Journal of Visualized Experiments

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

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61232R1
Full Title:	Improved Fecundity and Fertility Assay for Aedes aegypti Using 24 Well Tissue Culture Plates (EAgaL Plates)
Section/Category:	JoVE Biology
Keywords:	Aedes aegypti mosquito fitness egg number hatch rate fecundity fertility time-saving space-saving
Corresponding Author:	Hitoshi Tsujimoto, Ph.D. Texas A&M University College Station, Texas UNITED STATES
Corresponding Author's Institution:	Texas A&M University
Corresponding Author E-Mail:	htsujimo@tamu.edu
Order of Authors:	Hitoshi Tsujimoto, Ph.D.
	Zach N. Adelman
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	College Station, Texas, USA

TITLE:

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

AUTHORS AND AFFILIATIONS:

Hitoshi Tsujimoto[1], Zach N. Adelman[1]

[1]Department of Entomology, Texas A&M University, College Station, TX, USA

Corresponding Author:

Hitoshi Tsujimoto (htsujimo@tamu.edu)

Email Address of Co-Author:

Zach N. Adelman (zachadel@tamu.edu)

KEYWORDS:

Aedes aegypti, mosquito, fitness, egg number, hatch rate, fecundity, fertility, time-saving, space-saving

SUMMARY:

Described is a time and space-saving method to count eggs and determine hatch rates of individual mosquitoes using 24 well tissue culture plates, which can substantially increase the scale and speed of fecundity and fertility assays.

ABSTRACT:

Mosquitoes represent a significant public health problem as vectors of various pathogens. For those studies that require an assessment of mosquito fitness parameters, in particular egg production and hatch rates at the individual level, conventional methods have put a substantial burden on investigators due to high labor intensity and laboratory space requirements. Described is a simple method using 24 well tissue culture plate with agarose in each well and digital imaging of each well to determine egg numbers and hatch rates at an individual level with substantially reduced time and space requirements.

INTRODUCTION:

The control of mosquitoes to protect humans from vector-borne pathogens is an important public health goal, mainly due to the lack of effective vaccines for most of the pathogens carried by mosquitoes. Many studies aim to reduce mosquito fitness in conjunction with a field-applicable population reduction strategy[1-3]. This includes extensive studies to create transgenic mosquitoes and/or CRISPR/Cas9 knockout lines. Such population modification approaches require a detailed assessment of individual fitness parameters[4]. Conventional laboratory techniques to assess the fitness of female mosquitoes includes the individual containment of mated, blood-fed female mosquitoes in 100 mL containers[5], modified 50 mL conical tubes, or tubes for *Drosophila* rearing modified by providing moist surfaces using damp cotton and filter paper discs for oviposition (i.e., egg papers)[1,2,6,7]. Such methods require a relatively large space

(e.g., 30 cm x 30 cm x 10 cm: W x L x H for up to 100 *Drosophila* tubes) (**Figure 1**), and the manipulation of individual egg papers for counting eggs and hatching larvae, which can be labor intensive. This manuscript presents a method to count mosquito eggs and determine hatch rates using 24 well plates and agarose as an oviposition surface to circumvent these issues[8].

Concurrently, Ioshino et al.[9] described a detailed method using 12 and 24 well plates to perform egg counting obtained from individual females. Their protocol represented a significant improvement from conventional methods in saving time and space[9]. However, the protocol they described continues to use wet filter paper as a surface for oviposition, which requires unfolding each individual paper to get counts, as eggs are often found underneath or in folds. Their protocol also did not include the use of imaging technologies or a method for larval counting.

Presented is an improved method to perform fitness assays for egg number (i.e., fecundity) and hatch rate (i.e., fertility) using agarose as an oviposition surface in a 24 well tissue culture plate format for *Ae. aegypti* that oviposit on moist surfaces. These plates were named "EAgaL" plates, from **E**gg, **Aga**rose, and **L**arva. These 24 well plates provide individual mosquitoes with a minimal surface to lay eggs, thus simplifying and drastically reducing the time and effort needed to count and maintain eggs and hatched larvae for a few days. The EAgaL plate uses translucent agarose for the oviposition surface, which eliminates the need for handling egg papers and finding the eggs and larvae when hatched; photographing each well establishes a long-term archived record of the results and separates the counting process in both time and space from the rearing/handling process, where time is often limited.

PROTOCOL:

1. Plate preparation

1.1. Drill holes in 24 well tissue culture plate lids (4–6 holes per well) using a household drill with a ~1.6 mm (1/16 in) bit (**Figure 2**).

NOTE: These holes prevent water condensation from agarose to accumulate on the lid, where mosquitoes may lay eggs. The standard size of female *Ae. aegypti* ("Liverpool" strain) is ~3.11 mm wingspan. Reducing the size of the holes is recommended when using smaller mosquitoes to avoid escape from the plate.

1.2. On the day prior to the oviposition experiment, wash and rinse the plates thoroughly and soak them in 1–5% bleach for 30–60 min at room temperature. Rinse thoroughly under running deionized water and dry them.

NOTE: This process reduces the chance of fungi and bacteria growing on agarose.

1.3. Melt agarose at 2% in deionized water and immediately add 500 µL of the molten agarose to each well of the 24 well plates using a 1,000 µL pipette (**Figure 3A**,**B**). When the agarose begins to cool and clog the pipette tip, reheat the agarose and use a new pipette tip.

NOTE: Avoid touching the walls of the well with the pipette tip because it may leave a piece of agarose on the wall where a female may lay eggs, complicating the imaging and counting process.

1.4. Before use, dry out any condensation on the well walls overnight on a lab bench (**Figure 3C**,**D**).

NOTE: The timeline of the EAgaL plate assay from blood feeding to larval imaging is depicted in **Figure 4**, which is for colony mosquitoes (*Ae. aegypti* "Liverpool") reared under 14 h light:10 h dark light cycle, 27 °C, 80% relative humidity, 500 larvae in a 49.5 cm x 29.2 cm x 9.5 cm tray with 2 L of water), under insectary conditions. It is recommended that each laboratory test the EAgaL plate with the mosquitoes being used, especially when using different strains, different mosquito species, as well as different rearing protocols.

2. Mosquito feeding

NOTE: It is critical to use mosquitoes reared under uniform conditions for all treatment groups and control groups, because larval nutrition has an impact on mosquito fitness parameters[10,11]. Rear larvae under uncrowded conditions with sufficient food. Let female mosquitoes eclose in the presence of males so that mating is ensured, and mature for at least 3 days.

2.1. Remove any source of water and/or sugar from the female mosquitoes at least 16 h prior to blood feeding in order to enhance blood feeding.

2.2. Heat a water circulator for artificial feeding at 37 °C and feed female mosquitoes using vertebrate blood placed in artificial feeders for 15–30 min (**Figure 5A**).

2.3. Anesthetize mosquitoes with CO_2 or on ice, transfer them to a glass dish on ice, and select ones that are engorged with blood (**Figure 5B**) into a container provided with 30% sucrose water for more than 72 h, when females finish excretion and egg development.

NOTE: If the females are provided with a lower concentration of sucrose water, remove the sucrose water and any wet surfaces after 48 h to prevent the females from ovipositioning.

3. Oviposition

3.1. About 1 h prior to transferring mosquitoes to the plates, add 2–3 drops (~80–120 µL) of water into each plate well using a transfer pipette.

3.2. At least 72 h after blood feeding, knockdown mosquitoes with CO_2 or on ice, transfer them to a glass dish on ice, and individually place each mosquito on an inverted lid of the 24 well plate on ice (**Figure 6A**).

NOTE: This procedure has been applied from Ioshino et al.[9].

3.3. Once all 24 mosquitoes have been placed, cover the lid with an inverted plate bottom (**Figure 6B**), secure the lid and plate with a fresh, new rubber band and place in an environmental chamber (or rearing room) (**Figure 6C**) until mosquitoes recover (~10–15 min) and turn the plate right side up (**Figure 6D**).

3.4. Allow the female mosquitoes to oviposit for 24–48 h and remove females by releasing them from the plates into a large cage.

NOTE: If it is important to keep track on individual females, anesthetize them by chilling the plates and remove them individually on ice. Delayed oviposition and dark excretions that interfere with egg counting were observed when females were transferred to the plates earlier than 72 h post blood meal (PBM) (**Figure 7D**).

4. Egg counting

4.1. Check each well of the 24 well plate, as sometimes mosquitoes lay eggs on the wall of wells and at the margin of the agarose/plastic surface, where they are difficult to resolve in photographs. Using a wet paint brush, move the eggs laid on the wall and edge of agarose to the flat surface of the agarose so that all eggs are in a uniform plane and do not overlap with each other.

NOTE: The edge of the agarose is typically out of the camera's focal plane due to the surface tension of the agarose solution.

4.2. Using forceps, remove any broken legs, wings, and other particles in the wells that may interfere with imaging eggs (**Figure 7C**).

4.3. Insert white paper underneath the plate to increase the contrast with dark mosquito eggs prior to imaging using a stereomicroscope illuminator (**Figure 7A**).

4.4. Take an image of each well using a compact digital camera in microscope mode, which allows the user to focus on objects as close as 1 cm. This capability allows the camera to be placed directly on the plate to image eggs without a tripod or a stand (**Figure 7A**). To distinguish individual eggs laid in clumps, use the fine or super-fine mode to capture high-resolution images so that close-up details can be seen.

NOTE: Clear the memory card of the camera before use to prevent confusion between new and old images.

4.5. After photographing each well of a plate, take an image of the entire plate with an imaging order label to distinguish each plate later (**Figure 7E**).

4.6. Add a thin layer ~5 drops of water (~400 µL) with a transfer pipette to each well to prevent

its agarose plug and the eggs from drying and to induce embryo development and hatching. Pay attention to the water levels for the first few hours and check every day, because there may be water loss due to absorption by the agarose or evaporation. Add water when its level is too low for larvae to hatch.

NOTE: Evaporation of water occurs non-uniformly both within a plate and within a stack of plates. It has been observed that drying occurs in the following order: 1) corner wells (A1, A6, D1, D6) dry the fastest; followed by 2) wells at the outer edge of the plate (A2-A5, B1, B6, C1, C6, D2-D5); and last 3) wells inside. When plates are stacked, the top plate dries the fastest.

4.7. Transfer the images to a computer with ImageJ (Fiji) and rename the files for easier organization such as "[plate ID]_[well ID].jpg" (**Figure 8A**,**B**).

4.8. Create a spreadsheet file to record egg numbers (i.e., fecundity) and larval numbers and calculate hatch rate (i.e., fertility) (**Figure 9E**).

4.9. Open the images with ImageJ (Fiji)[12] and use the "**multi-point**" tool to mark each egg (**Figure 9A–C**); zoom-in or zoom-out using "**+**" or "**–**" key to count the egg clumps. After marking all the eggs, double-click the **multi-point icon** to bring up the number of marks (**Figure 9D**). Record the results in a spreadsheet.

5. Fertility assessment

NOTE: In 2 days, first instar larvae may begin to eclose in the wells. Wait for 3–5 more days before imaging/counting to ensure that all viable eggs hatch.

5.1. Prepare larval food by mixing 1/16 tablespoon (~168 mg) of ground fish food (i.e., normal mosquito larval diet) in 20 mL of water and waiting for large solid particles to settle (**Figure 10A–D**). Start adding food (the supernatant) to the wells that contain hatched larvae as soon as they appear, because they do not survive for long without food.

NOTE: Excess addition of food particles may interfere with imaging and counting of hatched larvae.

5.2. Approximately 5–8 days after addition of water to the wells, cool the plate by covering with crushed ice for 15–20 min to anesthetize larvae.

5.3. Take images of each well while keeping the plates on ice as was done with the eggs. For larval images, provide a dark background by inserting a black material underneath the plate to help enhance the contrast. After photographing each well of a plate, take an image of the entire plate with an imaging order label to distinguish each plate later on.

NOTE: Images are taken while the plates are on ice because larval movement may compromise the counting. The plates are reusable; freeze and remove the mosquitoes and agarose to clean

for the next use. The EAgaL plate is not suitable for rearing larvae to advanced stages.

