Video Article

Techniques for Investigating the Anatomy of the Ant Visual System

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URL: https://www.jove.com/video/56339
DOI: doi:10.3791/56339

Keywords: Environmental Sciences, Issue 129, Compound eye, ocelli, lens, ommatidium, rhabdom, crystalline cone, scanning electron microscopy, transmission electron microscopy, light microscopy

Date Published: 11/27/2017

Citation: Ramirez-Esquivel, F., Ribi, W.A., Narendra, A. Techniques for Investigating the Anatomy of the Ant Visual System. *J. Vis. Exp.* (129), e56339, doi:10.3791/56339 (2017).

Abstract

This article outlines a suite of techniques in light microscopy (LM) and electron microscopy (EM) which can be used to study the internal and external eye anatomy of insects. These include traditional histological techniques optimized for work on ant eyes and adapted to work in concert with other techniques such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM). These techniques, although vastly useful, can be difficult for the novice microscopist, so great emphasis has been placed in this article on troubleshooting and optimization for different specimens. We provide information on imaging techniques for the entire specimen (photo-microscopy and SEM) and discuss their advantages and disadvantages. We highlight the technique used in determining lens diameters for the entire eye and discuss new techniques for improvement. Lastly, we discuss techniques involved in preparing samples for LM and TEM, sectioning, staining, and imaging these samples. We discuss the hurdles that one might come across when preparing samples and how best to navigate around them.

Video Link

The video component of this article can be found at https://www.jove.com/video/56339/

Introduction

Vision is an important sensory modality for most animals. Vision is especially crucial in the context of navigation for pinpointing goals, establishing and adhering to routes, and obtaining compass information[1,2]. Insects detect visual information using a pair of compound eyes and, in some cases, one to three dorsally-placed simple eyes called ocelli[3,4,5].

The eyes of ants are of particular interest because, while ants are wonderfully diverse, they conserve some key characteristics across species. Despite dramatic variation in anatomy, size, and ecology, the vast majority of species are eusocial and live in colonies; as a result, different species face similar visual challenges in terms of navigating back and forth between a central place and resources. Across ants the same basic eye bauplan can be observed in animals ranging from 0.5-26 mm in body length, from exclusively diurnal to strictly nocturnal species, and from slow walking subterranean to leaping visual predators[6,7,8,9,10]. All of these staggering differences in ecology and behavior give rise to innumerable permutations of the same basic eye structures to suit different environments, lifestyles, and body-sizes[11,12]. As a consequence, studying the visual ecology of ants provides a veritable treasure trove of possibilities to the determined investigator.

Understanding the visual system of insects is essential in gaining an insight into their behavioral capabilities. This is apparent from integrative studies which nicely combine anatomy with ecology and behavior to a great success in a few insect groups (*e.g.*, references[13,14,15,16,17]). Though the field of ant navigation and ant behavior in general has been quite successful, very little emphasis has been placed on ant vision outside of a few selected species. Here, we will elaborate on the techniques involved in investigating eye design of ants. While we will focus on ants, these techniques can be applied, with slight modifications, to other insects, too.

Protocol

1. Specimen Preparation

NOTE: It is necessary to first understand the relative location of the compound eye and ocelli to each other and on the head. This can be achieved by acquiring images of the dorsal view of the head. For this, we recommend processing samples either for photomicrography or using SEM techniques. Below are steps involved in both processes.

1. **Specimen collection**
    1. Collect and store specimens directly into 70% ethanol. Collect different castes whenever possible.

   2. Label specimens with time, date, and place as well as any other relevant observations (*e.g.*, collected while foraging, mating aggregation, nest inside a twig, *etc.*)
   3. Collect enough specimens to have multiple replicates in each treatment.

2. **Photomicrography and Z-stacking**
   1. Air dry specimens and mount them on triangular point cards, using water soluble glue, and then on an insect pin. For details, see reference[18].
   2. Image using a high magnification stereomicroscope with a Z-stepper motor and a color camera.
   3. Use a diffuser to have uniform lighting for specimens.
   4. Capture images at different focal planes and save images in a lossless file format (such as tiff) and focus stack them using commercially available software.

3. **Scanning electron micrographs**
   NOTE: Thoroughly clean all tools and working surfaces with ethanol to avoid contamination of the specimen with dust and other particulates.
   1. Dehydrate specimens in ethanol overnight and air dry in a Petri dish.
   2. Use a sharp razor blade to separate the head from the remainder of the body.
   3. Mount the head at the required viewing angle (*e.g.*, dorsal facing up) on aluminum stubs using conductive carbon tape or tabs. Cut the carbon tape into thin strips and fold it to support the head capsule.
   4. Use a sputter coater to apply gold to the surface of the specimen for 2 min at 20 mA with a rotary stage. The time and current may need to be adjusted depending on the instrument.
   5. Transfer specimens to a new aluminum stub with fresh carbon tape or tabs.
      NOTE: The uncoated carbon will provide a black background but transferring specimens can damage the gold coating.
   6. Check that the specimen orientation is still correct using a dissecting microscope and adjust as necessary with a pair of fine forceps or similar tool. Take care not to scratch the gold coating; handle as little as possible.
   7. Load specimens onto the SEM stub holder, making note of the position of stubs and specimens relative to each other.
      NOTE: Some SEMs are equipped with a stage camera but many are not and it can be difficult to locate small specimens at high magnification.
   8. Image the specimens. Use a low accelerating voltage to avoid charging and a small aperture for good depth of field.
      NOTE: Settings are best optimized in consultation with a technician specialized in the particular instrument being used.

