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Imaging Cytonemes in *Drosophila* Embryos

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Abstract

Conserved morphogenetic signaling proteins disperse across tissues to generate signal and signaling gradients, which in turn are considered to assign positional coordinates to the recipient cells. Recent imaging studies in *Drosophila* model have provided evidence for a “direct-delivery” mechanism of signal dispersion that is mediated by specialized actin-rich signaling filopodia, named cytonemes. Cytonemes establish contact between the signal-producing and target cells to directly exchange and transport the morphogenetic proteins. Although an increasing amount of evidence supports the critical role of these specialized signaling structures, imaging these highly dynamic 200 nm-thin structures in the complex three-dimensional contour of living tissues is challenging. Here, we describe the imaging methods that we optimized for studying cytonemes in *Drosophila* embryos.

Keywords

Cytonemes; Cell-cell signaling; Morphogen; *Drosophila*; Embryo; Filopodia; Trachea; *Engrailed*; *Breathless*

1 Introduction

The body plan of a multicellular organism is determined by the concentration gradients of morphogens, such as Hedgehog (Hh), Wingless (Wg), Decapentaplegic (Dpp/Transforming growth factor β family protein), fibroblast growth factor (FGF), and epidermal growth factor (EGF). These proteins disperse from a restricted source and pattern a naïve field of cells into regions of distinct differentiation trajectories by inducing concentration-dependent differential gene expression [1–7]. However, the mechanisms by which signals disperse to form concentration gradients are poorly understood. Several contrasting models have been proposed to explain how morphogens disperse through tissues to establish a positional gradient. These include free-, restricted-, and facilitated-diffusion, transcytosis, localized translation of graded mRNA, and direct delivery through cytonemes [8–16]. In this chapter, we focus on imaging methods of *Drosophila* cytonemes that mediate direct delivery of signals in developing tissues. Recent research in vertebrate and invertebrate systems has revealed that many morphogenetic signals localize in long cellular membrane protrusions or cytonemes to travel target-specifically from the source to the recipient cells (reviewed in [17, 18]).

Cytonemes were first described in the *Drosophila* wing imaginal discs as thin actin-based filopodia that originate from the apical side of the GFP-marked wing disc cells and orient

toward the Dpp morphogen source at the center of the disc [19]. These cytonemes were found to be responsive to Dpp and contained the Dpp receptor Thickvein [20]. Several subtypes of *Drosophila* cytonemes were identified that showed signal-specific responses under the influence of either EGF, FGF, Dpp, and Hh [21]. Subsequent studies in the *Drosophila* air sac primordium (ASP), a wing disc-associated tracheal tube that grows in response to Dpp and Branchless (Bnl, an FGF family protein) produced in the wing disc, established that cytonemes are essential conduits of morphogens [21–23].

ASP cells extend signal-specific polarized cytonemes to establish cell-cell membrane contacts with the *bhl* and *dpp* sources in the wing disc (Fig. 1a, b). The ASP cells that express receptors for both Dpp and Bnl sort these receptors into distinct, signal-specific cytonemes (Fig. 1a). Signal-specific cytonemes were found to interact with components of the extracellular matrix (ECM), through which cytonemes navigate [24]. Cytonemes from the ASP that contact the Dpp source in the wing disc localize the Dpp receptor, receive Dpp, and transport receptor-bound ligand from the source to recipient cells. Many proteins, such as activated Diaphanous (formin, actin nucleator), Neuroglian (L1-CAM), and Capricious (LRR domain-containing membrane protein involved in neuronal matchmaking) localize at the cytoneme tips and were found to be essential for establishing signaling contacts with the source cells. Removal of their functions from ASP cells affected cytonemes or cytoneme contacts, and consequently led to a reduction in signal transfer and signal transduction in the ASP cells [23, 25]. These results provided first convincing evidence that cytonemes are essential for signal transport and signaling. Importantly, signal-producing cells also send cytonemes to deliver signals to the recipient cells. For example, Hh-producing cells in the *Drosophila* wing imaginal disc and abdominal histoblasts extend cytonemes to deliver Hh and were found to be essential for Hh signaling gradient [26].

