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1   **TITLE:**
2   Microinjection method for *Anopheles gambiae* embryos
3
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18 **KEYWORDS:**
19 Transgenesis vectors, gene expression, promoter analysis, mosquito, microinjection, genetic
20 engineering
21
22 **SUMMARY:**
23 Microinjection techniques are essential to introduce exogenous genes into the genomes of
24 mosquitoes. This protocol explains a method used by the James laboratory to microinject DNA
25 constructs into *Anopheles gambiae* embryos to generate transformed mosquitoes.
26
27 **ABSTRACT:**
28 Embryo microinjection techniques are essential for many molecular and genetic studies of insect
29 species. They provide a means to introduce exogenous DNA fragments encoding genes of interest
30 as well as favorable traits into the insect germline in a stable and heritable manner. The resulting
31 transgenic strains can be studied for phenotypic changes resulting from the expression of the
32 integrated DNA to answer basic questions or used in practical applications. Although the
33 technology is straightforward, it requires of the investigator patience and practice to achieve a
34 level of skill that maximizes efficiency. Shown here is a method for microinjection of embryos of
35 the African malaria mosquito, *Anopheles gambiae*. The objective is to deliver by microinjection
36 exogenous DNA to the embryo so that it can be taken up in the developing germline (pole) cells.
37 Expression from the injected DNA of transposases, integrases, recombinases, or other nucleases
38 (for example CRISPR-associated proteins, Cas) can trigger events that lead to its covalent
39 insertion into chromosomes. Transgenic *An. gambiae* generated from these technologies have
40 been used for basic studies of immune system components, genes involved in blood-feeding, and
41 elements of the olfactory system. In addition, these techniques have been used to produce *An.*
42 *gambiae* strains with traits that may help control transmission of malaria parasites.
43
44 **INTRODUCTION:**

45   Microinjection techniques have been used to experimentally manipulate organisms since the
46   early 1900s[1]. Microinjection has been used to study both basic biological functions and/or
47   introduce important changes in the biology of a desired organism. The microinjection technique
48   has been of particular interest to vector biologists and has been widely used to manipulate vector
49   genomes[2-11]. Transgenesis experiments in arthropod vectors often aim to make vectors less
50   efficient at transmitting pathogens by either enacting changes that decrease a vector's fitness or
51   increase refractoriness to the pathogens they transmit. Mosquitoes transmit a variety of human
52   pathogens and have a significant impact on morbidity and mortality worldwide. The *Anopheles*
53   genus of mosquitoes transmits the human malaria pathogen *Plasmodium spp.* Parasites. Genetic
54   engineering experiments with *Anopheles* have aimed to better understand the biology and
55   reduce the vectorial capacity of these mosquitoes in efforts to develop novel malaria elimination
56   strategies.
57
58   The mosquito vector that contributes the most malaria infections worldwide is the *Anopheles*
59   *gambiae* species complex*,* the African malaria vector. However, the majority of successful
60   transgenesis experiments have been performed on the malaria vector of the Indian subcontinent,
61   *Anopheles stephensi*. While plenty of laboratory adapted *Anopheles gambiae* strains exist, the
62   number of transgenic *Anopheles gambiae spp.* lines reported in the literature does not compare
63   to that of *Anopheles stephensi*. It is thought that the *Anopheles gambiae* embryo is more difficult
64   to inject and achieve successful transgenesis than *Anopheles stephensi*, although the reasons for
65   these differences are unknown. This protocol describes a method that has been proven to be
66   consistently successful in achieving transgenesis of *Anopheles gambiae* embryos via
67   microinjection. The protocol is based on a method previously developed by Herve Bossin and
68   Mark Benedict[12] with some additional details and alterations added that have been found to
69   increase efficiency of transgenesis.
70
71   **PROTOCOL:**
72
73   **1.   Preparing mosquitoes for microinjection**
74
75   1.1.   Seed a cage[13] (~5000 cm^3) with ~100 male and 200–300 female 1–2 day post eclosion
76   adult mosquitoes and allow them to mate for 2 days.
77
78   1.2.   After the mating period, provide mosquitoes a bloodmeal using either 2 mL of blood with
79   an artificial feeding device or live anesthetized animals depending on insectary practices[14]. The
80   following day provide mosquitoes a second blood meal to ensure that all mated females have
81   had opportunity to feed and that partially-fed mosquitoes have become fully-engorged.
82
83   1.3.   Utilize the mosquitoes for embryo collections 2–4 days after the second blood meal.
84
85   NOTE: Larval nutrition is important for adult robustness and reproductive fitness. See Benedict
86   et al. (2020) for information about how to rear healthy insects[14]. No significant differences in egg
87   laying have been observed when mosquitoes are fed on artificial feeder or live anesthetized
88   animals. Use whichever method is routinely used for *Anopheles gambiae* in insectary.

2.   Embryo preparation

2.1.   Use an aspirator to place 20–30 females in a transparent 50 mL conical tube that has been cut (with a band saw) to be open at both ends and is covered with latex dental film at one end and nylon mesh and filter paper at the other (**Figure 1**).

NOTE: The mosquitoes will deposit their eggs onto the filter paper and the nylon mesh will keep the filter paper securely in place. Mosquito eggs are cylindrical in shape, ~500 μm in length, ~200 μm in diameter at their widest point, and tapering at the anterior and posterior ends (**Figure 2**).

2.2.   Place the mosquito filled tube in a small (60 mm x 15 mm) Petri dish filled with double-distilled water (ddH$_2$O). Place the tube and dish in an incubator at 28 °C for 45 min (**Figure 3**).

2.3.   Remove the tube from the incubator and insert the tube into an empty cage. Gently tap the tube to allow mosquitoes to fly out and remove the tube from the cage once all mosquitoes have flown out.

2.4.   When the tube is free of mosquitoes, unscrew the bottom ring, remove the nylon, and use forceps to carefully remove the filter paper with the eggs from the tube. Place the egg laden filter paper in a plastic Petri dish (100 mm x 15 mm) containing a layer of filter paper moistened with ddH$_2$O.

2.5.   Observe the eggs. Eggs that have aged to the point where they are light gray in color (**Figure 4**) are ready for alignment.

2.5.1.   If eggs are still white, return them to the incubator and check the color every 5 min. White eggs are fragile, rupture easily, and do not survive well after the injection process resulting in low hatching.