5.4. Open images with ImageJ (Fiji) and use the "**multi-point**" tool to count by clicking on each larva. Over the 3–5 days period some larvae may have molted, especially in wells with low numbers of larvae. Therefore, when counting, exclude the shed cuticles (i.e., exuviae), which look like head-only larvae with a little bit of body, or shrunken larvae (**Figure 10E**). Record the results in the spreadsheet.

6. Perform analysis

6.1. Having collected all necessary data for the fecundity and fertility analysis, perform appropriate statistical analysis and create graphs using preferred software.

REPRESENTATIVE RESULTS:

Mosquitoes were injected with dsRNA targeting a candidate iron transporter (FeT) or control gene (EGFP), blood-fed, and measured for fecundity and fertility output using the EAgaL plate method, following the procedure described above.

Mosquitoes in which FeT expression was silenced following dsRNA injection exhibited a significant reduction in both egg number and hatch rate (**Figure 11A**–**C**). All control and treatment mosquitoes were placed in the EAgaL plates after 72 h PBM. FeT-silenced mosquitoes also exhibited delayed excretion and small and light-colored eggs (**Figure 12A**,**B**). Example results can also be found in Tsujimoto et al.[8].

FIGURE AND TABLE LEGENDS:

Figure 1: Fly tubes commonly used for mosquito fecundity/fertility assays. (**A**) A single tube with damp cotton and circular filter paper with sponge cap. (**B**) 100 tubes in a rack (~30 cm x 30 cm).

Figure 2: Drilling holes in a lid of 24 well tissue culture plate. (**A**) Drilling in process. (**B**) A lid with holes that prevent condensation.

Figure 3: Applying agarose (2% in water) into the wells. (**A**) Applying agarose using a 1,000 µL pipette. Note that the tip is not touching the wall of the well. (**B**) A plate containing agarose on the bottom. (**C**) Wall of a well right after agarose solidified. Note the condensation on the wall. (**D**) Wall of a well when condensation evaporated.

Figure 4: Timeline from blood feeding (day 0) to larval imaging (day 10–13).

Figure 5: Artificial blood feeding and selection of engorged females. (**A**) Blood feeding using artificial feeder. (**B**) Selection of engorged females on ice.

Figure 6: Transfer of females to EAgaL plate. (**A**) Transfer anesthetized females to an inverted plate lid on ice. (**B**) Then carefully place the inverted agarose-containing plate onto the inverted lid and (**C**) remove plate with lid attached from the ice and keep it an inverted position until

females have recovered from anesthesia. (**D**,**E**) Turn the female-containing plate around to the upward position. Note that lid and bottom part of the plate are held by a rubber band and that the bottom part is labeled.

Figure 7: Imaging of each well after females were removed. (**A**) Digital camera on top of an egg-containing 24 well plate for imaging. (**B**) Typical image of a well. (**C**) A well containing a leg, which must be removed. (**D**) Sample image of a well in which a female had been placed at 48 h PBM. Note the dark excretion marks, which can complicate egg counting (arrows). (**E**) Entire plate showing an imaging order label prepared following the imaging of all wells of the plate. This helps recognize wells during analysis.

Figure 8: Renaming the images for better organization. (**A**) Images of individual egg-containing wells including an image order label with the names automatically assigned by the camera. (**B**) The same images renamed with plateID_wellID.jpg format.

Figure 9: Counting eggs using "Fiji" (ImageJ2) software. (**A**) Screenshot of Fiji software showing the "**multi-point**" tool highlighted (turquoise square). (**B**) A well image with Fiji count marks on the eggs. (**C**) Zooming in helps when counting larger egg groups. (**D**) Double-clicking the "**multi-point**" tool icon on the main window shows count (red circle). (**E**) An example of a spreadsheet with hatch rate calculation formula.

Figure 10: Larval diet preparation and a well containing larvae and exuviae. (**A**) Ground fish food. (**B**) 20 mL of water in a cup. (**C**) Mixture of ground fish food and water. (**D**) Food/water settled for 15 min. Take supernatant of this mixture as larval food. (**E**) Well with molted larvae; representative exuviae are indicated by arrows.

Figure 11: Count data from a gene silencing experiment. Mosquitoes injected with dsRNA against putative iron transporter (FeT) exhibited significant reduction of (**A**) egg number, (**B**) larval number, and (**C**) hatch rate in comparison to control (EGFP).

Figure 12: Representative images of the wells showing additional phenotypes in gene silenced mosquitoes. dsFeT showed delayed excretion (dark brown marks) and small, lightly colored eggs. Two representative wells for each treatment are shown.

Table 1: Time requirements for completion of the fly tube method (FT) in comparison to the EAgaL plate method. (**1**) Prep: Time required to place cotton, pour water in, and insert filter paper disc into *Drosophila* rearing tubes (FT) versus pouring of agarose into wells of 24 well plate (EAgaL). (**2**) F_in: Time required to place individual mosquitoes into rearing tubes (FT) or wells of the 24 well plate (EAgaL). (**3**) F_out/E_img: Time required to release mosquito into a larger cage, remove, unfold egg paper, and image the eggs on the paper (FT) or release mosquitoes in a larger cage and image every well of the 24 well plate (EAgaL). (**4**) L_img: Time required to image larvae hatched in a small container (FT) or each well of the 24 well plate (EAgaL) after cold anesthesia. A total of 24 tubes were used for FT.

Table 2: Cost comparison between the EAgaL plate and FT. Top: Bulk costs for startup (assuming a drill and a drill bit can be provided by a researcher). Bottom: Estimated costs for one 24 well plate (EAgaL plate) and 24 FT. (**1**) Assuming slightly more than 15 mL of 2% = 0.3 g of agarose per plate, a total of 500 g of agarose can make 1,667 plates. (**2**) One sheet of paper can make 154 of ~38 mm diameter discs. With 100 sheets, 641.7 (15,400/24) sets of 24 tubes can be made.

DISCUSSION:

The EAgaL plate drastically reduces labor, time, and space to conduct individual fecundity and fertility assays in *Aedes aegypti* when compared to the FT method. Preliminary comparison between the FT method and the EAgaL plate resulted in shorter times for all steps (imaging technique was applied to the FT method) (**Table 1**). As a reference, an estimate of startup and time per assay (one 24 well EAgaL plate versus 24 FTs) costs are provided in **Table 2**.

There are a few points to consider when using the EAgaL plates. An initial concern was that mosquitoes placed in such a small space may not lay all eggs due to limited movement. To determine if this was the case, the mosquitoes were transferred collectively to a larger cage with an oviposition cup lined with a paper towel with water for an additional 48 h after they spent 48 h in the EAgaL plates. The mosquitoes did indeed lay additional eggs, but the average number of eggs per female was only 2.1, which does not result in any difference in the outcome of any statistical analysis in most, if not all, cases. These numbers are from more than 500 mosquitoes tested (data not shown). However, this may be solely for the *Ae. aegypti* "Liverpool" mosquito strain with the conditions described. Each laboratory may need to test whether this is the case for its mosquitoes and conditions.

For imaging, the single image of a camera attached to a stereomicroscope did not cover an entire well even at the lowest magnification. This required obtaining multiple images per well and in turn patching the images, or keeping track of eggs overlapped in multiple images of the same well. Both approaches severely complicated the analysis and significantly increased the labor involved. Moreover, due to the nature of a stereomicroscope, the camera angle is always slightly left or right from the perpendicular angle, which makes the left or right side wall block a part of the agarose surface.

Contamination by microorganisms, especially fungi, can be a problem during the assay. Although bleaching can minimize the contamination before the oviposition, fungi may be present in the insectary environment and carried by the mosquitoes themselves. In such cases, keeping insectary spaces clean may reduce the incidence. It is best for every lab to test an EAgaL plate to detect any potential issues.

Note that the EAgaL plate method was not designed to maintain mosquito cultures beyond the early larval stages. The average number of larvae per well was typically over 60, and it is not unusual to have more than 100 larvae per well. This creates crowded conditions, which result in a delay in development, lower pupation rate, and very small adults, which may compromise downstream studies.

Currently this method has only been tested with *Ae. Aegypti*. However, it is current being tested to expand its application to other species of *Aedes* and even other genera of mosquitoes such as *Anopheles* and *Culex*.

Because of the reduced time and space requirements for the EAgaL plate method, the fecundity and fertility assays can be scaled up to semi-high throughput (i.e., 5–10 plates or more per experiment). This feature of the EAgaL plate method may be extremely useful to assess the important fitness parameters of mosquitoes for insecticide testing, sterility evaluation, transgenesis, and gene editing studies.

ACKNOWLEDGMENTS:

We thank Texas A&M Agrilife Research Insect Vectored Diseases Grant Program for funding. We also thank the Adelman lab members for help on developing this method and suggestions when drafting the manuscript, as well as Kevin Myles lab members. We also thank the reviewers and editors for their help to make this manuscript better.

DISCLOSURES:

The authors declare no conflicts of interest for this study.

REFERENCES:

1. Bond, J. G. et al. Optimization of irradiation dose to *Aedes aegypti* and *Ae. albopictus* in a sterile insect technique program. *PLoS One.* **14** (2), e0212520 (2019).
2. Fernandes, K. M. et al. *Aedes aegypti* larvae treated with spinosad produce adults with damaged midgut and reduced fecundity. *Chemosphere.* **221**, 464-470 (2019).
3. Inocente, E. A. et al. Insecticidal and Antifeedant Activities of Malagasy Medicinal Plant (*Cinnamosma* sp.) Extracts and Drimane-Type Sesquiterpenes against *Aedes aegypti* Mosquitoes. *Insects.* **10** (11), E373 (2019).
4. Marrelli, M. T., Moreira, C. K., Kelly, D., Alphey, L., Jacobs-Lorena, M. Mosquito transgenesis: what is the fitness cost? *Trends in Parasitology.* **22** (5), 197-202 (2006).
5. da Silva Costa, G., Rodrigues, M. M. S., Silva, A. A. E. Toward a blood-free diet for *Anopheles darlingi* (Diptera: Culicidae). *Journal of Medical Entomology.* tjz217 (2019).
6. Gonzales, K. K., Tsujimoto, H., Hansen, I. A. Blood serum and BSA, but neither red blood cells nor hemoglobin can support vitellogenesis and egg production in the dengue vector *Aedes aegypti*. *PeerJ.* **3**, e938 (2015).
7. Gonzales, K. K. et al. The Effect of SkitoSnack, an Artificial Blood Meal Replacement, on *Aedes aegypti* Life History Traits and Gut Microbiota. *Scientific Reports.* **8** (1), 11023 (2018).
8. Tsujimoto, H., Anderson, M. A. E., Myles, K. M., Adelman, Z. N. Identification of Candidate Iron Transporters From the ZIP/ZnT Gene Families in the Mosquito *Aedes aegypti*. *Frontiers in Physiology.* **9**, 380 (2018).
9. Ioshino, R. S. et al. Oviplate: A Convenient and Space-Saving Method to Perform Individual Oviposition Assays in *Aedes aegypti*. *Insects.* **9** (3), 103 (2018).
10. Price, D. P., Schilkey, F. D., Ulanov, A., Hansen, I. A. Small mosquitoes, large implications: crowding and starvation affects gene expression and nutrient accumulation in Aedes aegypti. *Parasites, vectors.* **8**, 252 (2015).

11. Valerio, L., Matilda Collins, C., Lees, R. S., Benedict, M. Q. Benchmarking vector arthropod culture: an example using the African malaria mosquito, Anopheles gambiae (Diptera: Culicidae). *Malaria Journal.* **15** (1), 262 (2016).
12. Rueden, C. T. et al. ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics.* **18** (1), 529 (2017).