Cytonemes and cytoneme-like filopodial projections are abundant in developing tissues of vertebrate and invertebrate models [27, 28]. Almost all families of signaling proteins, including Hedgehog, EGF, WNT/Wingless, Notch/Delta, and FGF [29] are now reported to be localized in cytonemes or cytoneme-like cell projections [30–35]. The *Drosophila* system is an ideal model to characterize this conserved signaling mechanism, given the powerful genetic tools available to engineer and regulate gene expression, standardized genome editing methods, ease of histological studies and imaging, and several signaling systems that are well-characterized. To image the thin and fragile cytonemes in live tissues and visualize their signaling activities at cellular and subcellular resolutions, optimization of a noninvasive imaging method is the most critical requirement. So far, studies on cytonemes are reported from postembryonic developmental stages of *Drosophila* [21,23, 36]. Here, we describe the methods optimized for imaging cytonemes emanated from embryonic tracheal and *engrailed*-expressing epidermal tissues.

2 Materials

Make all stock buffers in sterile deionized water (dH₂O) and avoid contamination of the stock solutions by keeping them in 50 mL aliquots in sealed screw-capped bottles at the recommended temperature.

2.1 Equipment for Fly Pushing

1. Stereomicroscope with dual gooseneck fiber-optic illuminator (e.g., Standard Olympus SZ61 or Nikon SMZ745).
2. *Drosophila* workstation with CO₂ pad, as described by [37].
3. Incubator (25 °C, Peltier Refrigerated incubator).
4. Fly stocks: (1) tissue-specific *Gal4* or *LexA* driver lines (2) *UAS* or *LexAop* reporter lines that can tissue-specifically express membrane localized fluorescent protein (FP) reporter gene. For instance, CD8:GFP. Selection of fluorophore and driver is important (see **Note 1**). Stocks can be obtained through public stock centers:
 - a. <https://bdsc.indiana.edu/index.html>.
 - b. <https://kyotofly.kit.jp/cgi-bin/stocks/index.cgi>.

For example, here we used the following transgenic lines to express FP in tissue pattern: for trachea-specific expression—(1) *breathless-Gal4* (*btl-Gal4*) driver, (2) *UAS*-mCD8:GFP reporter [21, 38]; for epidermal expression—(1) *engrailed-Gal4* driver (*en-Gal4*) driver, (2) *UAS*-CD4:IFP2 [39].

2.2 Embryo Collection

1. FlyStuff grape agar premix (see **Note 2**).
2. Deionized water (dH₂O).
3. Dry baker's yeast. Make a thick paste of required amount of dry yeast with dH₂O in a beaker (see **Note 3**).
4. 50% sodium hypochlorite: 50% V/V sodium hypochlorite in deionized water. Make fresh.
5. 1× PBS buffer (pH 7.5).
6. PBST (embryo wash): 1% V/V Triton X-100 in 1× PBS (see **Note 4**).
7. Petri Dish, 60 × 15 mm².
8. Small embryo collection cage (Genesee Scientific, Fig. 2).
9. Metal spatula.
10. Fine-tip paintbrush.
11. Dissection watch glass.
12. Falcon cell strainer (fits 50 mL tube; pore size: 40–100 μm).

2.3 Preparing Live *Drosophila* Embryos

1. dH₂O.
2. Fine-tip paintbrush.

3. Forceps (Dumont no. 5, Fine Science Tools).
4. Microcentrifuge tubes (1.5–2 mL).
5. Glass bottom dishes, 35 mm petri dish, 14 mm glass diameter, No. 1 coverslip (MatTek # P35G-1.5-14-C).
6. Cavity slide (single concavity of 15–18 mm, depth 0.6–0.8 mm; L × W 76 mm × 26 mm, thickness 1.2–1.5 mm).
7. Halocarbon oil 27.
8. Kimwipes.
9. Needle.
10. Stereomicroscope (as described in Subheading 2.1).