2. Quantifying Facet Numbers and Diameters

1. **Cornea replicas**
   1. Use ants preserved in ethanol or mounted on a pin for this purpose (step 1.1.1).
   2. Mount the animal on an insect pin or on plasticine. If the head is relatively large, the remaining body parts can be removed.
   3. Use an insect pin or a fine toothpick to pick up a small drop of fast-drying colorless nail polish and quickly spread it over the eye. Ensure the pin does not scratch the eye. The nail polish should cover the entire eye and some of the surrounding head capsule.
      NOTE: It is important that the nail polish be of relatively uniform thickness across the eye.
   4. Leave the nail polish to set at room temperature.
   5. Once it is fully set, use a fine insect pin to gently lift the replica from the head capsule surrounding the eye.
   6. Use a fine pair of clean forceps to lift the replica, grasp the part of the replica that covers the head and not the eye.
   7. Ensure awareness of the orientation of the eye: the anterior, posterior, dorsal, and ventral region.
   8. Place the replica on a glass slide. Use a razor blade to trim the replica by carefully removing excess material around the eye. Use a needle or a pair of forceps to prevent the replica from moving.
   9. If the eye is very convex, use a razor blade to make 3-4 fine partial radial incisions around the edge to help 'flatten' the replica. If the eye is relatively 'flat', there is no need to make such incisions.
   10. Place a cover slip gently on the eye replica, ensuring that the orientation of the replica is known. Do not apply pressure as this can eliminate the corneal impression on the nail polish.
   11. Seal the coverslip using very little nail polish on four corners. If the nail polish flows between the cover slip and glass slides, it will damage the replica.
   12. Image the slide on a compound microscope.
       NOTE: If only some facets are in focus, then this suggests the eye replica is not flattened enough. Discard and start again from step 2.1.3.
   13. Import the image into freely available programs such as ImageJ/Fiji where the number of ommatidia and size of each ommatidia can be measured.
       NOTE: This method can be used to prepare replicas of ocelli, too. Since it is a single lens, we recommend keeping all the ocelli together in one replica.

3. Analyzing the Structure of the Eye

NOTE: To study the anatomy of the eye requires in most cases two complementary techniques of LM and TEM. The initial processing stages require similar techniques for both LM and TEM. The difference arises from the sectioning stage onwards. Processing samples requires the use of hazardous chemicals which must be handled with care and discarded responsibly. Use personal protective equipment, work in a fume hood, always read the safety data sheets(SDS), and carry out risk assessments before starting.

1. **Dissection**
   1. Anaesthetize specimens by cooling or by exposure to gaseous CO_2.

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1. CO_2 anesthesia is very fast (generally under 1 min) and care should be taken to avoid overexposure as this may result in death of the specimen. If using dry ice pellets (solid CO_2) avoid direct contact with specimens as this can cause cold burns.
2. Cold anesthesia is slower; 4 °C is sufficient, and colder temperatures are not recommended. Establish an appropriate cooling time for the species. Large or cold resistant ants such as bull ants may require >10 min to become fully immobilized, while smaller species may only need 1-2 min. Excessive cooling will kill the specimen (avoid direct contact with ice). Specimens should preferably be held in small, foam-stoppered, plastic containers and placed in an icebox where they can be observed rather than in an electric refrigerator or freezer.

2. Place specimen on a Petri dish, and adjust for viewing under a dissecting microscope. Working quickly is important to preserve anesthesia and to avoid degeneration of the tissue once incisions are made (this can happen within seconds).
3. Remove antennae with forceps. If working with a stinging insect, it is advisable to amputate the gaster first to avoid being stung.
4. Remove the mouth parts using a sharp razor blade; forceps may be used to hold the specimen down. Cut through the anterior part of the eye (large specimens) or as close to the eyes as possible (small specimens) without tugging on the brain as this may tear out the retina.
5. Prepare to open the head capsule. Angle the specimen so that the first incision is pointing up; this may be done either under the dissecting microscope while holding the specimen in forceps or under visual control while holding the specimen between the thumb and fore finger.
6. Make a transverse incision through the head to remove the ventral portion of the head; part of the ventral eye may be removed in large species to improve fixation and infiltration. The head should still be attached to the body at this point.
7. Sever the head capsule from the body by making a coronal incision just posterior to the compound eye.
8. Place the dissected head capsule with the compound eyes in ice cold fixative: 2.5% glutaraldehyde and 2% paraformaldehyde in phosphate buffer (pH 7.2-7.5).
Caution: Fixative is corrosive and toxic; wear appropriate protection and work in a fume hood.
NOTE: It is important to work quickly to arrest neural tissue degeneration. The dissection should be completed in 2 min or less (efficient dissection may require some practice).
NOTE: If the eye needs to be adapted to bright or dark conditions, then first expose the animals to the required light condition for a few hours. Carry out the dissections in the respective light conditions. Dissections can be carried out under red lights to simulate darkness.