Figure 1 (placeholder)

Click here to access/download;Figure;Fig1.jpg

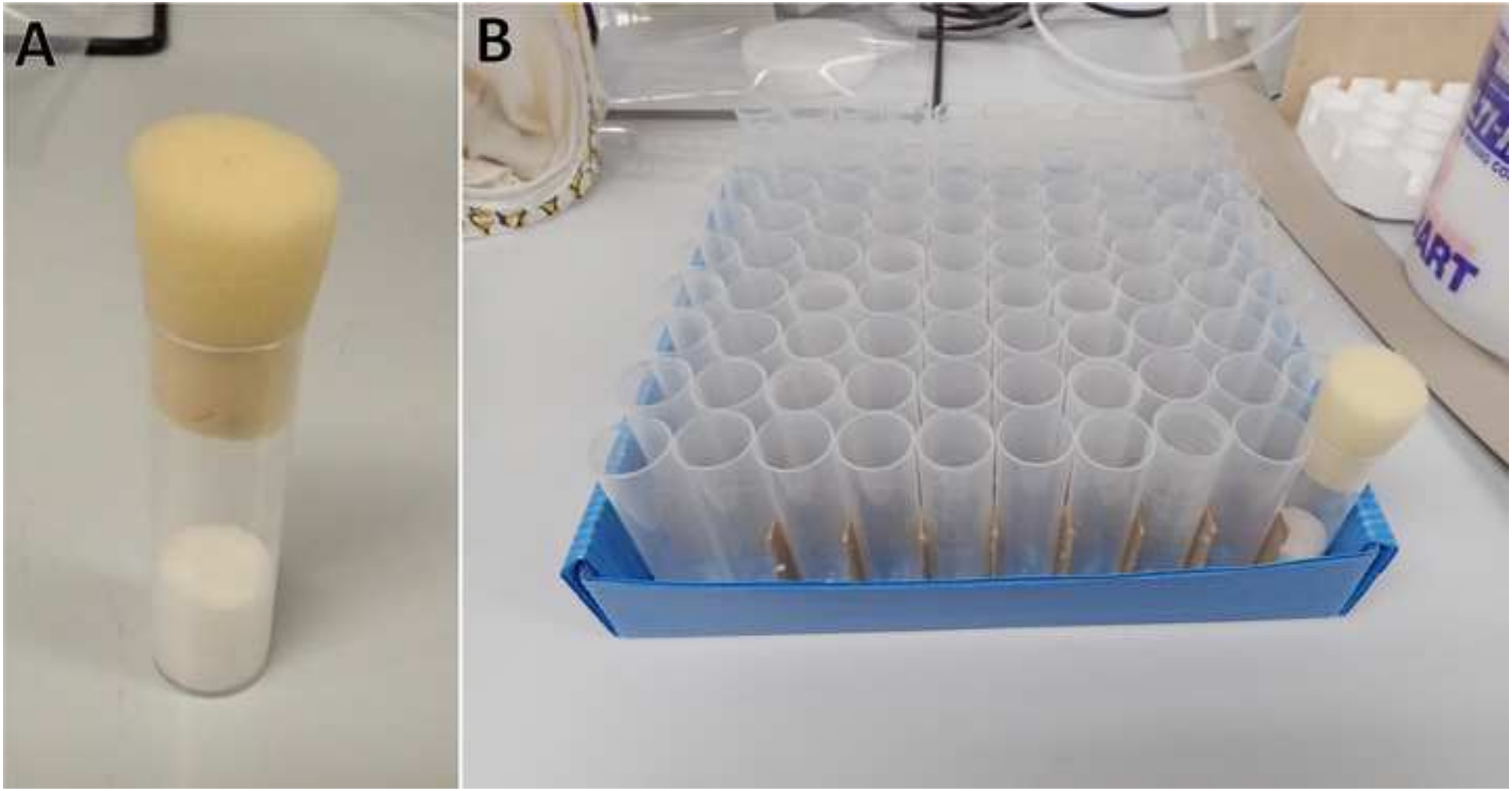

Figure 2 (placeholder)

Click here to access/download;Figure;Fig2.jpg

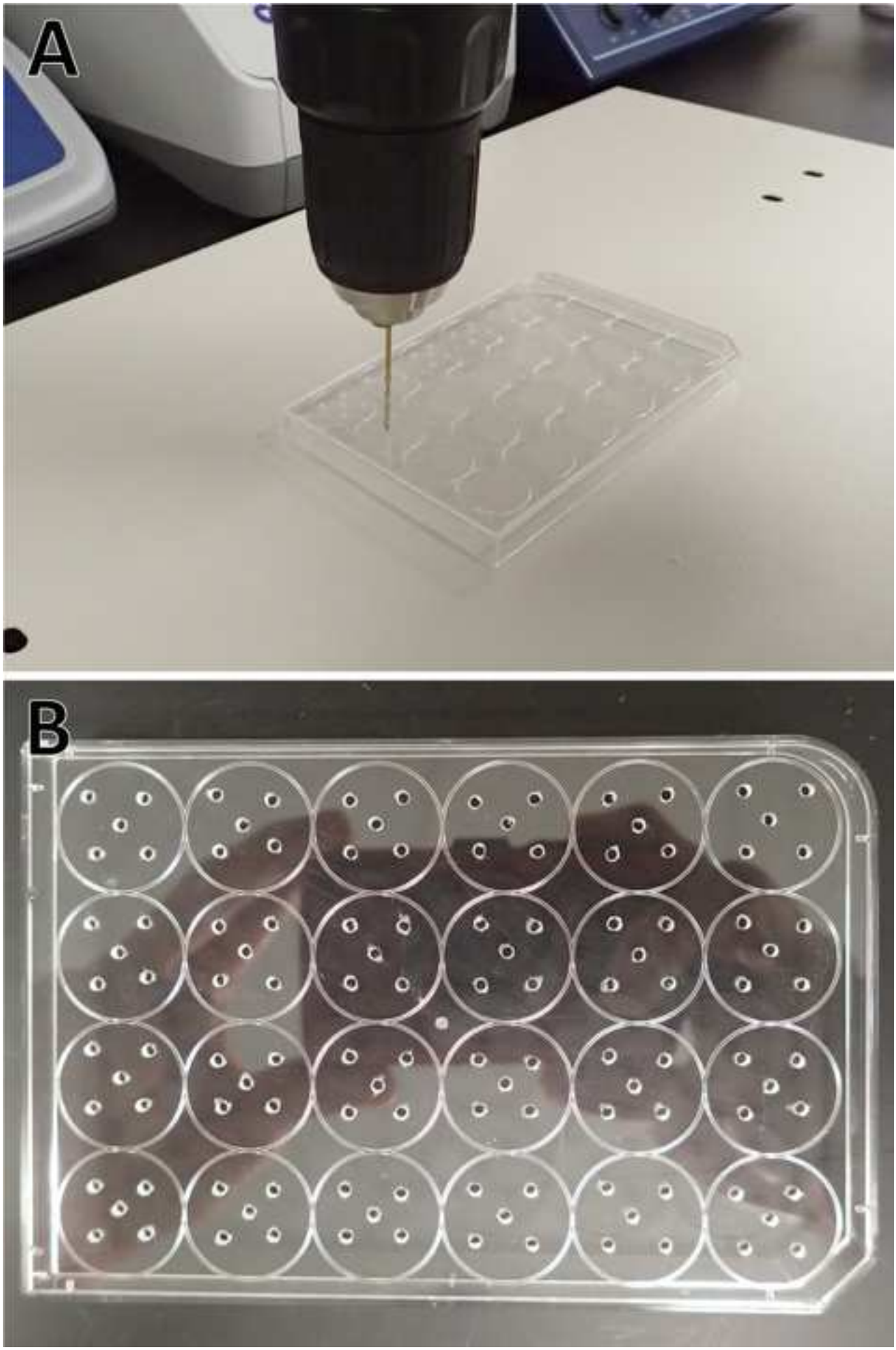

Figure 3 (placeholder)

Click here to access/download;Figure;Fig3.jpg

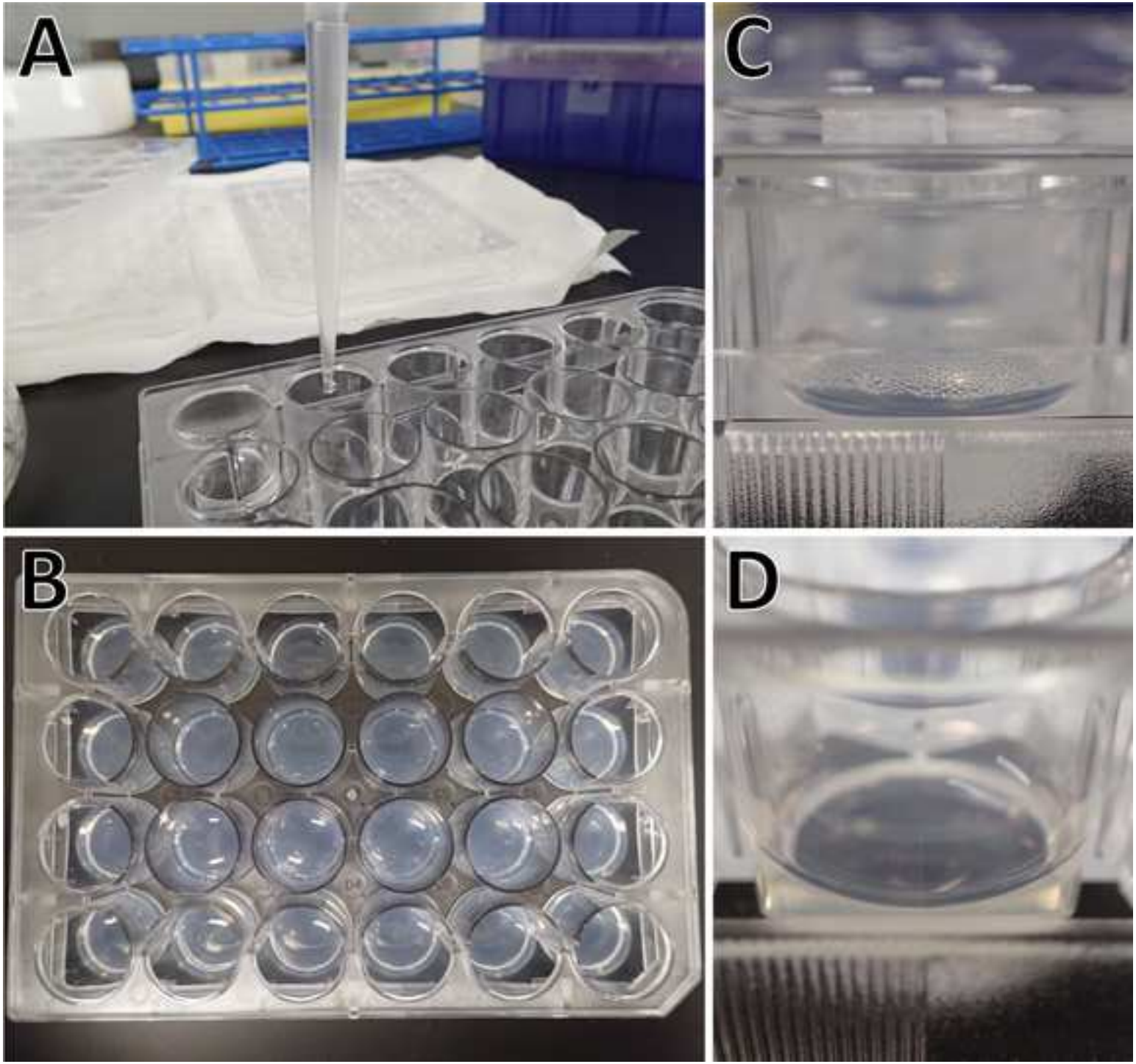

Figure 4 (placeholder)

