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Review article

A quick method to investigate the *Drosophila* Johnston's organ by confocal microscopy

Monalisa Mishra*

Department of Life Science, NIT Rourkela, Rourkela, Orissa, India

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ABSTRACT

Drosophila antennae is gaining attention to study the hearing molecules and its mechanism in last few decades. Various molecules required for the formation of hearing organ is conserved between *Drosophila* and human being. This suggests *Drosophila* can be used as a model organism to decipher the vertebrate hearing mechanism. In this context a protocol describing the fixation, sectioning and staining of antennae is lacking from the literature. The current paper describes various commercially available markers of the antennae to visualise it under confocal microscope.

Contents

1. Introduction

The *Drosophila* hearing organ is located in the antennae which is present in between two compound eyes (Fig. 1A). The antenna is formed of three different segments. The first antennal segment is a1, the second a2 or pedicel and the third a3, the funiculus. The hearing organ, also called Johnston's organ, is confined to a2 (Fig. 1B). It is composed of about two hundred [1] functional units called scolopidia (Fig. 2A). Each scolopidium is formed of five different types of cells: Scolopale cell, cap cell, ligament cell, dendritic cell and neuronal cell [2,3] (Fig. 2B). The sensory neuron has axon at the proximal region and apically it has a dendrite with outer segment having characteristic features of non-motile cilia [4]. The cilia contain a dilated structure known as ciliary dilation and are formed of paracrystalline like

* Tel.: +91 9583866756.
E-mail address: mishramo@nitrkl.ac.in

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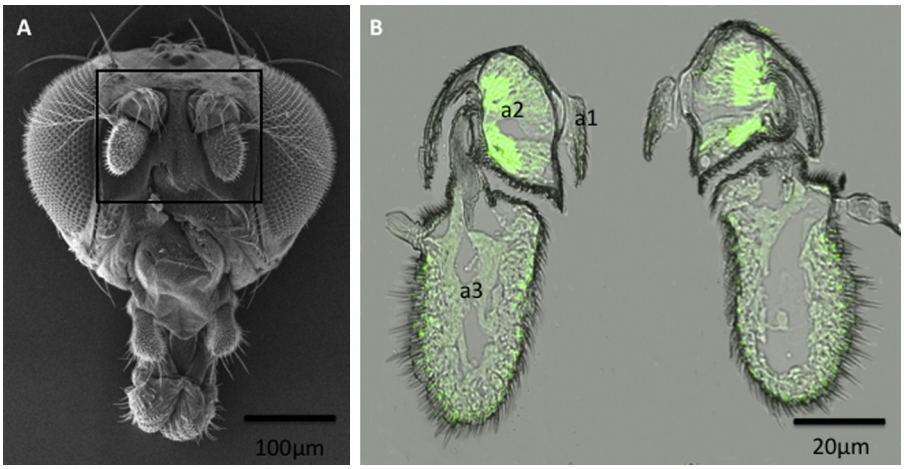

Fig. 1. Overview of the *Drosophila* antennae. (A) Scanning electron micrograph of the *Drosophila* head depicting the localisation of both the antennae marked in a rectangular box. (B) Cryosection of the antennae demonstrating various parts. Green colour in a2 represents for scolopidia, the function unit of Johnston's organ.

material (Fig. 2B). The basal part of the cilia contains ciliary rootlet, which extends from the base of the dendritic outer segment through inner segment into the perikarya. The scolopale cell forms an endolymph around the cilia and produces actin based rod, which provide strength to the scolopidia [5]. Apical to the scolopale cell is the cap cell, which is surrounded by attachment cell.

Drosophila Johnston's organ is gaining attention to study various hearing molecules of vertebrates [6–8]. Although various hearing mutants have been identified in *Drosophila* a complete protocol describing the analysis of Johnston's organ is missing from the literature. The current protocol describes the analysis of Johnston's organ of *Drosophila melanogaster* under confocal microscope.