2.4 Preparing Fixed *Drosophila* Embryos

1. 4% PFA: 4% V/V formaldehyde in PBS. Make fresh. Do not store.
2. Heptane.
3. Methanol (MeOH).
4. 0.1% PBST: 0.1% V/V Triton X-100 in 1×PBS.
5. 75% MeOH/25% (0.1% PBST).
6. 50% MeOH/50% (0.1% PBST).
7. 25% MeOH/75% (0.1% PBST).
8. Fine-tip paintbrush.
9. Microcentrifuge tube (1.5–2 mL).
10. Forceps.
11. Orbital shaker.
12. Parafilm M.
13. Needle.
14. Microscope slides.
15. Coverslips (22 mm² and 22 × 40 mm²).
16. Clear nail polish.
17. VECTASHIELD Antifade mounting medium.
18. Timer.

2.5 Image Acquisition and Analysis

1. Laser scanning confocal microscope (e.g., Leica SP5X with a low noise HyD hybrid detector and Z-galvo) either inverted or upright (*see Note 5*).

2. Yokogawa CSU-X1 spinning disc confocal microscope (PerkinElmer UltraView Vox) with an EMCCD camera (Hamamatsu) and a piezo-Z.
3. Stage: Universal mounting frame.
4. Long-range apochromat objectives (e.g., Leica 20×/multi-immersion 0.7 NA Plan Apo, 40×/oil 1.25 NA Plan Apo, and 63×/oil 1.4–0.6 NA plan Apo).
5. Multiple laser lines (e.g., 405, 440, 488, 514, 561, 640 nm, for Leica SP5X a white light laser can replace the individual laser lines between 470 and 670 nm).
6. Immersion oil with refractive index 1.518 at 23 °C (Zeiss Immersol™ 518F).
7. Confocal image acquisition software: Leica LASAF software; Volocity Improvision for PerkinElmer.
8. Image analyses software: ImageJ/Fiji.

3 Methods

Visualization of thin dynamic 100–200 nm diameter cytonemes requires a very high signal-to-noise ratio. A high signal can be achieved by expressing fluorescent membrane markers, such as CD8:GFP, or CD8:mCherry, mCherryCAAX, or CD4:IFP2 under a strong tissue-specific *Gal4* or *LexA* driver (see **Notes 1** and **6**). For instance, in this method, we marked tracheal cell-membrane by driving CD8:GFP with a trachea-specific *btl-Gal4*, and epidermal engrailed-expressing cells by driving CD4:IFP2 (Infrared fluorescent protein) with *en-Gal4*. Fluorescent-labeled cytonemes are efficiently visualized when they extend over a non-fluorescent and less autofluorescent background. A relatively higher number of cytonemes is observed in live embryos than in the fixed-tissue preparations.

3.1 Genetic Crosses for Tissue-Specific Expression

1. Expand the driver (e.g., *btl-Gal4*) and reporter (e.g., *UAS*-CD8:GFP) lines to collect a large number of flies.
2. Prepare ~50 young female virgin *btl-Gal4* flies, as well as ~30 male *UAS*-CD8:GFP flies (see **Note 7**).
3. Anesthetize male and female flies by placing them on CO₂ pad (as recommended in [37] for fly pushing).
4. Put a small scoop of yeast paste with a spatula at the center of the grape juice agar plate.
5. Place females and males together in the small collection cage and close the lid containing the grape juice agar plate. Do not flip the cage when the flies are in anesthetized condition to avoid them sticking to the food (see **Note 8**).
6. Flip the cage with the plate side down when the flies wake up.
7. Incubate at 25 °C, which is ideal for egg laying.
8. Replace the old grape juice agar plate with new ones containing a scoop of yeast paste every 12–24 h for at least 3 days. To replace the old plate, flip the cage

with its mesh side down facing a CO₂ pad. Once the flies are anesthetized, open the cage lid and replace the old plate.

