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## Injecting Gryllus bimaculatus eggs

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**TITLE:**

Injecting *Gryllus bimaculatus* Eggs

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Injection, dsRNA, RNA interference, Genomic Modification, Evolution, Development, CRISPR/Cas

**SUMMARY:**

Here we present a protocol to inject cricket eggs, a technique which serves as a foundational method in many experiments in the cricket, including, but not limited to, RNA interference and genomic manipulation.

**ABSTRACT:**

Altering gene function in a developing organism is central to different kinds of experiments. While tremendously powerful genetic tools have been developed in traditional model systems, it is difficult to manipulate genes or messenger RNA (mRNA) in most other organisms. At the same time, evolutionary and comparative approaches rely on an exploration of gene function in many different species, necessitating the development and adaptation of techniques for manipulating expression outside currently genetically tractable species. This protocol describes a method for injecting reagents into cricket eggs to assay the effects of a given manipulation on embryonic or larval development. Instructions for how to collect and inject eggs with beveled needles are described. This relatively straightforward technique is flexible and potentially adaptable to other insects. One can gather and inject dozens of eggs in a single experiment, and

survival rates for buffer-only injections improve with practice and can be as high as 80%. This technique will support several types of experimental approaches including injection of pharmacological agents, in vitro capped mRNA to express genes of interest, double-stranded RNA (dsRNA) to achieve RNA interference, use of clustered regularly interspaced short palindromic repeats (CRISPR) in concert with CRISPR-associated protein 9 (Cas9) reagents for genomic modification, and transposable elements to generate transient or stable transgenic lines.

## INTRODUCTION:

The ability to modify the genome or influence gene expression in organisms is the basis for the design of many types of experiments testing functional causality. It is also critical for the comparative and evolutionarily-relevant work that genomic and non-genomic modification techniques be available in organisms outside traditional genetic laboratory animal model systems (e.g., *Mus musculus*, *Danio rerio*, *Drosophila melanogaster*, and *Caenorhabditis elegans*). Whether it is the desire to understand organismal diversity<sup>1</sup> or one's adherence to Krogh's principle, that for every biological question there is an organism best suited to its solution<sup>2,3</sup>, the ability to modify genomes or influence the gene expression is essential for modern experimental designs.

The cricket *Gryllus bimaculatus* is an emerging model system. Used for the last century in neuroethology experiments<sup>4</sup>, the last two decades have witnessed an increased experimental interest in the cricket, particularly focused on the evolution and development of this organism<sup>5</sup>. The cricket is a hemimetabolous insect that branches basally to well-studied holometabolous insects, such as *D. melanogaster* and *Tribolium castaneum*<sup>6</sup>. Due to its useful position on the evolutionary tree, scientists are interested in asking modern, sophisticated experimental questions in this insect, which has led to a growing interest in adapting molecular tools for the use in *G. bimaculatus*.

Injections of molecular reagents into cricket eggs can be used for genomic modification experiments as well as non-genomic manipulations of gene expression in embryos. For example, transgenic *G. bimaculatus* carrying eGFP insertions have been created using the transposase *piggyBac*<sup>7,8</sup>. Investigators have successfully created knockout *G. bimaculatus* using Zinc-finger nucleases (ZFNs) and transcription activator-like (TAL) effector nucleases (TALENs) to introduce double-stranded breaks in specific genomic regions<sup>9</sup>. Though ZFNs and TALENs allow site-specific targeting in animals beyond the big four model systems, these reagents have quickly been surpassed by the CRISPR/Cas9 system, which is simpler to use, more efficient, and highly flexible<sup>10</sup>. CRISPR has been used in *G. bimaculatus* to produce knock-out<sup>11</sup> as well as knock-in lines<sup>12,13</sup>. In addition to genomic modification, dsRNA can be injected into eggs to knock down mRNA expression in developing embryos, allowing investigators to understand the role of specific transcripts throughout development<sup>14,15</sup>. Some limited details on how to inject cricket eggs have been published previously<sup>12</sup>.

Here, we describe a detailed protocol for injecting early *G. bimaculatus* eggs. This protocol is effective and easily adaptable to various laboratory settings, injection materials, and possibly to other insects. While additional details for designing and implementing genomic modification and

knockdown experiments have been published elsewhere<sup>12,13</sup>, these approaches will ultimately rely on the injection protocol detailed here.

## PROTOCOL:

### 1. Hardware setup and preparation of materials

NOTE: Please see **Table 1** and **Table of Materials** for preparation of solutions, reagent, and equipment details.

1.1 Set up a dissecting microscope in order to see eggs and guide the injection needle. (**Figure 1A** shows a dissecting microscope equipped with fluorescence.) Fluorescence capability is advantageous but not necessary.

1.2 Position a 3-axis micromanipulator to maneuver the injection needle into place (**Figure 1A**).

1.3 Set up a microinjector with tubing and a needle holder to inject small amounts of material (**Figure 1A**). Optionally use a foot pedal which is not shown in the figure.

1.4 Design and create a stamp to create wells for eggs (**Figure 1C**) by printing the inverse of the desired pattern, either with a 3D printer or a laser engraver.

NOTE: The 3D printed sample stamp shown is 4.5 cm x 5 cm with 128, 3 cm x 1 cm x 1 mm protrusions for creating individual wells. Details on how to make a variety of customizable molds have been published elsewhere<sup>16</sup>.

1.5 Prepare 10x Injection Buffer, HEPES buffered saline (HBS), and a stock solution of Tetramethylrhodamine Dextran dye and store at 4 °C.

1.6 Prepare experimental solutions to be injected, such as dsRNA, CRISPR/Cas9<sup>12</sup>, transposable elements, in vitro capped mRNA<sup>7,8</sup>, pharmacological agents<sup>17</sup>, ZFNs, or TALENS<sup>9</sup>.

