Submission ID #: 61924
Scriptwriter Name: Bridget Colvin
Project Page Link: https://www.jove.com/account/file-uploader?src=18886868

Title: Sand Fly (Phlebotomus papatasi) Embryo Microinjection for CRISPR/Cas9 Mutagenesis

Authors and Affiliations: Isabelle Louradour[1], Kashinath Ghosh[1], Ehud Inbar[1], David L. Sacks[1], Channa Aluvihare[2], and Robert A. Harrell II[2]

[1]Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health
[2]University of Maryland Insect Transformation Facility, The Institute for Bioscience and Biotechnology Research

Corresponding Author:
Robert A. Harrell II
harrelr@umd.edu

Co-Authors:
isabelle.louradour@nih.gov
kashinghosh@gmail.com
einbar@sanaria.com
dsacks@niaid.nih.gov
aluvihar@umd.edu

Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **Y**, **Olympus SZX-12 and Zeiss Stemi, both with camera port head**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **N**

3. Interview statements: Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements must be filmed outside if weather permits.

4. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **Y, 11 mi**

Protocol Length
Number of Shots: **31**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Robert Harrell**: Targeted mutagenesis has been adapted to sand flies only recently and is a crucial technique for understanding the role and function of genes of interest in these vector insects **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Isabelle Louradour**: Embryo microinjection is a very important step in insect genome editing, but it needs to be adapted to the species that you are working with, which in our case is the sand fly **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Channa Aluvihare**: Compared to other insect species, sand fly embryos are very small, take a long time to develop, and are particularly sensitive to the level of humidity **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

Protocol

2. Embryo Collection and Micromanipulation

2.1. Five days before the injection, blood feed female sand flies as for routine colony maintenance **[1-TXT]**.

2.1.1. WIDE: Talent feeding flies **TEXT: See text for sand fly colony maintenance details**

2.2. One day post feeding, capture the blood-fed females in plaster pots with a side port in groups of 100-150 **[1]** and feed the files with a 30%-sucrose solution **[2]**.

2.2.1. Talent adding flies to pot

2.2.2. Talent adding sucrose to pot

2.3. On day 5 post blood-feeding, place a white piece of moist filter paper into the egg-laying chamber **[1-TXT]** and use a mouth aspirator to collect **[2]** and transfer about 10 females from the plaster pot to the egg-laying chamber **[3-TXT]**.

2.3.1. Talent placing paper into chamber **TEXT: Do not moisten plaster pots d 2-3 post blood-feeding**

2.3.2. Flies being collected through side port *Videographer/Video Editor: Shot will be used again*

2.3.3. Flies being added to chamber *Videographer: Important step* **TEXT: Moist substrate will induce flies to ovaposit**

2.4. After 30-60 minutes remove the petri dish and filter paper from the egging chamber, **[1-TXT]**, taking care that the filter paper remains moist **[2]** and continuing to collect new groups of females and embryos throughout this period **[3]**.

2.4.1. Talent placing paper into dish. **TEXT: Embryos can be used for injection for up to 3 hours**

2.4.2. Paper being tested and/or paper being moistened

2.4.3. Use 2.3.2. Talent collecting flies

2.5. When all of the embryos have been collected, use a stereomicroscope **[1]** and a very fine paintbrush to carefully transfer about 50 embryos onto a individual pieces of moist black filter paper placed microscope slides topped with a single coverslip per slide **[2]**.

2.5.1. Talent placing slide and paper under microscope

2.5.2. SCOPE: Eggs being placed onto paper on slide *Videographer: Important step*

2.6. Add enough water to each filter paper to keep the embryos moist without floating or being sucked under the coverslips **[1]** and line the embryos against the coverslips **[2]**.

2.6.1. SCOPE: Water being added to paper

2.6.2. SCOPE: Embryos being lined against coverslip

3. Embryo Injections

3.1. Two to three hours after the start of the egg collection, place a slide under a dissecting microscope **[1]** and use the paintbrush to carefully add water to the back end of the filter paper **[2]**.

3.1.1. WIDE: Talent placing slide under microscope

3.1.2. Talent adding water to filter paper

3.2. Back load 0.5-1 microliters of load injection mix into a microinjection needle **[1-TXT]** and mount the needle into a pneumatic injection controller set to 30 pounds per square inch of pressure under the microscope **[2]**.

3.2.1. Talent adding injection mix to needle **TEXT: Injection mix: guide RNA 80 ng/microliter + recombinant Cas9 protein 300 ng/microliter**

3.2.2. SCOPE: Needle being loaded onto holder

3.3. Depress the trigger to expel any air from the needle tip **[1]**.

3.3.1. Talent depressing foot trigger

3.4. When the air has been released, slowly increase the hold pressure **[1]**. When the injection material starts to flow, decrease the hold pressure until it is just below the point of the injection mix flowing from the needle **[2]** and insert the needle into the side of the first embryo **[3]**.

