Journal of Visualized Experiments

Dissection and Immunostaining of Larval Salivary Glands from Anopheles gambiae Mosquitoes

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62989R3
Full Title:	Dissection and Immunostaining of Larval Salivary Glands from Anopheles gambiae Mosquitoes
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TITLE:

Dissection and Immunostaining of Larval Salivary Glands from *Anopheles gambiae* Mosquitoes

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SUMMARY:

The adult mosquito salivary gland (SG) is required for the transmission of all mosquito-borne pathogens to their human hosts, including viruses and parasites. This video demonstrates efficient isolation of the SGs from the larval (L4) stage *Anopheles gambiae* mosquitoes and preparation of the L4 SGs for further analysis.

ABSTRACT:

Mosquito salivary glands (SGs) are a requisite gateway organ for the transmission of insect-borne pathogens. Disease-causing agents, including viruses and the Plasmodium parasites that cause malaria, accumulate in the secretory cavities of SG cells. Here, they are poised for transmission to their vertebrate hosts during a subsequent blood meal. As adult glands form as an elaboration of larval SG duct bud remnants that persist beyond early pupal SG histolysis, the larval SG is an ideal target for interventions that limit disease transmission. Understanding larval SG development can help develop a better understanding of its morphology and functional adaptations and aid in the assessment of new interventions that target this organ. This video protocol demonstrates an efficient technique for isolating, fixing, and staining larval SGs from *Anopheles gambiae* mosquitoes. Glands dissected from larvae in a 25% ethanol solution are fixed in a methanol–glacial acetic acid mixture, followed by a cold acetone wash. After a few rinses in phosphate-buffered saline (PBS), SGs can be stained with a broad array of marker dyes and/or antisera against SG-expressed proteins. This method for larval SG isolation could also be used to collect tissue for *in situ* hybridization analysis, other transcriptomic applications, and proteomic studies.

INTRODUCTION:

Malaria is a major public health threat causing almost 230 million infections and an estimated 409,000 deaths in 2019[1]. The majority of deaths are in sub-Saharan Africa and are caused by the parasite *Plasmodium falciparum*, whose insect vector is *Anopheles gambiae*, the subject of this video demonstration. Although the numbers indicate a significant drop in annual death rate since the turn of the century (>300,000 fewer annual deaths), the promising decreases in disease rates observed from 2000 to 2015 are tapering, suggesting the need for new approaches to limiting disease transmission[2]. Among promising additional strategies for controlling and possibly eliminating malaria is targeting mosquito vector capacity using CRISPR/Cas9-based gene-editing and gene-drive[3-5]. Indeed, it is the targeting of the mosquito vector (through the expanded use of long-lasting insecticide-treated bed nets) that has had the greatest impact on reducing disease transmission[6].

Female mosquitoes acquire Plasmodium gametocytes from an infected human during a blood meal. Following fertilization, maturation, midgut epithelium traversal, population expansion, and hemocoel navigation in their obligate mosquito hosts, hundreds to tens of thousands of Plasmodium sporozoites invade the mosquito SGs and fill the secretory cavities of the constituent secretory cells. Once inside the secretory cavities, the parasites have direct access to the salivary duct and are thus poised for transmission to a new vertebrate host upon the next blood meal. Because SGs are critical for the transmission of malaria-causing sporozoites to their human hosts, and laboratory studies suggest that SGs are not essential for blood-feeding, mosquito survival, or fecundity[7-9], they represent an ideal target for transmission-blocking measures. Adult mosquito SGs form as an elaboration of "duct bud" remnants in the larval SGs that persist beyond early pupal SG histolysis[10], making the larval SG an ideal target for interventions to limit adult-stage disease transmission.

Characterizing the larval stage of SG development can help develop not only a better understanding of its morphology and functional adaptations but can also aid in assessing new interventions that target this organ through gene editing of key SG regulators. Because all previous studies of larval salivary gland architecture predate immunostaining and modern imaging techniques[10,11], we have developed a protocol for isolating and staining salivary glands with a variety of antibodies and cell markers[12]. This video demonstrates this approach to the extraction, fixation, and staining of larval SGs from *Anopheles gambiae* L4 larvae for confocal imaging.

PROTOCOL:

1. Preparation of solutions and tools

1.1. Preparation of dissection solution

1.1.1. To prepare dissecting solution, add 2.5 mL of 100% ethanol to 7.5 mL of distilled H_2O in a 15 plastic tube. Invert the tube 3 times to mix.

NOTE: This solution can be stored at room temperature for several weeks.

1.2. Preparation of 10x phosphate-buffered saline (PBS) stock

1.2.1. To prepare 10x PBS stock, add 17.8 g $Na_2HPO_4 \bullet 2H_2O$, 2.4 g KH_2PO_4, 80 g NaCl, and 2 g KCl to 800 mL of deionized water. Mix with a stir bar on a stir plate until the solids have fully dissolved. Adjust the final volume to 1 L with purified water.

NOTE: This solution can be stored at room temperature for several months.

1.3. Preparation of 1x PBS

1.3.1. Add 10 mL of 10x PBS to 90 mL of H_2O in a sterile glass bottle. Close the lid tightly and invert 3x to mix.

NOTE: The solution can be stored at 4 °C for several weeks.

1.4. Preparation of fixative.

1.4.1. Add 600 µL of methanol to 200 µL of glacial acetic acid. Make the solution fresh each time.

1.5. Construction of putty plate (a tissue culture dish filled with silicone rubber)

1.5.1. Mix the components (1:50) of epoxy (1 g) and water (50 mL) and pour the mixture into a Petri dish. Wait for the components to dry before using.

NOTE: Once constructed, the putty plate can be used for years.

2. Gland dissection (**Figure 1A**)

2.1. Collect late-stage L4 larvae (~10 days post hatch; **Figure 2**) from feeding trays using a plastic transfer pipette.

NOTE: See this helpful online manual for standard mosquito husbandry and larval culture[13].

2.1.1. Place a putty plate on the stage of a dissecting microscope and transfer larvae onto the putty plate.

NOTE: When aliquoting larvae for initial dissection, it is helpful to transfer ~10 larvae onto the slide at one time using a plastic transfer pipette.

2.2. Place a drop of dissection solution (1:3 EtOH:H_2O) onto the putty plate, separate from the 10 larvae.

2.3. Use a plastic transfer pipette or disposable glass pipette to place one L4 larva into the 25% EtOH drop.

2.4. Take forceps (#5), one in each hand, and with the non-dominant hand, grip the head of the larva. Using the dominant hand, grasp the larva with forceps just below the head and gently pull with minimal constant force, such that the head detaches from the rest of the body and the glands remain attached to the head. Discard the body portion of the larva carcass.

NOTE: When dissecting, set a paper towel nearby to collect the larvae carcasses.

2.5. Collect the head/glands into 1 mL of 1x PBS in a 1.5 mL microcentrifuge tube. Use 1 mL of PBS for ~40 dissected glands with their attached heads. Wait for the heads/SGs to sink to the bottom of the buffer.

3. Fixation for antibody staining (**Figure 1B**)

3.1. Drain the PBS starting from the top of the microcentrifuge tube using a glass transfer pipette, avoiding the sticky mosquito tissues and removing as much of the PBS solution as possible without damaging the tissues. Replace the solution with 800 µL of a 3:1 mixture of methanol to glacial acetic acid. Place the tube at 4 °C overnight (12–24 h recommended; 19 h preferred).