1.7 Place double-stick tape on several layers of standard lab tape on a glass microscope slide in order to create a platform 1 mm or so above the glass slide, which will be used for holding needles for the assessment.

1.8 Prepare a cricket container approximately 40 cm x 60 cm in size with a lid possessing a mesh-covered opening for air circulation.

1.9 Add food, water, and sheltering materials.

1.10 Add several dozen healthy male and female adult crickets. Identify adult cricket by the presence of wings.

NOTE: A range of post-imaginal ages may increase egg yields. The density of crickets should be relatively high in order to be able to collect enough eggs for an experiment, but not so crowded that cannibalism occurs. For example, approximately two dozen healthy crickets, with a roughly equal male to female ratio, in a container of the size noted above should be sufficient to collect about 200 eggs in a one to two h period. Crickets can be kept at room temperature, but development and behavior will be optimal if crickets are maintained in a warm (23.5 - 26 °C), humid (40 - 60% relative humidity) incubator.

## 2. Making needles for injection

2.1. Use Borosilicate Glass Capillaries with filament (see the **Table of Materials** for size details). Prepare the pull needles on the same day, or up to a few days before injections are completed.

2.2. Place a capillary glass into the puller, center the glass on the filament, and tighten the knobs to secure the glass in place.

2.3. Set the puller temperature near the glass ramp temp (-5 °C to +10 °C).

2.4. Use a single-step, high-heat protocol to pull a long taper with a small opening but avoid melting the ends closed.

NOTE: For example, when using the micropipette puller detailed in the **Table of Materials** equipped with a box filament, use the following parameters for glass with a ramp temperature of 478: Heat 478; Pull 45; Vel 75; Del 120. Optimal conditions for producing a needle with the shape shown in **Figure 2B** should be determined empirically by each user.

### 2.5. Bevel needle tips in preparation for the injections.

2.6. Wet the spinning grinder plane of the beveler (**Figure 2A**) with dripping water. Use either reverse osmosis (RO) or diethylpyrocarbonate (DEPC)-treated distilled water.

2.7. Place the pulled needle into the beveler and turn to a 20° angle.

2.8. Lower the needle until it just touches the grinding plane and bevel for 2 to 3 min.

2.9. Assess the bevel by placing the beveled needle on double-stick tape that has been adhered to a glass microscope slide.

2.10. Place the slide holding the needle on the stage of a compound microscope equipped with a camera and image acquisition software.

2.11. Acquire an image of the needle tip using a 20x objective.

2.12. Use the imaging software to measure the interior lumen diameter of the needle just proximal to the beveled opening (**Figure 2C**). The optimal size is  $10\ \mu\text{m} \pm 2\ \mu\text{m}$ .

2.13. Discard needles that have an opening below  $8\ \mu\text{m}$  and above  $12\ \mu\text{m}$ .

2.14. Store the needles in a container with a lid to avoid getting dusty. Use a strip of wax or similar material to elevate the needles off the bottom of the container to prevent breaking the tips (**Figure 2D**).

### 3. Egg collection and preparation

3.1. Maximize the number of eggs laid by depriving crickets of any moist laying material (water vials, egg dishes) overnight or for at least 8-10 h before attempting to collect eggs.

NOTE: Since females have the ability to internally store sperm, a differently timed procedure may be necessary if carefully timed fertilization is experimentally essential<sup>18</sup>.

3.2. Make 40 mL of 1% agarose in water and pour into a 10 cm petri dish.

3.3. Place an egg-well stamp on the surface of the agarose before it solidifies.

3.4. Remove the stamp, once the agarose is solidified, to reveal the wells (**Figure 1C**).

3.5. Fill the agarose well plate with HBS containing 1% Penicillin/Streptomycin.

3.6. Place the lid on the dish, wrap in parafilm and store at  $4\ ^\circ\text{C}$ .

NOTE: Dishes can be stored for a few days at  $4\ ^\circ\text{C}$  but should be warmed up to room temperature before use to avoid condensation.

3.7. Make an egg collection dish for crickets to lay eggs in by filling a 35 mm petri dish with white playground sand (**Figure 1B**).

NOTE: The sand specified in the **Table of Materials** is of the appropriate grain size. If the sand grains are too large, the sand will damage the eggs.

3.8. Cover the egg dish with a square of paper towel cut to be approximately 7 cm x 7 cm, and place on the top of dish with sand. Through this paper towel cover the filled dish with tap water.

3.9. Tilt the petri dish and gently squeeze the top to remove excess water.

3.10. Tuck the corners of the paper towel square under the dish and place the egg dish in the inverted lid, which will help keep the paper towel cover in place.

3.11. Place the egg dish into the cricket bin and allow the adult females to oviposit eggs for one to two h.

NOTE: The relatively short time here ensures that all the eggs will be similar in the stage of development at the time of injection. If injection before cellularization is important, injections should occur within 14 h of egg laying<sup>19</sup>.

3.12. Meanwhile, allow the agarose dish (from step 3.6) to warm to the room temperature in preparation for holding eggs for injection.

3.13. Remove egg collection dish, now containing freshly laid eggs, from the cricket bin, and remove the paper towel cover. Place a strainer with a pore size of 0.5 - 1 mm over a beaker of at least 500 mL capacity (**Figure 1B**).

3.14. Rinse the contents of the egg-laying dish (sand and eggs) into the strainer under gently running tap water letting the sand grains fall through the strainer mesh into the water in the beaker below but leaving the cricket eggs in the strainer basket.

3.15. Fill a container with RO water and place it in a tray. Invert the strainer over the container and tap it against the dish to dislodge the eggs into the water. The eggs will sink to the bottom of the container.