Click here to access/download;Figure;Fig4.jpg

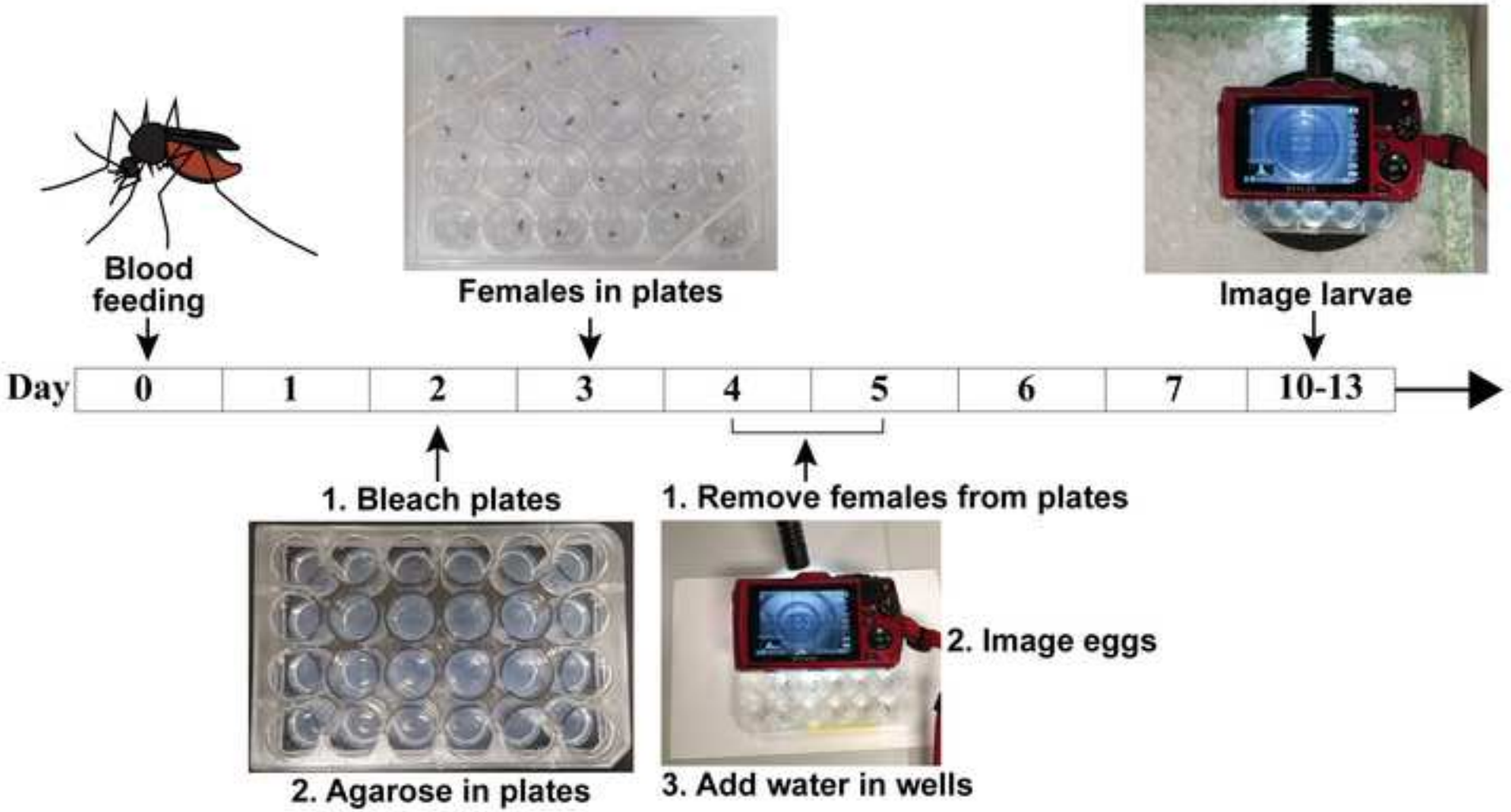

Figure 5 (placeholder)

Click here to access/download;Figure;Fig5.jpg

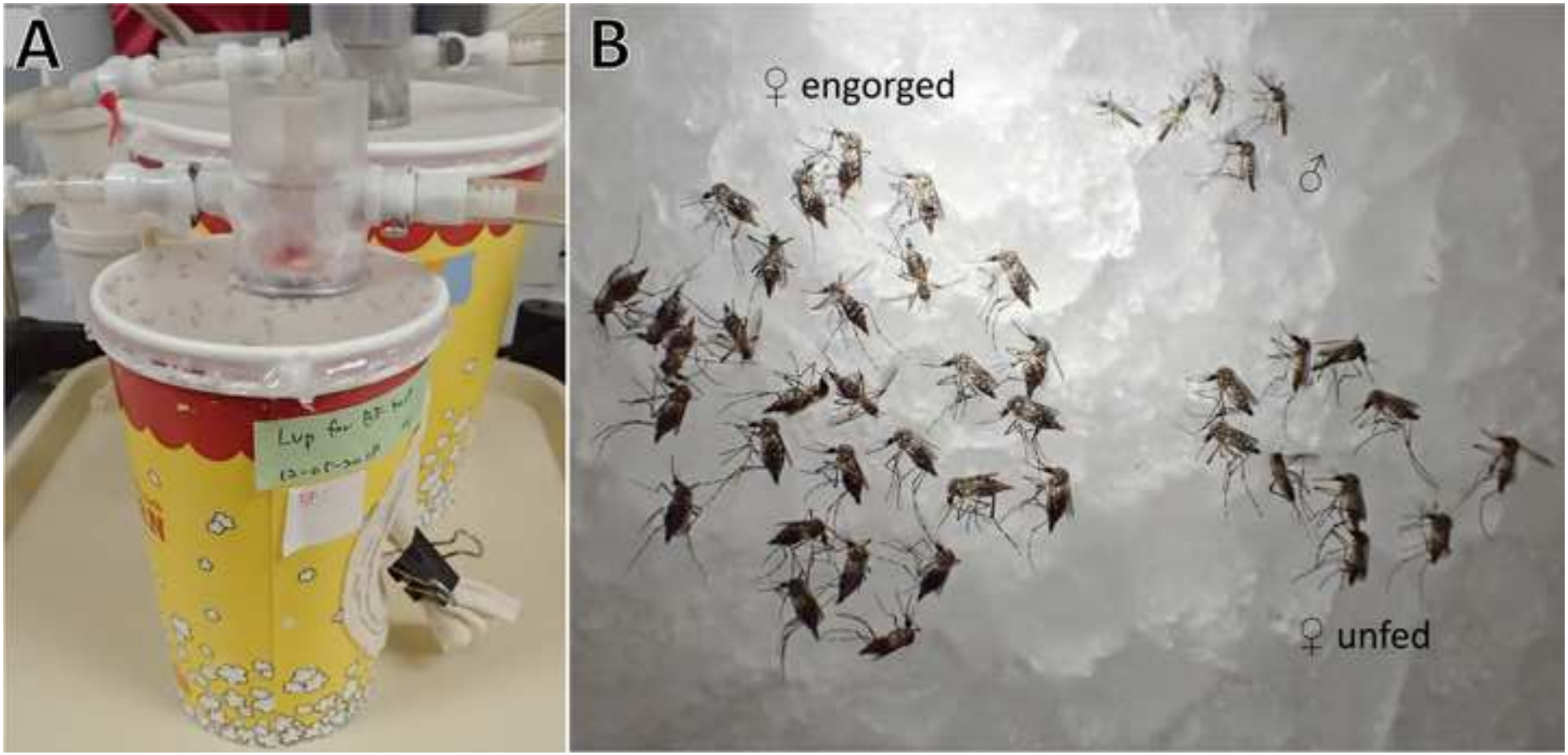

Figure 6 (placeholder)

Click here to access/download;Figure;Fig6.jpg

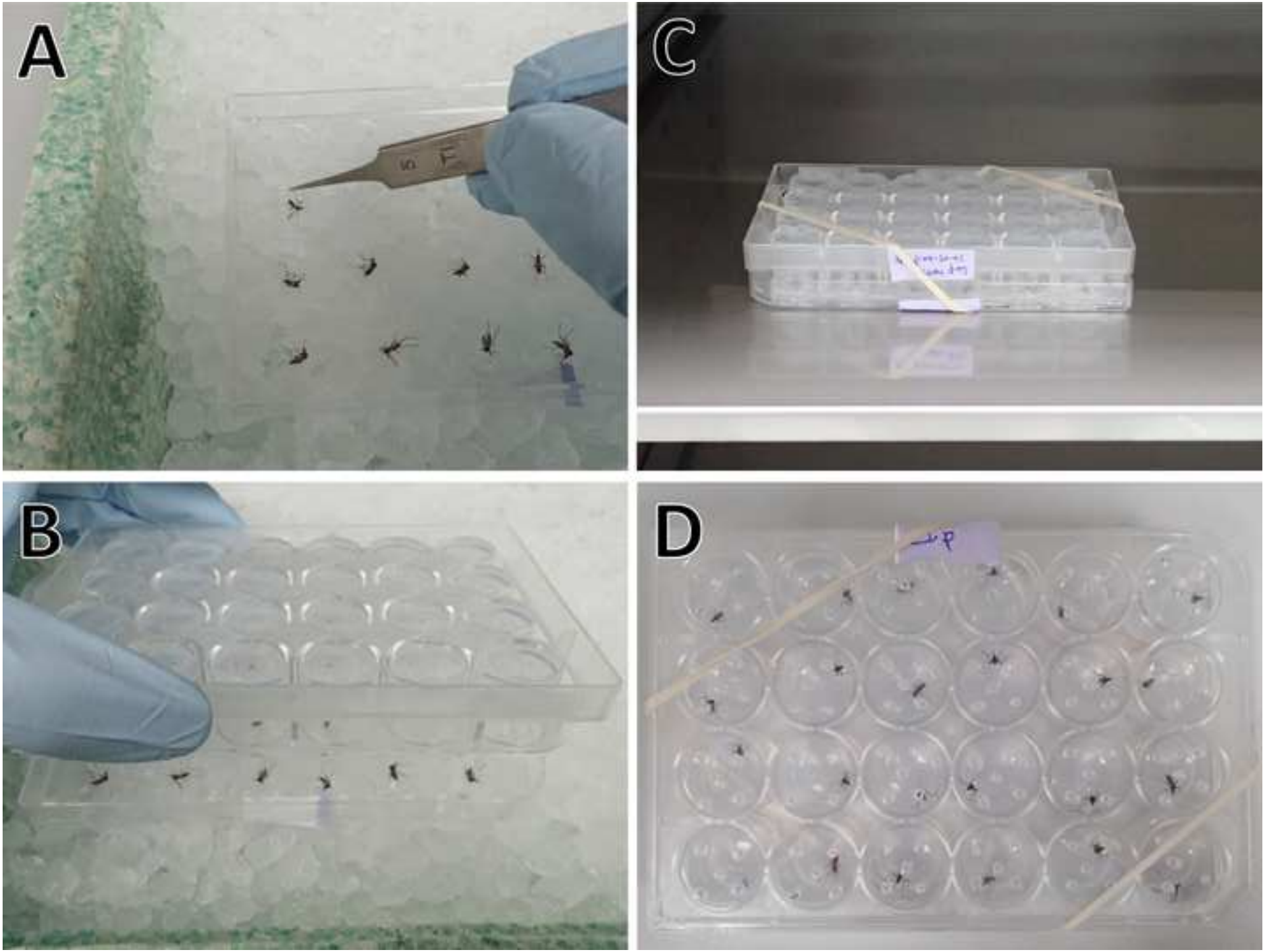

Figure 7 (placeholder)