3.2. The following day, drain the solution and replace it with 1 mL of cold 100% acetone. Leave for 90 s.

2.3 Remove the acetone and gently rinse the tissues three times with 1 mL of 1x PBS each time.

NOTE: The samples should float slowly up with the flow, then fall back to the bottom of the tube by the time each PBS addition is complete.

4. Immunostaining (**Figure 1B**)

4.1. Add primary antibody (e.g., Rab11) at the appropriate dilution (1:100) in 200 µL of the total volume of 1x PBS. Swirl the tube contents gently with a pipette tip. Incubate overnight at 4 °C.

4.2. Remove the primary antibody solution and wash three times with 1 mL of 1x PBS (gently pipetting the solution in and out of the pipette).

4.3. Add secondary antibody (Alex Fluor 488 Goat anti-Rabbit) at the appropriate dilution (1:200) in 200 µL of total volume. Swirl the tube contents gently with a pipette tip. Incubate at room temperature for 90 min.

4.4. Add dyes, such as Nile Red (lipids, 5 µL of 1 µg/µL) and/or Hoechst (DNA, 3 µL of 1 µg/µL), into 200 µL of PBS at this stage. Incubate at room temperature for an additional 60 min. Wash gently three times with 1x PBS.

5. Mounting stained glands for microscopy (**Figure 1C**)

5.1. Pipette 200 µL of 100% glycerol onto a microscope slide.

NOTE: As glycerol is viscous, leave the pipette tip in the liquid until it reaches the appropriate volume. No tissue shrinking was observed when going straight into 100% glycerol. However, researchers can go through 30% and 50% glycerol washes in the tubes before moving the samples into 100% glycerol.

5.2. Transfer the stained heads (up to 20 per slide) with the attached glands (along with other internal structures) to the microscope slide using a soft brush (**Figure 3A**). Spread the samples out so that they are evenly distributed along the glass slide.

NOTE: When depositing glands on a cover slide with a brush, forceps can be used to gently orient the SGs so that they are all oriented in the same direction.

5.3. Viewing under a dissecting microscope, separate the larval head from the glands using two pairs of forceps and carefully pulling the two tissues in opposite directions.

NOTE: As it is challenging to completely isolate the SGs, simply remove the heads, leaving the SGs and associated internal structures. Even if the SGs remain associated with other tissues, they are easily recognized when stained with Hoechst and other markers (**Figure 3B,C**).

5.4. Remove and discard the heads and repeat the separation process for each SG.

NOTE: When removing heads, set a paper towel nearby to collect them.

5.5. Gently place a 1.5 mm thick coverslip on the top (avoiding air bubbles) and seal with clear nail polish.

5.6. Store at 4 °C in a light-proof container.

NOTE: Prepared slides of stained glands can be kept at 4 °C in the dark for up to six months to one year without any notable changes in quality. If glycerol starts to leak as the months go by, gently lift the coverslip and pipette in more glycerol to fill holes.

REPRESENTATIVE RESULTS:

Salivary glands are relatively easy to dissect from all stage 4 larvae. Male and female larvae can be distinguished at the late L4 larval stage by a red stripe along the dorsal thorax of females but

not males (**Figure 2**). We also observe that antennal morphology is much more elaborate in male than in female L4 larvae (**Figure 2**), similar to the differences observed in this structure in adult mosquitoes. Along with the considerable overall growth during the L4 stage, the salivary glands also form a lumen during L4[12]. Salivary glands isolated from early L4 stage larvae stained with Hoechst reveal the proximal and distal lobes separated by a narrow constriction (**Figure 3B,C**). The forming lumen will extend all the way from the salivary duct at the proximal-most end (not shown) through the distal lobe. The forming lumen can be seen in the immunostained distal salivary gland of a mid-to-late L4 larva (**Figure 4**). The apical domains of the secretory cells surrounding the forming lumen have intense Nile Red staining (**Figure 4B,C**) suggestive of microvilli-like structures. Also observed close to the apical surface is Rab11 staining (**Figure 4C,D**, arrows). Rab11 localizes to apical recycling endosomes. The Rab11 staining that also accumulates along the basal surface of the gland is an artefact due to the stickiness of the basal membrane. Similar background staining is common with immunostaining of both larval and adult salivary glands and has been mistaken for bona fide signal.

FIGURE AND TABLE LEGENDS:

Figure 1: Cartoon visualization of the immunostaining process from gland dissection through slide preparation. Abbreviations: SGs = salivary glands; PBS = phosphate-buffered saline; MeOH = methanol; DAPI = 4',6-diamidino-2-phenylindole; Abs = antibodies; LH = left hand; RH = right hand.

Figure 2: Male and female L4 stage *Anopheles gambaie* larvae. (**A, B**) Early L4 larvae. (**C, D**) Late L4 Larvae. There is considerable growth during the L4 stage. Females (**A, C**) have been described as having a distinguishing red stripe down the dorsal thorax (**C**; black arrow) that is not present in males (**B, D**), but also their antennae are much less elaborate (frilly) than those of male larvae from both early and late L4 (white arrows in enlarged images). Scale bars = 1 mm.

Figure 3: Early L4 salivary glands. (**A**) Early L4 female larval head with salivary glands and other internal organs still attached. Salivary gland is outlined. Scale bar = 50 µm. (**B, C**) Isolated larval glands stained with Hoechst. (**B**) Merge of fluorescent and Nomarski images showing the shape of the proximal and distal lobes and the blue Hoechst staining in nuclei. (**C**) Hoechst fluorescent image highlights nuclei only. Scale bar in bottom panels = 20 µm.

Figure 4: Immunostained distal L4 salivary gland. (**A**) Gland has been stained with Hoechst (nuclear DNA, blue) and (**B**) Nile Red (membrane marker, red); immunostained for Rab11 (apical recycling vesicles, green) (**C**). (**D**) The merged image. Note that a lumen is present at the stage shown (**B–D**). The arrows in **C** and **D** point to the expected Rab11 staining of vesicles near the apical surface that overlaps the Nile Red-positive vesicular staining in the merge (white arrows). Non-specific background staining around the basal surface (asterisks) is also observed, a common type of background observed in larval and adult salivary glands. Scale bars = 20 µm.

DISCUSSION:

The protocol described herein was adapted from a Drosophila SG dissection protocol and an adult mosquito dissection protocol[14-16]. However, most markers did not penetrate the basement

membrane (data not shown) when using the adult dissection and SG staining methods. Adaptations of the adult protocol included dissecting the glands in a 25% EtOH solution, washing the glands with a combination of MeOH and glacial acetic acid, and having a 90 s acetone wash. In the original adult protocol, adult SGs were dissected in 1x PBS[14-16]. Dissecting in a 25% EtOH solution helped preserve the SGs and prevented damage during the staining periods. When performing the initial dissection, it was easiest to orient the larval SGs with the head facing the non-dominant hand and having the dominant hand gently pull outwards (because the larvae are very actively moving). The improved tissue penetration achieved by washing the glands with a MeOH: glacial acetic acid solution suggests that the lipid content in the larval SG basement membrane and/or cellular plasma membranes is distinct from that of the adult SG. The 90 s acetone wash improved the clarity of glands for imaging. Although the fixation period was recommended to be at least 19 h (overnight), longer periods worked just as effectively.