3.16. Cut the tip off a P1000 pipette tip with scissors to make an opening approximately 3 mm in diameter. Place this tip on a P1000 pipettor and use it to transfer the eggs from the container to the agarose egg mold dish, transferring as little water as possible.

3.17. Use plastic tweezers to line up eggs in agarose wells filled with HBS plus 1% Penicillin/Streptomycin. Each egg will sink to the bottom of an individual well. Cover with the petri dish lid until ready to inject.

#### 4. Prepare the microinjector

4.1. Attach the microinjector to a compressed air or gas source (this protocol was optimized using either compressed air or N<sub>2</sub>).

NOTE: If using a portable air tank, fill to at least 75 psi. The tank will lose air pressure throughout the injections and may need to be refilled; injections become difficult below 40psi.

4.2. Turn on the microinjector. Set the injection time to 0.17 s and the pressure to 10 - 15 psi.

NOTE: The exact pressure needed will depend on the opening of the needle and must be determined empirically for every round of injection and/or new needle used.

4.3. Ensure that the BALANCE knob of the microinjector is turned to 0 (typically fully counter-clockwise) and that the microinjector is pressurized and in the **injection** mode.

## 5. Injections

5.1. Filter about 500  $\mu$ L of 10x Injection Buffer using a 1 mL syringe and a 0.45  $\mu$ m syringe filter.

5.2. Mix injection reagents (the details that follow are for the injection of dsRNA prepared as described in<sup>12</sup>).

NOTE: It may be advisable to design and perform several controls, especially when one is first learning this technique. Poking eggs without injecting, injecting buffer + dye alone, or injecting buffer + dye + a control reagent (such as eGFP dsRNA) are all good tests of how disruptive various elements of the injection protocol might be. See **Figure 3** for the survivability of a number of different controls.

5.3. Start with a 4  $\mu$ L dsRNA aliquot.

5.4. Add 0.5  $\mu$ L of the filtered 10x Injection Buffer to the dsRNA aliquot.

5.5. Add 0.5  $\mu$ L of 50 mg/mL Tetramethyl rhodamine Dextran dye stock solution. If working on a dissecting scope without fluorescence, use Phenol Red instead.

5.6. Keep all materials on ice for the duration of the injection period.

5.7. Load the injection solution into an injection needle using 20  $\mu$ L loading tips and a p10 pipet.

5.8. Draw up a 1.5  $\mu$ L injection solution and insert the loading tip into the wide end of the injection needle.

5.9. Eject solution as far down into the needle as possible and eliminate air bubbles by flicking gently; be careful not to break the needle.

5.10. Place the dish of eggs under the dissecting microscope and select a low magnification of around 10x (1x magnification viewed through 10x eye objectives).

5.11. Insert the needle into the injection housing and tighten.

NOTE: Take care that the needle is properly and firmly inserted into the housing. To do this, pull the wide end of the needle back out of the metal housing, bringing the silicon tubing in the housing with it. Adjust the silicon tubing so that the glass end of the needle protrudes just beyond the silicon. If this is not done, the silicon tubing may get pinched between the glass and the metal housing, blocking the end of the needle.



5.12. Carefully insert the injection housing with an attached needle into the micromanipulator. Be aware of the sharp end of the needle and be careful that it is not bumped into surfaces and broken.

5.13. While looking at both the eggs and the needle through the microscope, move the needle near the eggs in the top left corner of the dish grid.

5.14. Lower the needle until the tip enters the HBS plus 1% Penicillin/Streptomycin buffered solution in the dish.

5.15. Center the needle in the field of vision and move the egg dish so that the needle is a few mm closer to the edge of the dish than the eggs.

5.16. Do not obstruct the view of the grid of eggs with the needle.

5.17. Set the microscope to the filter appropriate for Rhodamine so it is possible to observe the fluorescence in the needle and focus on the tip of the needle.

5.18. On the microinjector, slowly turn the BALANCE knob clockwise until the injection solution starts to leak out of the needle into the suspension solution. The balance number on the screen of the microinjector will begin to increase, though the numerical value is not important. Next, turn the knob back counterclockwise slightly just until the dye stops leaking out of the needle.

NOTE: It is critical to set this balance each time a new needle is used. Without the balance set appropriately, the microinjector will continue to inject for some time after the injection is triggered. It is even possible that a single push of the injection button with an unbalanced needle will result in the expulsion of the entire contents of the loaded needle.

5.19. With the needle centered in the field of view, move the egg dish so that the needle is aimed at the egg to be injected first. It is recommended that injections start in one corner of the grid of arranged eggs, for example, the upper left corner.

5.20. Adjust the magnification to about 50x; at this magnification, a single egg will fill most of the field of view.

5.21. Use both the micromanipulator and one's hand on the egg dish to move the needle into position for the injection.

5.22. Advance the needle using the micromanipulator and insert the tip of the needle into the first egg to be injected.

5.23. Insert the needle at 20 - 30% egg length from the posterior (blunter) end of the egg (**Figure 1E**), perpendicular to the long axis of the egg.

5.24. Inject solution either with the injection foot pedal or the injection button on the microinjector. A small bolus of fluorescent material inside the egg will indicate a successful injection (**Figure 1D**).

NOTE: It is not uncommon for some yolk to be ejected from the egg during injection (**Figure 1F**), and this does not necessarily indicate that the injected egg will be inviable.

5.25. Retract the needle from the egg. If the egg is unintentionally lifted out of its well upon retraction of the needle, use small forceps to push the egg back into its well while retracting the needle.

5.26. If the needle clogs during injection, remove the needle from the egg and, keeping the needle submerged in the HBS plus 1% Penicillin/Streptomycin solution covering the eggs, clear the clogged needle using either the **Clear** function or by pushing the inject button.