3.4.1. SCOPE: Pressure being increased

3.4.2. SCOPE: Material flowing/pressure being decreased

3.4.3. SCOPE: Needle being inserted *Videographer: Important/difficult step*

3.5. Using the coverslip behind the embryo as a backstop, deliver a small amount of injection mix into the embryo **[1]** before gently removing the needle **[2]**.

3.5.1. SCOPE: Mix being injected *Videographer: Important/difficult step*

3.5.2. SCOPE: Needle being removed

3.6. Immediately after removing the needle, press the injector to remove any backfilled material from the needle tip **[1]** and proceed to the next embryo **[2-TXT]**.

3.6.1. SCOPE: Injector being pressed

3.6.2. SCOPE: Needle being inserted into second embryo *Videographer: Important step* **TEXT: Check paper moisture and needle clearance between injections**

3.7. When all of the embryos have been injected, count the number of injected embryos to keep a running tally **[1]** and add water to the filter paper so that the coverslip floats **[2]**.

3.7.1. Shot of injected embryos *Video Editor: please add numbers of embryos to suggest counting*

3.7.2. Water being added to paper/coverslip floating

3.8. Then use a probe to hold the filter paper in place **[1]** while pulling the coverslip away from the embryos **[2]** and use filter paper to blot away the excess water from the slide **[3]**.

3.8.1. Paper being held in place

3.8.2. Coverslip being removed

3.8.3. Water being blotted

3.9. Transfer the filter paper onto a stack of moist filter paper in a new Petri dish **[1]** and manually transfer each embryo onto moistened, small-size plaster pots without crowding **[2]**.

3.9.1. Talent placing filter paper onto filter paper stack

3.9.2. Embryo being placed into pot *Videographer: Important step*

3.10. Then cover the pots with a screen **[1]** and store the pots in a 26-degree incubator at a 70% humidity **[2]**.

3.10.1. Pot being covered with screen

3.10.2. Talent placing pot(s) into incubator

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
2.3.3., 2.5.1., 2.6.2., 3.4.3., 3.5.1., 3.9.2.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
Steps 3.4.3., and 3.5.1. are the most difficult steps, success is ensured in step 3.3.3 by making sharp needles that allow easy insertion, In step 3.4.1 success is ensured by monitoring the needle to make sure materials is going into the embryo and that material is not leaking out of the embryo.

Results

4. Results: Representative Identification of Mosaic G_0 Adults and Mutations of Interest

4.1. As with most successful microinjection protocols, a good microinjection needle suited to the embryos being injected is important **[1]**.

4.1.1. LAB MEDIA: Figure 1

4.2. Good, sharp needles can easily penetrate the embryo without allowing the material to escape post-injection **[1]**.

4.2.1. LAB MEDIA: Figure 1 *Video Editor: please emphasize Figure 1A*

4.3. The pulled needle should not have a taper that is too long **[1]**. Otherwise, the lumen of the needle becomes very narrow for a major portion of the taper, making it difficult to get the injection pressure high enough to force the material through the needle **[2]**.

4.3.1. LAB MEDIA: Figure 1 *Video Editor: please emphasize Figure 1B*
4.3.2. LAB MEDIA: Figure 1 *Video Editor: please emphasize end of needle in Figure 1B/add/emphasize bracket at end of needle in Figure 1B*

4.4. Post injection-reared embryos that are healthy should be retained **[1]**, while damaged, fungus-contaminated, desiccated, or deformed embryos should be discarded **[2]**.

4.4.1. LAB MEDIA: Figure 4B *Video Editor: please emphasize normal development images*
4.4.2. LAB MEDIA: Figure *Video Editor: please emphasize abnormal embryos to discard images*

4.5. Potential mutant alleles can be identified in post injection reared-individuals by PCR analysis of the region surrounding the expected cutting sites **[1]**.

4.5.1. LAB MEDIA: Figure 4C *Video Editor: please emphasize bands below wt band in G0 lane*

4.6. After mutant line establishment using the injected embryos **[1]**, genotyping PCR allows the identification of homozygous mutant sibling crosses **[2]**.

4.6.1. LAB MEDIA: Figure 5A

4.6.2. LAB MEDIA: Figure 5 *Video Editor: please emphasize rectangle around cross 2 lanes/bands in cross 2 lanes*

Conclusion

5. Conclusion Interview Statements

5.1. **Robert Harrell**: It is essential to have a good needle, to be gentle when manipulating and injecting the embryos, and to keep the embryos hydrated throughout the procedure **[1]**.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (2.5.1., 3.4.3., 4.1.1.)

5.2. **Isabelle Louradour**: Genome editing in sand flies is just getting started. This technique is really a first step that should be followed by more complex gene modification strategies **[1]**.

5.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*