2. **Specimen processing**
   1. Keep the specimens in the fixative at room temperature with motion on an orbital shaker, for 2 h. Large specimens may require longer fixation times.
   2. Remove the fixative and dispose it appropriately. Wash specimens in room temperature phosphate buffer (3 times, 5 min each) on the shaker.
   NOTE: The phosphate buffer comprises of 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.244 g KH_2PO_4 in 1 L of distilled H_2O (pH 7.2).
   3. Remove the phosphate buffer and add 2% OsO_4. Place the specimen jar on the shaker in the fume hood for 1-2 h. This is a post-fixation step to fix fats and also provide contrast for TEM.
   NOTE: Osmium fixation times are subject to specimen size; as a rough rule of thumb, calculate 1 h of fixation per 1 mm^3.
   4. Remove the osmium solution and dispose of it appropriately. Wash specimens in room temperature buffer (3 times, 5 min each) on the shaker.
   5. Dehydrate the specimens by placing them in increasing concentrations of ethanol or acetone; for example, 50, 70, 80, and 95% for 10 min each and finally 100% (2 times, 15 min each). Place the specimens on the shaker between solution changes.
   NOTE: If necessary, specimens may be stored in 70% ethanol overnight.
   6. Drain the ethanol and add 100% acetone. Leave it for 20 min on the shaker (skip this step if dehydrating it in acetone). Replace with fresh acetone and leave it for an additional 20 min.
   7. Infiltrate the tissues with resin using the following ratios of acetone to resin: 2:1 (3 h), 1:1 (overnight), 1:2 (4 h), and pure resin (overnight). At each step leave specimens on the shaker inside the fume hood, and cap the container for all but the last two steps.
   NOTE: Resin is too viscous to be drained so specimens must be moved to a new disposable container at each step.
   8. Prepare blocks to mount the samples. Blocks can be custom made by cutting acrylic glass into small rectangular blocks (1.5 x 0.5 x 0.3 cm). Blocks can also be made by pouring epoxy resin (there are many commercial kits available) into a silicon mold and then cure it in the oven for 12-14 h at 60 °C.
   Caution: Uncured (liquid) resin is carcinogenic and should be returned to the oven until fully hardened.
   9. Place the block vertically in the mold. Carefully take the specimens from the liquid resin, allow excess resin to drain, and place the specimen on the top of the block. A small amount of additional resin can be used to bind the specimen to the block.
   10. Label the block. Print the paper labels and embed it in the block or attach it to a block face.
   11. Keep the mold with the embedded blocks in the oven for 12-14 h at 60 °C.
   12. Store the specimen block in a clean envelope. This can be stored in this manner over several months to years.
   13. Leave the used containers, dirty gloves, and other contaminated equipment in the fume hood to allow acetone to evaporate completely (minimum 12 h).
   14. Cure resin in the oven before discarding disposable equipment or scraping resin off other items such as forceps.

3. **Sectioning**
   1. Observe the block under the dissecting microscope to ensure that the specimen orientation is appropriate for the sectioning plane.
   2. If the orientation is not appropriate, use a jeweler's saw to cut out the specimen and re-orient using fragments of set resin and fresh resin to re-seat the specimen. The head may also be split into two halves to section the two eyes separately. Cure resin again before proceeding.
   3. Mount the block on the removable microtome chuck. Remove the chuck and place on holder.
   4. Trim the resin block using a razor blade under the dissecting microscope.
   Caution: Do not do this when the chuck is mounted on the microtome arm as it can jolt the arm and damage the bearings.
   5. Remount the chuck onto the microtome arm and angle the specimen.
   6. Mount the knife on the holder at the appropriate angle (0° for glass knives, see manufacturer's instructions for diamond knives).

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NOTE: Glass knives can be made cheaply with a glass knife maker but must be replaced periodically as they lose their edge; high quality diamond knives can be purchased but are expensive, require special care, and are not suitable for beginners.