2. Materials and methods

2.1. Fixation

Anaesthetise the fly for 1 min using CO_2. Cut the heads with a razor blade and pull out the proboscis. Place the heads (20) in an Eppendorf tube (1.5 ml) with Stefanini's fixative (480 µl of water + 220 µl of 37% formaldehyde + 150 µl of picric acid + 150 µl of 0.5 M PIPES). In the final solution the formaldehyde and PIPES concentration will be 4% and 75 mM respectively. Keep the Eppendorf tube for 40 min on ice for fixation. Remove the fixative and wash the heads with Phosphate Buffered Saline (PBS) (pH 7.4), three times 20 min each wash. Add 10%

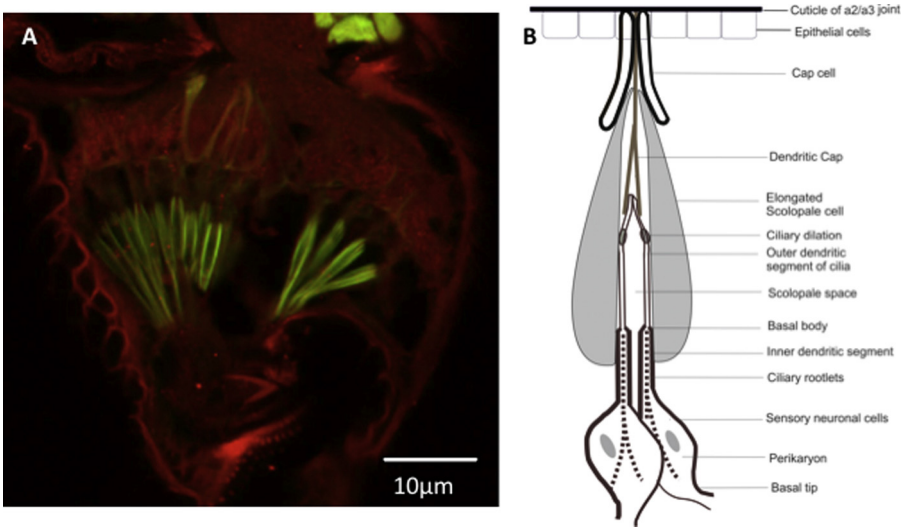

Fig. 2. Structure of the *Drosophila* Johnston's organ. (A) Cryosection through the Johnston's organ stained with F-actin (green, labels the scolopale rods) and anti-HRP (red) stains the neuron. (B) Graphical representation of the scolopidia. Cap cell (CC), dentritic cell (DC), scolopale rod (SR), scolopale cell (SC), cilia (Ci), ciliary rootlet (Cr), and nerve (Ne).

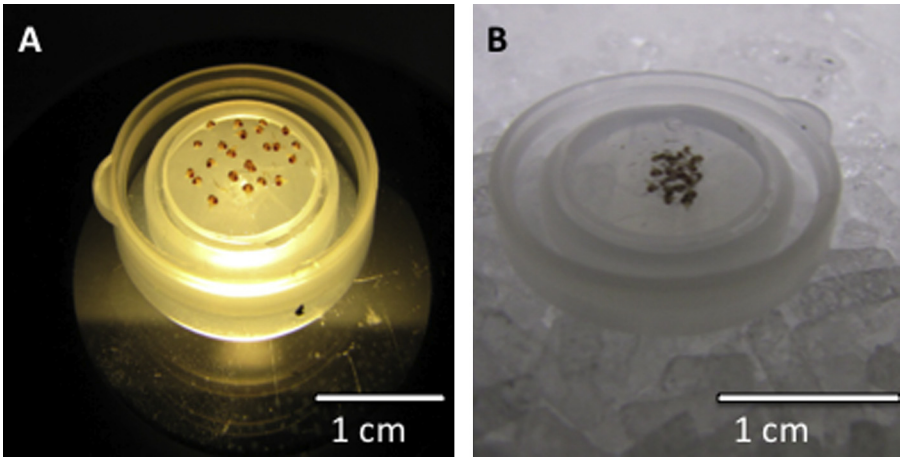

Fig. 3. Steps to embed the heads in mounting media. (A) Fly heads are put in the cap containing mounting media. Heads are arranged in such a way that eyes are looking towards the upside. (B) The cap containing fly heads and mounting media are put on dry ice and allowed to freeze.

sucrose solution (AppliChem, Cat. No. 57-5-1 diluted with PBS to 10%) to the Eppendorf tube and keep it rotating for one hour at room temperature. Change to 25% sucrose solution and keep it rotating (shaker/rotater for agitation) in 4 °C for overnight. Transfer the heads to the boat and align the heads in such a way that the eyes are looking towards upside (Fig. 3A). Place the boat on dry ice and allow it to freeze (Fig. 3B). Store the boats at −80 °C for future use.

