Chapter 2

In Vivo Imaging of Hedgehog Transport in *Drosophila* Epithelia

Irene Seijo-Barandiarán, Isabel Guerrero, and Marcus Bischoff

Abstract

The Hedgehog (Hh) signaling pathway is a regulator of patterning, cell migration and axon guidance during development as well as of homeostatic events in adult organs. It is highly conserved from *Drosophila* to humans. In many contexts during development, Hh appears to function as a morphogen; it spreads from producing cells to trigger concentration dependent responses in target cells, leading to their specification. During production, Hh undergoes two lipid modifications resulting in a highly hydrophobic molecule. The processes that create lipid-modified Hh for release from producing cells and that move it to target cells in a graded manner are complex. While most of the work done trying to explain Hh gradient formation is based on immunohistochemical studies in steady state, in vivo imaging in intact organisms is the finest technique to study gradient formation in real time. Both the wing imaginal disc epithelium and the adult abdominal epidermis of *Drosophila* are well suited for in vivo imaging. They allow us to observe the behavior of cells and fluorescently labeled proteins, without interfering with development. Here, we describe in vivo imaging methods for these two epithelia, which allowed us to study Hh transport along specialized cytoplasmic protrusions called cytonemes.

Key words In vivo imaging of Hedgehog signaling, Morphogen dispersion in *Drosophila*, Cell communication, Cytonemes, Exosomes

1 Introduction

Hh is essential for the morphogenesis of most organs in the fly and for many metazoan tissues. During development, Hh acts as a morphogen in many contexts. Hh disperses from producing to receiving cells in a graded manner, activating different target genes depending on the concentration they receive. Thus, cells close to the source of production will receive higher Hh levels than cells far from the source, and this will regulate tissue growth and cell fate specification [1]. The mechanism by which morphogens are transported from producing

Electronic supplementary material: The online version of this chapter (doi:10.1007/978-1-4939-2772-2_2) contains supplementary material, which is available to authorized users.

Natalia A. Riobo (ed.), *Hedgehog Signaling Protocols*, Methods in Molecular Biology, vol. 1322,
DOI 10.1007/978-1-4939-2772-2_2,

to receiving cells and how morphogen gradients are formed is still under debate [2, 4, 5].

Hh is posttranslationally modified by the addition of cholesterol [6] and palmitic acid [7]. This confers high hydrophobicity to this signaling protein and tethers it to lipid membranes, thus impairing its spreading by free diffusion [6]. There are different models that explain how lipid-modified Hh is secreted and transported from producing to receiving cells: restricted diffusion [8], planar transcytosis [9], argosomes [10], exosomes [11, 12], lipoprotein particles [13], and cellular protrusions, called cytonemes [3, 14, 15].

The mechanisms of Hh gradient formation have been studied extensively in the *Drosophila* wing imaginal disc. The wing disc is a flattened sac made of two layers of closely juxtaposed polarized epithelial cells—the columnar cells of the disc proper and the squamous cells of the peripodial membrane (Fig. 1a, b). Four cell populations with different affinities divide the disc proper in four compartments: anterior (A), posterior (P), dorsal (D), and ventral (V) [16]. Hh is produced in the P compartment and moves across the A/P compartment border decreasing in concentration as it spreads into the A compartment (Fig. 1b) [6, 17], where it patterns the central region of the wing [18, 19]. The Hh receptor Patched (Ptc) is a readout of Hh signaling; it is upregulated in a graded manner in the A compartment, depending on the graded distribution of the Hh signal [20, 21].

In the larval and adult abdominal epidermis, Hh also acts as a morphogen and is expressed in the P compartment [22, 23]. The adult abdominal epidermis is formed during metamorphosis, when the adult histoblasts replace the larval epithelial cells (LECs) [24]. It comprises a sequence of successive segments with Ptc being expressed in A compartment cells anterior and posterior to each P compartment (Fig. 1c, d). The pattern of Ptc expression in the histoblasts is only established during morphogenesis of the adult tissue [3, 22, 23, 25]. Both the larval and the adult abdominal epidermis of *Drosophila* are excellent systems for in vivo imaging [26], with the adult fly hatching after up to 30 h of imaging.

The behavior of cells and proteins during cell-to-cell communication, in wild type or under different mutant conditions, has mainly been studied using in vitro and ex vivo techniques, which are based on fixation and immunostaining. These techniques can only give a static view and also may lead to variable results, due to changeable conditions during fixation and staining. In vivo approaches, on the other hand, allow the observation of processes that happen in the context of a living organism without the need of fixation and staining. Importantly, they also permit the analysis of changes over time (e.g., changes in cell behavior, protein concentration or distribution).