7. Fill the knife boat with distilled water using a syringe equipped with a filter (0.45 µm pore size).
8. Fill the boat until the water level reaches the edge of the knife; the meniscus may be convex in other areas of the boat.
9. Drain the boat until the meniscus is very slightly concave but still reaches the edge of the knife. The level of the water can be adjusted at any point but always away from the edge of the knife.
10. Carefully bring the knife towards the specimen and align the block to the knife. This is best done slowly, periodically checking the proximity of the knife through the eyepiece and from the side.
    NOTE: Check the instrument handbook for specific instructions as instruments vary.
11. Set the section thickness on the microtome. Selecting the correct thickness will be dependent on specimen size, region of interest and the kind of knife being used.
12. If using a glass knife select a higher setting (*e.g.*, 4 µm), if a lot of material must be cut away before reaching the area of interest. If a diamond knife is being used or if the specimen is very small, 1-2 µm may be more appropriate.
13. Start the "cutting" (cranking the microtome wheel) when the knife is close but not yet cutting into the specimen to perform the last part of the approach. Sections should start appearing within a few rotations; if not, stop and very carefully bring the knife a little closer.
14. Adjust the section collecting thickness (1 µm for semi-thin sections) when approaching the region of interest.
15. Collect any sections using an eyelash tool.
    NOTE: Eyelash tools can be made with an eyelash mounted onto a thin stick with nail polish.
16. If a lot of material must be removed, allow the sections to accumulate and remove *en masse* by removing the knife and flushing it out with water. If using a glass knife, this may be an appropriate time to change to a fresh section of the knife or to a new knife.
17. Place a series of small droplets of distilled water on a slide using a Pasteur pipette or ideally, the filter equipped syringe.
18. Carefully float the collected section on the eyelash onto the water droplet.
19. Collect sections like this until it is appropriate to check the depth of sectioning.
    NOTE: Although it can be tedious, it is always best to check often.
20. Place the slide on a hotplate set to 60 °C. Allow all water to evaporate and the section to adhere to the slide.
21. Dye sections with toluidine blue for 10-60 s (staining time will vary with section thickness). Dispense the dye with a syringe equipped with a filter (as above).
22. Place a drop of the dye on one end of the slide and spread it using the side of the needle without touching or scraping the sections. Place the slide on the hotplate for approximately 10-20s.
23. Rinse the slide by spraying it with distilled water in a wash bottle and place it on the hot plate to dry it.
24. Check under the compound microscope and image them.
25. Repeat until region of interest is reached.
26. For ultra-thin sections: set the cutting thickness between 40-60 nm to collect TEM sections.
27. Cut about 3-5 sections and check thickness using an interference color chart. Sections should reflect light grey when viewed at an angle.
    NOTE: Chloroform fumes can be pipetted over sections to relax any creases. This is most relevant to experienced users, and beginners need not worry. Too much chloroform released too close to the section can damage sections.
28. Pick up a Formvar-coated, copper, slot grid held with forceps with the Formvar side up. Be careful not to puncture the Formvar coating.
29. Dip the grid into the boat edgeways and away from the sections, then bring the grid up parallel to the surface under the sections. If necessary use the eyelash to guide the sections over the grid.
30. Carefully dab around the tip of the forceps with filter paper to soak up water trapped between the arms. If this is not done, water tension can pull the grid up between the arms or make it stick to one side. This can result in contamination or mechanical damage.
31. Carefully remove excess water from the grid itself by standing the grid edgeways on filter paper.
32. Place the grid in a grid holder.
33. Repeat until enough sections have been collected.
34. Semi-thin sections can be imaged directly with any compound microscope equipped with a camera. Immersion oil can be placed directly onto the sections. Slides should be stored in a slide box to prevent discoloration.

4. **Staining ultra-thin section for TEM contrast**

NOTE: The following steps should be carried out under cover as the dyes are light and CO_2 sensitive. Furthermore, EM dyes use heavy metals to produce contrast and are therefore hazardous substances. Appropriate care must be taken when handling these stains.

1. Cover a few large Petri dishes with aluminum foil to block light. Work under these for the following steps. Partially uncover them to allow working space but place the covers back on as soon as possible.
2. Cut a piece of wax film and carefully place five droplets of 6% saturated uranyl acetate on it using a Pasteur pipette.
    1. To prepare the solution, mix 2 g uranyl acetate with 100 mL 50% methanol in distilled water, and filter the solution before using. The solution cannot be stored and should be made fresh each time[19].
3. Carefully pick up the TEM grids with forceps and balance on top of dye droplets with the section side down. Leave for 25 min.
4. Rinse grids individually by rapidly dipping them in and out of distilled water; progress through four different vials of distilled water.
5. Place five droplets of lead citrate on a fresh piece of film. Arrange a few NaOH pellets around the dye droplets (this absorbs atmospheric CO_2 to prevent precipitation of lead carbonate).
    1. To make the lead citrate solution, prepare bi-distilled water by boiling distilled water for 0.5 h. Allow the water to cool and in a sealable container, add 0.3 g lead citrate to 100 mL of the bi-distilled water. Add 1 mL 10 M NaOH, seal the container tightly, and shake until dissolved[19].
6. Place the grids on the dye drops as described in 4.3 and cover. Stain for 5 min.
7. Rinse in distilled water as before by dipping the grids in and out of distilled water 20 times. Progress through three vessels of distilled water.
8. Soak up excess water with filter paper and allow the grids to dry in a grid box.

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9. Image in a TEM at low accelerating voltage.

Representative Results

The methods described here enable detailed study of the simple and compound eyes of ants. Imaging the dorsal view of the head using Z-stack photomicrography techniques allows one to obtain an overview of the layout of the visual system (**Figure 1**). This is good preparation for dissections and to determine the required sectioning angle. This technique is also useful for taking measurements such as head width, eye length, and ocellar lens diameters. SEM imaging also gives detailed overview images but additionally allows acquisition of high magnification and high resolution images. Particular regions of interest in the eye can be examined in detail and variations in lens shape can be identified (**Figure 2**). SEM images are especially useful for resolving ants with small eyes and ocelli. The cornea replicas provide information on the shape, size, and number of lenses in each eye (**Figure 3**). Semi-thin sections imaged using LM techniques allow investigation of the gross internal anatomy of the eye (**Figure 4** and **Figure 5**); this includes the thickness of the lens, diameter of the crystalline cone, presence of a crystalline cone tract, shape, width, and length of the rhabdom, mapping the dorsal rim area, and location of the primary and secondary pigment cells. This technique can be nicely complemented by ultra-thin sections imaged using TEM, which allows for determining the ultrastructure especially, the microvillar orientation (**Figure 4**) and the quantifying smaller structures (*e.g.,* width of the constricted crystalline cone tract, **Figure 5**).