2.2. Sectioning

Add a drop of mounting media to the sample holder. Take out the frozen block with the tip of forceps and freeze the sample holder inside the cryotome (Fig. 4A). Once it is frozen (Fig. 4B), trim out the excess mounting media from different corners and give the block a trapezoid shape (Fig. 4C). The trapezoid shape will help to collect serial

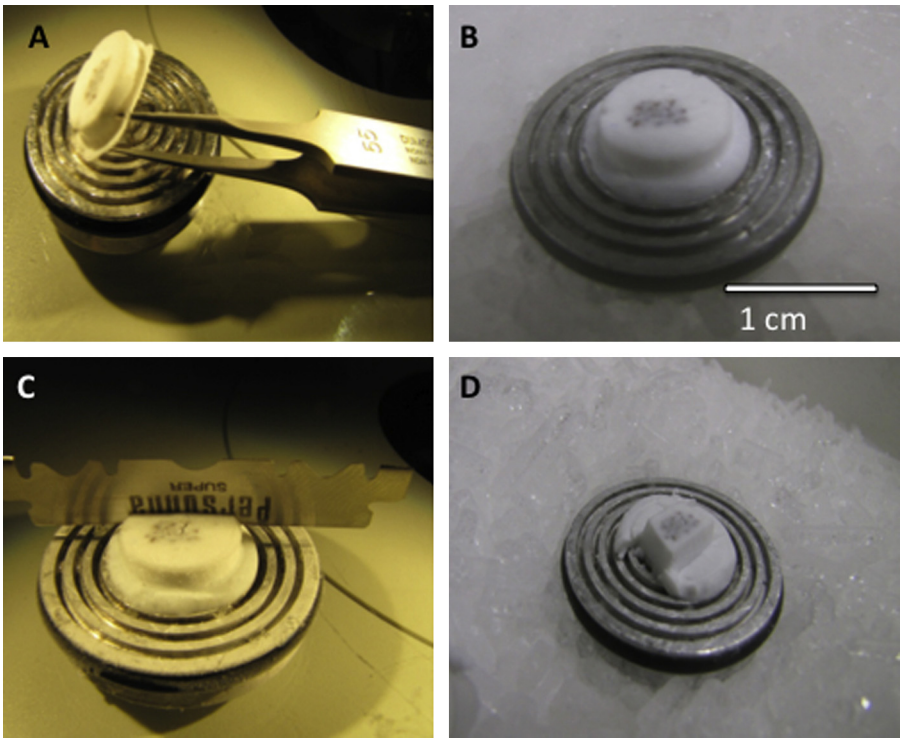

Fig. 4. Steps to align the sample for cryo-sectioning. (A) Frozen sample is taken with a forecep and add to the sample holder containing little amount of mounting media. (B) Sample holder alongwith the sample was frozen over dry ice. (C) Frozen sample was cut with the help of a razor blade to give a trapezoid shape. (D) Block was shaped as trapezoid after proper trimming.

Table 1
Primary antibodies used to label different structures in the Johnston's organ.

Structure to be stained	Primary antibody	Source	Dilution	Reference
Any green fluorescent protein (GFP)-tagged fusion protein	Rabbit anti-GFP	Molecular Probes	1:1000	[3]
Scolopidia	Alexa Fluor 488 Phalloidin	Invitrogen	1:50	[4]
Neuron	Goat anti-HRP (Cy3/Cy5-conjugated)	Dianova	1:100/1:500	[9–11]
	Mouse anti-Futsch (22C10)	Developmental Studies Hybridoma Bank (DSHB)	1:25	
Scolopale space	Mouse anti-Spacemaker (21A6)	DSHB	1:50	[12]
Junctions	Rat anti-DE-Cadherin (DCAD2)	DSHB	1:25	M.M. unpublished
	Rat anti-DN-Cadherin (DN-EX#8)	DSHB	1:5	
Cilia	Mouse anti-acetylated tubulin	Sigma	1:10,000	[13]
Rootlet	Rabbit anti-actinin Rabbit anti-centrin	Sigma	1:200	[17,18]
Basal body	Rabbit anti-γ tubulin	Sigma	1:200	[14]
Basolateral membrane	Mouse anti-Na^+/K^+-ATPase (a5)	DSHB	1:25	M.M. unpublished