The morphology of the SGs can vary widely depending on when the larvae are dissected in the 2-day long L4 stage. In early L4 dissections (day 1), the lumen appears much smaller[12]. In late L4 dissections (second half of day 2), the lumen is large, and the cells are elongated. We found that the optimal period for working with larvae was the L4; L1–L3 SGs are simply too small for manual dissections. In imaging results, glands dissected at mid-to-late-L4 showed smaller cells mixed with larger, laterally elongated cells, particularly in the distal sac.

Previous reports on *Anopheles* SG development have provided much guidance for the current studies. These reports have included illustrations of dissected embryonic and larval SGs[17,18], detailed microscopic analysis of dissected glands[19,20], and transcriptomic studies[20-22]. The features of the *Anopheles stephensi* SG were reported during the 1950s by two different groups[10,11]. Many of the morphological features of the larval glands were described, including the overall organization of the SG, the relative positions of distinct cell types within the organ (duct, adult SG precursors, and the larval proximal and distal secretory sacs)[10,11]. Both groups also reported additional morphometric data, including the number and distribution of secretory cells within each sac and their nuclear size[10,11]. Rishikesh showed that, like the Drosophila larval SGs, the larval SG chromosomes from *Anopheles stephensi* are polytenized[10]. These important foundational overviews described broad biological aspects of larval *Anopheles* SGs.

The method described herein should be useful for studying not only larval SGs of *An. gambiae* but can also apply to those studying other species of mosquitos. Indeed, the same protocol has been successfully utilized (unpublished) with *An. stephensi*, a species frequently studied in the laboratory. One limitation to the protocol is the limited number of antibodies that have been specifically generated against mosquito proteins. Although using Drosophila antibodies for highly conserved proteins has circumnavigated this limitation[12], the field could benefit from more mosquito protein-specific antibodies. Understanding larval SG cellular and molecular biology can contribute to new control or target strategies and allow for the discovery of new candidate target genes for SG disruption.

ACKNOWLEDGMENTS:

We would like to thank the Johns Hopkins Malaria Research Institute for access to and rearing of *An. gambiae* larvae.

DISCLOSURES:

The authors have no conflicts of interest to disclose.

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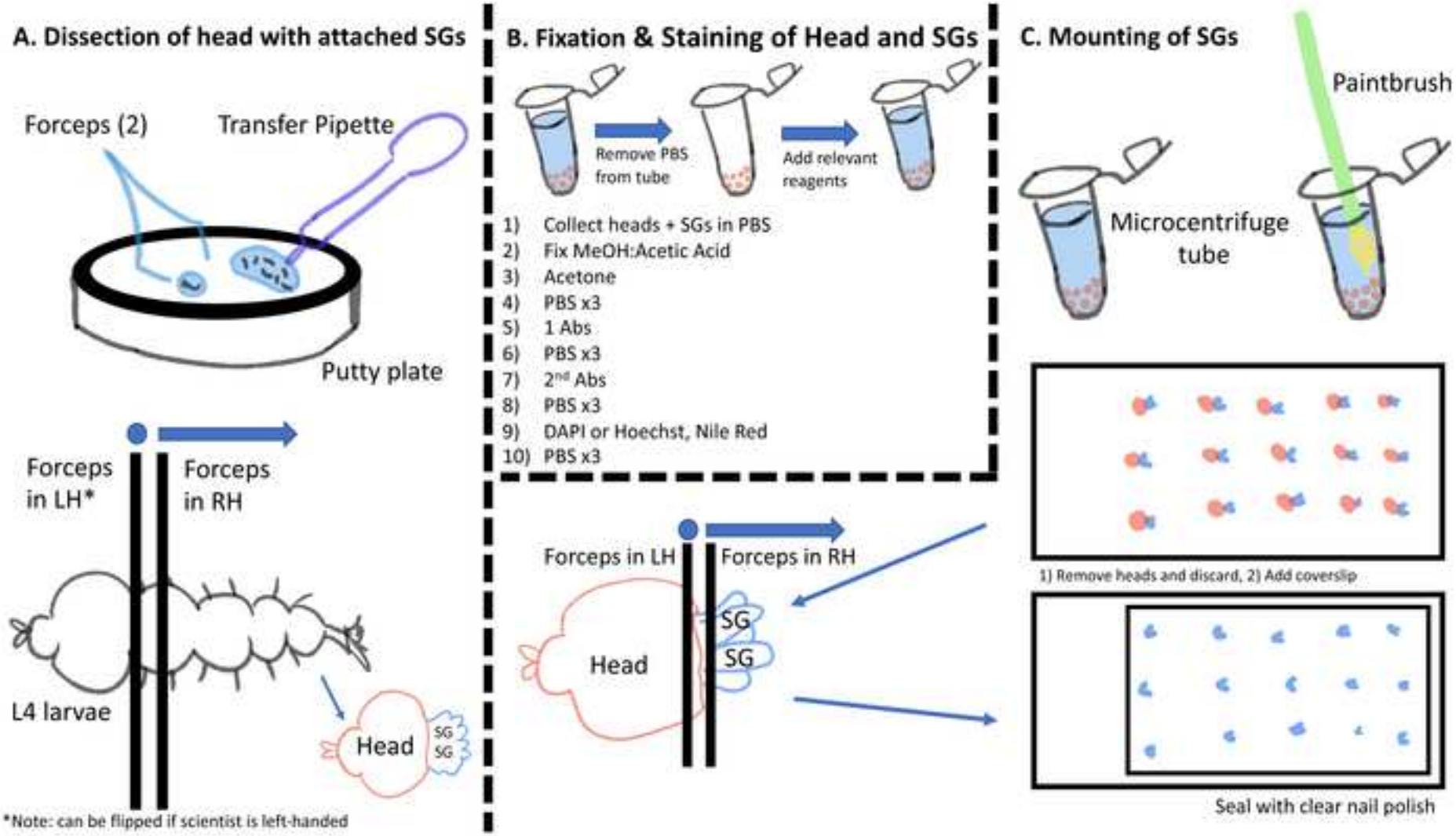
Figure 1