9. Start embryo collection from 3rd–4th day when most of the female flies start laying eggs (*see Note 9*).

3.2 Embryo Collection and Dechorionization

1. Incubate plates for 8–14 h to harvest embryos of all stages. For tracheal cytonemes, mostly the embryos between stage 11–15 are required (*see Note 10*). Epidermal cytonemes are visualized from the *engrailed*-expressing cells between embryonic stage 8–16.
2. Follow **step 8** of Subheading 3.1 to collect the plate to be harvested for embryos and replace with a new plate for future collection.
3. Use a small spatula or a brush to remove the yeast paste and dead flies from the agar plate.
4. Add some dH₂O covering the surface of the plate, and use the brush to gently dislodge all the embryos from grape juice agar plate into the water.
5. Transfer them by pouring into the cell strainer and keep them hydrated.
6. Rinse embryos with water for 30 s.
7. Put the cell strainer with embryos in a dissection watch glass dish containing 50% sodium hypochlorite. Dechorionize embryos by slightly rotating the cell strainer till most embryos float up, indicating that they are dechorionized (this takes about 3 min).
8. Rinse embryos thoroughly with flowing dH₂O for at least 1 min to remove residual bleach (*see Note 11*).
9. Rinse embryos with embryo wash (1% PBST).
10. Rinse embryos with dH₂O to thoroughly wash off all the Triton X-100 (*see Note 12*).
11. For **steps 9** and **10**, make sure to wash all the embryos on the side of the strainer down onto the center of the mesh. This facilitates their efficient transfer to the next step.
12. All the procedures are carried out at room temperature.
13. Immediately follow fixation (*see Subheading 3.3*) or live imaging (*see Subheading 3.4*) protocol.

3.3 Embryo Fixation

1. All the following procedures are carried out at room temperature.
2. Prepare fixation solution in a 1.5 mL microcentrifuge tube in the fume hood by mixing 500 µL 4% PFA and 500 µL heptane.

3. Use a paintbrush to transfer the dechorionated embryos from the mesh into a 1.5 mL Eppendorf tube containing the fixation solution.
4. Tightly seal the tube with Parafilm to avoid spillage, and place it on top of the orbital shaker, secure with tape, and shake vigorously at high speed for 20 min.
5. Embryos will be between the two-phase layers. With a pipette, gently remove as much of the lower aqueous phase as possible, without taking the embryos at the interphase.
6. Add 800 μ L of 100% MeOH to the tube and immediately shake vigorously for 1 min. This step removes the vitelline membrane.
7. Let the tube stand for a minute when the devitellinized embryos sink to the bottom (*see Note 13*).
8. Remove the top layer including the embryos at the liquid interface and with part of MeOH containing embryos that do not sink.
9. Rinse three times with 800 μ L MeOH to ensure complete removal of heptane and embryos with partial devitellinization.
10. Embryos in MeOH can be stored at -20°C . However, we do not recommend this step, because, freshly fixed embryos yield the best fluorescent detection. Directly go to the next step.
11. Rehydrate embryos with three washes of 5 min each: 75% MeOH–25% (0.1% PBST), 50% MeOH–50% (0.1% PBST), 25% MeOH–75% (0.1% PBST).
12. Wash two times in 0.1% PBST, 2 min each.

3.4 Preparing Live Embryo for Imaging

1. Following dechorionization of the embryos (described in **step 11** of Subheading 3.2), transfer them in a small watch glass containing 1 $\times$ PBS.
2. Monitor under a stereomicroscope and remove the dead embryos with forceps.
3. Place the glass bottom dish on a black background under the stereomicroscope.
4. Use a paintbrush to transfer the embryos to the center of the glass in the glass bottom dish (*see Note 14*).
5. Remove the excess liquid from the glass that got transferred along with the embryos.
6. Add a drop of halocarbon oil 27 to cover the embryos completely (*see Note 15*).
7. Use needle or forceps to gently spread the embryos in the oil and make a single layer of the embryos. Avoid spreading them close to the edge of the glass. Pipette out extra oil. The indication of a good mounting is when the single-layered embryos firmly sit on the glass, rather than floating in the oil (Fig. 3a).
8. Place a piece of wet kimwipes in the dish surrounding the glass bottom to retain moisture. Put on the lid of the plate (*see Notes 16 and 17*).