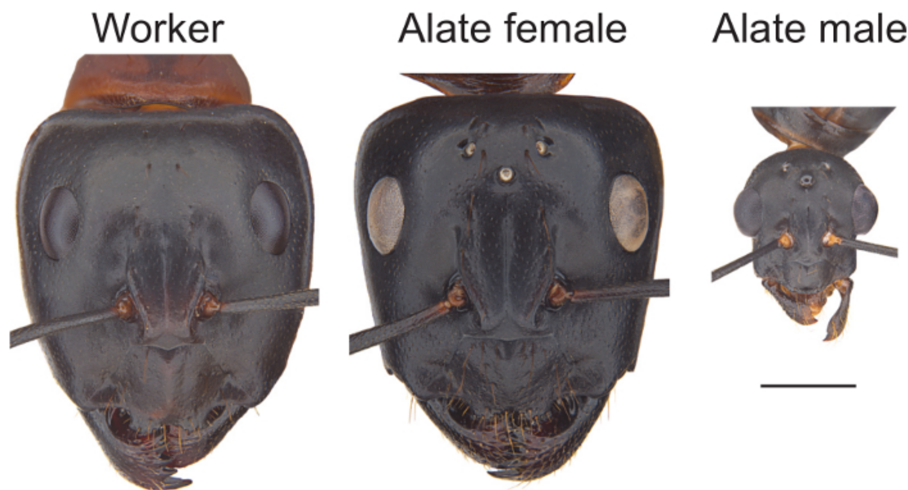

Figure 1: Z-stack photomicrographs of the three castes of the Australian sugar ant, *Camponotus consobrinus*. This provides an overview of the layout of the visual system in all three castes. Adapted from reference[20]. Scale bar = 1 mm. Please click here to view a larger version of this figure.

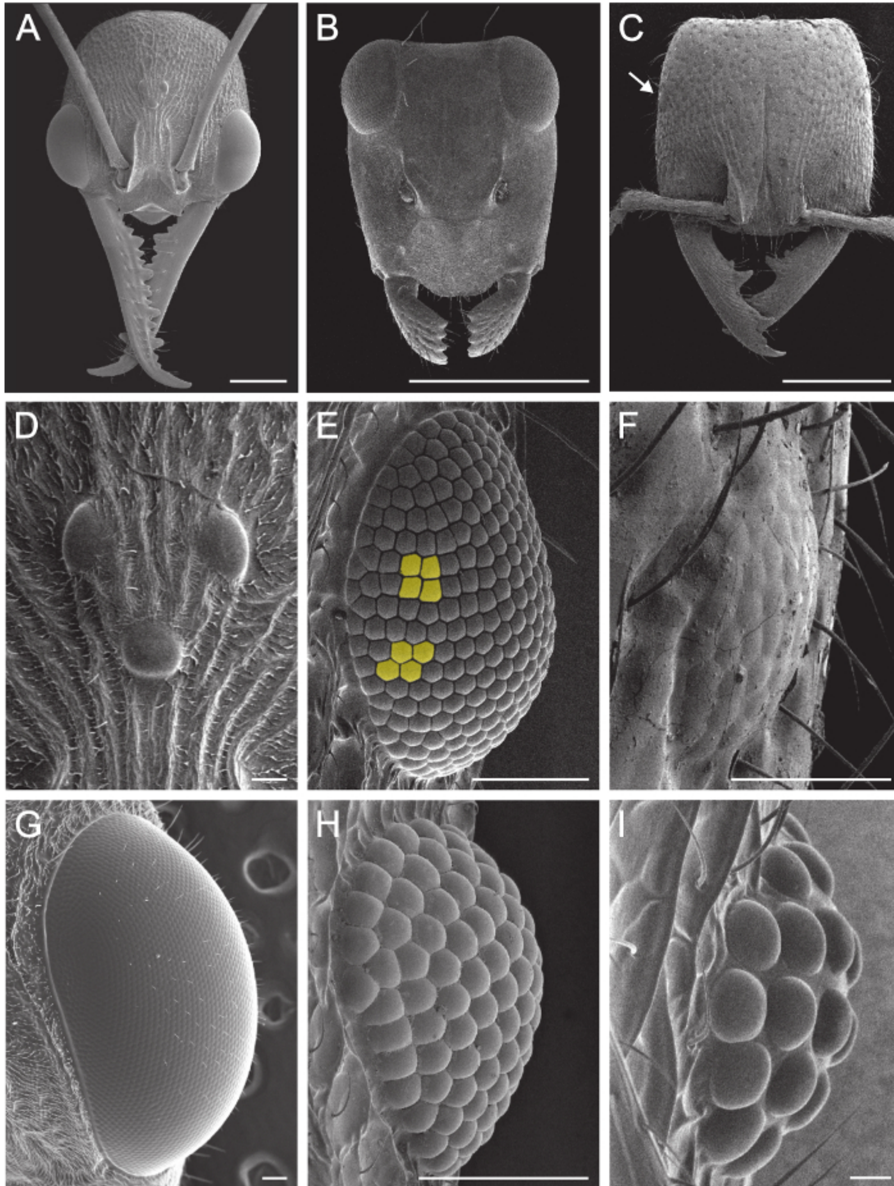

Figure 2: Scanning electron micrographs of the ant visual system demonstrating the imaging capabilities of this technique. Top row shows different eye positions and eye sizes in: (**A**) *Myrmecia nigriceps*; (**B**) *Opisthopsis pictus*; and (**C**) *Amblyopone australis* (note the very small eyes, white arrow). Images acquired at high magnification showing: (**D**) the three simple eyes in workers of *Myrmecia nigriceps;* different sized compound eye in (**E**) *Rhytidoponera metallica* (note the different shaped ommatidia in different regions of the compound eye in yellow), (**F**) *Amblyopone australis*, (**G**) *Myrmecia pyriformis,* (**H**) *Orectognathus clarki*, and (**I**) *Pheidole* species. Scale bars = 1 mm (A-C), 100 µm (D-H), 10 µm (I). Please click here to view a larger version of this figure.