Figure

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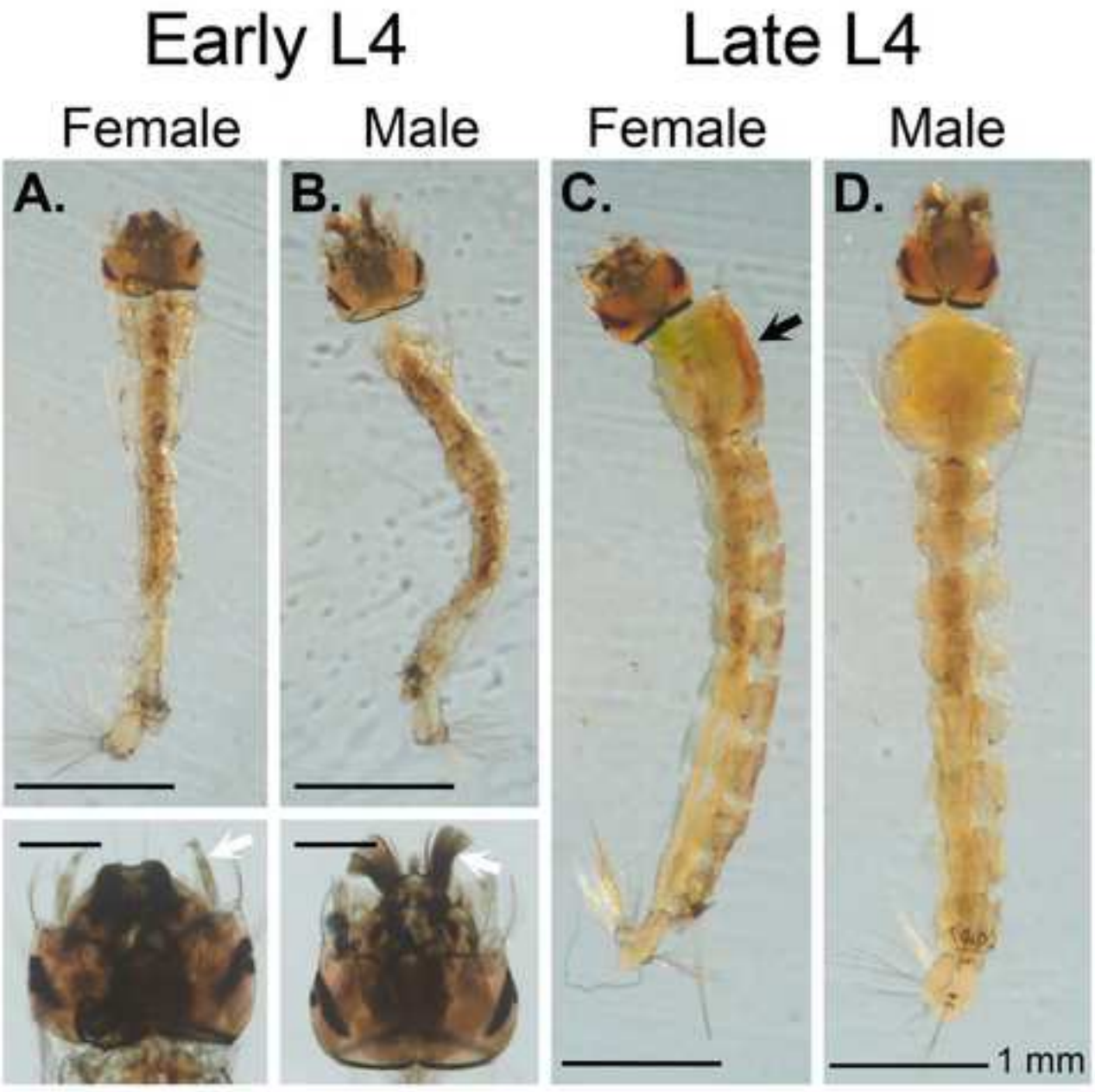

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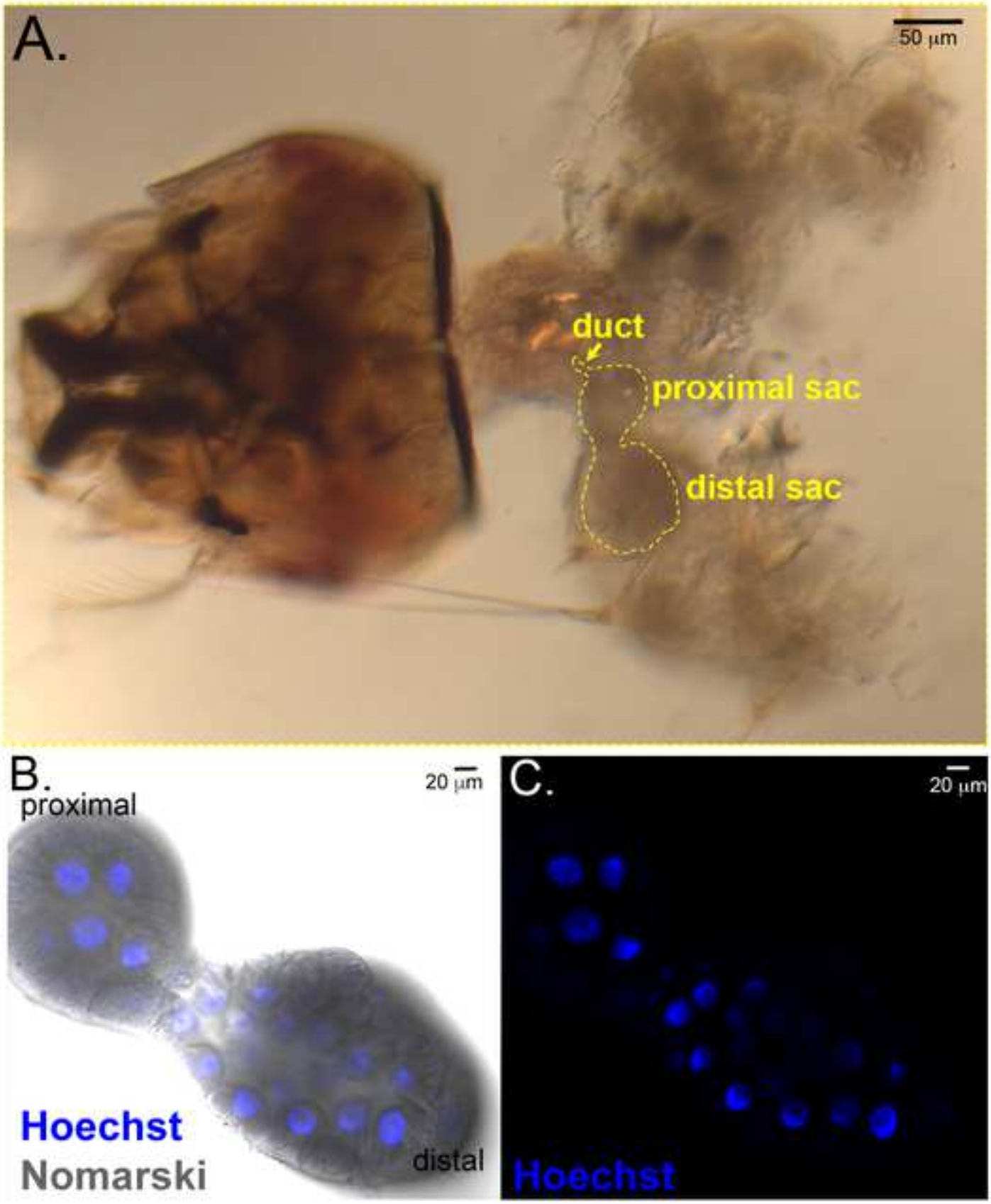