sections (Fig. 4D). Take 12 μm sections from the frozen block. The first sections will be the transverse sections of the compound eye, which can be recognised in the mounting media from their shape. Trim few more sections from the eye. After sectioning the eye, the antenna with the Johnston's organ will appear. It can be detected as two dots in the mounting media. Start collecting the section at this point on a super frost plus glass slide. Due to the organisation of the Johnston's organ, transverse sections will appear first, followed by longitudinal sections.

2.3. Immunohistochemistry

Transfer the slides to a slide box and add 500 μl of PBS to each individual slide. Rinse the PBS and add 500 μl of PBST (PBS containing 1% Tween-20) three times 15 min in each wash. Block the slide by adding 500 μl of blocking (5% Normal Horse Serum diluted with PBST) solution. Dilute the primary antibody (Table 1) with the blocking solution. Add 500 μl of the diluted primary antibody to the slide. Keep the slide at 4 °C overnight in the dark. Keep the slide-containing chamber moist (with water) in order to avoid drying of the antibody solution. The next day, bring the slides to room temperature and remove the primary antibody. Wash the slide with 500 μl of PBST, three times 15 min each wash. Dilute the secondary antibody (Table 2) with the blocking solution to the appropriate concentration. Add 500 μl of the secondary antibody solution along with Alexa 488-conjugated phalloidin and/or Cy3- or Cy5-conjugated anti-Horse Radish Peroxidase (HRP). Keep the slides in the secondary antibody in the dark for two hours at room temperature. Remove the secondary antibody and rinse the slide with 500 μl of PBS, three times for 15 min each wash. Mount the slide using 100 μl of Vectashild to it. Put the coverslip and seal it with nail polish. Slides are ready to observe under the confocal microscope (Zeiss LSM META 510).

3. Results and discussions

Various primary and secondary antibodies used to label the Johnston's organ are optimised in this protocol. Proteins are tagged with GFP for their subcellular localisation. Commercially available GFP antibody is used to localise the GFP-tagged version of the protein in Johnston's organ (Table 1). Scolopidia are the functional unit of the Johnston's organ and it is highly enriched with actin. Alexa-conjugated pholloidin is used in this protocol to visualise the scolopidia. Pholloidin binds to filamentous actin thus make the scolopidia visible (Fig. 5A). The optimisation of the pholloidin is mentioned in Table 1. Basal to the scolopidia are the neurons. Anti-HRP is used as a marker to stain the neuronal membrane and recognise the sugar residues of multiple glycoproteins [9–11] (Fig. 5B). Anti-HRP antigens further accumulate in the ciliary dilation and sensory dendrites in wild-type scolopidia [12]. Besides anti-HRP, anti-Fustch (22C10) also labels the neurons and can be substituted for anti-HRP. Use of these two dyes will help to label the scolopidia completely (Fig. 5C). Scolopale cell produces an endolymph that surrounds the cilia. The scolopale space is further known to be filled with extracellular matrix. Spacemaker/Eys is the protein which is known to fill this space [12]. The expression pattern of spacemaker is also varified in Johnston's organ (Fig. 6A). Two non-motile cilia are embedded in the scolopale space. Various genes are responsible for the formation of cilia in Johnston's organ. Acetylated tubulin, a common marker of cilia [13] is used to label the cilia of Johnston's organ. The base of the cilia is known as the basal body. Basal body is formed from centriole and it is an array of microtubule.

Table 2
Secondary antibodies used to label Johnston's organ.