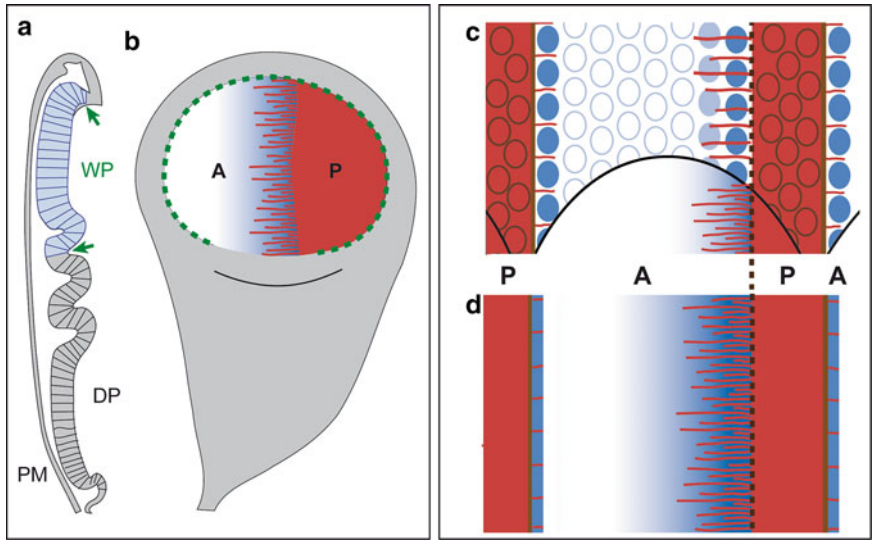

Fig. 1 Schematic representation of Hh signaling in the *Drosophila* wing imaginal disc and abdominal epidermis. (**a**, **b**) Schemes depict Hh expression domain and the Hh morphogenetic gradient in the wing imaginal disc. (**a**) Transversal section of the wing imaginal disc showing the two juxtaposed epithelia: squamous peripodial membrane (PM) and columnar disc proper (DP). (**b**) Longitudinal DP section of the wing imaginal disc showing the P compartment (*red*) that expresses Hh, which disperses towards the A compartment (*blue*) in a graded manner. This leads to high concentrations of Hh close to the A/P compartment border and lower concentrations far from the source of production. The wing pouch (WP) is delimited by *green arrows* in A and by *green dotted line* in **b**. Note cytonemes emanating from the P compartment (*red*) are present in areas of Hh signaling of the A compartment. (**c**, **d**) Scheme depicting Hh signaling and cytonemes in one abdominal segment (after Bischoff et al. [3]). Hh and Ptc expression in histoblasts and LECs of A and P compartment (*red*) is shown. (**c**) During histoblast spreading, both histoblasts and LECs express Hh in the P compartment. The Ptc gradient can be observed in the A compartment in both histoblasts and LECs as well as in a row of LECs at the segment boundaries. Cytonemes are present in areas of Hh signaling. (**d**) Adult tissue. Hh is expressed in the P compartment. Ptc is expressed in a gradient in the A compartment as well as in a narrow strip at the segment boundaries. Cytonemes are present in areas of long-range Hh signaling, but not at the segment boundaries, where only local signaling is occurring

In this chapter, we describe in vivo imaging techniques for a better characterization of cell and protein behavior in the context of cell-to-cell communication, namely the imaging of wing imaginal discs, both ex vivo and within the living larva, and the abdominal epidermis (Fig. 2). We have used these techniques to analyze the formation of the Hh morphogen gradient and how exosomes containing Hh move along cytonemes from Hh producing cells to Hh receiving cells [3, 12]. We showed in vivo that cytoneme formation and the establishment of the Hh morphogen gradient correlate in space and time [3]. Furthermore, we showed that Hh-containing exovesicles move along these filopodia-like protrusions [12].