2.5.2.   Eggs that are dark gray or black have aged too much. Do not use them.

3.   Embryo alignment

3.1.   Cut a piece of nylon blotting membrane (2 cm x 1 cm) with a razor blade, making sure that the edge is neatly cut.

NOTE: If the edge is not straight, the eggs will not stay affixed to the membrane properly during the injection process.

3.2.   Put a membrane on a glass cover slide and cover the membrane with a piece of filter paper (2 cm x 2 cm), leaving ~1 mm of the membrane filter uncovered (**Figure 5**).

NOTE: Ensure microscope slides are clean before use and use clean forceps to manipulate the

133 membrane filter.

134

135 3.3. Wet the filter paper with deionized H_2O as eggs will die if desiccated for prolonged periods
136 of time.

137

138 NOTE: Do not put too much water on the paper because the embryos will move during the
139 injection process, but make sure that there is enough to prevent the embryo from drying (**Figure**
140 **6A, B**). Excess water can be removed by absorbing it with a piece of filter paper.

141

142 3.4. Gently transfer 30–50 embryos to the edge of the membrane with a paintbrush (size #0).
143 Use the brush to align the eggs vertically along the membrane by gently rolling them over on the
144 slide so that the ventral side (convex) is facing upward.

145

146 3.5. Orient all the eggs in the same direction so that when the eggs are observed under the
147 microinjection microscope, the posterior end (more pointed, **Figure 6A, B**) is in a down position
148 and forms an angle of ~15° with the needle (**Figure 6C**). Line the entire edge of the membrane
149 with eggs (30–50) and place the slide under the microscope for injection.

150

151 NOTE: Choose the eggs carefully. Discard those that are white (too young or undeveloped) or
152 dark gray (too old and will break the needle), and the abnormal ones (**Figure 7**). Make sure the
153 filter paper remains moist at all times.

154

155 **4. DNA preparation**

156

157 4.1. Purify plasmid DNAs for injection, at the minimum, with an endotoxin-free DNA plasmid
158 maxiprep kit following the manufacturer's protocols.

159

160 4.2. Resuspend the DNA in PCR-grade water and centrifuge at 17,100 x *g* in a refrigerated
161 centrifuge for 30 min at 4 °C. Then, transfer the pellet to a new, clean 1.5 mL conical tube.
162 Carefully micro-pipette to avoid transferring undesired particulate matter from the column.

163

164 4.3. Perform a second precipitation of DNA using a two-fold volume of 100% ethanol. Before
165 injection, clean the DNA using a column according to manufacturer's protocol.

166

167 4.4. Resuspend the DNA in 300–500 µL of PCR-grade water for a final plasmid concentration
168 of 1000 ng/µL. Mix the plasmid cocktail in the injection buffer for a final plasmid concentration
169 of 300–400 ng/µL and make 10–20 µL aliquots.

170

171 NOTE: DNA can be stored at -20 °C. DNA microinjection aliquots can be stored at -20 °C, or at -80
172 °C if using RNA components. Do not exceed 800 ng/µL of DNA in the injection mix because
173 viscosity will cause the needles to clog.

174

175 **5. Needle preparation**

176

177 5.1. Make the needles by pulling 10 cm quartz capillaries using a programmable micropipette
178 puller.

179
180 5.2. Examine the needle under microscope to ensure that the tip is formed well. Ensure that
181 the needle setting on the programmable puller yields a tip that starts to taper ~0.5 mm from the
182 terminal end and ends in a fine point (see **Figure 6**). Needles that break easily usually have too
183 long of a taper.

184
185 NOTE: Conditions might differ depending on the needle puller (The authors will make available
186 on request the settings for the programmable puller they use. Journal restrictions prevent us
187 from citing a specific brand). Needles can be pulled by hand, but this requires a skill-set not
188 available in most laboratories.

189
190 **6. Embryo microinjection**

191
192 6.1. Use a micro-loader tip to fill the needles with 2 µL of DNA mixture. Insert the needle into
193 the needle holder and connect the automated pressure pump tubing (**Figure 8**).

194
195 6.2. Important: Align the needle so that it makes an angle of 15° with the plane of the slide
196 (**Figure 8**).

197
198 6.3. Open the needle tip by carefully touching the first egg and inject it by inserting the tip of
199 the needle ≤ 10 µm in the posterior pole. A successful injection will lead to a small movement of
200 the cytoplasm within the egg.

201
202 6.4. Use the microscope coaxial stage controls to move to the next egg to continue injection.

203
204 6.4.1. To ensure that the needle tip remains open and has not clogged, press the **Inject** button
205 before entering another embryo and visualize the small droplet at the opening of the needle.

206
207 6.4.2. If needle gets clogged, press the **Clear** button to clear the needle and repeat the droplet
208 visualization test. Adjust the pressure as needed if the needle tip opening gets slightly bigger to
209 ensure that the size of the droplet stays small.

210
211 6.4.3. Inject ~40–50 eggs with one needle.

212
213 NOTE: Make sure the filter paper stays moist at all time. Keep sufficient back-pressure on the
214 needle to keep it cleared. A needle that cannot be unclogged must be replaced with a new
215 needle. Embryo placement, needle insertion and injection are made easier with microscopes that
216 have coaxial controls for horizontal movement of the stage (**Figure 9**).

217
218 6.5. After injections are complete, rinse the eggs off into a glass container lined with filter
219 paper and filled with 50 mL of deionized H_2O (**Figure 10**).