Secondary antibody	Source	Dilution
Donkey anti-rabbit, Alexa 488 conjugated	Invitrogen	1:200
Donkey anti-mouse, Alexa 647 conjugated	Invitrogen	1:300
Goat anti-mouse, Alexa 488 conjugated	Invitrogen	1:250
Goat anti-mouse, Alexa 568 conjugated	Invitrogen	1:350
Goat anti-mouse, Alexa 647 conjugated	Invitrogen	1:300
Goat anti-rat, Aleax 647 conjugated	Invitrogen	1:300
Goat anti-rat, Alexa 568 conjugated	Invitrogen	1:500
Goat anti-rat, Alexa 647 conjugated	Invitrogen	1:300
Goat anti-rabbit, Alexa 488 conjugated	Invitrogen	1:250
Goat anti-rabbit, Alexa 568 conjugated	Invitrogen	1:300
Goat anti-rabbit, Alexa 647 conjugated	Invitrogen	1:300
Donkey anti-mouse, Cy2- or Cy3-conjugated	Dianova	1:100
Donkey anti-rabbit, Cy3 conjugated	Dianova	1:100
Donkey anti-rat, Cy2- or Cy3-conjugated	Dianova	1:100
Donkey anti-gp, Cy3-conjugated	Dianova	1:100

The molecule, which is responsible for shaping the basal body is gamma tubulin. Taking the evolutionary conservation of gamma tubulin into account, gamma tubulin is used to verify the localisation of basal body in Johnston's organ (Fig. 6D) [14]. In Johnston's organ there are four basal bodies. The distal basal bodies are known to be responsible for the cilia formation whereas the proximal two basal bodies are known to be responsible for the formation of rootlet. Rootlet is required for the long term stability of vertebrate hearing and photoreceptor organ [15,16]. Molecules known to mark rootlet are centrin and alpha-actinin [17,18]. Commercially available antibodies are used to label the rootlet (Fig. 6C). To define the scolopidia more, baso-lateral domain marker like Na^+/K^+-ATPase [19,20] is