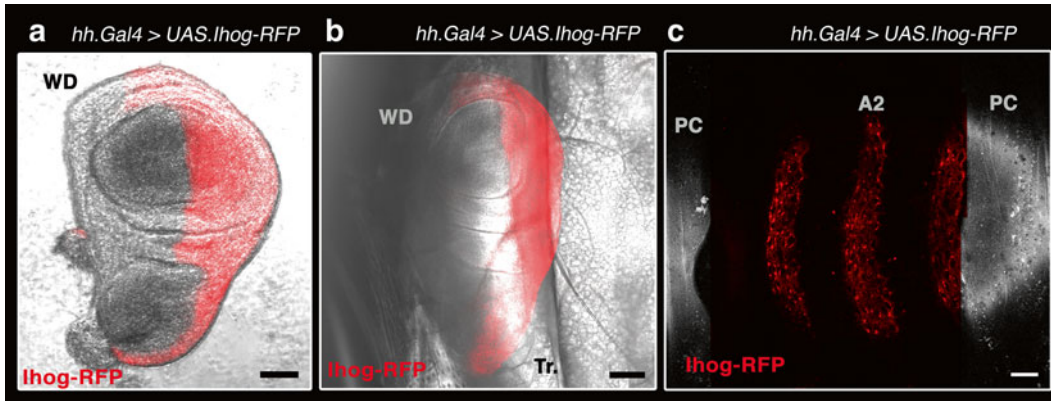

Fig. 2 In vivo imaging of third instar larval wing imaginal disc and pupal abdominal epidermis. In each instance, the P compartments are labeled with *hh.Gal4 > UAS.Ihog-RFP*. Anterior is to the *left*. (**a**) Ex vivo wing disc (WD). Merge of transmitted light and RFP channel. (**b**) In vivo wing disc (WD) in third instar larva. The second thoracic lateral tracheal branch (Tr.) of the larva is also visible. Merge of transmitted light and RFP channel. (**c**) Dorsal view of a pupa, in which a window has been made in the pupal case (PC) for in vivo imaging. The P compartments of segments A1, A2, and A3 are shown

2 Materials

2.1 Ex Vivo Imaging of Drosophila Wing Imaginal Discs

1. *Drosophila* third instar larvae grown using standard protocols [27].
2. 1× PBS buffer: 4.3 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4.
3. Media composition: M3 insect medium was supplemented with 2 % FBS (Invitrogen #10106-169) and 0.5 % penicillin–streptomycin (Invitrogen #15140-122) and 0.1 μg/ml 20-hydroxyecdysone (Sigma-Aldrich #H5142). Reserve half of the prepared medium to add methylcellulose (M0387-100G; Sigma) at a concentration of 2.5 % wt/vol.
4. Chamber: standard microscope slide, double-sided sticky tape, 24 × 40 mm and 15 × 15 mm coverslips (both No. 1).
5. Poly-lysine (Sigma-Aldrich #P6282) and sterile water.
6. Forceps and needles.
7. A confocal laser-scanning microscope with optics for imaging bright field and fluorescence, such as Zeiss LSM710 upright.

2.2 In Vivo Imaging of Drosophila Wing Imaginal Discs in Living Larvae

1. *Drosophila* third instar larvae from fly stocks grown using standard protocols [27].
2. 1× PBS buffer.
3. Liquid glue: heptane and double-sided sticky tape.
4. Voltalef oil 10 S (VWR International, Cat No. 24627188).
5. Chamber: standard microscope slide, double-sided sticky tape, 24 × 40 and 20 × 20 mm coverslips (both No. 1).

6. Forceps and needles.
7. A confocal laser-scanning microscope with optics for imaging bright field and fluorescence, such as Zeiss LSM710 upright.

2.3 In Vivo Imaging of Drosophila Pupal Abdomen

Preparation of Pupae for Imaging

1. *Drosophila* pupae from fly stocks grown using standard protocols [27]. Staging of pupae is performed after Bainbridge and Bownes [28].
2. Deionized water.
3. Paintbrush with cutoff bristles.
4. Double-sided sticky tape.
5. Standard microscope slide.
6. Forceps and micro knife (or hypodermic needle).
7. 200 µl pipette with tip.
8. Dissecting microscope (e.g., Leica S6E).

Preparation of Imaging Chamber

1. Standard microscope slide and coverslip (22 × 50 mm, No. 1).
2. Parafilm M.
3. Vaseline (pure petroleum jelly); melted in a heating block (e.g., Techne Dry-Block).
4. Paint brush.
5. Forceps.
6. Deionized water.
7. Whatman paper (3MM).
8. Humid chamber: 90 mm diameter petri dish with wet tissue, sealed with Parafilm M.
9. Voltalef oil 10 S (VWR International, Cat No. 24627188).
10. A confocal laser-scanning microscope, such as Leica SP8.