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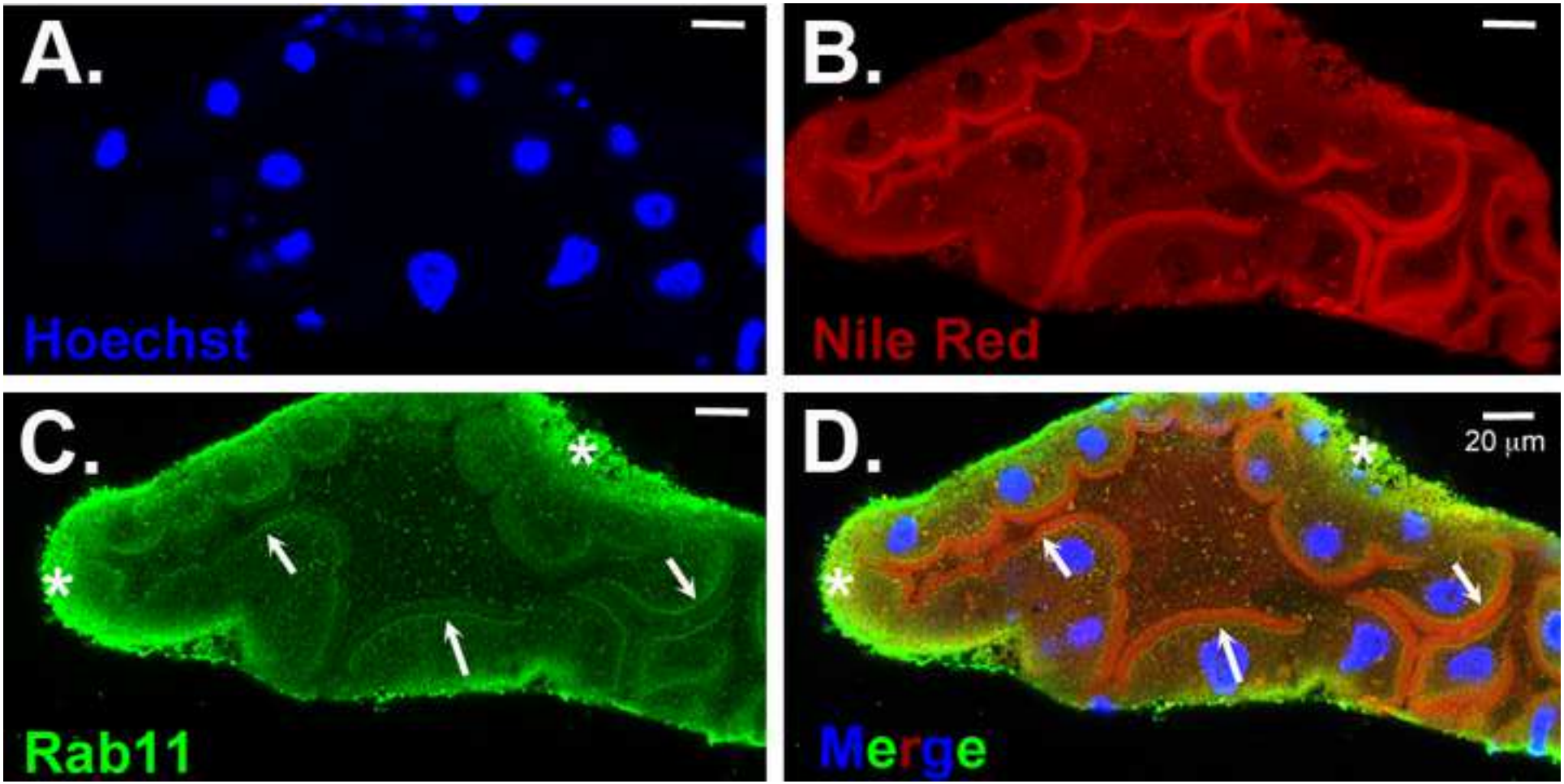

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Table of Materials

Table 1. Materials and Reagents 082221.xlsx

29 July 2021

Dear Editor,
I believe we have addressed all of the editorial comments and the suggestions by the reviewers. We appreciate how much the manuscript has improved with these changes. I am including specific responses after each comment in italics.

Thanks,

Debbie Andrew

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. *Done.*
2. Please revise the following lines to avoid previously published work: 181-182, 204-218. *Done.*
3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). *Done.*
4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Eppendorf, etc.
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5. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks
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Done.
7. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
Done.
8. Line 71: Please include a citation which can help readers to understand how the mosquitoes are prepared for laying eggs and hatching.
Done.
9. Line 73: Please define what is “Putty Plate”.
Done.
10. Line 88/91: Please specify the volume of the solution used.
Done.
11. Line 98/102: Please specify the dilution of antibodies used in this experiment.

We have provided a reference for all of the antibodies that we have used with this protocol, both primaries and secondaries..

12. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
I think we have provided video footage related to each step in the protocol.
13. Please consider providing reaction set-ups and solution compositions as Tables in separate .xls or .xlsx files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text.
None of the solutions have complicated formulas, thus we would prefer to leave them in the text.
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d) The significance with respect to existing methods
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19. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material. *Done.*

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Chiu et al. describe a procedure for investigating the salivary glands of late larval stages of the African malaria mosquito, Anopheles gambiae using immunostaining. The authors are experts in the study of salivary gland development and function in Drosophila melanogaster and, over the last few years, have leveraged that expertise and the well developed technical resources available for studying that system against salivary gland development and function in Anopheles gambiae. Their technical insights are valuable and I support the creation of a video protocol.

Overall the procedure appears straightforward but visualizing the initial dissection steps and the ultimate step of separating the processed salivary glands from the head are likely to be extremely helpful to anyone attempting this for the first time.

Note that I have only seen the text version of this and on page 14 the authors list videos in addition to figures. I did not see any videos.

Major Concerns:

A general concern is that the introduction could be a bit more scholarly. These comments are intended to be helpful:

A few comments the authors might consider:

1) They use malaria fatality rates to emphasize the significance of malaria as a public health problem and say "we have a long way to go….". They might mention that the bulk of the fatalities are caused by Plasmodium falciparum malaria in Africa and transmitted by Anopheles gambiae - the subject of their video. They might consider looking at Feachem et al (2019) 10.1016/S0140-6736(19)31139-0 and perhaps referencing it to support their statement that "we have a long way to go…" or more explicitly say something about the need for new tools and approaches. *We have made these changes and cited the paper mentioned.*

2) They say 'one attractive strategy …is to target transmission by the mosquito vectors of the disease'. For all intents and purposes mosquitoes are the only way to contract malaria. They authors might consider looking at Bhatt et al (2015) 10.1038/nature15535. This paper nicely illustrates that targeting the vector has been the most impactful approach to reducing the incidence of and deaths from malaria between 2000 and 2015. So targeting the vector is not just 'one ' attractive strategy but 'the' most impactful and important strategy and this is not likely to change in the foreseeable future. *We have made these changes and cited the paper mentioned.*

3) The authors talk about 'irradicating' (sic) malaria but perhaps controlling and eliminating are more appropriate. *We have made this change.*

4) They say "Following fertilization, maturation, population expansion , midgut epithelium traversal….". There is no Plasmodium parasite population expansion in the midgut. Ookinetes do not undergo mitosis prior to traversing the epithelium. *We have altered the text to more accurately capture the sequence of events.*