3.5 Preparing Fixed Embryos for Imaging

1. Put microscope slide on a black background.
2. Remove most of the PBST from the tube, leave about 100 μ L of PBST.
3. Use clean scissors or blade to cut the tip off a p200 pipette tip, pipet embryos with PBST and move them to the microscope slide.
4. Remove the extra PBST from the slide, remove bubbles.
5. Add the mounting solution (two drops of VECTASHIELD) on top of the embryos (*see Note 18*).
6. Use a needle to spread embryos in the mounting solution, try to center and align the embryos to avoid overlap.
7. Use Kimwipe to remove extra PBST at the edges of the slide.
8. Gently put the coverslip from one side to another to avoid bubble (Fig. 3b).
9. Use nail polish to seal the coverslip. Dry for 2 min.
10. Slides can be stored at 4 °C for future imaging, but freshly prepared samples are the best for detection of cytonemes.

3.6 Image Acquisition and Processing

1. Set up the imaging condition using the confocal microscope software. The laser scanning microscope is suitable for deep tissue low noise imaging; it allows imaging from a selected ROI and zoom-in using zoom factor (most often 2–3 $\times$). On the other hand, a spinning-disc microscope enables high-speed, high-resolution, wide-field imaging (*see Note 19*).
2. Add a drop of immersion oil on 40 $\times$ or 60 $\times$ objectives depending on the resolution required. Both objectives are suitable for imaging cytonemes. We recommend starting with using 40 $\times$ objective with 2–3 $\times$ zoom factor to avoid FP quenching. 60 $\times$ imaging is useful for subcellular resolutions.
3. Position a 10–20 $\times$ air objective in the light path.
4. Place the glass-bottom dish (**step 8** of Subheading 3.4) or the slide (**step 9** of Subheading 3.5) on the universal mounting frame of the microscope stage.
5. Locate an embryo using the 10–20 $\times$ bright field objective.
6. Switch to epifluorescence and identify the right stage and orientation of the tissue of interest [40] (*see Note 20*).
7. Switch to the oil objective already with immersion oil and quickly bring the sample to focus (*see Note 21*). Use epifluorescence or bright-field for focusing.
8. Switch to the laser system using the software and quickly select a region of interest (ROI) to image cytonemes in live mode. Usually growing tips of the dorsal and ventral branches project many cytonemes toward the FGF (fibroblast

growth factor) source situated ahead of them (Fig. 4) [38]. Cytonemes from cells in the *en* stripes extend toward the neighboring cells (Fig. 5).

9. Start acquiring images. Start with a reasonably low laser power in live mode (**step 8**). For EMCCD camera: adjust exposure time and EM gain according to the level of GFP/RFP expression in the tissues and efficiency of cytoneme detection.
10. For efficient visualization of cytonemes, focus mostly at the edge of the fluorescent tissue (*see Note 22*).
11. Open confocal images in Fiji/ImageJ to perform Z-projection of selected sections and adjust brightness and contrast to visualize cytonemes. Convert multicolor images to RGB and save with a scale bar. For a single color, apply the desirable change in intensity and save the image with a scale bar.

4 Notes

1. Membrane marking is critical for efficient detection of the thin 200 nm cytonemes. However, various FP fused proteins also marked cytonemes with variable efficiency [17,19,20,23,28].
2. Mix one pack of the grape juice agar powder in 450 mL deionized water in a 2-L beaker. Microwave on high for 2 min, stir well, and repeat the microwave step for two more times, 1 min each, followed by stirring to remove all the clumps. If necessary, repeat the heating and stirring steps until a clump-free homogeneous mix is formed. Usually, a homogeneous mix is obtained when the liquid is completely boiled and starting to bubble. Observe to avoid a spillage during microwave.
3. Mix dry baker's yeast with a bit of water in a plastic beaker, mold it with a metal spatula until it forms a homogenous thick paste. Do not make the paste too watery to avoid flies getting stuck in it. Keep the yeast paste at 4 °C, covered with aluminum foil, for maximum 2 weeks. Fresh yeast paste induces flies to lay more eggs.
4. Triton X-100 is very viscous, so to take up the right amount, cut the narrow end of the micropipette tip and slowly take up the liquid without bubbles. Make sure that the viscous liquid is not sticking to the outer surface of the tip. Mix by a gentle stirring on a magnetic stirrer and avoid bubbles.
5. A standard scanning confocal microscope such as Leica SPE is sufficient to image cytonemes [21]. Motorized x-y stage is not required, but it enables time-lapse imaging from multiple embryos. A stage-top incubator is not required as *Drosophila* embryos develop efficiently at room-temperature between 20 and 25 °C. A fast optical-Z scan is important to capture the cytonemes present deep inside the tissues. Scanning confocal provides better resolution for deep-tissue imaging.