3 Methods

For in vivo imaging, protein components of exosomes or cytonemes or proteins implicated in the release, transport, or reception of Hh, were fused to a fluorescent tag. These proteins were expressed in a restricted area of the wing imaginal disc or in a group of histoblasts and LECs under the control of a P compartment specific driver, using the GAL4-UAS system [29]. A good tool for visualizing cytonemes is the expression of fluorescently labeled Interference Hedgehog (Ihog), an Hh co-receptor, which stabilizes cytonemes in the basal parts of wing disc and abdominal epithelia [3, 12, 30, 31]. In addition, membrane markers, such as CD4-Tomato

and Myr-RFP, are also good markers to visualize cytonemes and exosomes [3].

Isolated wing discs, third instar larvae, and pupae are all imaged in small chambers. These are assembled using microscope slides and/or coverslips as bottoms and lids, with various spacers in between.

3.1 Ex Vivo Imaging of Drosophila Wing Imaginal Discs

1. Making a chamber (on the day before imaging): use one layer of double-sided sticky tape to surround a 5 × 5 mm area on a 24 × 40 mm coverslip. Put a drop of poly-lysine on the area (*see* **Note 1**). Once the drop has dried, wash the area with sterile water and keep the chamber at 4 °C.
2. The next steps are done at 4 °C on ice (*see* **Note 2**). Place *Drosophila* third instar larvae in 1× PBS buffer and wash three times with 1× PBS.
3. Transfer larvae to M3 medium and dissect wing imaginal discs. Place the dissected discs in 25 μl M3 medium in the 5 × 5 mm area of the chamber and add another 25 μl M3 medium with methylcellulose (*see* **Note 3**). Finally, close the chamber with a 15 × 15 mm coverslip and place it on a standard microscope slide.
4. Image wing discs with a confocal laser-scanning microscope (*see* **Note 4**) (Fig. 3a).

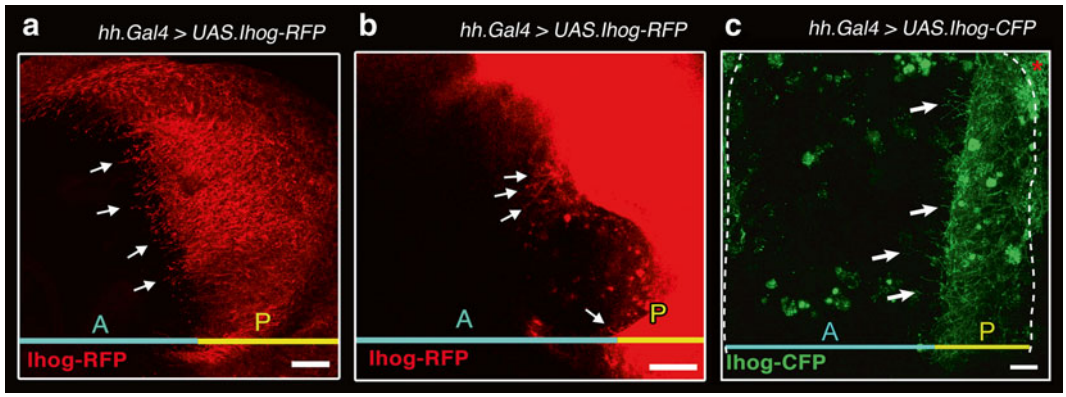

Fig. 3 Cytonemes in wing imaginal discs and the abdominal epidermis. Cytonemes are visualized by driving fluorescently labeled *Ihog* in the P compartment. A and P compartments indicated by *cyan* and *yellow lines*, respectively. Bars, 20 μm. Anterior compartment is oriented to the *left*. (**a**) Basal view of the disc proper of an ex vivo wing imaginal disc. Cytonemes (*arrows*) grow from P compartment cells towards A compartment cells, thereby crossing the compartment border. (**b**) Basal view of the disc proper of an in vivo disc proper in a living third instar larva. Cytonemes are pointing anteriorly into the A compartment (*arrows*). (**c**) Abdominal epithelium approx. 40 h after puparium formation. Histoblasts are labeled with Ihog-CFP. Along the compartment border, cytonemes point anteriorly (*arrows*). Segment A2 shown (indicated by *hatched line*). *Asterisk* indicates a LEC, which has not yet been replaced during morphogenesis

3.2 In Vivo Imaging of Drosophila Wing Imaginal Discs in Living Larvae

1. Making the chamber: use two layers of double-sided sticky tape to surround a 15 × 15 mm area on a 24 × 40 mm coverslip.
2. The next steps are done at 4 °C on ice (*see* **Note 2**). Place *Drosophila* third instar larvae in 1× PBS buffer and wash three times with 1× PBS.
3. Put a drop of liquid glue in the 15 × 15 mm area of the chamber and press the larva on it until the glue gets dry (*see* **Note 5**). Add a drop of Voltalef oil on the larva to avoid desiccation during imaging. Finally, seal the chamber with a 20 × 20 mm coverslip.
4. Image wing discs with a confocal laser-scanning microscope (*see* **Notes 6** and **7**) (Fig. 3b).