5) They reference Sterling et al 1973 to support a claim that salivary glands are not essential for mosquito survival or fecundity. That paper is a classic EM study of infected salivary glands and does not report evidence regarding the essential or non essential role

of salivary glands. The authors are encouraged to support that statement with references. Have a look at Mellink et al (1981) Mosquito News 41:115-119 and Ribeiro et al (1984)10.1242/jeb.108.1.1. More recently Yamamoto et al (2016) 10.1371/journal.ppat.1005872 might be useful. *We have altered the text a bit and included these references.*
The robustness of the protocol and its range of application are not well documented and this limits it potential impact and usefulness. *We cite the paper where this protocol has been applied (and as stated in response to (7), we have tested the protocol in one other spp of mosquito).*
6) The protocol is limited to L4 larvae. They talk about the optimal stage which suggests that this protocol is limited in applicability. *We have clarified that L4 is optimal because of its larger size and easier dissections.*
7) The authors state that the method can also apply to other species of mosquito. Have they actually tried it with other species. An. stephensi, Aedes aegypti, Culex spp? If so, then data demonstrating the robustness of the method would be useful. *We now include that we have used this protocol for one other spp of mosquitoes and it works well. It has not been tested beyond this.*

Reviewer #2:
Manuscript Summary:
The authors provide a protocol for dissection and staining of larval salivary glands from Anopheles mosquitoes. The protocol is well-written and straightforward to follow. I feel that with the protocol and appropriate accompanying video, I would be able to successfully isolate and stain the salivary glands.

Major Concerns:
none

Minor Concerns:
There are a few minor things that could be improved for better clarity:
(1) In the "Introduction" I believe the intention is a "strategy for eradicating malaria" rather than "irradicating" it. *Repaired.*
(2) The reference to the Chiu et al IMB paper is listed as both 2021 and 2020 *Repaired.*
(3) While the protocol states that the glands remain attached to the head until separated following staining, the text in many steps refers just to the salivary glands (§2.1 "without damaging the SGs", §2.3 "the glands should float slowly up", etc.). This is confusing as it makes it sound as if they have been separated already. *Repaired.*
(4) It would be helpful if one of the figures clearly showed the dissected head+salivary gland unit, with the SG clearly marked. Presumably this will be in the video, but it's an important aspect and a still in the manuscript is I think warranted. *This is not something we could manage in the time frame.*
(5) What size cover slip is recommended? 22x22 mm square? Or maybe 22x50 rectangular which is more in accord with the drawing. *This information has been provided.*
(6) More detail is required on the "putty plate." All it says is to mix the epoxy and water—there is no indication as to what sort of epoxy is preferred (we used to use Sylgard for this

type of thing). Also, what size petri dish is considered optimal? *This is now described and the epoxy we used and the plates we used are in the table of reagents with the company and product number.*
(7) In the Discussion, there is some discussion about the optimal stage for dissection, but it is never really made clear. Earlier in the paper is says "late L4" while the discussion here seems to me to imply more of mid-L4. Nor is it made clear how to distinguish the relevant timing. *See response to reviewer 1.*

Reviewer #3:
The objective of the study is clear and is unexpected. For the first time I expected to conduct the technique on the salivary gland (SG) of mosquito adults, and not on the salivary glands of fourth-instar larvae, due to the medical importance of adult SGs in the transmission of pathogens. The protocol is efficient and opens further horizons for the scientific community. For example, it would help in evaluating new interventions that target SG through gene editing of key SG regulators. Discussion is adequate; however, there are some suggestions/speculations that need references. The paper meets the scope of the journal and is worthy to be published in Journal of Visualized Experiments.

Reviewer #4:
Manuscript Summary:
The manuscript by Michelle et al., describes a simple methods of isolation and stain9ng of larval mosquito salivary glands. The technique is well described and will be highly appr3ciated by the community. The authors show familiarity with the technique that is demonstrated by multiple important details highlighted by the authors. I recommend this article to be published in the JoVE journal.

Minor Concerns:
Introduction
third paragraph:
…editing of key salivary gland regulators…. Change to …editing of key SG regulators…. *Done*

Protocol
Step 4.1 indicates use of 100% glycerol. Sometime small soft tissues have the tendency to shrink in 100% glycerol therefore multiple groups use first 20%, 50%, 70% (5 min each) until placed into the 100% glycerol. Did the authors observed such a phenomenon? *As indicated (now) in the manuscript, we did not observe this shrinking.*

TITLE:
Dissection and Immunostaining of Larval Salivary Glands from *Anopheles gambiae* Mosquitoes

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SUMMARY:
The adult mosquito salivary gland (SG) is required for the transmission of all mosquito-borne pathogens to their human hosts, including viruses and parasites. This video demonstrates efficient isolation of the SGs from the larval (L4) stage *Anopheles gambiae* mosquitoes and preparation of the L4 SGs for further analysis.

ABSTRACT:
Mosquito salivary glands (SGs) are a requisite gateway organ for the transmission of insect-borne pathogens. Disease-causing agents, including viruses and the Plasmodium parasites that cause malaria, accumulate in the secretory cavities of SG cells. Here, they are poised for transmission to their vertebrate hosts during a subsequent blood meal. As adult glands form as an elaboration of larval SG duct bud remnants that persist beyond early pupal SG histolysis, the larval SG is an ideal target for interventions that limit disease transmission. Understanding larval SG development can help develop a better understanding of its morphology and functional adaptations and aid in the assessment of new interventions that target this organ. This video protocol demonstrates an efficient technique for isolating, fixing, and staining larval SGs from *Anopheles gambiae* mosquitoes. Glands dissected from larvae in a 25% ethanol solution are fixed in a methanol–glacial acetic acid mixture, followed by a cold acetone wash. After a few rinses in phosphate-buffered saline (PBS), SGs can be stained with a broad array of marker dyes and/or antisera against SG-expressed proteins. This method for larval SG isolation could also be used to collect tissue for *in situ* hybridization analysis, other transcriptomic applications, and proteomic studies.

INTRODUCTION:

Malaria is a major public health threat causing almost 230 million infections and an estimated 409,000 deaths in 2019[1]. The majority of deaths are in sub-Saharan Africa and are caused by the parasite *Plasmodium falciparum*, whose insect vector is *Anopheles gambiae*, the subject of this video demonstration. Although the numbers indicate a significant drop in annual death rate since the turn of the century (>300,000 fewer annual deaths), the promising decreases in disease rates observed from 2000 to 2015 are tapering, suggesting the need for new approaches to limiting disease transmission[2]. Among promising additional strategies for controlling and possibly eliminating malaria is targeting mosquito vector capacity using CRISPR/Cas9-based gene-editing and gene-drive[3-5]. Indeed, it is the targeting of the mosquito vector (through the expanded use of long-lasting insecticide-treated bed nets) that has had the greatest impact on reducing disease transmission[6].

Female mosquitoes acquire Plasmodium gametocytes from an infected human during a blood meal. Following fertilization, maturation, midgut epithelium traversal, population expansion, and hemocoel navigation in their obligate mosquito hosts, hundreds to tens of thousands of Plasmodium sporozoites invade the mosquito SGs and fill the secretory cavities of the constituent secretory cells. Once inside the secretory cavities, the parasites have direct access to the salivary duct and are thus poised for transmission to a new vertebrate host upon the next blood meal. Because SGs are critical for the transmission of malaria-causing sporozoites to their human hosts, and laboratory studies suggest that SGs are not essential for blood-feeding, mosquito survival, or fecundity[7-9], they represent an ideal target for transmission-blocking measures. Adult mosquito SGs form as an elaboration of "duct bud" remnants in the larval SGs that persist beyond early pupal SG histolysis[10], making the larval SG an ideal target for interventions to limit adult-stage disease transmission.