6. Earlier studies used CherryCAAX, CD8:Cherry, CD4:IFP2, CD2:GFP, and CD8:GFP to image cytonemes from larval tissues [21,23,24, 39,41]. We successfully imaged the embryonic cytonemes using all these constructs.
7. The number of parent flies needed depends on the required size of the embryo collection. The recommended size is more than sufficient for live cell imaging. The way of setting up genetic crosses is variable, and depends on the needs and the availability of flies. The cage size can be adjusted accordingly from small to mini or large size (Genesee Scientific). A higher number of parent flies provide better chances to get synchronized embryos within a certain time window. For a repeated collection of the embryos, establishing a genetically combined or recombined fly stock harboring the driver and the reporter genes is convenient and recommended (e.g., recombined *btl-Gal4*, *UAS-CD8:GFP* homozygous stock on second chromosome [38]).
8. The small size cross can also be set up in tubes for 2 days before transferring them to the cage.
9. Well-fed, healthy, 10-day-old females with big ovary-filled abdomen start to lay many eggs.
10. Tracheal cytonemes were best observed during stages 11–15 when the primary tracheal branches migrate in response to the Branchless signal, and they cover relatively less surface area than the old embryos [42]. Modify the collection window based on the required developmental stage.
11. Sodium hypochlorite is highly toxic and a thorough wash is required for time-lapse imaging.
12. The dechorionated embryos should be processed immediately for imaging or fixation. Do not leave the dechorionated embryo in the strainer for long. Dehydration in the embryos leads to abnormal cytoneme patterns and tissue morphology leading to death.
13. If less than 50% of the embryos do not sink, for subsequent experiments, try preparing fresh 50% bleach for dechoriation or increase the shaking speed and time in the devitellinization step.
14. Imaging embryos at the junction of the glass-bottom and the plastic disc leads to unwanted diffraction of light.
15. Oil allows oxygen exchange and prevents dehydration.
16. A humid chamber is required, because the high fluidity of halocarbon oil 27 is not effective against dehydration, especially for long duration imaging.
17. An inverted microscope is desirable for long-term imaging using the setup described here. For upright confocal microscopes, adopt an alternative hanging drop method. However, the method is suitable only for a short duration time-lapse imaging. In this method, the live embryos are transferred on a small drop of Halocarbon oil 700 (or 27) that is placed at the center of a coverslip. Align the

embryos at the center with a needle. Remove excess oil but keep sufficient amount to cover the embryos. Add four tiny drops of halocarbon oil at the four corners of the coverslip. Place a cavity slide right on top of the coverslip such a way that the embryos on the coverslip are at the center of the concavity and are not squished. Press the slide against the coverslip so that they stick to one another. Flip the slide and temporarily seal the edge of the coverslip with rubber cement (Electron Microscopy Sciences). In this hanging drop preparation, the surface tension of the oil helps the embryo to stick to the coverslip. This alternative method is also suitable for an inverted microscope.