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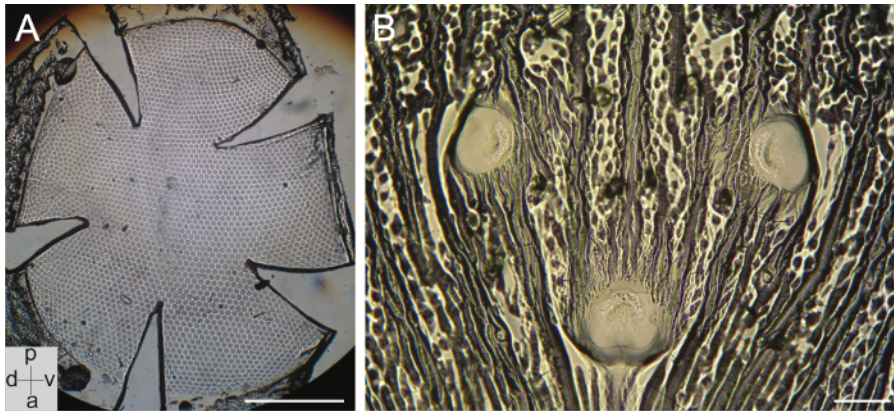

Figure 3: Cornea replicas of ant eye and ocelli. (**A**) Replica of the compound eye of a worker of *Myrmecia nigriceps*. The convex replica was flattened by making incisions. The inset indicates posterior (p), anterior (a), and dorsal (d), ventral (v) axes. (**B**) Replica of the ocelli of worker of *Myrmecia tarsata*. Scale bars = 0.5 mm (A), 10 µm (B). Please click here to view a larger version of this figure.

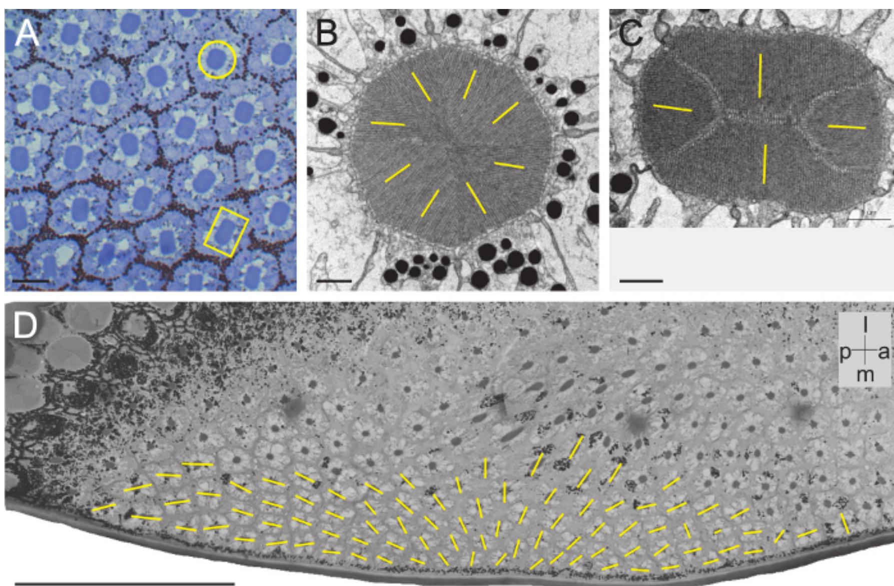

Figure 4: LM and EM images of rhabdom cross-sections. (**A**) Cross-section of distal rhabdoms in *Myrmecia nigriceps* stained in toluidine blue can be used to distinguish rhabdoms that are circular or rectangular in shape. Transmission electron micrographs show: (**B**) multiple orientations of microvilli in the circular rhabdom and (**C**) microvilli oriented in two opposite directions in the rectangular shaped rhabdom. (**D**) Using light microscopy, the long axis of the rectangular rhabdoms are mapped to show a fan-like organization in the dorsal region of the eye in a queen of *Camponotus consobrinus*; inset indicates posterior (p), anterior (a), and lateral (l), medial (m) axes. Panel D adapted from reference[20]. Scale bars = 10 µm (A), 1 µm (B-C), 100 µm (D). Please click here to view a larger version of this figure.

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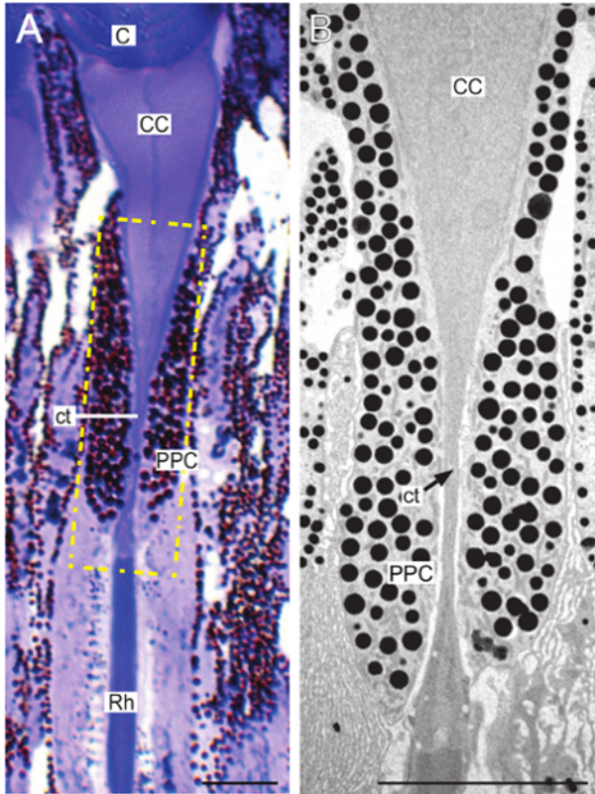