220

221 NOTE: Embryos will start hatching 2 days post-injection and may take as long as 3–5 days.
222 Hatched first instar larvae must be transferred immediately (check 2 times daily) to a clean
223 container with water and food.
224
225 **REPRESENTATIVE RESULTS:**
226 A representative example of the application of the microinjection protocol described can be
227 found in Carballar-Lejarazú et al[5]. The intent here was to insert an autonomous gene-drive system
228 into the germline of a laboratory strain, G3, of *An. gambiae*. The system was designed to target
229 the *cardinal* ortholog locus (*Agcd*) on the third chromosome in this species, which encodes a
230 heme peroxidase that catalyzes the conversion of 3-hydroxykynurenine to xanthommatin, the
231 last step in ommochrome biosynthesis (**Figure 11**). Homozygous gene drive-containing
232 mosquitoes are loss of function mutants with larvae, pupae and newly-emerged adults having a
233 red-eye phenotype. A plasmid, pCO37, containing the drive system expresses *Streptococcus*
234 *pyogenes* Cas9 (SpCas9) endonuclease under control of the regulatory elements of the *An.*
235 *gambiae nanos* gene ortholog, a 23-nucleotide guide RNA (gRNA) under the control of an *An.*
236 *gambiae* U6 gene promoter and a 3XP3-CFP dominant marker cassette expressing the cyan
237 fluorescent protein for identifying transgenic progeny. *Agcd* DNA fragments homologous to
238 genomic regions flanking the gRNA target cut site make possible homology-directed repair- (HDR)
239 mediated integration.
240
241 Seven-hundred eighty wild-type *An. gambiae* embryos were injected with the pCO37 plasmid
242 along with SpCas9 protein. One-hundred forty-six injected embryos (18.7%; G_0) survived to the
243 adult stage and were outcrossed subsequently to wild-type members of the opposite sex.
244 Fluorescent microscope screening of the next generation (G_1) progeny identified three of 3,428
245 (0.09%) that were positive for the CFP marker gene, only two of which, a male and female,
246 survived. Molecular approaches (gene amplification, DNA sequencing) verified the accurate
247 insertion of ~10 kb of DNA and the resulting strain was designated AgNosCd-1. Extensive follow-
248 up analyses showed that AgNosCd-1 was highly-efficient at drive, created few resistant alleles
249 and had no major genetic load (fitness costs) as a result of the integration and expression of the
250 gene-drive system[5]. The pCO37 plasmid serves as backbone for developing population
251 modification strains of *An. gambaie* to prevent malaria parasite transmission.
252
253 **FIGURE AND TABLE LEGENDS:**
254 **Figure 1: Egg collection device.** The egg collection device is composed of filter paper, nylon mesh,
255 dental film and a retrofitted 50 mL conical tube. The device allows mosquitoes to be deposited
256 by aspirator securely into the container via the slit in dental film. The filter paper and nylon mesh
257 allow water to be absorbed, which provides a damp, but not flooded surface, for oviposition.
258
259 **Figure 2: *Anopheles gambiae* embryos. A**) A batch of embryos at varying stages of maturation.
260 **B**) The arrow indicates the posterior pole, the location within the embryo that contains germline
261 cells, which are the intended targets for transformation. The scale bar represents ~1 mm.
262
263 **Figure 3: Egg collection.** Once the mosquitoes are placed inside the egg collection device it is
264 moved to a Petri dish with distilled water. Adjust the water volume so that the water level rises

265   to ~1/4 the height of the cap. The egg collection device and Petri dish are then placed in a
266   darkened incubator and mosquitoes allowed to oviposit for a total of 45 min.

268   **Figure 4: Embryo development.** The embryos are only suitable for injection during a short time
269   window. Eggs that are injected prematurely in development are too delicate and will suffer
270   damage and not hatch. Eggs that are injected later in their development become hardened and
271   can break the needle causing too much genetic material to be injected in the egg. Additionally,
272   injections later in development are less likely to cause permanent changes to the germline as
273   pole cells rapidly undergo further replication and differentiation. Pictures were taken every 10
274   min after oviposition time of 45 min. The asterisks (*) indicate eggs that are suitable for injection.

276   **Figure 5: Membrane slide assembling.** The eggs are aligned along the nylon membrane, which
277   provides a support structure so that the eggs do not move during the injection process. The filter
278   paper serves as a reservoir for water to ensure that the eggs stay moist throughout the injection.
279   Place the filter paper and nylon membrane close to the edge of the glass slide allowing room for
280   both eggs and the water that submerges them. If the membrane and filter paper are too far from
281   the edge of the slide achieving the correct angle between embryos and needle will be difficult.

283   **Figure 6: Embryo orientation for microinjection. A**) Embryos are aligned with the posterior poles
284   lower than the anterior, running down the membrane. **B**) The embryos are immersed in water;
285   adjust the width of the water line above the egg to be approximately an eighth to a quarter the
286   width of an average embryo. **C**) Close up image of needle angle with embryos. The scale bar
287   represents ~1 mm.

289   **Figure 7: Abnormal embryos.** Embryos that appear abnormal in coloration or texture will not
290   hatch; do not inject them. Embryos that are yellow in color will not properly melanize. Reduced
291   hatching has also been observed in smooth embryos that do not have normal-appearing
292   eggshells (cuticles).

294   **Figure 8: Needle position during the microinjections. A**) Wide-angle view of the injection stage.
295   **B**,**C**) Position the needle so that the aligned embryos form an angle of 15° with the injection
296   needle. Do not elevate the arm of the microinjection instrument that holds the needle
297   significantly higher than the microscope stage to ensure proper angling between the needle and
298   slide containing the embryos.

300   **Figure 9: Co-axial controls. A**) The co-axial controls (arrows) allow for 3-dimensional movement
301   of the needle to ensure precise placement of the needle for each injection. It is important to use
302   co-axial controls to raise the needle vertically when switching slides so that the needle does not
303   collide with the new slide when putting it into place on the stage. Lateral co-axial controls allow
304   for accurate penetration of the needle into the posterior pole.

306   **Figure 10: Chamber for egg hatching. A**,**B**) Carefully wash injected embryos into a cylindrical
307   container filled to a quarter depth with distilled water and lined with filter paper. **C**,**D**) Movement
308   of the container causes the eggs to naturally adhere to the filter paper. Before leaving the