Click here to access/download;Figure;Fig7.jpg

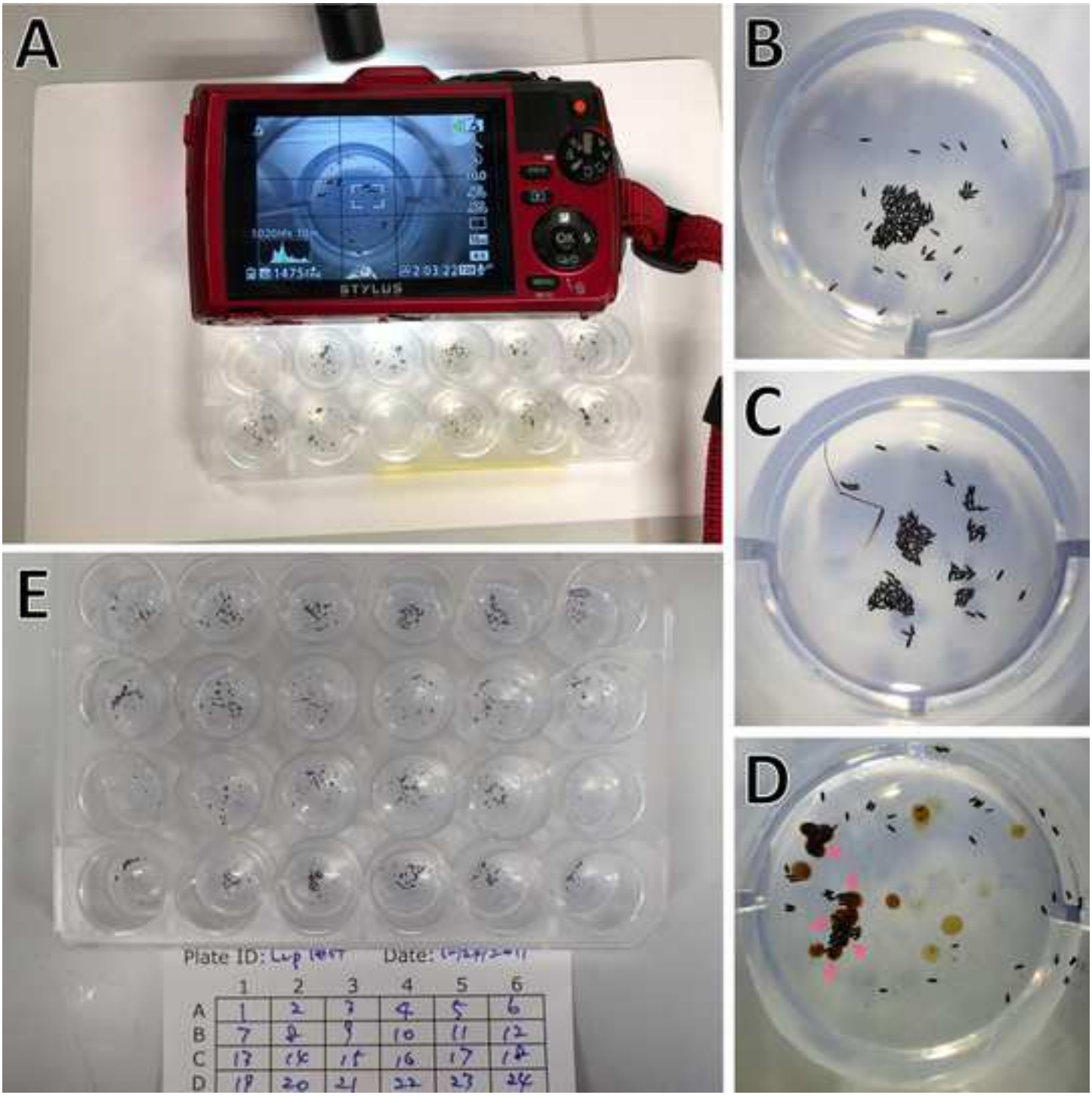

Figure 8 (placeholder)

Click here to access/download;Figure;Fig8.jpg

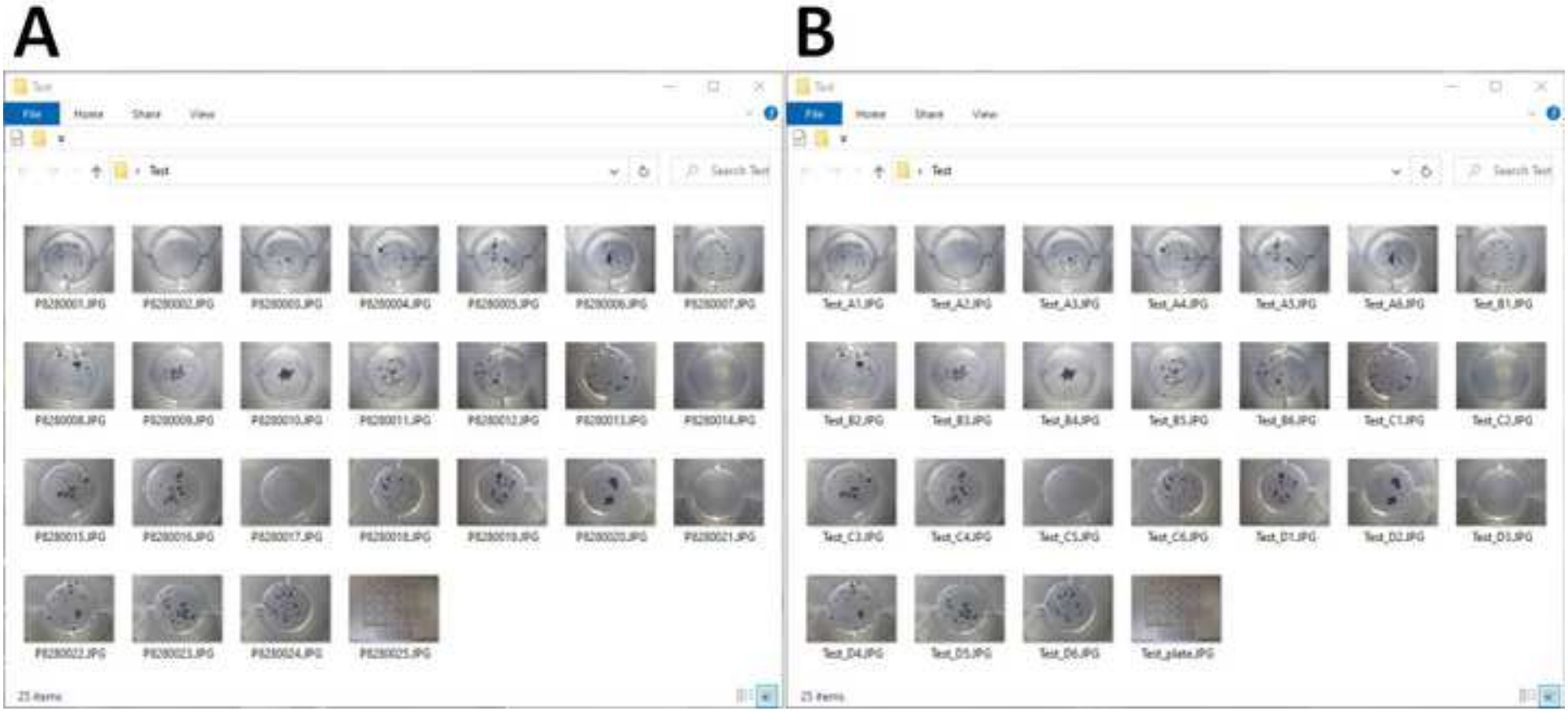

Figure 9 (placeholder)

Click here to access/download;Figure;Fig9.jpg

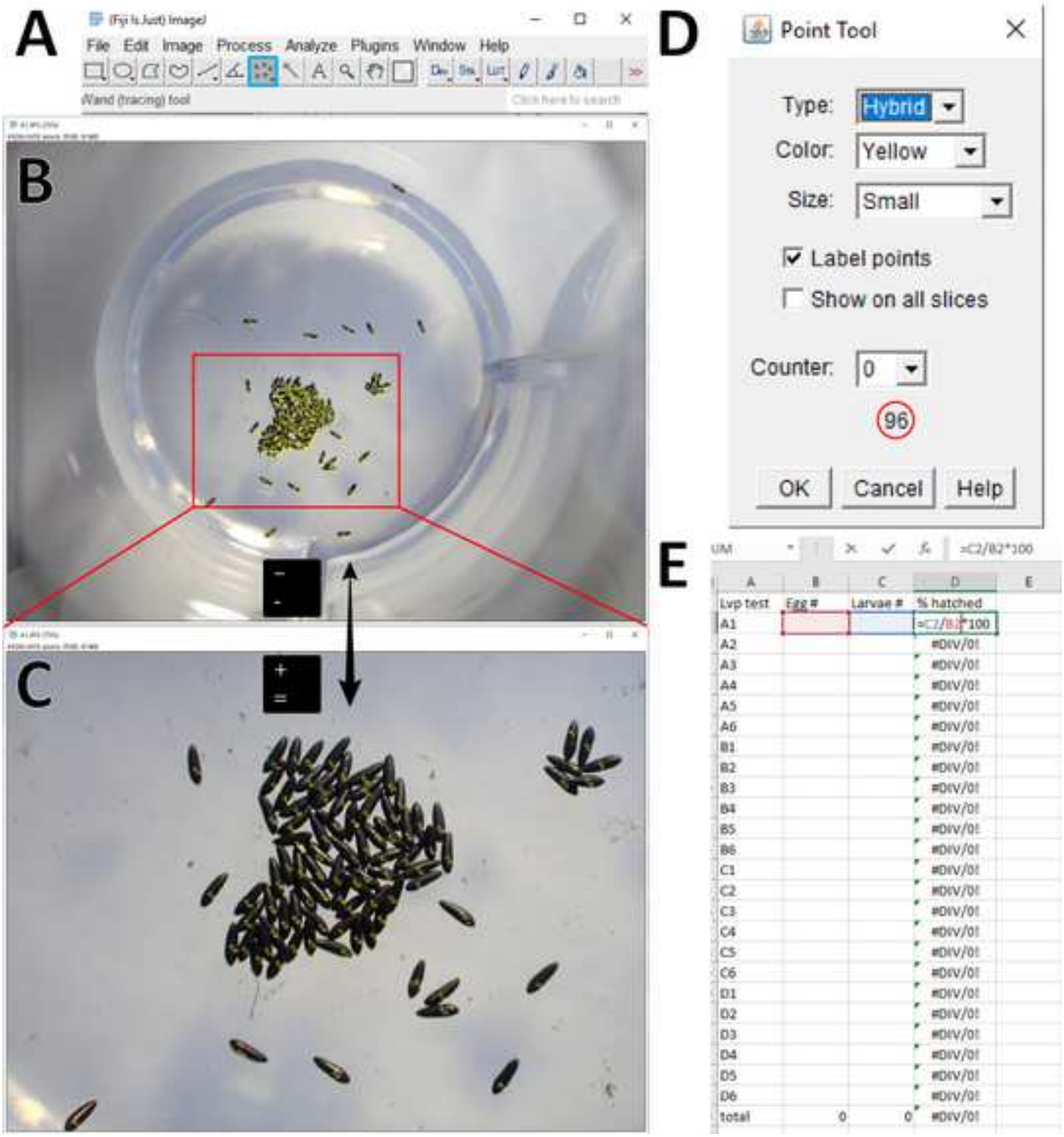

Figure 10 (placeholder)

Click here to access/download;Figure;Fig10.jpg

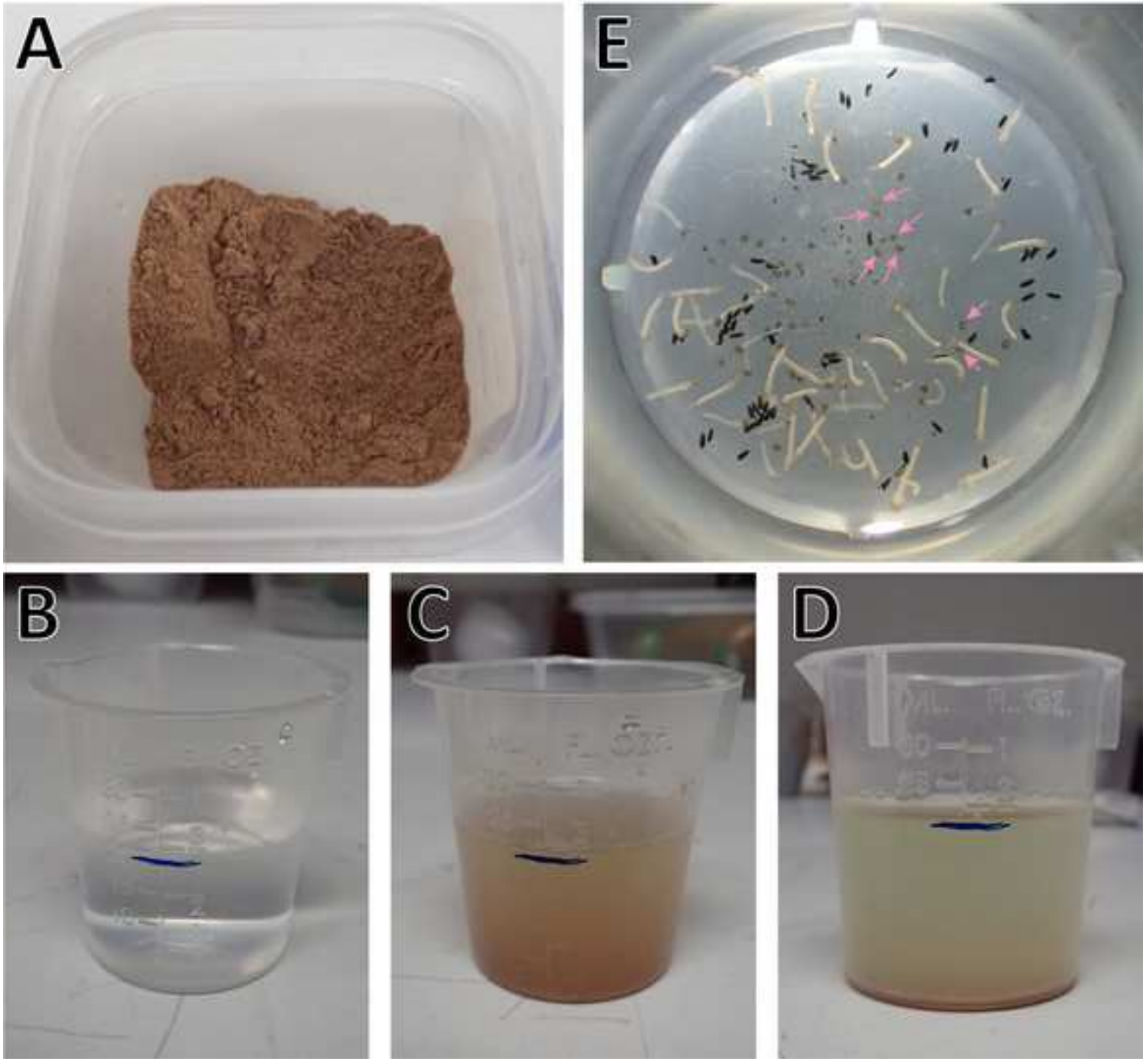

Figure 11 (placeholder)