5.27. Continuing to use the same needle, repeat the injection procedure for as many eggs as possible. At first, this may be only about 20 or 30 eggs but will increase to over 100 with practice.

5.28. If the needle becomes clogged and cannot be cleared, discard the needle, fill a new needle and begin again (i.e., return to step 5.7).

5.29. Store the dish of injected eggs in a warm incubator (23.5 – 26 °C) that is slightly humid (to minimize the evaporation) until the embryos have reached the desired stage of development for the analysis.

5.30. At least every 24 h, remove eggs that are no longer viable (**Figure 3A,B**), and replace the suspension solution with fresh HBS plus 1% Penicillin/Streptomycin.

5.31. Two to three days after injection, transfer the eggs by gentle pipetting or with plastic forceps to paper towels moistened with HBS plus 1% Penicillin/Streptomycin in a lidded petri dish to continue development in the warm, humid incubator.

5.32. Monitor the eggs, and remove eggs that are no longer viable each day (**Figure 3A,B**).

#### **REPRESENTATIVE RESULTS:**

Crickets readily lay eggs in the moist material, and providing adequate material, such as moist sand or dirt, induces them to lay a large number of eggs. This is especially effective if crickets are first deprived of egg-laying material for 8-10 h. Eggs laid in clean sand can be easily separated, collected (**Figure 1B**) and placed into custom-designed egg wells for injection (**Figure 1C**). A dissecting microscope, a microinjector, and a micromanipulator (**Figure 1A**) can be used to inject individual eggs with a variety of materials (**Figure 1D**). Eggs are injected near the posterior end, at a point approximately 20-30% along the anterior-posterior axis (**Figure 1E**). Distinguishing the more pointed anterior end from the more blunted posterior end can be difficult, as these differences are often subtle (**Figure 1E**). Rolling an egg from side to side often helps to reveal

which end is which. After removal of the injection needle, egg yolk is sometimes ejected (**Figure 1F**), though this does not appear to compromise the health of the developing embryo inside.

One of the most critical steps in this protocol is the preparation of beveled needles. After pulling long, slender needles they must be beveled to a 20° angle and have an opening diameter of 10 μm ( $\pm 2 \mu\text{m}$ ). If the diameter is too large, the injection solution will leak out of the needle, and these needles will create large holes in eggs, decreasing survival rates. Narrower needles increase survivability, but if they are too narrow, they tend to clog easily, which makes injections slow and frustrating. With a needle diameter of  $10 \mu\text{m} \pm 2 \mu\text{m}$ , it is possible to inject upwards of 100 eggs with a single needle without having the needle clog.

Needles can be beveled with a micro grinder or beveller (**Figure 2A**). These pieces of equipment can be obtained with a microscope attached, or one could add a dissecting scope on an independent stand for models that do not include the microscope. A small micromanipulator holds the needle, which can be tilted to the desired angle and lowered to the surface of a rotating grinding plane, which is moistened with dripping water. An example of a pulled, but unbeveled needle can be seen in **Figure 2B**, and a needle with a 20° bevel is shown in **Figure 2C**. Needles must then be stored safely to avoid damage and to be kept clean (**Figure 2D**).

Setting the balance and injection pressure appropriately on the microinjector should allow one to inject a comparable amount of material into each egg injected with the same needle. Optimization of injection and balance pressures will be necessary when changing to a needle with a different diameter. This optimization will maximize the consistency of injection pulses.

The survival rate is one important parameter for assessing the success of these experiments. Most dead eggs can easily be identified by sight as follows: the yolk and embryonic tissue within the egg begin to clump unevenly (**Figure 3A**) and eventually most of the yolk will migrate to one side of the egg (**Figure 3B**). When assessed after 4-6 days (equivalent to stages 8-15)<sup>19</sup>, greater than 85% of uninjected eggs survived while eggs injected with experimental reagents generally had a lower survival rate (**Figure 3C**). Controlling for all aspects of the injection itself, including the needle puncture, the introduction of dye and buffer, or the presence of vehicle or dsRNA, is required in order to understand the true effects of the experimental manipulation attempted (**Figure 3C**). Depending on the experimental manipulation, a higher level of lethality may be expected, and one may need to alter the timing of the assay or the timing of injection in order to assess any non-lethal effects of the manipulation.

The way one assesses the phenotypic results of injection depends on what was injected. For example, phenotypic changes as a result of specific mRNA knockdowns may be obvious at the gross anatomy level. For example, the gene *Enhancer of zeste* (*E(z)*) normally suppresses *Hox* genes including *Ultrabithorax* (*Ubx*). Injecting dsRNA for *E(z)* knocks down *E(z)* function and releases *Ubx* suppression, resulting in ectopic *Ubx* expression. This leads to the transformation of five pairs of ventral appendages (antenna, maxilla, labium, T1 and T2 leg) into T3 leg-like appendages, due to the derepression of *Ubx* in those segments<sup>20</sup>. In a second example, it is possible to use GFP expression to assay the successful insertion of a transgene. For example,

when a cDNA encoding an eGFP-tagged *Histone 2B* gene under the control of a *G. bimaculatus* *Actin* promoter was inserted into the cricket genome using *piggyBac* transposase, it became possible to visualize each nucleus in the egg using fluorescence to excite the eGFP-tagged His2B protein (**Figure 4C,D**). In this case, it was possible to monitor the movement of nuclei over time<sup>7</sup>.

