techniques to induce apoptosis in a subset of fluorescently-labeled epithelial cells that are genetically targeted for cell death[10]. Combining this strategy with our method to image actin dynamics in the epidermis should facilitate studies of the early steps leading to apoptotic cell extrusion. Together, the information gained from these experiments will add significantly to our understanding of epithelial cell extrusion and its medical significance.

Disclosures

Animal Welfare Assurance

All procedures performed in this protocol using the zebrafish, *Danio rerio*, have been approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Utah (Animal Welfare Assurance #10-07017).

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