Click here to access/download;Figure;Fig11.png

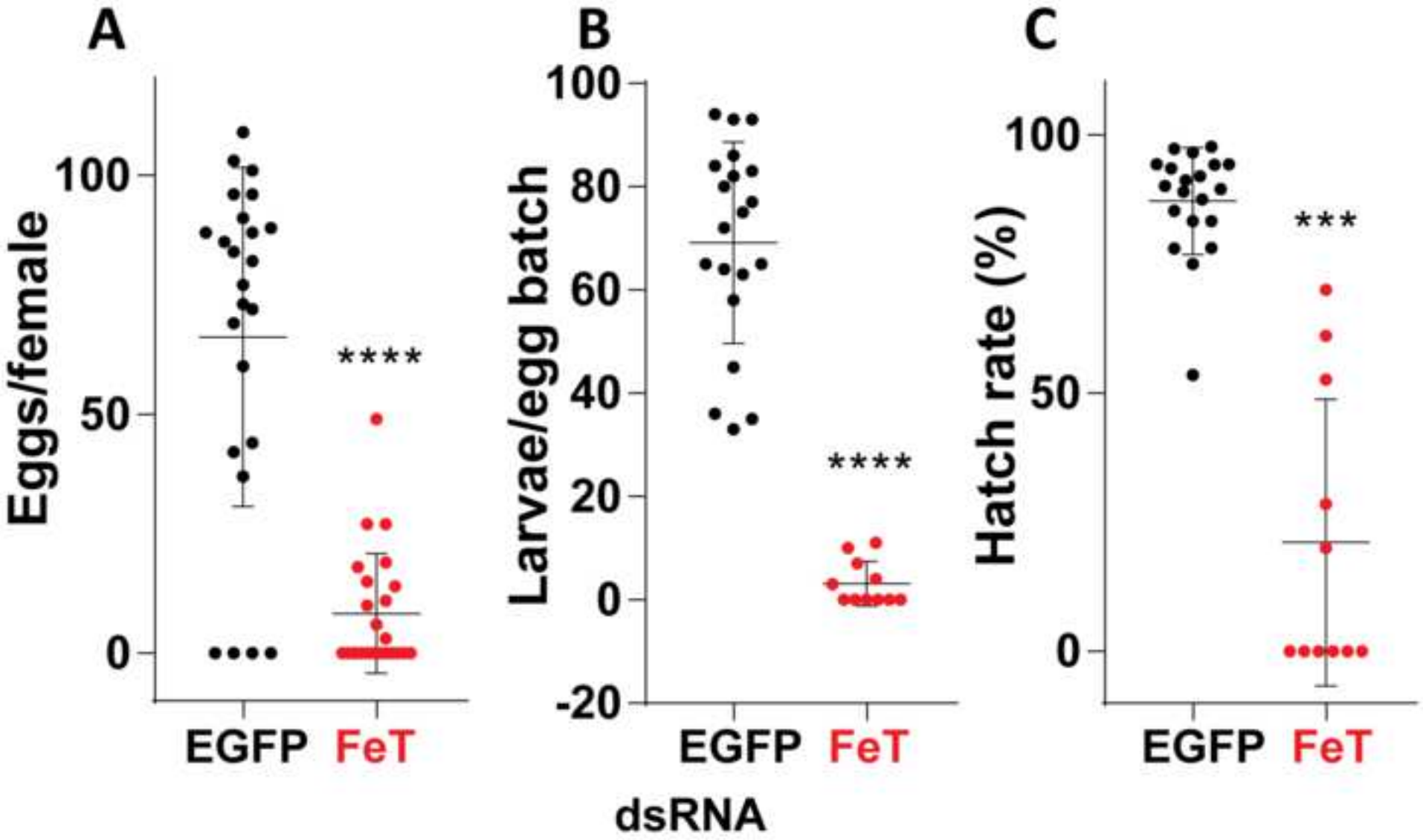

Figure 12 (placeholder)

Click here to access/download;Figure;Fig12.jpg

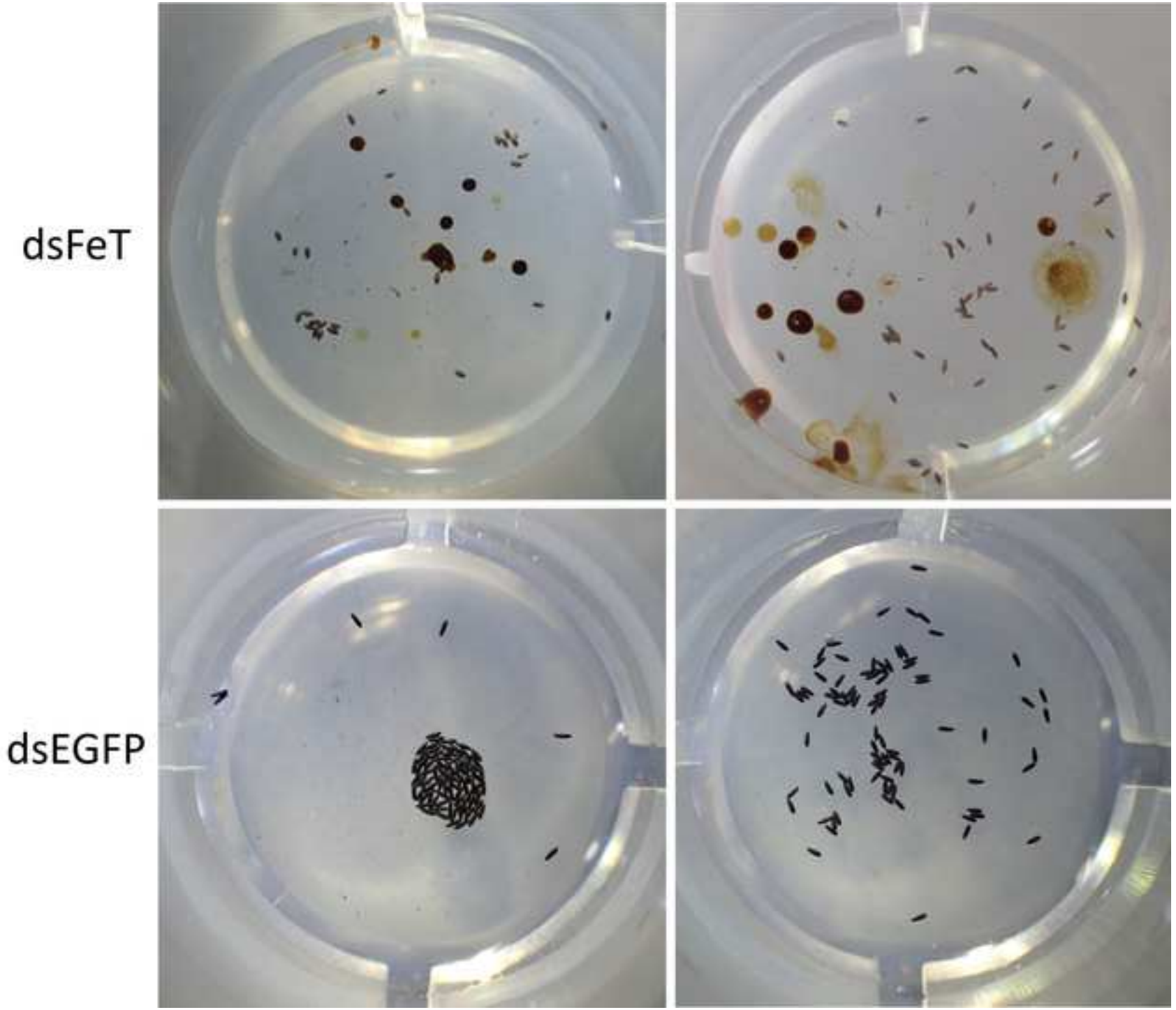

Figure 1


Click here to access/download
Video or Animated Figure
Fig1.svg

Figure 2

Click here to access/download

Video or Animated Figure

Fig2.svg

Figure 3

Click here to access/download

Video or Animated Figure

Fig3.svg

Figure 4


Click here to access/download
Video or Animated Figure
Fig4.svg

Figure 5


Click here to access/download
Video or Animated Figure
Fig5.svg

Figure 6


Click here to access/download
Video or Animated Figure
Fig6.svg

Figure 7


Click here to access/download
Video or Animated Figure
Fig7.svg

Figure 8


Click here to access/download
Video or Animated Figure
Fig8.svg

Figure 9


Click here to access/download
Video or Animated Figure
Fig9.svg

Figure 10


Click here to access/download
Video or Animated Figure
Fig10.svg

Figure 11


Click here to access/download
Video or Animated Figure
Fig11.svg

Figure 12


Click here to access/download
Video or Animated Figure
Fig12.svg

Table 1

	FT	EAgaL
(1) Prep	20:29.0	3:49.3
(2) F_in	3:55.0	1:56.0
(3) F_out/E_img	43:44.3	15:28.3
(4) L_img	38:03.5	9:30.5

Table 2

	EAgaL plate			Fly tubes
Item	**Price**	**Quantity**	**Item**	**Price**
24 well plates	$45.61	Case of 50	Chromatography paper sheets	$103.63
Agarose	$250.97	500 g	Fly tube racks + tubes	$68.45
Compact digital camera	$375.00		Fly tube plugs	$66.10
			Cotton balls	$104.27
Startup total	**$671.58**		**Startup total**	**$342.45**

Unit cost (a 24 well plate or 24 tubes)

Item	**Price**	**Note**	**Item**	**Price**
A 24 well plate	$0.91	price/50	Chromatography paper sheets	$0.16
Agarose per plate	$0.15	500 g = 1667 plates (1)	Fly tube racks + tubes	$3.29
			Fly tube plugs	$7.93
			Cotton balls	$1.25
Unit total	**$1.06**		**Unit total**	**$12.63**

;

Quantity

46 x 57 cm 100 sheets/PK
5 trays of 100
Case of 200
Case of 2000

Note

641.7 sets of 24 tubes worth (2)
(price/500) x 24
(price/200) x 24
(price/2000) x 24

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.6 mm Φ drill bit			alternatively heated nails can be used
1000 µL pipette tips (long)	Olympus plastics	24-165RL	
24-well tissue culture plate	Thermo Scientific	930186	clear, flat-bottom with ringed lid plates
Agarose	VWR	0710-500G	
Compact digital camera	Olympus	TG-5/TG-6	
Computer (Windows, Mac or Linux)			
Deionized water			
Fiji (imageJ) software			download from: https://fiji.sc/
Forceps	Dumont		sharp forceps may break mosquito's body
Glass Petri dishes	VWR		
Household bleach			
Household electric drill			
illuminator for stereomicroscope (gooseneck)			
P-1000 pipette	Gilson		
paint brushes			
Rubber bands			
SD card			to record digital camera images (DSHC, SDXC should be better)
Spreadsheet software (Microsoft Excel)	Microsoft		Any spreadsheet software works
TetraMin fish food	Tetra		ground with coffee grinder, blender or morter & pestle
Transfer pipetts	VWR	16011-188	

Response to reviewers' comments

Editorial comments:
Changes to be made by the author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. Title: Please revise to avoid the use of punctuation (colon, dash, etc.).
Done.
3. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.
Done.
4. References: Please do not abbreviate journal titles; use full journal name.
Done.
5. Figure 5, Figure 6 and Figure 9: Please line up the panels better. Some panels are off-set. Please ensure that the panels are of the same dimensions if possible.
We tried, please check.