#### FIGURE AND TABLE LEGENDS:

**Table 1:** Solution recipes and notes.

**Figure 1: Egg injection protocol overview.** (A) A typical set up that can be used for egg injections. (B) Eggs are separated from sand via a sieve. (C) Eggs are held for injection in small wells made by placing a mold into warm, liquid agarose. (D) Fluorescence in the needle and in five injected eggs can be seen through the fluorescent dissecting microscope. (E) An uninjected egg. The anterior end (slightly pointed; left) and posterior end (more blunted; right) are distinguishable from each other. The region most suitable for injection, near the posterior end, is shown with the bracket. (F) An egg two days after injection. The injection site is visible as a slight discoloration, and a small amount of expelled yolk is evident (arrow). Scale bars D: 1 mm; E and F: 0.5 mm.

**Figure 2: Needles are beveled using a micro grinder or beveller.** (A) An example of a micro grinder with a circular, spinning grinding surface moistened by dripping RO water (water drips from the syringe shown in the top of the image). (B) Before beveling, pulled needles are long and narrow. (C) After beveling to 20°, a sharp injection needle is created. Note the 10 µm diameter of the inner lumen. (Red scale bars are 10 µm). (D) Pulled, beveled needles can be stored in a Petri dish with a lid and held in place using dental wax.

**Figure 3: Injections can decrease the rate of survival.** (A) Dead and dying eggs are obvious upon visual inspection. (B) Over time, the material will migrate to one side of the egg. (C) Comparison of survival rates four to six days after egg laying for a variety of conditions, including: uninjected eggs (n=6 experiments); eggs punctured with a 9 µm needle but uninjected (n=2 experiments); eggs injected with buffer and dye (n=13 experiments); eggs injected with buffer, dye, and plasmid vector (n=2 experiments); eggs injected with buffer, dye, and DMSO (n=11 experiments); and eggs injected with buffer, dye, and control dsRNA (n=22 experiments). The type of control dsRNA used included dsRNA against eGFP or dsRed. The numbers at the base of each bar note the total number of surviving eggs / total eggs used for each condition. Error bars represent standard error of the mean. Scale bar for A and B shown in B = 1 mm.

**Figure 4: Assessing injection results.** (A-B) Injection of *Enhancer of Zeste (E(z))* dsRNA alters the normal expression of *Ubx* mRNA (wild type shown in (A) and leads to the transformation of antennae (AN) and mouthparts (Mx, Lb) into leg-like appendages (B). (C-D) Injection of early eggs with the transposable element *piggyBac* and histone2B-eGFP produces transgenic embryos expressing GFP in all nuclei. Location of nuclei can be observed 8 h after egg laying (C) and 20 h after egg laying (D). Abbreviations: An: Antenna; Mx: maxilla; Lb: labium; T1–3: thoracic legs 1 to 3; RNAi: RNA interference; Gb: *G. bimaculatus*; Act: actin; H2B: Histone H2B; GFP: green fluorescent protein. Scale bars: 200 µm for A and B; 500 µm in E and F. These experimental results were originally described elsewhere<sup>7,20</sup>.

## DISCUSSION:

The two main challenges with this technique are the related issues of optimal needle size and survivability. Though smaller needles improve survivability, needles with narrower lumens have a greater degree of capillary forces at work, which makes it more likely that yolk will move into the needle causing it to clog. In the best case, blockages can be cleared simply by injecting another egg or by clearing the needle as described above. One can also attempt to increase the balance pressure on the microinjector until the yolk is pushed out of the needle. The other major challenge with this technique is survivability. In our hands, survival rates for eggs injected with experimental solutions are typically between 40 and 80%. Survival rates can be as low as 10% for those beginning to learn this technique but will improve with practice. Negative controls, as described above, are important for researchers to accurately assess changes in survival rate with practice, given that the introduction of various materials can lower survival rates (**Figure 3C**).

The timing of injections is an important consideration for each experimental approach as well. Older eggs are more difficult to inject than younger ones, and in general, survival rates are higher when injecting younger rather than older eggs. The introduction of the needle and injected material at later stages can lead to unintended anatomical damage. With experience, however, injection of two- to three-day old eggs is possible. Targeting the dorsal side of the egg at these stages helps to avoid damaging the embryo, which first forms on the ventral side. Targeting the posterior end may increase reagent access to the first nuclear divisions and germ band formation, which occur near this end of the egg<sup>19,21</sup>. If one is attempting to manipulate the genome, with the aim of achieving either widespread somatic or germline transmission of the genome modification, injections should be performed before cellularization, which starts approximately 14 h after egg laying<sup>19</sup>. If injecting dsRNA to knockdown mRNA levels, the time window for injections should be determined based on how early in development one wishes to knock down a gene of interest. While the effects of RNAi likely last for some time, dsRNA needs to be introduced in advance of the time at which one plans to assess any changes in phenotype. Keeping track of the time elapsed during injection and the progression of embryonic development may be important to ensure that the proper stage eggs are being injected. Regardless of what material is injected, developmental delays following injection are common, although the degree of the delay may vary depending on the injectant.

The protocol presented here relies on a number of relatively expensive pieces of equipment. It should be possible, however, to inject eggs successfully without the use of some or even all of these items. For example, there are a variety of microinjectors on the market, and some may be less expensive than the one suggested in the **Table of Materials**. It is also possible to simply use a syringe to inject an appropriate amount of material, though this would take some practice. Instead of an expensive micromanipulator, one could use a variety of materials to hold the needle steady and allow a slow, controlled advance, and a simple plasticine design has been used by others<sup>22</sup>. It may also be possible to avoid the use of a beveller, though in our experience proper beveling can make an enormous difference to the success of injections. It may be possible to sharpen a pulled needle by dragging the tip of the needle by hand across the finest sand paper,

assessing this process under a microscope. Regardless, sharpened needles will make injections easier and improve survivability.