Characterizing the larval stage of SG development can help develop not only a better understanding of its morphology and functional adaptations but can also aid in assessing new interventions that target this organ through gene editing of key SG regulators. Because all previous studies of larval salivary gland architecture predate immunostaining and modern imaging techniques[10,11], we have developed a protocol for isolating and staining salivary glands with a variety of antibodies and cell markers[12]. This video demonstrates this approach to the extraction, fixation, and staining of larval SGs from *Anopheles gambiae* L4 larvae for confocal imaging.

PROTOCOL:

1. Preparation of solutions and tools

1.1. Preparation of dissection solution

1.1.1. To prepare dissecting solution, add 2.5 mL of 100% ethanol to 7.5 mL of distilled H_2O in a 15 plastic tube. Invert the tube 3 times to mix.

Commented [A1]: Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
I think we have provided video footage related to each step in the protocol.

Editor: I highlighted the steps based on the videos you have uploaded. But as you have not provided videos for all steps, those steps are unhighlighted and hence, the continuity is lost. Also, given that your title has immunostaining in it, you don't have any video on the immunostaining aspect (steps or images) so this part is not represented in your paper or video. Please provide videos for this also. The video production department has checked the videos and found them to be of good quality. A comment/question has been inserted about the time-lapse video; please check that. I have included AuthorProducedVideo criteria as a PDF attachment.

NOTE: This solution can be stored at room temperature for several weeks.

1.2. Preparation of 10x phosphate-buffered saline (PBS) stock

1.2.1. To prepare 10x PBS stock, add 17.8 g $Na_2HPO_4 \bullet 2H_2O$, 2.4 g KH_2PO_4, 80 g NaCl, and 2 g KCl to 800 mL of deionized water. Mix with a stir bar on a stir plate until the solids have fully dissolved. Adjust the final volume to 1 L with purified water.

NOTE: This solution can be stored at room temperature for several months.

1.3. Preparation of 1x PBS

1.3.1. Add 10 mL of 10x PBS to 90 mL of H_2O in a sterile glass bottle. Close the lid tightly and invert 3x to mix.

NOTE: The solution can be stored at 4 °C for several weeks.

1.4. Preparation of fixative.

1.4.1. Add 600 µL of methanol to 200 µL of glacial acetic acid. Make the solution fresh each time.

1.5. Construction of putty plate (a tissue culture dish filled with silicone rubber)

1.5.1. Mix the components (1:50) of epoxy (1 g) and water (50 mL) and pour the mixture into a Petri dish. Wait for the components to dry before using.

NOTE: Once constructed, the putty plate can be used for years.

2. Gland dissection (**Figure 1A**)

2.1. Collect late-stage L4 larvae (~10 days post hatch; **Figure 2**) from feeding trays using a plastic transfer pipette.

NOTE: See this helpful online manual for standard mosquito husbandry and larval culture[13].

2.1.1. Place a putty plate on the stage of a dissecting microscope and transfer larvae onto the putty plate.

Commented [A2]: Video 1

NOTE: When aliquoting larvae for initial dissection, it is helpful to transfer ~10 larvae onto the slide at one time using a plastic transfer pipette.

2.2. Place a drop of dissection solution (1:3 EtOH:H_2O) onto the putty plate, separate from the 10 larvae.

Commented [A3]: Video 2

2.3. Use a plastic transfer pipette or disposable glass pipette to place one L4 larva into the 25% EtOH drop.

2.4. Take forceps (#5), one in each hand, and with the non-dominant hand, grip the head of the larva. Using the dominant hand, grasp the larva with forceps just below the head and gently pull with minimal constant force, such that the head detaches from the rest of the body and the glands remain attached to the head. Discard the body portion of the larva carcass.

NOTE: When dissecting, set a paper towel nearby to collect the larvae carcasses.

2.5. Collect the head/glands into 1 mL of 1x PBS in a 1.5 mL microcentrifuge tube. Use 1 mL of PBS for ~40 dissected glands with their attached heads. Wait for the heads/SGs to sink to the bottom of the buffer.

3. Fixation for antibody staining (Figure 1B)

3.1. Drain the PBS starting from the top of the microcentrifuge tube using a glass transfer pipette, avoiding the sticky mosquito tissues and removing as much of the PBS solution as possible without damaging the tissues. Replace the solution with 800 µL of a 3:1 mixture of methanol to glacial acetic acid. Place the tube at 4 °C overnight (12–24 h recommended; 19 h preferred).

3.2. The following day, drain the solution and replace it with 1 mL of cold 100% acetone. Leave for 90 s.

2.3 Remove the acetone and gently rinse the tissues three times with 1 mL of 1x PBS each time.

NOTE: The samples should float slowly up with the flow, then fall back to the bottom of the tube by the time each PBS addition is complete.

4. Immunostaining (Figure 1B)

4.1. Add primary antibody (e.g., Rab11) at the appropriate dilution (1:100) in 200 µL of the total volume of 1x PBS. Swirl the tube contents gently with a pipette tip. Incubate overnight at 4 °C.

4.2. Remove the primary antibody solution and wash three times with 1 mL of 1x PBS (gently pipetting the solution in and out of the pipette).

4.3. Add secondary antibody (Alex Fluor 488 Goat anti-Rabbit) at the appropriate dilution (1:200) in 200 µL of total volume. Swirl the tube contents gently with a pipette tip. Incubate at room temperature for 90 min.

Commented [A4]: Video 3

Commented [A5]: Videos 4A & 4C

Commented [A6]: Video 5

Commented [A7]: Video 6

Commented [A8]: Video 7

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Commented [A10]: Video 9

Commented [A11]: Video 10

4.4. Add dyes, such as Nile Red (lipids, 5 µL of 1 µg/µL) and/or Hoechst (DNA, 3 µL of 1 µg/µL), into 200 µL of PBS at this stage. Incubate at room temperature for an additional 60 min. Wash gently three times with 1x PBS.

5. Mounting stained glands for microscopy (**Figure 1C**)

5.1. Pipette 200 µL of 100% glycerol onto a microscope slide.

NOTE: As glycerol is viscous, leave the pipette tip in the liquid until it reaches the appropriate volume. No tissue shrinking was observed when going straight into 100% glycerol. However, researchers can go through 30% and 50% glycerol washes in the tubes before moving the samples into 100% glycerol.

5.2. Transfer the stained heads (up to 20 per slide) with the attached glands (along with other internal structures) to the microscope slide using a soft brush (**Figure 3A**). Spread the samples out so that they are evenly distributed along the glass slide.

NOTE: When depositing glands on a cover slide with a brush, forceps can be used to gently orient the SGs so that they are all oriented in the same direction.

5.3. Viewing under a dissecting microscope, separate the larval head from the glands using two pairs of forceps and carefully pulling the two tissues in opposite directions.

NOTE: As it is challenging to completely isolate the SGs, simply remove the heads, leaving the SGs and associated internal structures. Even if the SGs remain associated with other tissues, they are easily recognized when stained with Hoechst and other markers (**Figure 3B,C**).

5.4. Remove and discard the heads and repeat the separation process for each SG.

NOTE: When removing heads, set a paper towel nearby to collect them.