Reviewers' comments:

Reviewer #1:
Thank you for your time and helpful comments regarding our manuscript, especially the extensive grammatical correction.
Manuscript Summary:

The paper describes a novel assay format to analyze the fecundity/fertility of single bloodfed mosquito females (Ae. aegypti) in a 24 well plate format. Using 24 well plates in conjunction with a camera for automatic image processing including the counting of objects will 1) substantially reduce the space required when using conventional fecundity assays (using 24 mosquito cartons, or Drosophila breeding tubes) and 2) reduce the time period required for data collection.
We did not use automatic image processing; all egg/larva counting are done manually. This is the reason why we mentioned that it "can be scaled up to **semi**-high throughput" in the discussion.
The idea behind this study is valid (as long as the scenario described under point 4.1 is not frequently occurring) especially when large groups of females need to be analyzed, and the novel assay design including its protocol seem to be straight-forward and easy to reproduce. The images used in the paper are of high quality and the data look solid and convincing.
As long as there is no condensation on the wall, oviposition on the wall is minimal, but some mosquitoes oviposit on the agarose/wall margin, we always need to move eggs from the margin to the lower flat agarose surface. This is also the reason we did not term EAgaL plate method is "high-throughput" and specifically included step 4.1.

Major Concerns:
none

Minor Concerns:

The text, however, needs improvement, apparently due to language problems.

Abstract:
Line 26: For those studies that require an assessment of…..at the individual level, conventional…….on investigators due to high labor intensity and laboratory space requirements.
Line 30: ……..tissue culture plates…….at the individual level……
Introduction:

Line 36: ..due to the lack……to protect humans from vector-borne pathogens.
Line 37: ….reduce the mosquito's fitness in conjunction with a field-applicable population reduction strategy.
Line 38: Such population modification approach requires a detailed assessment of individual fitness parameters .
Line 41: ..the individual containment….and filter paper discs…….
Line 45:…..of individual egg papers for counting and hatching, which can be labor-intensive.
Line 47: …24-well plates to perform egg counting obtained from individual females.

Line 49: surface for oviposition.
Line 50: the unfolding of each ….
Line 51: …not include the use of imaging technologies or an alternative method for larva counting.
Line 54:…that oviposit on…
Line 56:….with a minimal surface…
Line 57:……and maintaining hatched larvae…, thereby drastically reducing time and effort.
Line 59: …as oviposition surface, …….for handling egg papers and finding the eggs on those papers.
Line 74:…oviposition experiment…
Line 78;…..to each well…
Line 83:….eggs, complicating the imaging and counting process.
Line 85:…any condensation on the walls of the 24-well plates…
Line 88: …differ from other mosquito stains or species…
Line 93:…under uniform…line 94:…. Has an impact on…fitness parameters. Rear larvae under non-crowded conditions…
Line 109:…from ovipositioning
Line 111: oviposition:
Line 114: leave out: "in each well of the plates"
Line 123:…to oviposit….
Line 124: in case it is important to keep track on individual females…..
Line 128: ….incomplete oviposition….
Line 129:…to the plates…
Line 136: ….do not overlap with each other.
Line 147: ..in microscope mode…focus on objects…
Line 158:…to each well to prevent its agarose plug and eggs from drying.
Line 187: when larvae have appeared.
Line 189:…interfere with imaging and counting of hatched larvae.
Line 191:…by covering the plates…
Line 194:….similar way like taking images…
Line 208: Over the 3-5 day period,…
Line 220:…dsRNA targeting a candidate…
Line 223:….in which FeT expression was silenced….. reduction in both egg number and….
Line 238:…. the wall of the well. (B) A plate containing agarose.
Line 240: …when condensation has evaporated.
Line 245: ….Transfer of females to…
Line 246: (B) The carefully place the inverted agarose containing plate onto the inverted lid and (C) remove plate (with lid attached) from ice and keep it in an inverted position until females have recovered from anesthesia. (D-E) Turn the female containing plate around to the upward position. Note that lid and bottom part of the plate are held together by a rubber band.
Line 250: Imaging of each well after females had been removed. (A) Digital camera on top of an egg containing 24-well plate……. (D) Sample image of a well in which a female had been placed at 48hPBM; note the dark excretion marks which can complicate the egg counting (arrows). (E) Entire plate showing an image order label, which was prepared following the imaging of all wells of the plate.
Line 256: (A) Images of individual egg containing wells including image order labels and sample designations.
Line 260: ..using "Fiji" (Image J2) software. (A) Screenshot of Fiji software showing the "Multipoint" tool……
Line 262: …helps when counting larger egg groups.
Line 265: Larva diet preparation and a well containing larvae and exuviae.
Line 267: ..of this mixture as…
Line 277: Time requirements for completion of the fly tube method (FT) in comparison to the EAgal plate method. (1) Prep: time required to …….into Drosophila rearing tubes (FT) versus pouring of agarose into wells of 24-well plate (EAgaL). (2) Time required to place ..into rearing tubes (FT) or wells of the 24-well plates (EAgaL).
Line 280: (3) Time required to……..remove, unfold egg paper
Line 281:…image every well….
Line 282: (4) Time required
Line 283:… or each well of……
In the table: (1) Prep; (2) F_in, (3) ….also: show FT (instead of "Tubes") column before EAgal column to match text in description.
All have been addressed in the main manuscript.

Reviewer #2:
Thank you for taking time to review and comment on our manuscript.
Manuscript Summary:

The manuscript entitled "EAgaL plate": Improved fecundity and fertility assay for Aedes mosquitoes using 24-well tissue culture plates" (JoVE61232) details a protocol that adds to a previous 24-well tissue culture plate method to collect eggs from individual females in a space-saving way. The EAgaL plate has benefits compared to the previously described method (Ioshino et. al. 2018) in that it facilitates the egg counting process, potentially reduces the time required, and allows for the eclosion of the eggs in the plate for fecundity and fertility assays, which can reduce human error. The authors also discuss some limitations of the method.

Overall the manuscript is well written and contains all the necessary details and pictures to easily follow the protocol. The method described is definitively useful for mosquito research and I recommend its publication in JoVE. I only have few comments and questions detailed as "minor concerns".

Minor Concerns:

a) 1.3: do the tips need to be sterile/autoclaved? Have the authors considered using tips with "cut tips" in order to facilitate the process of pouring the agarose?
Yes, we always use autoclaved tips which minimize the chance of potential contamination, especially mold. For the use of “cut tips”, we have not considered it. It may work better, but it also increases the time to prepare and the chance of contamination (if cut happens after autoclaving).
b) 1.3: have the authors tried other concentrations of agarose? Why did they choose 2%?
We have not tried agarose other than 2%, because 2% as we feel with our fingers is solid enough and lower percentage might trap mosquitoes.
c) 1.3.1: it would be useful to have more details about their rearing conditions as a reference point (i.e. duration of light cycle, rearing temperature)
A brief description was added in the manuscript.
d) 3.1/3.2: why are the 2-3 drops of water added to the agar? Does the added water drip on the females while the plate is upside-down?
It is to compensate for lost humidity during the time that the plate is out on the lab bench. The plate without addition of water worked fine. Some (or sometimes all) water has been absorbed in agarose. The added water has never dripped on the females while in the inverted position. Remaining water on the agarose seemed to help females to lay eggs in the center (where water is) of the wells instead on the margins.
e) 3.3: have the authors tried leaving the females in the plates for longer than 48 h? The authors mention incomplete egg laying if the females are transferred earlier than 72h post blood meal. Is this due to drying out of the agarose? Have the authors tried leaving these plates in conditions with higher humidity to prevent drying of the agarose, or tried other concentrations of agarose?
We have not tried that as well, because we were afraid that embryos may lose viability with excess drying at such an early point of development. We mis-worded here, it was not actually “incomplete egg laying”, because these mosquitoes did not lay all the eggs for the first 24 h, and laid eggs for the following 24 h. This suggests that the agarose did not lose the function as egg laying surface. Under our insectary conditions (27 °C, 80% RH), increasing humidity was not necessary. Higher humidity, especially saturated humidity (100%) may cause condensation on the wall and the lid where females lay eggs.
f) 4.4: the authors should state that for taking pictures of the individual wells is also possible to use a camera attached to a stereo microscope (if the magnification allows it).
We have tested the stereo-microscope camera. The problems are: (1) View in camera (not from eyepiece) does not cover the entire well, which in turn requires acquisition of multiple images for each well. This increases the numbers of pictures and labor to patch images (or keep tracking which eggs were counted when using multiple pictures per well). A camera attached on a stereo-microscope (in our case Leica M165 FC with Leica DFC 3000G camera) does not get a view from perpendicular angle (slightly towards the right with our scope perhaps due to the nature of stereo-microscope), which makes the right side wall block some part of agarose. Our eye-piece camera (Amscope) verified that when using right eye piece, it looked slightly from the right angle (right wall blocking), and when using left eye piece, it looked slightly from the left angle (left wall blocking).
We think that it is a useful information and added to the discussion section.
g) 4.6: authors mention that the water levels get lower, if the plates are kept at higher humidity, would this reduce the evaporation? Can more water be added to the wells to reduce the need for checking?
It is possible to reduce evaporation by keeping the plates at higher humidity, but not possible to completely prevent from happening. Adding more water may reduce the need for checking. One caution is that the deeper the water is,

the more space for the larvae to be present, meaning, focusing on top of water as well as bottom in the same picture becomes more difficult. Keeping the water depth shallow is better for imaging purpose.
h) 4.9: have the authors considered using the automatic particle counting option in ImageJ?
Yes, we are aware of the automatic particle counting options. However, those counting technologies at present can be applied only for egg counting and do not reach beyond “estimation”, which may be pretty close to the real numbers, but not exact counts. Automatic counting also may increase learning curve, which may reduce acceptance of many readers.
i) 5.1: have the authors considered the possibility of storage of eggs in this setup and synchronous hatching at a later date?
This can be an option. To do this one will need to test viability of the eggs without addition of water for control and treatment. We think that such a possibility may be tested for each researcher’s demands.
j) 5.2.1: the authors state that the plate is not "designed to maintain mosquito cultures beyond early larval stages". Can the larvae be recovered and transferred to a larger container if it is necessary to measure survival to adult stage?
That also is a possibility. As we stated above, it should be tested elsewhere on demand.
k) Fig. 11, dsFeT: both wells have blood in them, indicating that the females were transferred into the plate before the recommended 72hs period. Even though these figures are just to exemplify the use of the method, it would be useful if the authors could explain the reason of the difference between the treatment and control wells.
Sorry for the confusion. In this experiment, we followed the standard procedure for the EAgaL plate method; both dsFeT and dsEGFP females were transferred to the EAgaL plate at 72 hPBM. The results showing not only reduce number of eggs but also delayed excretion, which is not the case for dsEGFP. That is why Fig 11 legend states “delayed excretion”. Added text in the main body to clarify.
l) Table of material/equipment: the authors state that "any 24-well plate should work". Due to personal experience placing Aedes aegypti females in 24-well plates, I don´t believe that any plate will work. It is necessary to use plates with rings in the lid in order ensure that the well is closed well enough to avoid the females from escaping and moving into neighboring wells.
We appreciate your useful information. We revised to “clear, flat-bottomed with ringed lid plates”.
m) Could the authors add a figure of a timeline to make the process clearer? It is unclear if the plates are made before the females are bloodfed or after the females are bloodfed.
Yes. That is a good point and we added a figure of the assay timeline (from bloodfeeding to larval imaging).
n) Could the authors comment about the possibility of fungi growing on the agar during the study?
Added a comment.
o) If it is not necessary to eclose the eggs, how long can the plates be kept before taking the pictures for egg counting?
It all depends on the condition of storage. The EAgaL plates with eggs may be kept at –20 °C, which may change the texture of agarose.