This injection technique is flexible and can be used for a variety of different manipulations. Here, we have shown results for dsRNA and piggyBac-dependent genomic modification experiments, but it is also possible to use CRISPR/Cas or other approaches with this technique. In the experiments included here, resulting phenotypes were obvious and easy to assess. Some experiments may require the use of immunohistochemistry or other visualization tools in order to see the effects of experimental manipulation. One alternative to injections is the use of electroporation, which can be used to introduce DNA or other macromolecules into cells and tissues *in vivo*. Electroporation can be used in older cricket eggs where embryos have already formed in order to target specific regions<sup>8</sup>, but injection is more useful when the experiment requires that the entire developing embryo is targeted. This injection technique should also be easily adaptable for use in eggs from a variety of different hemimetabolous and holometabolous insects, ideally improving the ability to undertake meaningful comparative studies.

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#### DISCLOSURES:

The authors have nothing to disclose.

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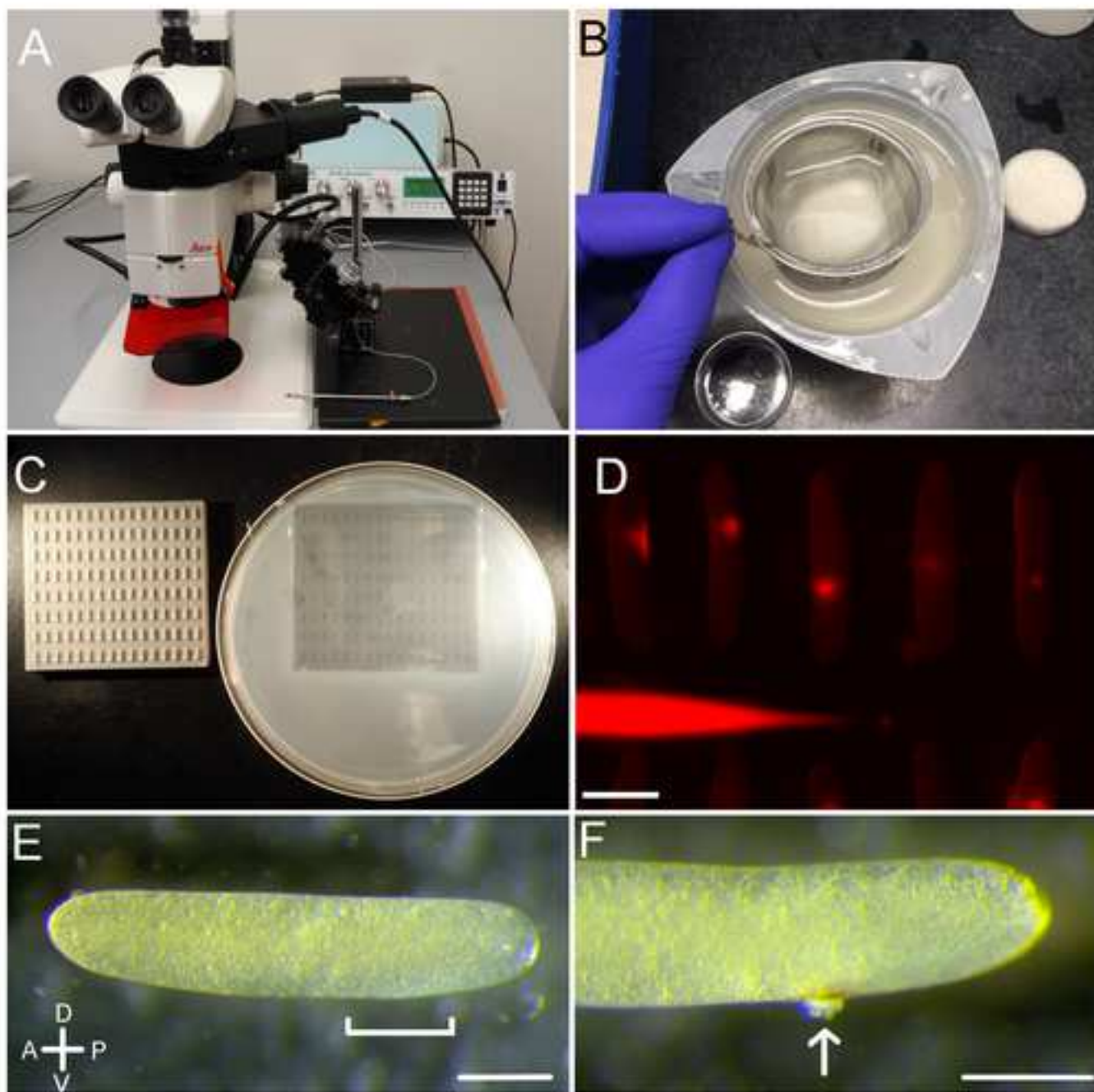
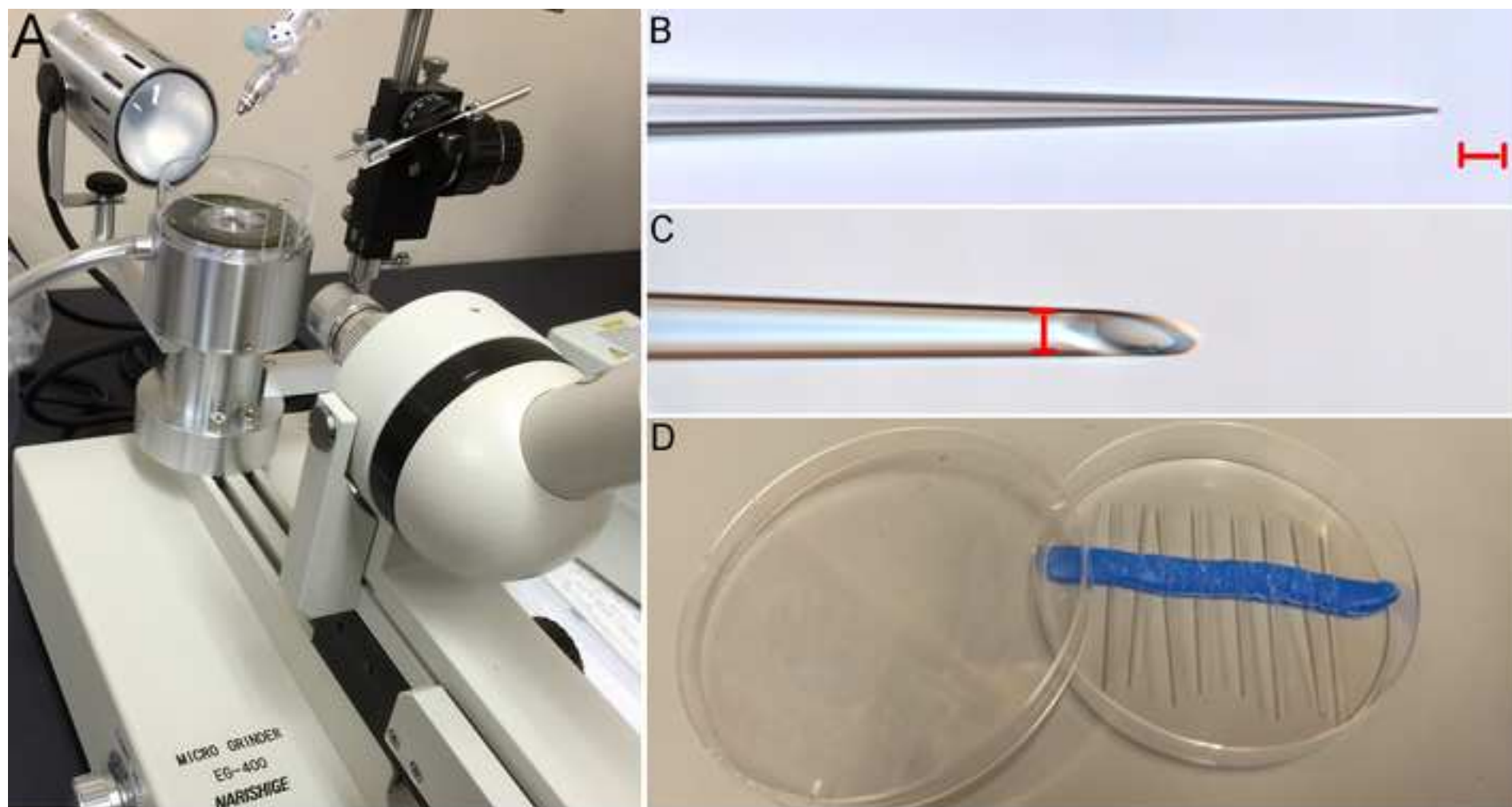
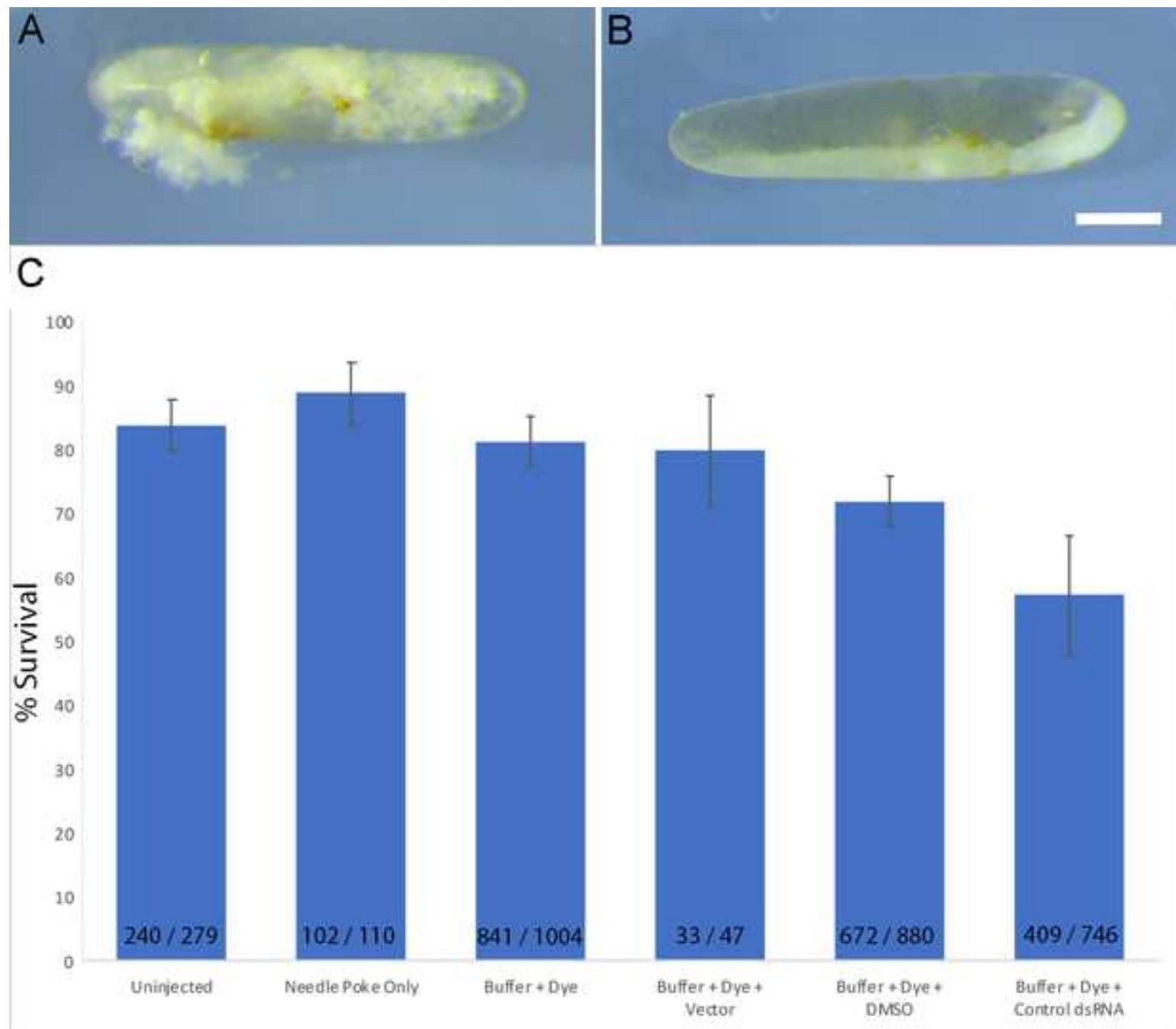


