Submission ID #: 61232
Scriptwriter Name: Anastasia Gomez
Project Page Link: https://www.jove.com/account/file-uploader?src=18675808

Title: Improved Fecundity and Fertility Assay for Aedes aegypti Using 24 Well Tissue Culture Plates (EAgaL Plates)

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all set.**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Hitoshi Tsujimoto:** This procedure allows mosquito researchers to easily address their mosquito's reproductive fitness at the individual level.

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

1.2. **Hitoshi Tsujimoto:** Compared to conventional methods, this protocol significantly reduces the time, space and labor required to test mosquito's fecundity and fertility at the individual level.

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

Protocol

2. Plate Preparation and Mosquito Feeding

2.1. Begin by preparing plates for the oviposition experiment **[1]**. Drill 4 to 6 holes per well in the lid of a 24-well culture plate using a household drill with a 1.6-millimeter bit **[2]**.

2.1.1. WIDE: Establishing shot of talent at the lab bench with the drill and the plate.

2.1.2. Talent drilling holes in the lid.

2.2. One day before the experiment, wash and rinse the plates **[1]** and soak them in 1 to 5% bleach for 30 to 60 minutes at room temperature **[2]**. Then, rinse the plates thoroughly under running deionized water and dry them **[3]**.

2.2.1. Talent washing the plate.

2.2.2. Talent putting the plate in a container with bleach.

2.2.3. Talent rinsing the plate under running water.

2.3. Melt 2% agarose in deionized water **[1]** and immediately add 500 microliters of the molten agarose to each well of the 24-well plate **[2]**. Leave the plates on the lab bench overnight to dry out any condensation **[3]**.

2.3.1. Talent taking a bottle of agarose out of the microwave.

2.3.2. Talent adding agarose to a few wells.

2.3.3. Plate on the lab bench.

2.4. At least 16 hours before the blood feeding, remove any source of water or sugar form the female mosquitoes **[1]**. On the day of the feeding, heat a water circulator to 37 degrees Celsius **[2]** and feed the mosquitoes with vertebrate blood placed in artificial feeders for 15 to 30 minutes **[3]**.

2.4.1. Talent removing water or sugar from the mosquitoes.

2.4.2. Talent starting the water circulator.

2.4.3. Talent placing the mosquitoes in the artificial feeder.

2.5. Then, transfer the mosquitoes to a glass dish on ice **[1]**, select the ones that are engorged with blood **[2]**, and place them into a container with 30% sucrose water. Allow at least 72 hours for the females to finish excretion and egg development **[3]**.

2.5.1. Talent transferring mosquitoes to a dish on ice.

2.5.2. ECU: Talent sorting the mosquitoes.

2.5.3. Talent adding mosquitoes to the container with sucrose water.

3. Ovipositioning and Egg Counting

3.1. About 1 hour before transferring mosquitoes to the 24-well plate, use a transfer pipette to add 2 to 3 drops of water into each well **[1]**. Knockdown mosquitoes with carbon dioxide and transfer them to a glass dish on ice **[2]**, then individually place each mosquito on an inverted lid of the 24-well plate **[3]**. Videographer NOTE: 3.1.3- The author wants to use the 2nd take (inverted lid).

3.1.1. Talent adding water to a few wells on the plate.

3.1.2. Talent transferring mosquitoes to the dish on ice.

3.1.3. Talent placing mosquitoes on the inverted lid.

3.2. Once all 24 mosquitoes have been placed, cover the lid with an inverted plate bottom **[1]**, secure it with a fresh, new rubber band **[2]**, and place it in an environmental chamber until mosquitoes recover **[3]**, then turn the plate right side up **[4]**. *Videographer: This step is important!*

3.2.1. Talent covering the lid with the plate bottom.

3.2.2. Talent securing the lid to the plate with a rubber band.

3.2.3. Talent placing the plate in the environmental chamber.

3.2.4. Talent flipping the plate over.

3.3. Allow the female mosquitoes to oviposit for 24 to 48 hours **[1]**, then remove the females by releasing them from the plates into a large cage **[2]**.

3.3.1. Mosquitoes in the plate.

3.3.2. Talent releasing the mosquitoes into a large cage.

3.4. Before counting the eggs, check each well for eggs that are on the well walls and at the margin of the agarose and plastic surface, where they are difficult to resolve in photographs **[1]**. Use a wet paintbrush to move these eggs to the flat surface so that all eggs are in a uniform plane and do not overlap each other **[2]**. *Videographer: This step is important!* Videographer NOTE: 3.4.1 and 3.4.2 are combined as 1 clip.