309 container for hatching, ensure any eggs that have adhered higher up the filter paper are gently
310 washed back down to water level. The blue oval marks a number of deposited eggs to show
311 relative size.
312
313 **Figure 11. *Anopheles gambiae cardinal* (*Agcd*) gene ortholog, pCO37 gene drive construct and**
314 **resulting phenotypes.** Top) *Agcd* gene: maroon blocks, exons (E1-4); empty blocks, 3'- and 5-
315 untranslated regions (UTR); thick black line, introns and intergenic DNA. Middle) pCO37 plasmid:
316 homology arms from *Agcd* gene: maroon blocks, blue blocks, dominant marker gene components
317 (3XP3 and CFP); tan blocks, drive components (*nanos* promoter and SpCas9 protein-encoding
318 sequences); green blocks, guide RNA components (U6 promoter and gRNA sequence). Genes and
319 features of pCO37 are not to scale. Recombination (maroon arrows) resulting from homology
320 directed repair (HDR) initiated at the SpCaS9/gRNA-mediated cut site (green arrowhead) results
321 in integration of the gene drive construct. Bottom) resulting visible phenotypes of gene drive
322 integration: (left) CFP+ (white/blue arrow) and homozygous *Agcd*-mutant (red arrow) phenotypes
323 in larvae and *Agcd*-mutant (red arrow) phenotypes in pupae. (right) Homozygous *Agcd* mutant
324 phenotype "red eye" in adults. Adapted from Carballar-Lejarazú *et al.* (2020) and used with
325 permission[5].
326
327 **DISCUSSION:**
328 With the increased availability of precise and flexible genetic engineering technologies such as
329 CRISPR/Cas9, transgenic organisms can be developed in a more straightforward and stable way
330 than previously possible. These tools have allowed researchers to create transgenic strains of
331 mosquito vectors that are very close to achieving the desired properties of either refractoriness
332 to pathogens (population modification) or heritable sterility (population suppression). However,
333 to develop the most safe and stable genetically modified mosquitoes possible, additional
334 transgenic lines need to be created and evaluated so that optimal lines are selected for field
335 studies. The protocol described can increase the efficiency of generating *Anopheles gambiae*
336 transgenics so that additional candidates can be developed for population modification and
337 suppression strategies.
338
339 The microinjection procedure described utilizes equipment and materials that are likely available
340 in a lab that routinely performs microinjections of mosquitoes or other arthropod vectors.
341 Maintaining attention to detail throughout the protocol is important. Rushing through steps such
342 as selecting eggs by quality or skipping steps such as the second DNA precipitation will impair
343 results and cause reduced hatching and reduced transformation efficiency. In addition, many
344 difficulties with the procedure can arise if care is not taken in mosquito husbandry. Mosquitoes
345 that are reared in crowded conditions or with a lack of nutrients will lay less eggs of poorer
346 quality[14], which creates bottlenecks in the microinjection workflow and will increase the time
347 needed to reach a desired number of injected embryos.
348
349 Unlike methods described for the microinjection of *Anopheles stephensi*[12], this method does not
350 require oil submersion of the embryos and more embryos can be injected in a shorter time
351 without the additional steps required by the oil-based method. A limitation to this method
352 compared to the oil-based method is poorer visualization throughout the microinjection process.

353 To ensure that injection material or embryo cytoplasm is not flowing back into the needle,
354 attention must be kept to the pressure on the needle at all times throughout the injection
355 process.
356
357 The African malaria vector has been historically difficult to genetically manipulate compared to
358 other *Anopheles spp.,* yet it is the largest contributor to malaria cases worldwide. Novel tools are
359 needed to aide malaria elimination efforts and transgenic mosquitoes designed to either modify
360 or suppress mosquito populations can play an important role in achieving elimination. To select
361 the best transgenic candidates for field-based malaria elimination studies, a number of lines
362 should be evaluated so they can be compared and selected for optimal efficacy and safety[15]. The
363 genetic and molecular building blocks needed to create transgenic lines with enhanced
364 refractoriness or suppression are characterized and available. This protocol will aide in increasing
365 the speed at which these refractory or suppressive lines can be created so that validation efforts
366 may begin to select a candidate for field trials in the near future.
367