3.3 In Vivo Imaging of Drosophila Pupal Abdomen

Preparation of Pupae for Imaging

1. Collect pupae with wet paintbrush with cutoff bristles from the walls of a culture tube and stick them on double-sided tape on a standard microscope slide with the dorsal side up.
2. Dry pupae for around 30 min at room temperature.
3. Make a small hole in the pupal case in the space between thorax and abdomen dorso-laterally using a micro knife or a hypodermic needle. Start at this hole to peel a window in the pupal case in your area of interest using forceps.
4. Add some water on the pupae with the pipette and remove them carefully from the sticky tape with forceps to transfer them to the imaging chamber.

Preparation of Imaging Chamber and Imaging

1. Stick two strips of Parafilm "M" spacers (50 × 4 mm; six layers thick) on the long sides of a standard microscope slide (*see* **Note 8**).
2. Put pupae in the center of the slide.
3. Add two small pieces (ca. 5 × 5 mm) of wet Whatman paper in the vicinity of the pupae to provide humidity.
4. Add some water to cover the pupae.
5. Put a coverslip (22 × 50 mm, No. 1) on the spacers; fix it with melted Vaseline all way along the spacers using a paintbrush.
6. Seal the two open sides of the chamber with Voltalef oil.
7. Image pupae with confocal laser scanning microscope (*see* **Note 9**) (Fig. 3c; Supplementary Video 1).
8. Remove Voltalef oil after filming and keep slide in a humid chamber until flies hatch.

4 Notes

1. We use the poly-lysine to prevent imaginal disc movement during imaging, as it will attach the wing imaginal discs to the coverslip.
2. Working at 4 °C will confer a better manipulation of larvae, as they will not move. This condition also prevents medium contamination.
3. Methylcellulose is used to make the medium more viscous.
4. The wing discs survive imaging for up to 14 h under the used conditions. We use Caspase3 immunostaining after imaging to check that cells do not undergo apoptosis due to the culture conditions.
5. The liquid glue is made of double-sided sticky tape that is kept overnight in a bottle of heptane until all the glue has dissolved in the heptane. Then the tape is removed. The glue is used to prevent larval movement in the imaging chamber as much as possible. Depending on the manufacturer of the sticky tape, the glue might be toxic to the larvae. Therefore, it might be necessary to trial a few different tapes.
6. Wing discs expressing a fluorescent protein are easily visualized in the living larvae situated on the second thoracic lateral tracheal branch. Due to interference of the larval integument and tissue, imaging of cellular structures in the wing imaginal disc is challenging and high laser power is required. Imaginal discs that are positioned close to the epidermis with little tissue below are best to image.
7. Due to residual larval movement, time-lapse imaging is limited to a few minutes under these conditions. Larvae can be kept in the imaging chambers for around 30 min. After imaging, the larvae are transferred into standard fly food vials, in which they often pupate and produce adult flies, indicating that imaging is not impairing larval development.
8. The number of layers of Parafilm M can be adjusted depending on the size of the pupae, which might vary depending on growing conditions and genotype.
9. Due to the nature of the chamber, there might be considerable drift of the plane of focus during the course of a time-lapse recording. We take this into account by starting the recording 30 μm above the top of the specimen.

Acknowledgements

We are very grateful to Carmen Ibáñez for technical assistance. We also thank the confocal microscopy facility of the CBMSO for skillful technical assistance. Work was supported by grant BFU2011-

25987 from the Spanish MINECO and by an institutional grant to the CBMSO from the Fundación Areces to I.G. I.S. was financially supported by an FPI fellowship of the Spanish MINECO.

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http://www.springer.com/978-1-4939-2771-5

Hedgehog Signaling Protocols
Riobo, N.A. (Ed.)
2015, XVI, 200 p. 42 illus., 28 illus. in color., Hardcover
ISBN: 978-1-4939-2771-5
A product of Humana Press