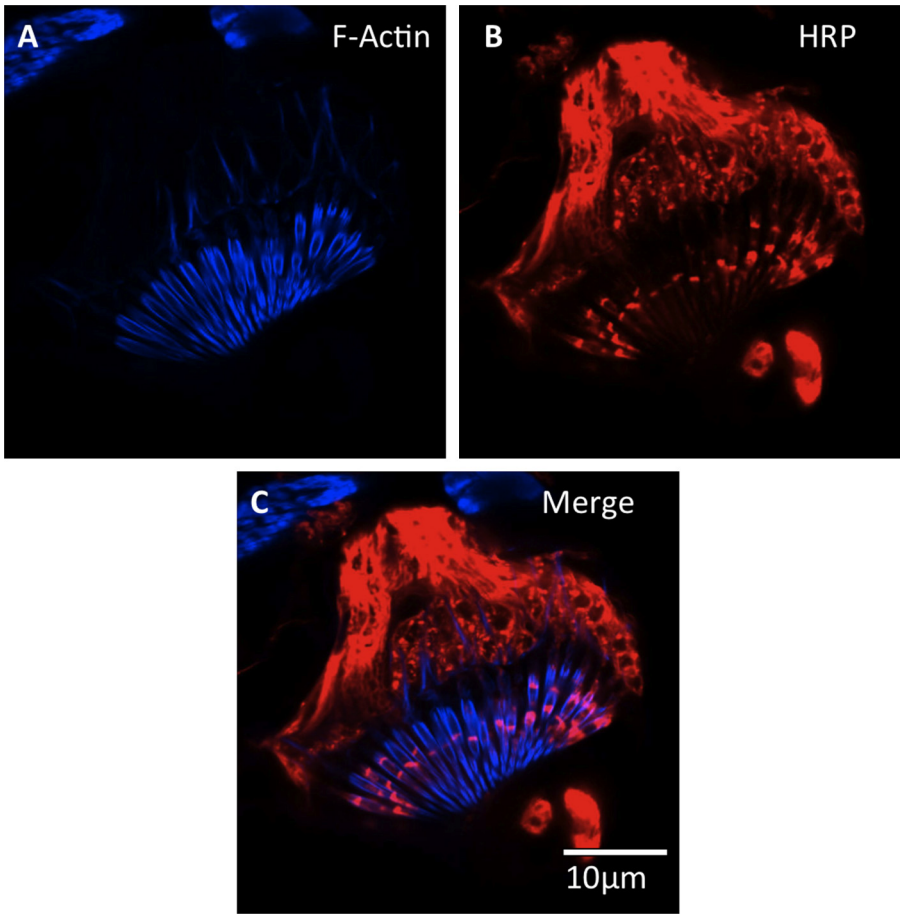

Fig. 5. Cryosection of the antennae stained with antibodies. Cryosection of the antennae counter stained with (A) Alexa conjugated phalloidin to make the scolopidia visible. (B) HRP stains the neuron. (C) Merged picture showing the entire scolopidia.

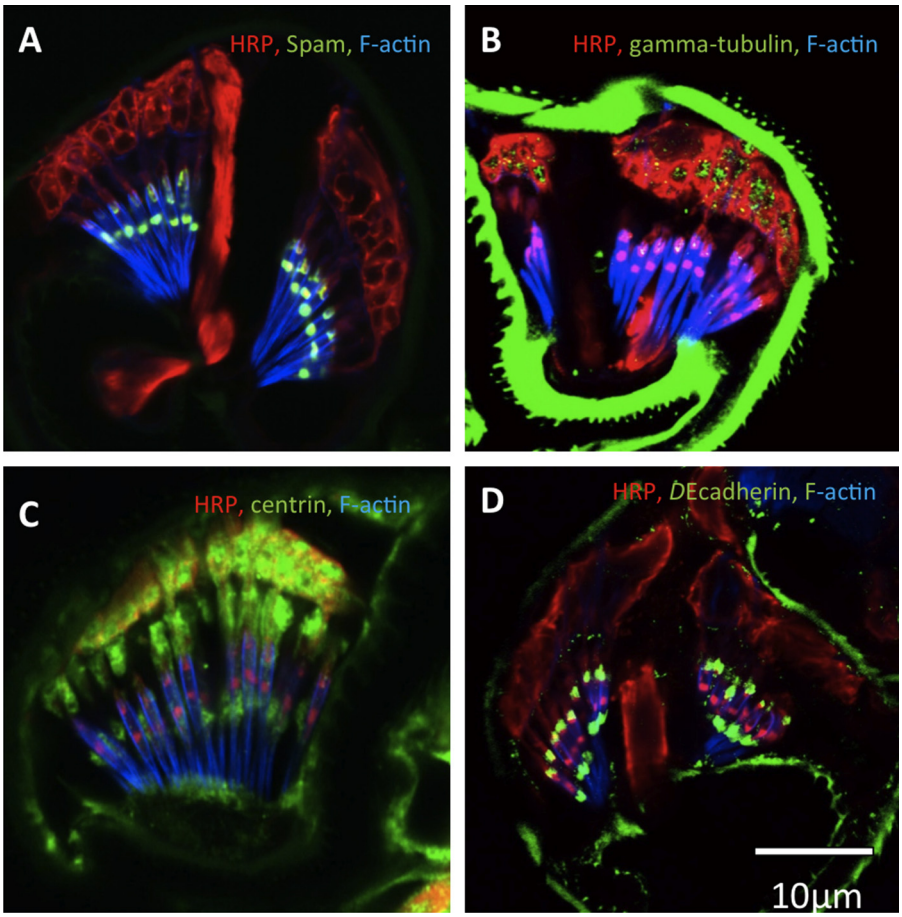

Fig. 6. Cryosection of the antennae stained with various JO protein. Cryosection of the antennae counter stained with alexa conjugated phalloidin, HRP and other antibodies. (**A)** Stained with spacemaker, (B) with gamma tubulin, (C) alpha-actinin and (D) *D*E cadherin.

investigated for this organ. Since junctions are indispensible for the proper functioning of an organ the localisation of junction was traced using known commercially available antibodies like *D*E cadherin and Armadillio. *D*E cadherin staining reveals presence of junctions in Johnston's organ (Fig. 6D).

The optimisation of various primary and secondary antibodies used in this study will help to analyse various parts of antennae. This protocol will help to characterise a mutant antenna phenotype.

Conflict of interest

There is no conflict of interest in this study.

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