373 **DISCLOSURES:**
374 The authors have nothing to disclose.
375

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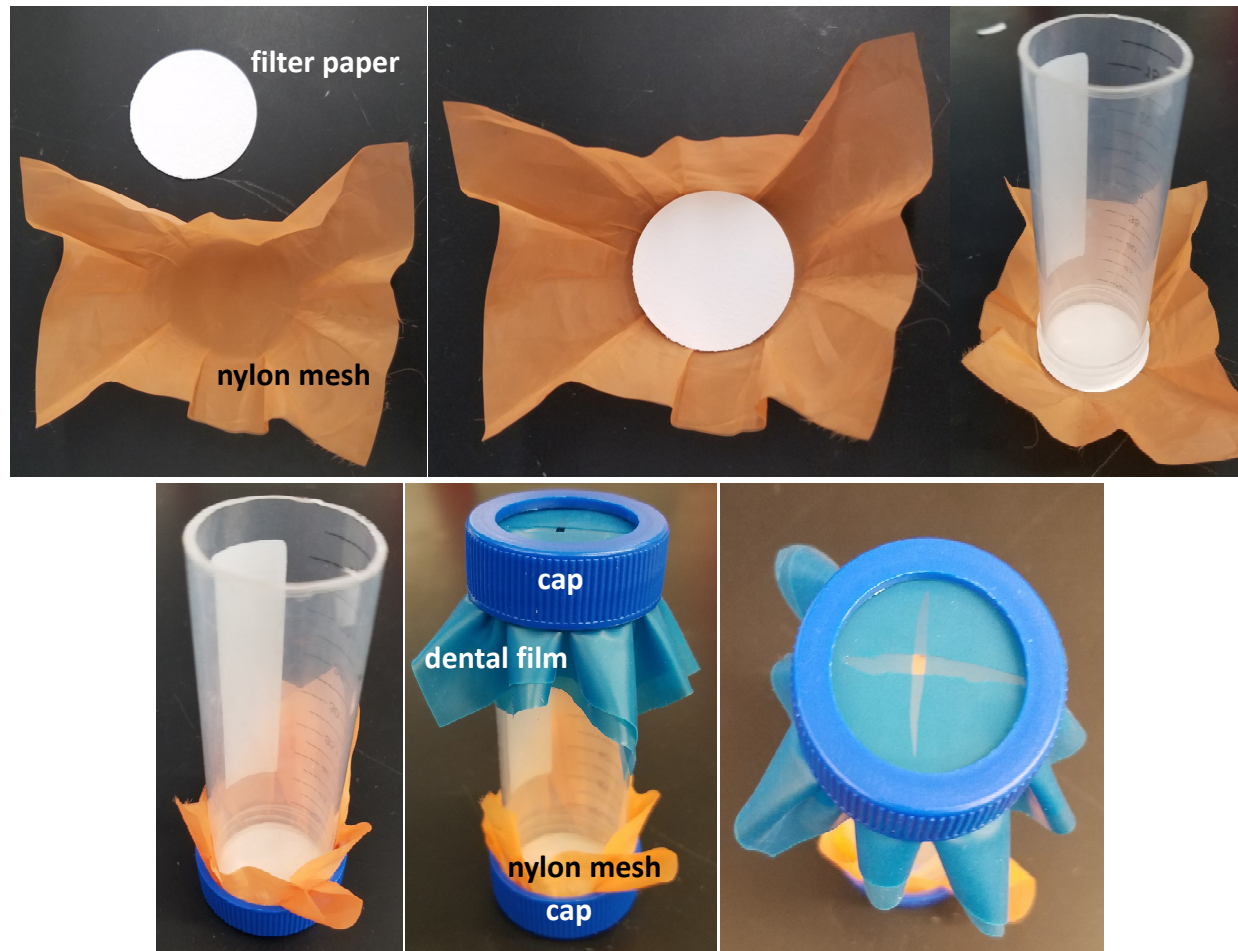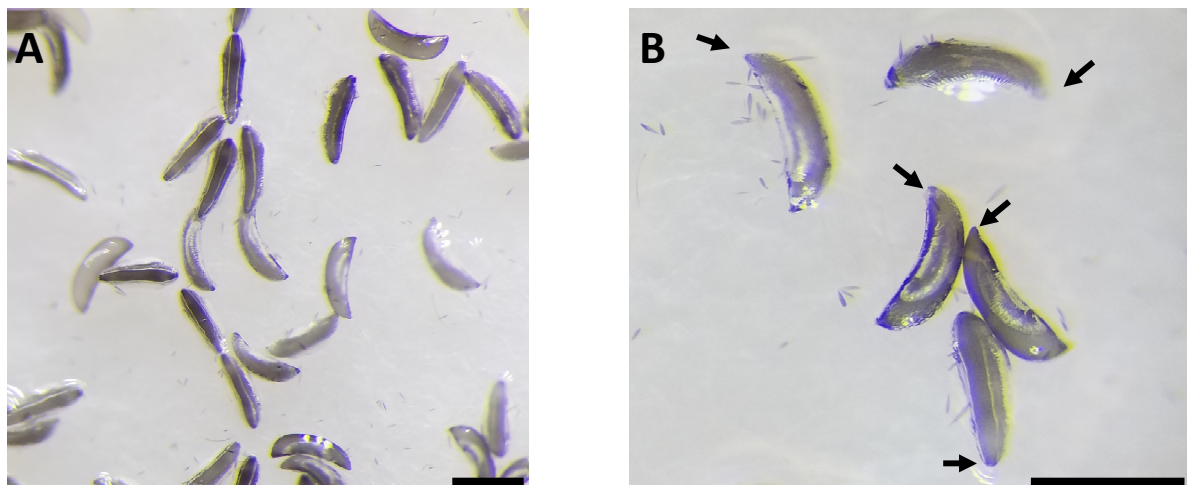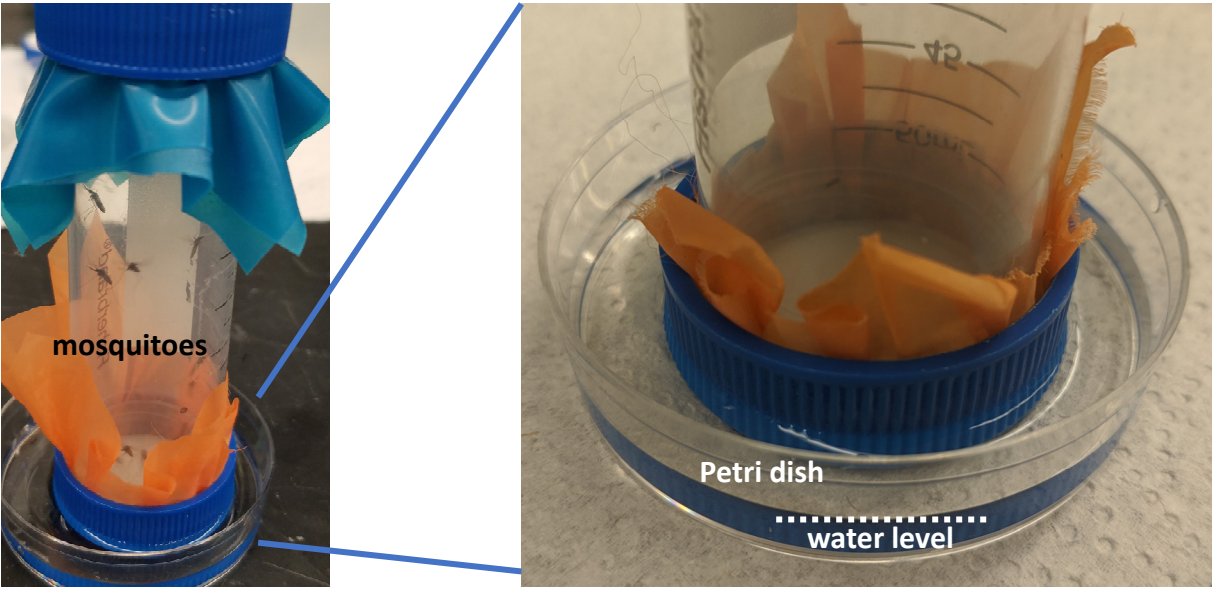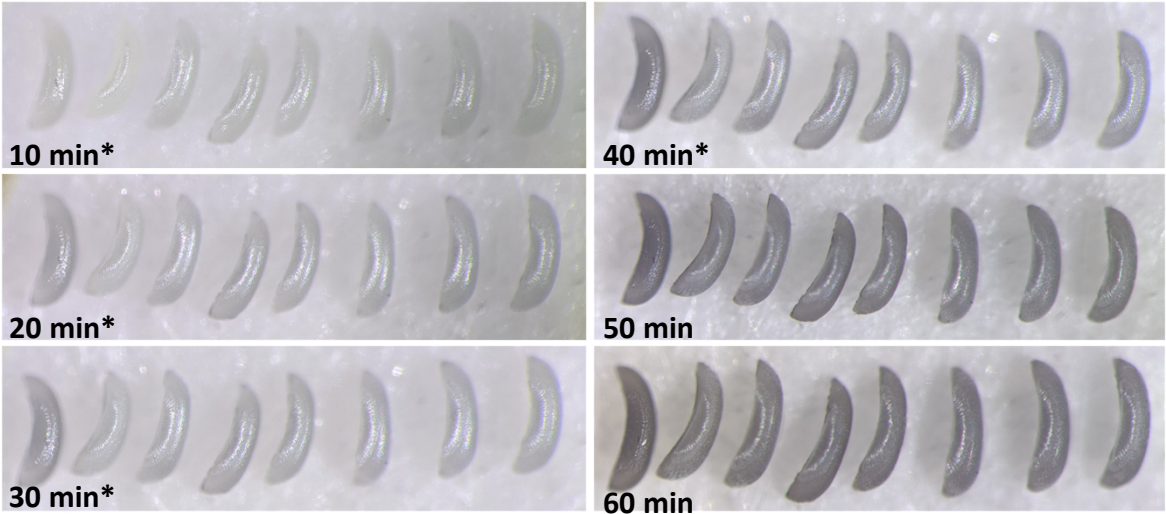414