3.4.1. ECU: Well with eggs on the side.

3.4.2. ECU: Wet paintbrush moving the eggs from the side to the flat surface.

3.5. Use forceps to remove any broken legs, wings, and other particles in the wells that may interfere with imaging eggs **[1]**. Set a compact digital camera in microscope mode, which allows the user to focus on objects as close as 1 centimeter **[added 3.5.2]** and take an image of each well **[3]**. *Videographer: This step is important!*

3.5.1. Talent using forceps to remove unwanted particles from the wells.

3.5.2. Added shot: Talent sets the camera to the "microscope mode."

3.5.3. Talent taking a picture of a well.

3.6. After photographing each well of a plate, take an image of the entire plate. Use an imaging order label to distinguish each plate later **[1]**. Add about 5 drops of water to each well to prevent its agarose plug and the eggs from drying and to induce embryo development and hatching **[2]**.

3.6.1. Talent taking photograph of the entire plate.

3.6.2. Talent dripping water into a few wells.

3.7. Transfer the images to a computer and rename the files with the plate and well IDs for easier organization **[1]**. Open the images with ImageJ **[2]** and use the **multi-point** tool to mark each egg, zooming in or out to count the egg clumps **[3]**.

3.7.1. SCREEN: 61232_screenshot_1. 0:19 – 0:46. *Video Editor: Speed up between 0:22 and 0:39.*

3.7.2. SCREEN: 61232_screenshot_2. 0:10 – 0:17.

3.7.3. SCREEN: 61232_screenshot_2. 0:17 – 0:30. *Video Editor: Speed up between 0:17 and 0:25.*

3.8. After marking all the eggs, double-click the **multi-point icon** to bring up the number of marks and record the results in a spreadsheet **[1]**.

3.8.1. SCREEN: 61232_screenshot_2. 1:30 – end.

4. Fertility Assessment

4.1. Start adding food to the wells that contain hatched larvae as soon as they appear **[1]**. Approximately 5 to 8 days later, anesthetize the larvae by covering the plate with crushed ice for 15 to 20 minutes **[2]**.

4.1.1. Talent adding food to wells with larvae.

4.1.2. Talent covering the plate with crushed ice.

4.2. Insert a black material underneath the plate to enhance the contrast **[1]** and take images of each well while keeping the plates on ice **[2]**. After photographing each well, take an image of the entire plate with an imaging order label **[3]**. *Videographer: This step is difficult and important!*

4.2.1. Talent inserting black material under the plate.

4.2.2. Talent taking a photo of a well.

4.2.3. Talent taking a photo of the entire plate.

4.3. Open images with ImageJ and use the **multi-point** tool to count the larvae. Larvae can vary in shape, angle, and focus **[1]**. Exclude the shed cuticles, which look like head-only larvae with a little bit of body, or shrunken larvae **[2]**. Record the results in the spreadsheet **[3]**.

4.3.1. SCREEN: 61232_screenshot_3. 0:15 – 0:30.

4.3.2. LAB MEDIA: Figure 10 E. *Video Editor: Emphasize the defective larvae to which the pink arrows are pointing.*

4.3.3. SCREEN: 61232_screenshot_3. 1:38 – end.

Results

5. Results: FeT Gene Silencing

5.1. Mosquitoes were injected with double stranded RNA targeting a candidate iron transporter, FeT *(spell out 'F-E-T')*, or a control gene, EGFP **[1]**. Then, they were blood-fed and measured for fecundity and fertility output using the EAgaL *(pronounce 'eagle')* plate method **[2]**.

5.1.1. LAB MEDIA: Figure 11. *Video Editor: Emphasize the red data points when VO says "candidate iron transporter, FeT" and the black data points when VO says "control gene, EGFP".*

5.1.2. LAB MEDIA: Figure 11.

5.2. Mosquitoes in which iron transporter expression was silenced exhibited a significant reduction in both egg number and hatch rate **[1]**. FeT-silenced mosquitoes also exhibited delayed excretion and small and light-colored eggs **[2]**.

5.2.1. LAB MEDIA: Figure 11. *Video Editor: Emphasize A and C.*

5.2.2. LAB MEDIA: Figure 12.

Conclusion

6. Conclusion Interview Statements

6.1. **Hitoshi Tsujimoto:** Taking good pictures is the critical step for this method, because the quality of the pictures and clarity of individual eggs or larvae directly affects the quality of the results.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.5.2. – 3.6.1 or 4.2.2 – 4.2.3.*