Figure 5: LM and EM images of an ommatidium in a light-adapted eye of *Myrmecia tarsata*. (**A**) Longitudinal section of an ommatidium showing the cornea (C), crystalline cone (CC), cone tract (ct), rhabdom (Rh) and primary pigment cells (PPC). (**B**) Dashed rectangular box in panel A from a different section viewed under a TEM to quantify the narrow width of the cone tract. Adapted from reference[21]. Scale bars = 10 µm. Please click here to view a larger version of this figure.

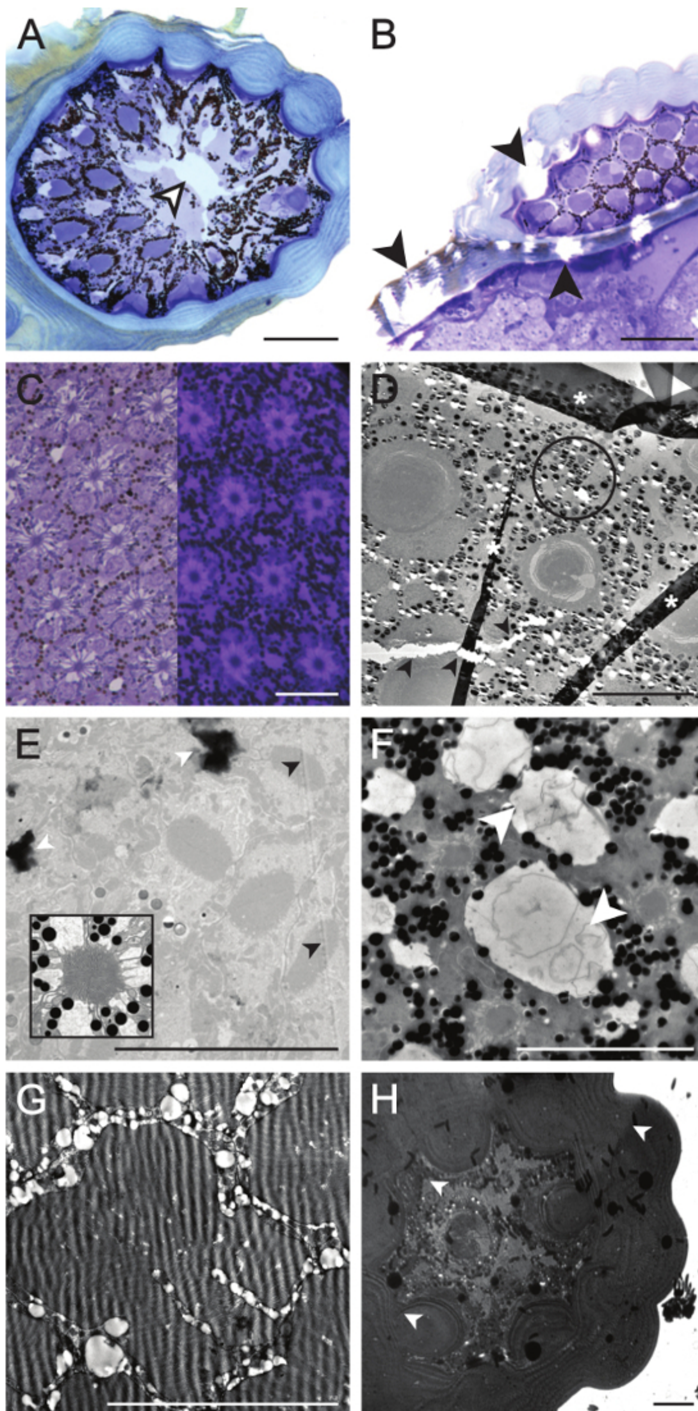

Figure 6: Common problems with semi-thin and ultra-thin sections (fixation and infiltration, cutting and staining). (**A**) Poor fixation of tissue due to inadequate penetration (arrow) in a semi-thin section of *Pheidole* species; (**B**) ripping during sectioning in *Iridomyrmex calvus* (semi-thin); (**C**) perfect staining (left) and over staining (right) with toluidine blue in *Myrmecia croslandi*; (**D**) pigment (circle) and tissue ripping during sectioning (arrows) due to poor matching of the resin and tissue density (resin too soft). Folding of the section (asterisks), can happen when collecting sections from the knife boat; (**E**) poor contrast due to insufficient staining (compare to inset), lead citrate crystals (white arrows) from exposure to CO_2, and vertical knife mark (black arrows); (**F**) holes in the tissue (white arrows) caused by poor fixation in a *Melophorus hirsutus* compound eye; (**G**) resin too soft, leads to chitter when sectioning seen as vertical ripples in the section; (**H**) section too thick (~100 nm) resulted in dark image with poor contrast, contaminated distilled water lead to bacteria and particulate matter scattered throughout the section (white arrows) in *Pheidole* species. Scale bar = 25 µm (A-B), 10 µm (C-H). Please click here to view a larger version of this figure.