Figure 1

Figure 2

Figure 3

Figure 4

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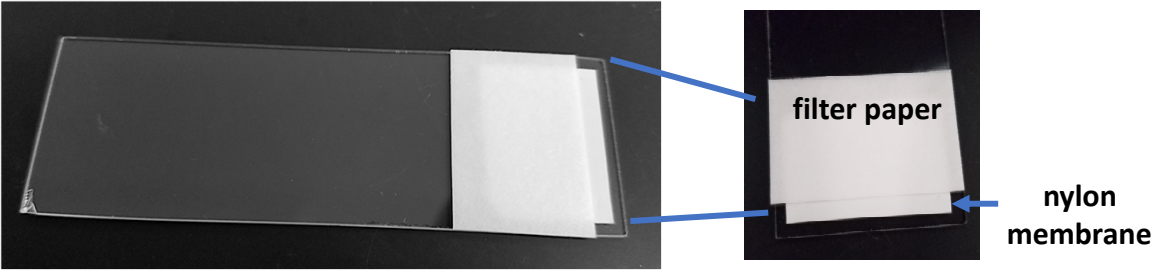
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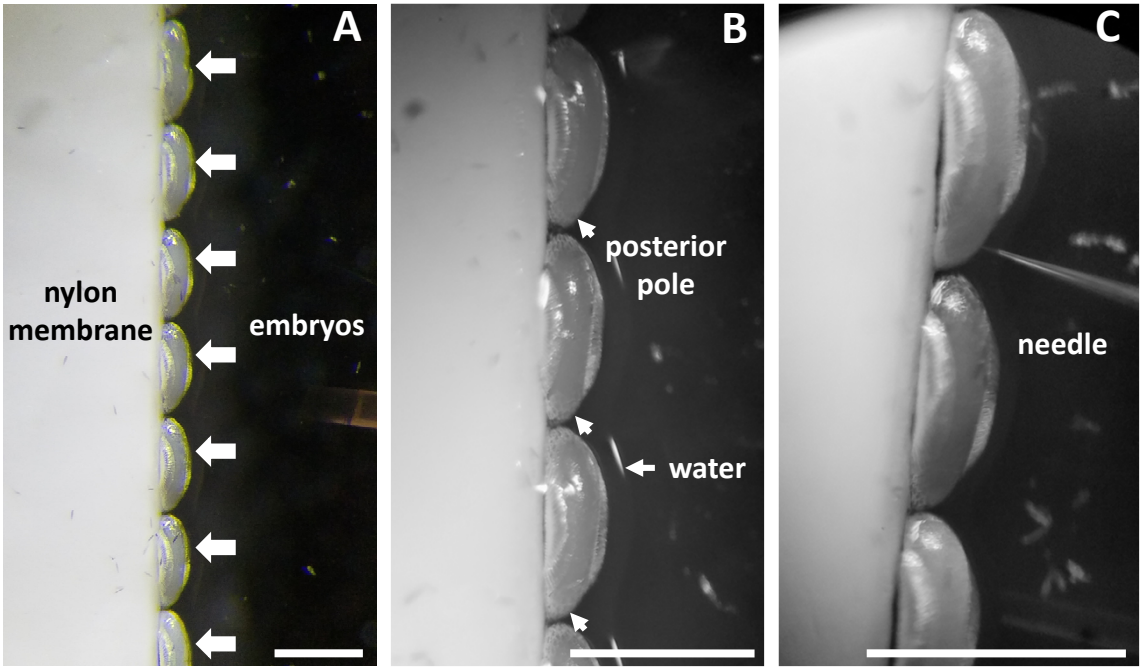
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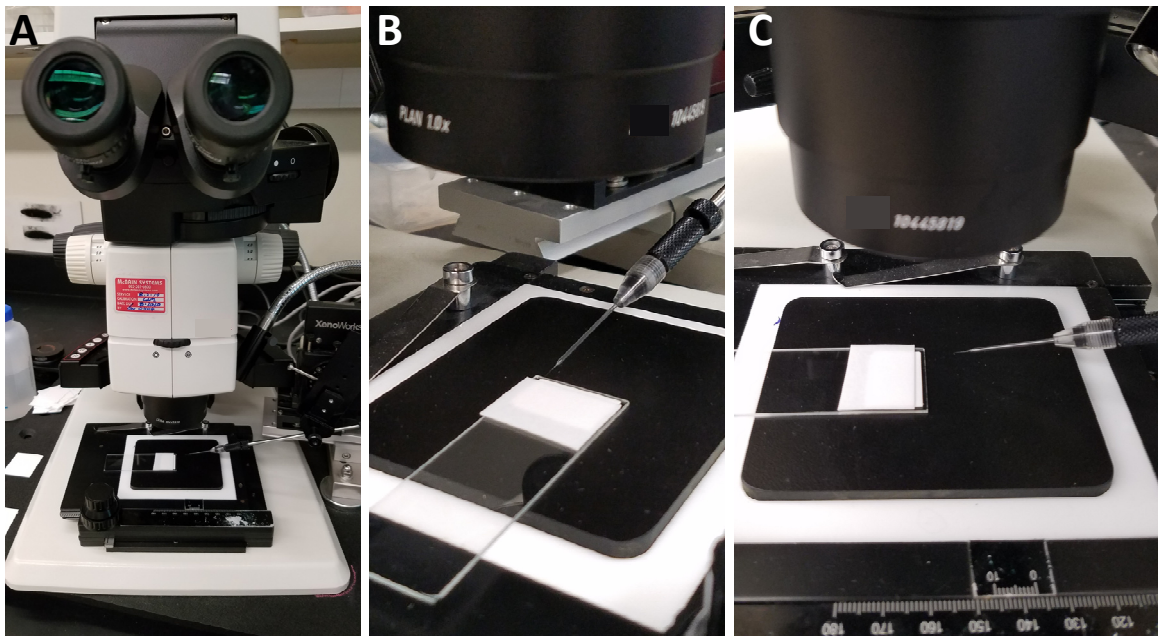
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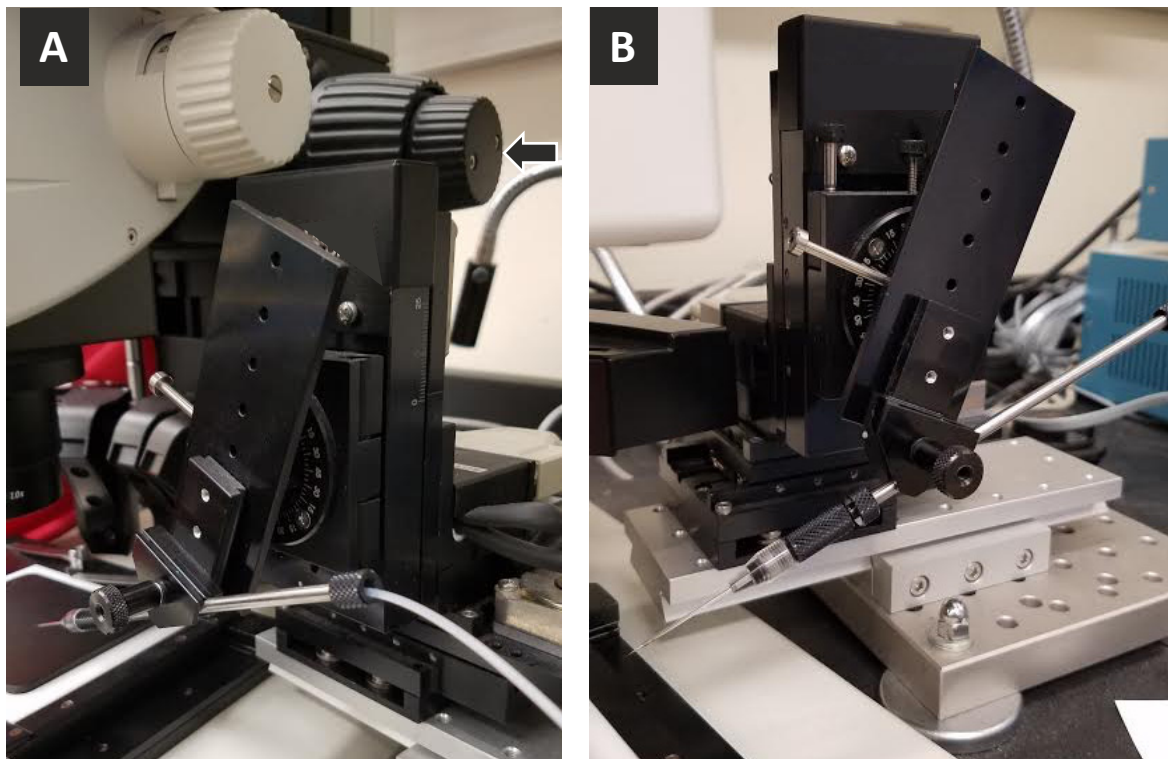
Figure 10
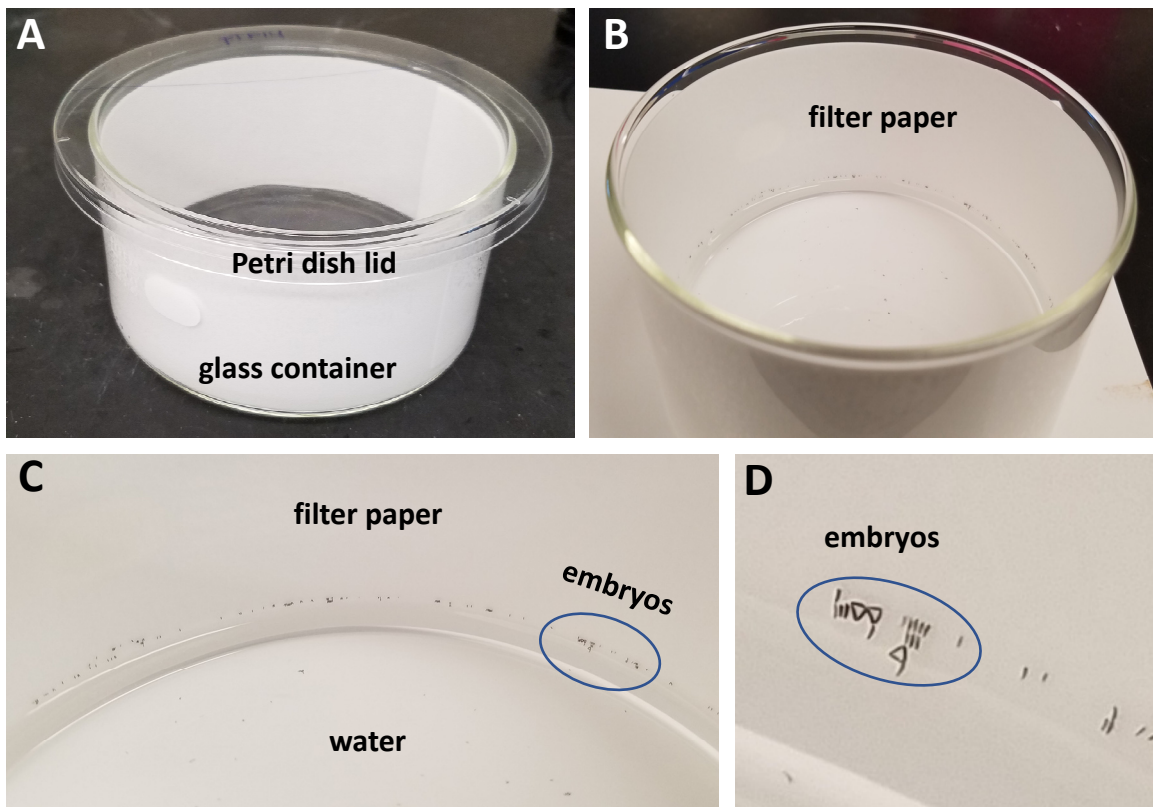
Figure 11
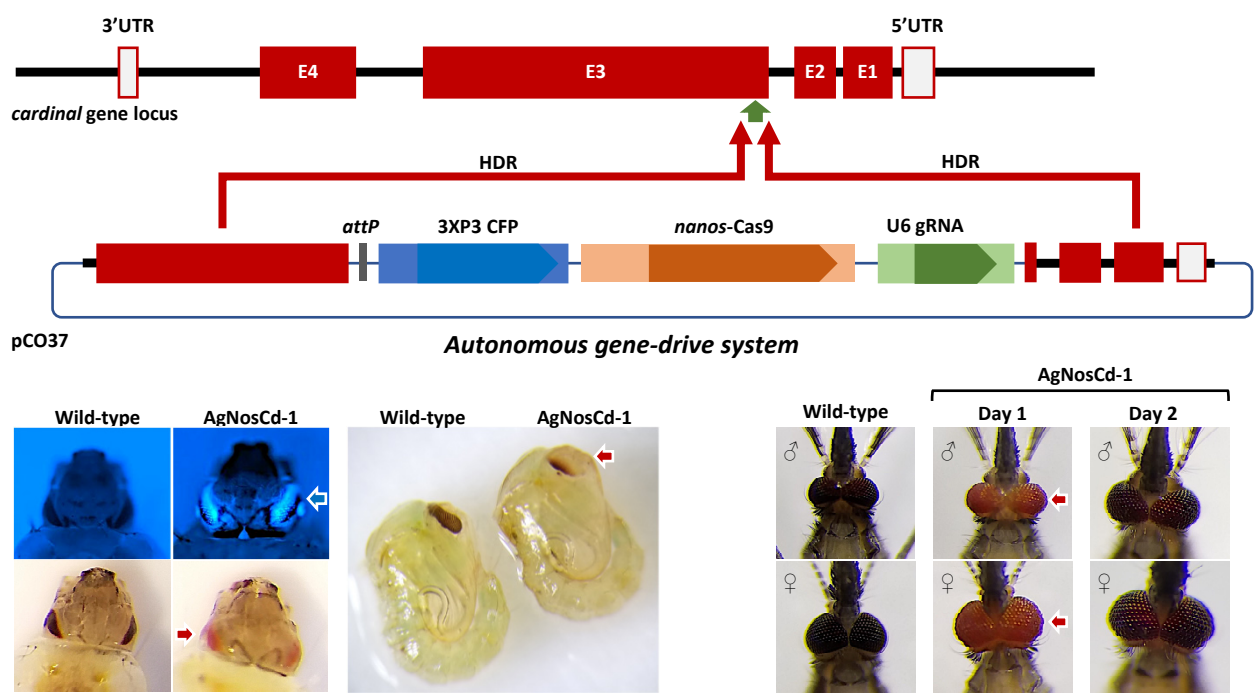
Name	Company	Catalog Number	Comments
10x Microinjection Buffer	-	-	1 mM $NaHPO_4$ buffer, pH 6.8, 50 mM KCl
Blotting membrane (Zeta-Probe GT Genomic Tested Blotting Membrane)	Bio-Rad		Neatly and straightly cut into 2x1 cm piece
Conical tubes 50 ml (disposable centrifuge tube, polypropylene)	Fisher Brand		Ends cut
De-ionized or double-distilled water (ddH_2O)	Mili-Q		In a wash bottle
Dissecting microscope	Leica	Leica MZ12	For embryo alignment
EndoFree Plasmid Maxi kit	Qiagen	12362	
Forceps			No. 5 size