5.5. Gently place a 1.5 mm thick coverslip on the top (avoiding air bubbles) and seal with clear nail polish.

5.6. Store at 4 °C in a light-proof container.

NOTE: Prepared slides of stained glands can be kept at 4 °C in the dark for up to six months to one year without any notable changes in quality. If glycerol starts to leak as the months go by, gently lift the coverslip and pipette in more glycerol to fill holes.

REPRESENTATIVE RESULTS:
Salivary glands are relatively easy to dissect from all stage 4 larvae. Male and female larvae can be distinguished at the late L4 larval stage by a red stripe along the dorsal thorax of females but

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Commented [A13]: Video 12

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Commented [A18]: Video 17

Commented [A19]: Video 18: Timelapse

Comment from Video Production team member: There is a timelapse included and I wonder if there is a real-time version. Some cameras have a timelapse mode which if used would preclude the possibility of there being an 'original', but if it was created in post-production, we'd be interested in having it.

not males (**Figure 2**). We also observe that antennal morphology is much more elaborate in male than in female L4 larvae (**Figure 2**), similar to the differences observed in this structure in adult mosquitoes. Along with the considerable overall growth during the L4 stage, the salivary glands also form a lumen during L4[12]. Salivary glands isolated from early L4 stage larvae stained with Hoechst reveal the proximal and distal lobes separated by a narrow constriction (**Figure 3B,C**). The forming lumen will extend all the way from the salivary duct at the proximal-most end (not shown) through the distal lobe. The forming lumen can be seen in the immunostained distal salivary gland of a mid-to-late L4 larva (**Figure 4**). The apical domains of the secretory cells surrounding the forming lumen have intense Nile Red staining (**Figure 4B,C**) suggestive of microvilli-like structures. Also observed close to the apical surface is Rab11 staining (**Figure 4C,D**, arrows). Rab11 localizes to apical recycling endosomes. The Rab11 staining that also accumulates along the basal surface of the gland is an artefact due to the stickiness of the basal membrane. Similar background staining is common with immunostaining of both larval and adult salivary glands and has been mistaken for bona fide signal.

FIGURE AND TABLE LEGENDS:

Figure 1: Cartoon visualization of the immunostaining process from gland dissection through slide preparation. Abbreviations: SGs = salivary glands; PBS = phosphate-buffered saline; MeOH = methanol; DAPI = 4′,6-diamidino-2-phenylindole; Abs = antibodies; LH = left hand; RH = right hand.

Figure 2: Male and female L4 stage *Anopheles gambaie* larvae. (**A, B**) Early L4 larvae. (**C, D**) Late L4 Larvae. There is considerable growth during the L4 stage. Females (**A, C**) have been described as having a distinguishing red stripe down the dorsal thorax (**C**; black arrow) that is not present in males (**B, D**), but also their antennae are much less elaborate (frilly) than those of male larvae from both early and late L4 (white arrows in enlarged images). Scale bars = 1 mm.

Figure 3: Early L4 salivary glands. (**A**) Early L4 female larval head with salivary glands and other internal organs still attached. Salivary gland is outlined. Scale bar = 50 µm. (**B, C**) Isolated larval glands stained with Hoechst. (**B**) Merge of fluorescent and Nomarski images showing the shape of the proximal and distal lobes and the blue Hoechst staining in nuclei. (**C**) Hoechst fluorescent image highlights nuclei only. Scale bar in bottom panels = 20 µm.

Figure 4: Immunostained distal L4 salivary gland. (**A**) Gland has been stained with Hoechst (nuclear DNA, blue) and (**B**) Nile Red (membrane marker, red); immunostained for Rab11 (apical recycling vesicles, green) (**C**). (**D**) The merged image. Note that a lumen is present at the stage shown (**B–D**). The arrows in **C** and **D** point to the expected Rab11 staining of vesicles near the apical surface that overlaps the Nile Red-positive vesicular staining in the merge (white arrows). Non-specific background staining around the basal surface (asterisks) is also observed, a common type of background observed in larval and adult salivary glands. Scale bars = 20 µm.

DISCUSSION:

The protocol described herein was adapted from a Drosophila SG dissection protocol and an adult mosquito dissection protocol[14-16]. However, most markers did not penetrate the basement

membrane (data not shown) when using the adult dissection and SG staining methods. Adaptations of the adult protocol included dissecting the glands in a 25% EtOH solution, washing the glands with a combination of MeOH and glacial acetic acid, and having a 90 s acetone wash. In the original adult protocol, adult SGs were dissected in 1x PBS[14-16]. Dissecting in a 25% EtOH solution helped preserve the SGs and prevented damage during the staining periods. When performing the initial dissection, it was easiest to orient the larval SGs with the head facing the non-dominant hand and having the dominant hand gently pull outwards (because the larvae are very actively moving). The improved tissue penetration achieved by washing the glands with a MeOH: glacial acetic acid solution suggests that the lipid content in the larval SG basement membrane and/or cellular plasma membranes is distinct from that of the adult SG. The 90 s acetone wash improved the clarity of glands for imaging. Although the fixation period was recommended to be at least 19 h (overnight), longer periods worked just as effectively.

The morphology of the SGs can vary widely depending on when the larvae are dissected in the 2-day long L4 stage. In early L4 dissections (day 1), the lumen appears much smaller[12]. In late L4 dissections (second half of day 2), the lumen is large, and the cells are elongated. We found that the optimal period for working with larvae was the L4; L1–L3 SGs are simply too small for manual dissections. In imaging results, glands dissected at mid-to-late-L4 showed smaller cells mixed with larger, laterally elongated cells, particularly in the distal sac.

Previous reports on *Anopheles* SG development have provided much guidance for the current studies. These reports have included illustrations of dissected embryonic and larval SGs[17,18], detailed microscopic analysis of dissected glands[19,20], and transcriptomic studies[20-22]. The features of the *Anopheles stephensi* SG were reported during the 1950s by two different groups[10,11]. Many of the morphological features of the larval glands were described, including the overall organization of the SG, the relative positions of distinct cell types within the organ (duct, adult SG precursors, and the larval proximal and distal secretory sacs)[10,11]. Both groups also reported additional morphometric data, including the number and distribution of secretory cells within each sac and their nuclear size[10,11]. Rishikesh showed that, like the Drosophila larval SGs, the larval SG chromosomes from *Anopheles stephensi* are polytenized[10]. These important foundational overviews cover various biological aspects of larval *Anopheles* SGs.

The method described herein should be useful for studying not only larval SGs of *An. gambiae* but can also apply to those studying other species of mosquitos. Indeed, the same protocol has been successfully utilized (unpublished) with *An. stephensi*, a species frequently studied in the laboratory. One limitation to the protocol is the limited number of antibodies that have been specifically generated against mosquito proteins. Although using Drosophila antibodies for highly conserved proteins has circumnavigated this limitation[12], the field could benefit from more mosquito protein-specific antibodies. Understanding larval SG cellular and molecular biology can contribute to new control or target strategies and allow for the discovery of new candidate target genes for SG disruption.

ACKNOWLEDGMENTS:

We would like to thank the Johns Hopkins Malaria Research Institute for access to and rearing of *An. gambiae* larvae.

DISCLOSURES:
The authors have no conflicts of interest to disclose.

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