18. It is important to add enough amount of mounting solution to avoid overlapping of embryos. The amount of solution depends on the number of embryos.
19. An optimized condition for imaging cytonemes in scanning confocal microscope: Imaging dimension (XYZCT); X-Y size: 512X512 pixels; Z-volume: 20–40 μm with 0.2–0.35 μm step size (Cytonemes are very thin, about 0.2 μm in thickness); laser power: <20% with white light laser; pinhole size: airy 1; dwell time: set it up during imaging based on the required speed; HyD gain: 100%; scan speed: default (can be bidirectional scan); averaging for 2–3 times. For spinning disc: camera exposure time 20 ms (<100 ms), EM Gain 300. When acquiring time-lapse images, set up a Z volume 3–5 μm up or down from the tissue in focus to accommodate continuous imaging of the tissue in a growing embryo.
20. Embryonic trachea adopts a developmental stage-specific morphology and can reliably be used as a staging marker.
21. Ensure that the oil objectives have sufficient immersion oil to avoid light scatter.
22. The fixed embryos showed less number of cytonemes than the live ones. Few advantages of the fixed embryos are: (1) the embryos are closer to the coverslip, so high-resolution images can be achieved easily, and (2) the fixation process reduces autofluorescence.

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Fig. 1.

Drosophila ASP cytonemes. **(a)** A drawing depicting the third instar larval wing disc and air sac primordium (ASP). The ASP cells project cytonemes (green) to contact the *bnl* (blue) and *dpp* sources (pink) in the disc for receiving signals. **(b)** Live image of CD8:GFP marked cytonemes emanating from the growing tip region of an ASP; the image in **(b)** corresponds to an area represented by the dashed square in **(a)**

Fig. 2. Embryo collection apparatus. **(a)** Embryo collection cage. **(b)** Grape juice agar plate fits in the plastic cap of the embryo collection cage. **(c)** Cell strainer used for embryo dechorionization

Fig. 3.

Cartoon illustrating the steps for embryo imaging preparation. **(a)** Live embryo imaging preparation (description in Subheading 3.4): 1. use a paintbrush to transfer embryos to the center of the cover glass; eliminate excess liquid; 2. add a drop of Halocarbon oil 27 on top of the embryos; 3. gently spread embryos in the oil and make a single layer of embryos; 4. place some wet tissue in the dish surrounding the glass bottom microwell. **(b)** Slide preparation for fixed embryos (description in Subheading 3.5): (1) Leave about 100 µL of PBST in Eppendorf tube with embryos; cut off the tip of a p200 pipette tip, pipette embryos onto a microscope slide; remove the extra PBST liquid from the slide. (2) Add two drops of the VECTASHIELD on top of the embryos. (3) Use a needle to gently spread the embryos in a single layer, gently lower the coverslip (arrow) onto the embryos in VECTASHIELD without including an air bubble

Fig. 4.

Examples of cytonemes from fixed and live embryonic trachea. **(a)** Confocal images of a fixed embryo at stage 14 showing dorsal tracheal branches (green), each growing toward a dorsal *bnl*-source (red); genotype: *btl-Gal4,UAS-CD8:GFP/+; bnl-LexA,lexO-CherryCAAX/+*. **(b)** A stage 14 embryo showing cytonemes from the dorsal (1) and ventral (2) tracheal branches without marking the *bnl*-source; genotype: *btl-Gal4; UAS-CD8: GFP*. Top panel, a z-projection image of 34 optical sections across 51 μm depth of a whole embryo, imaged in fixed condition with a 40× objective in Leica SP5X. Bottom panels, images of cytonemes from a dorsal (1) and a ventral (2) branch of a live stage 14 embryo; these cytonemes project toward the *bnl*-source (*see* panel a); imaging condition: 40× objective with 2× zoom factor, focusing a growing tip of a branch. **(c)** Schematic drawing of a stage 14 trachea metamere (green) and the *bnl*-sources (red), arrows point to the dorsal (1) and the ventral (2) branches as shown in **(b)**

Fig. 5.

Example of cytonemes from live embryonic epidermal cells that express *engrailed* (*en-Gal4*). **(a)** Schematic drawing of a stage 12–13 embryo with engrailed-expressing stripes. **(b)** CD4:IFP2 expressing cells in the *engrailed* stripes project short cytonemes; 40× magnification with a 2× zoom of ROI function; the region shown in **(b)** corresponds to the dashed square in **(a)**; scale bar, 10 μ m