Glass container	Pyrex	No. 3140	125 x 65
Glass slide	Fisher Brand	No. 12-549-3	75x26 mm
Incubator	Barnsted Lab-line	Model No. 150	28 °C
KCl			50 mM
Latex dental film	Crosstex International	No. 19302	
Microinjector	Sutter Instrument		XenoWorks Digital Microinjector
Microloader Pipette tips	Eppendorf		20 µL microloader epT.I.P.S.
Micromanipulator	Sutter Instrument		XenoWorks Micromanipulator
Micropipette	Rainin		20 µL
Micropipette puller	Sutter Instrument	Sutter P-2000 micropipette puller	
Microscope	Leica	DM 1000 LED or M165 FC	For microinjection
Minimum fiber filter paper	Fisher Brand	No. 05-714-4	Chromatography Paper, Thick
Mosquitoes	MR4, BEI Resources		*Anopheles gambiae*, mated adult females, blood-fed 4-5 days post-eclosion
$NaHPO_4$ buffer			1 mM, ph 6.8
Nylon mesh			
Paint brush	Blick	No. 05831-7040	Fine, size 4/0
Petri dish			Plastic, (60x15 mm, 90x15 mm)
Quartz glass capillaries	Sutter Instrument	No. QF100-70-10	With filament, 1 mm OD, ID 0.7 10 cm length
Water PCR grade	Roche	No. 03315843001	

Responses to editorial comments 2nd revision

1. We cannot have references in the abstract. Please remove them from the abstract and include them in the introduction.

Acknowledged and complied.

2. Please include the details of the endotoxin-free DNA plasmid maxiprep kit used in the Table of Materials.

Done.

3. Figure 10: Please define what the blue oval represents in the Figure Legends.

Done.

4. Please note that we require at least one representative result obtained by the application presented in the protocol to show in the results section of the video. Please provide a results figure from the cited previous publication along with the explicit copyright permission to reuse the figure. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]." Discuss the figure in the representative results, and include the description in the Figure Legends.

Acknowledged and complied. An excerpt from the PNAS instructions about figure reproduction is included in the resubmission materials.

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