Reviewer #3:
We appreciate your time and expertise to review our manuscript.
The present article describes an improved protocol for the Oviplate method by using agarose as an oviposition substrate and applying Image J to count the number of eggs laid/hatched larvae. It is an interesting improvement in the method of individual analysis, which deserves to be propagated to the scientific community, especially because of the use of agarose in the wells. However, I consider that some major points need to be addressed before this article can be accepted.

General comments:

The major critique regards the specific improvements. Undoubtedly, the authors brought innovation, but the authors are also claiming about "space-saving method" and "the use of 24-well tissue plates", which were not the specific advances they have contributed. The use of 24-well tissue culture plate has already been demonstrated elsewhere. Actually, the authors have referenced this method in lines 47 to 51. In this sense, I think the authors can strengthen the wording of the text, highlighting what is real innovation. To clarify my point, see the two questions below:

1- What is the space-saving modification that "EAgal plate" has improved in comparison to the oviplate method? Is the use of a 24-well tissue culture plate for Aedes aegypti oviposition assay an advance?
This is a detailed version of the method described in Tsujimoto, et al., 2018, Frontiers in Physiology https://doi.org/10.3389/fphys.2018.00380. Many people encouraged us to present this in a more visible way and we

were invited to submit a manuscript to JoVE by a guest editor. Therefore, we committed to invest our efforts to prepare this manuscript.
According to the JoVE policy, they accept “novel methods, innovative applications of preexisting techniques and gold standard protocols”, which is in “Objective” section in “PEER REVIEW” page in JoVE website (https://www.jove.com/publish/peer-review/), copy-pasted below:

> “JoVE serves the research community as a scientific methods journal for efficient dissemination, reproduction and discussion of experimental approaches in biological, medical, chemical and physical research. Video is an effective publication format as it ensures more efficient transfer of information than traditional text articles. JoVE publishes novel methods, innovative applications of existing techniques and gold standard protocols in a scholarly video and text format. Detailed text and representative results accompany every video.”

We consider JoVE is not a place to present “which comes first” like arguments, rather it is a journal to present a method in more visible and learnable way. We clarified this in the introduction.
An innovation of the EAgaL plate method is not only using agarose as oviposition substrate, but also using the tissue-culture plates as egg viability tests, when it is compared to the oviplate. Because the oviplate method did not describe larval assay, the EAgaL may be able to reduce space for hatching eggs from individual females (in comparison to tube method).
Specific comments by section:

Title:
a. The manuscript title ""EAgaL plate": Improved fecundity and fertility assay for Aedes mosquitoes using 24-well tissue culture plates" can be improved. I would suggest some options:
-""EAgaL plate": Improving the oviplate method for fecundity and fertility assay in Aedes aegypti."
- ""EAgaL plate": Agarose as an oviposition substrate and digital counting improves oviplate method for fecundity and fertility assay in Aedes aegypti.
We changed the title in a way to reflect point b below, but try not to include “oviplate” not to give readers an impression that we disgrace the oviplate method in the title.
These two would be more accurate in my opinion since:
a. The present work shows an improvement of the oviplate method that already uses 12- and 24-well plate as well. "using 24-well tissue culture plates", is not an advance per se for individual oviposition in Aedes aegypti.
At the time we developed the EAgaL plate method, we aimed to improve from conventional fly tube method. Publication chronology (please see acceptance and published dates for Tsujimoto et al., 2018) serves as evidence of this.
b. The method was only tested for Aedes aegypti in the present article. Although it is likely to work with Aedes albopictus and other Aedes species, this needs to be experimentally demonstrated.
We agree, and feel presenting this protocol in detailed form will be the best way to disseminate this technique to others working on related species.

Keywords and Summary:
1- The "space-saving" descriptor and this quality was not an advance that this article brought up. The use of 24-well tissue culture plates, the substantial increase of the assay scale has already been shown in the literature. The authors need to be precise in highlighting the new improvements that they have created with the agarose and downstream counting protocol.
As noted above, the method we describe here is a detailed version of a protocol we used in 2018, and extends other protocols by including space-saving larval assays as well as egg counts.

Introduction section:
1- Line 43-44 - "…Such method requires…. (Figure 1).". The oviplate article that the authors have referenced has shown the same information about the space regarding the Drosophila tubes. Hence, Figure 1 is not necessary if the authors just refer to this comparison.
Above argument also applies here and we are comparing the EAgaL plate method to the fly tubes. To be clear on this point, we would like to keep the Fig 1.
2- Line 47-51 - The authors precisely described the negative aspects of the oviplate method and these are the drawbacks overcame by the present work, more than the space improvements. I understand this is the valid justification for the proposed work.
We appreciate the complement.
3- Line 55 - "…. (e.g., Aedes)", it would be better to mention this broad use of the method after testing it for at least for a couple more species.
Removed this notion to reflect the reviewer’s suggestion.

4- Line 54 - "We introduced an improved method to… in a 24-well tissue culture plate format..., the plates are not an innovation in the literature. The authors should explore the other characteristics of the method that make it unique so far.
Please see our comments in general comment section.
5- Line 56 - The authors state the use of the 24-well plates again. Would EAgal plate method work for 12-well plates?
We did not test 12-well plates.

Protocol section:
Line 72 - The holes' idea is interesting. The authors let the readers aware of the size. This is a good point. An addition here would be the standard size of the reared mosquitoes. Tiny ones (malnourished ones could escape as well). The authors have commented about this issue in lines 93 to 96, but it is good to include this information here as well.
Size of the holes are roughly the size of the drill bit used (1.6 mm diameter). Our mosquito size and comment for the readers added.
Line 74 - The authors described a bleaching procedure. Why is it necessary? An explanation should be included.
Bleach treatment is to minimize a potential contamination that may grow on agarose. Description added.
Line 88 - "….different species…", I am not sure about mentioning other species since the protocol was tested for Aedes aegypti only.
We tried to mention applicability here. Modified to clarify.
Line 101 - The information about the feeder device could be included in the table of materials.
Our feeder devise is custom made. Not available commercially.
Line 113 - A standard volume of water would be better than "2-3 drops of water" since the counting will be compared among wells.
Modified accordingly.
Lines 116 to 121 - Ioshino et al 2018 already discussed the procedure of putting females on the inverted lid and the reason why it needs to be done in this way. I suppose the female's wings can get stuck on the agarose surface before they wake up. The authors have not explained the reason why and have not referenced this procedure. The figures 5A and 5B show similar pictures of the same procedure reported in Ioshino et al. The only modification was the lid on the ice (I suppose the holes prevent the condensation so females are not stuck on the lid). This is the modification in the procedure that needs to be highlighted.
Added a note that this procedure has been applied from Ioshino et al.
Unlike Ioshino et al. described, even mosquitoes were directly placed in the wells mosquitoes are fine. We have never seen mosquitoes stuck on the agarose and cannot get up. Dry lid for such a short time (a few minutes) in our lab (we keep mosquitoes in rearing chambers; prep room is at normal lab temperature) did not cause enough condensation for the mosquitoes to stick.
The rubber band can be a convenient way to do it but it can rupture eventually. Do the authors think that it would be better to have the lid and the plate be held together by applying a piece of tape?
As long as we use fresh rubber bands, they have never been snapped over 10-13 days of entire experiment. However, it is a good point and we added a note for the readers.
Line 125-126 - Can the chilling procedure jeopardize the eggs? What would be an alternative to that?
We have never observed reduction of viability of eggs by chilling on ice (we have never used below freezing temperature for chilling). Alternative may be using CO_2, but it would be complicated as it is difficult to knockdown by gassing in each well; using a chamber to fill CO_2 with EAgaL plate may work.
Line 163 to 166 - Would this issue be a concern when comparing the hatching rates among wells and among plates. Do the authors think that putting the plates inside a humid chamber (or even a sealed container with cotton balls soaked in water) would overcome this drawback?
As far as viability, we do not think the difference in water levels for each well make difference in viability, unless wells dry.
Line 207 to 211 - The authors have mentioned in the discussion section that it is usual to have more than 100 larvae per well. In this situation, how is the accuracy of the counting when the chilled larvae are twisted in each other (clumped)?
Clumping can be resolved manually with a fine forceps or probe prior to taking pictures.

Discussion section:
287- …"space to conduct". This is not an improvement. I agree that the other points are. The authors could argue that it saves time in comparison to the flytube/oviplate method in terms of preparation. In the last case, the filter paper procedure may require longer preparation. What are the authors' thoughts?
Please see the comments in General Comments section.

However, how faster is to run all the protocol to count automatically in comparison to counting them visually (a lot of steps are necessary - from the pictures to marking each of them and recording the results). Is it still faster than visually counting the eggs? Is this more reliable? I addressed this last question in the specific comments below.
I do appreciate the idea of photographing each of the wells, so the results can always be revisited.
When it comes to compare automatic counting vs manual counting, speed is not the only factor. Accuracy is the major factor to be considered. Since we are not introducing automatic counting, it is not discussed.

Lines 291 to 300 - It was nice that the authors addressed a potential limitation. Please, includes "data not shown" if this is the case.
Modified accordingly.

Specific comments:
1- Regarding the costs of the EAgal plate, how does the use of agarose increase the costs in comparison to Flytubes and Oviplate methods? Did the authors estimate it? This is not included in the article.
1. We do not compare the EAgaL plate method to the Oviplate method.
2. We did not mention “cost-saving”, but added the price estimation of the EAgaL plate and fly tubes in the Discussion along with Table 2.

2- The authors did not include a comparison between the results obtained with the automatic counting protocol and the regular manual (visual) counting method. Is the automatic one reliable? More reliable than the manual one? It would be nice to include this comparison for at least one plate analyzed by both methods.
We did not compare automatic counting and manual counting, and this protocol was not developed using automatic counting. We have tried, but none had been reliable and increase the learning curve.

3- The authors did not compare the proposed method with the gold standard ones (flytube/oviplate). They mentioned that females did not retain a significant amount of eggs when the agarose substrate was offered (lines 296 to 297), however, a rigorous comparison could show that this substrate is better than filter papers.
We have compared between the EAgaL plate and fly tubes and did not compare with the oviplate because of the reason we mentioned above. Our speculation is the space constraint regardless to the substrate. Moreover, the Oviplate article did not address if this issue is the case for the Oviplate.

4- What is the authors' opinion about the proposed method - is it a completely new method, or is it a significant improvement on the oviplate method by using agarose? Is it an advance that deserves a new name representing a new method (EAgal plate) since it is still applying a tissue culture plate to oviposition assays? What are the authors' thoughts about this aspect?
With our colleagues’ encouragement, lab’s support and JoVE’s purpose, we strongly believe that making EAgaL plate method more visible (than Tsujimoto et al., 2018) will benefit the mosquito research community. Whether it deserves the publication in JoVE is determined by the reviewers and the editor.

5- Can larvae eat the agarose substrate? Can they penetrate in the layer? Would these two issues be a concern for downstream use of the hatched larvae?
We did not observe larvae have consumed agarose or chewing down agarose. We speculate that since mosquito larvae are filter feeders and do not have ability to grind or crush agarose to fine particles.

6- The authors have used Fiji (ImageJ) software to count eggs and larvae. In the introduction section, no background information about automatic egg counting was provided for the readers. There are some methods already showing the automatic counting protocols in the literature (e.g., Gaburro J, Duchemin JB, Paradkar PN, Nahavandi S, Bhatti A. Assessment ofICount software, a precise and fast egg counting tool for the mosquito vectorAedes aegypti. Parasit Vectors. 2016), some of them applying ImageJ for the same purpose (e.g., Mains JW, Mercer DR, Dobson SL. Digital image analysis to estimate numbers of Aedes eggs oviposited in containers. J Am Mosq Control Assoc. 2008; Ross PA, Endersby-Harshman NM, Hoffmann AA. A comprehensive assessment of inbreeding and laboratory adaptation in Aedes aegypti mosquitoes. Evol Appl. 2018). How does the proposed ImageJ method compare to the others?
Thank you for the references. Testing these methods (ICount is no longer publicly available) did not give us satisfactory results and we thought that including automatic counting in the protocol may increase the learning curve (and confusion) and reduce acceptance by many readers.