Discussion

The suite of methods outlined above allow for an effective investigation into the optical system of ants and other insects. These techniques inform our understanding of sampling resolution, optical sensitivity, and potential polarization sensitivity of the eye being studied. This knowledge

provides an important foundation for physiological and behavioral investigation into their visual capabilities. Furthermore, while the methods detailed here have focused on ant visual systems, these techniques can be used on other insects, albeit with slight modifications in the protocol (*e.g.*, increasing duration of fixation and infiltration in thicker tissues). Slightly modified protocols have been used to characterize the visual systems of a variety of insects including cicadas[22], flies[14], bees[23], wasps[24], butterflies[25], and moths[26]. Although most of the techniques outlined here have been in use for some time, this article takes the opportunity to bring them together in the context of studying the ant's optical system and compare alternative techniques and describe common pitfalls.

There are many imaging techniques currently available that have overlapping applications and it can be difficult to assess which technique is appropriate for the task at hand. A relevant example here is choosing a technique for overview imaging. The external morphology of the head and eye and relative positioning of the optical system on the head can be done using SEM or photomicrography. The strengths and weaknesses of these techniques have been reviewed[27], however, there are some special considerations when imaging eyes. When imaging the relative positioning and size of the eyes, both techniques have their advantages and disadvantages. SEM images lack color information and hence where pigmentation is relevant photomicrography is better. However, SEM images can illustrate fine structures such as inter-ommatidial hairs and facet boundaries in greater detail and even reveal surface features not visible under photomicrography techniques (*e.g.*, ocellar lenses, surface sculpturing of compound eye lenses). SEM is a versatile technique when it comes to exploratory imaging and identifying features of interest because it can operate on a large range of specimen sizes while still retaining very high resolution throughout this range. However, it is not as widely accessible as a dissection microscope and requires a higher level of expertise. There is often no single way of obtaining the information one requires. In such a scenario, it is useful to consider what is available and where it is most important to invest resources.

Nail-polish replicas of the cornea have proven to be most useful in obtaining the most accurate measure of facet numbers and facet diameters. This has now been used in a variety of insects[11,22,28,29]. While the quality of the images acquired from an SEM is far superior, the curvature of the eye prevents accurate measurements of the whole facet array. Mapping the facet size and facet distribution should also be feasible from scans acquired from micro-computed tomography[5].

In both the LM and TEM techniques, it often is difficult to know whether the sample has been prepared and processed well until the final stage of imaging. To avoid complications, it is important to establish good practices such as maintaining clean working spaces and tools, preparing fresh solutions regularly, and thoroughly filtering water. Contaminants that are invisible to the naked eye can ruin EM samples. For this reason, it can be useful to wipe down surfaces and instruments using a solvent, such as ethanol or acetone, and a non-lint producing wipe. This is most relevant when sectioning, staining EM sections, and when preparing SEM samples. Similarly, distilled water sources can present problems and introduce contaminants so it is always best to check filters, change them regularly, and always use freshly filtered water (do not store). Most fixatives, stains, and embedding materials cannot be stored indefinitely and it is important to label all solutions with the date of preparation. It is important to take a systematic approach and set aside enough time to carry out protocols without interruptions.

Adapting techniques to different species is always a matter of trial and error. When working within Formicidae, the main differences lie in the size of the animal and the muscle mass within the head. Ants with more musculature in their head will typically take longer to fix. With very large ants, it is best to remove the mandibular muscles, trachea, and mandibular glands, while ensuring minimum interference with the neural tissue. In small ants and those with few mandibular muscles, it is possible to achieve adequate fixation by just removing the mandibles and exposing the clypeal region. In these cases, small holes using minutiae pins can be made on the head to improve fixation.

It is important to note that environmental conditions can also affect preparations. Hot and humid environments (especially field stations in the tropics) can prove to be a challenge during the infiltration stage. Warm conditions can lead resins to partially polymerize prematurely resulting in the unused resin becoming increasingly more viscous. In this case, the best option is to store the resin in small, single use, containers in the fridge or freezer. Cooling fixatives can be helpful to counter faster tissue decay in warm conditions. However, cooled solutions will disperse more slowly which means that treatment times should be extended to ensure proper penetration.

With these cautions in mind, investigation into the optical system of ants and other insects can prove very rewarding. Studying the visual system allows us to estimate the size of visual fields, interommatidial angles, optical sensitivity, and sampling resolutions. Understanding the anatomy of the eye informs our understanding and interpretation of animal behavior. For example, anatomy allows us to make predictions on the visual capabilities of animals such as whether they are diurnal or nocturnal, which may not have been previously documented. Given the current knowledge about the visual system of handful of ants, we hope our methods will inspire biologists and myrmecologists to investigate the compound eye and ocelli in ants to further our understanding.

Disclosures

The authors declare no competing interests.

Acknowledgements

We are grateful to Jochen Zeil, Paul Cooper and Birgit Greiner for sharing their knowledge in insect anatomy and for demonstrating several of the techniques we have described here. We are grateful to the talented and supportive staff at the Centre for Advanced Microscopy at ANU and The Microscopy Unit at MQU. This work was supported by a graduate scholarship to FRE and grants from the Australian Research Council (DE120100019, FT140100221, DP150101172).

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