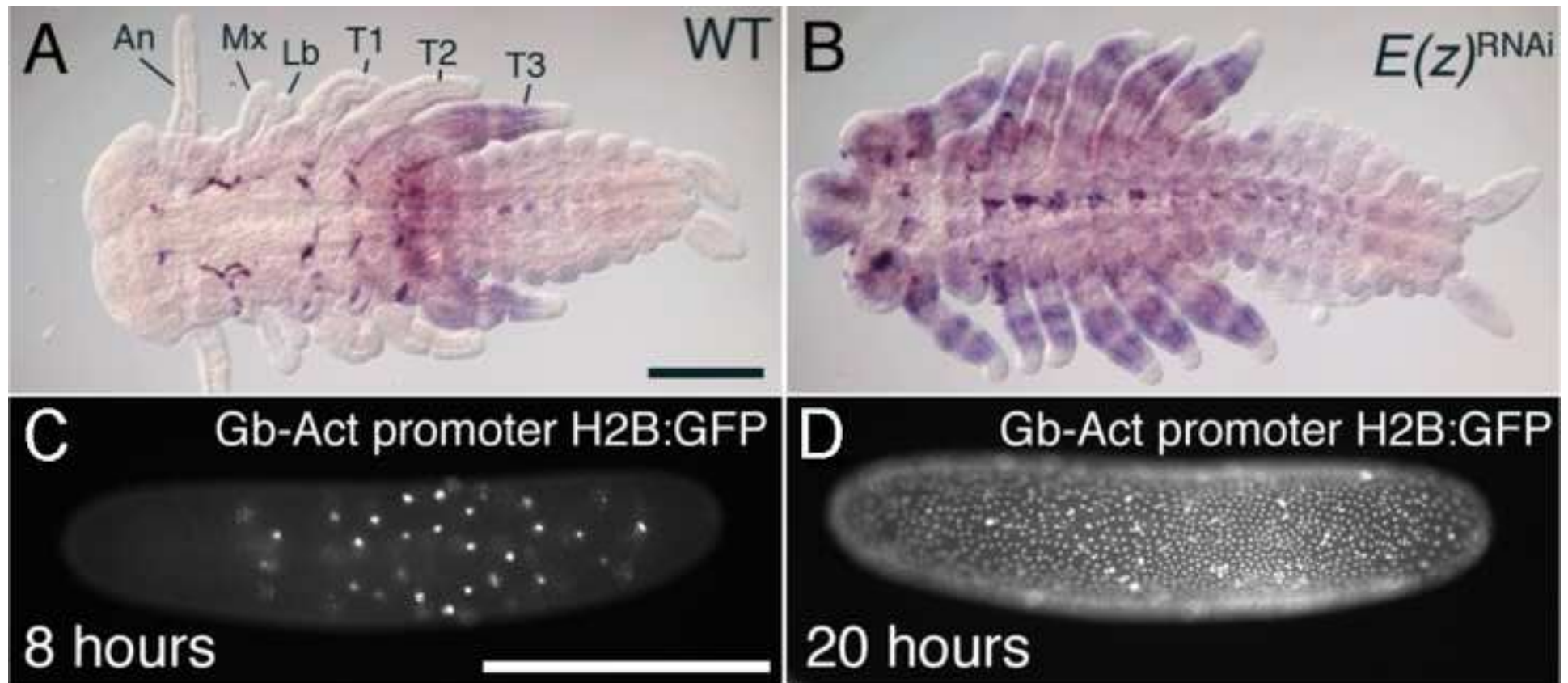


Figure2

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Name of Solution	Components	Comments/Description
10X Injection Buffer Solution	In 1L H <sub>2</sub> O add: 0.8 g NaCl, 0.09 g Na <sub>2</sub> HPO <sub>4</sub> , 0.04 g KH <sub>2</sub> PO <sub>4</sub> , 2.98 g KCl	Store stock at 4°C. Filter a small amount immediately before use with a 1 mL syringe equipped with a 0.45 µm syringe filter. Repeat as needed to filter approximately 500 µL of Injection Solution. Keep filtered Injection Solution on ice for the duration of the experiment.  pH to 7.0 and store at 4°C.
HEPES buffered saline	In 1L H <sub>2</sub> O, add: 8.77 g NaCl, 5.2 g HEPES,	
Tetramethyl Rhodamine Dextran (10,000 MW) dye stock solution	Rhodamine dye from Thermofisher	This dye is often sold as a lyophilized powder. In this case, make up a stock solution of 50 mg/ml with water. Centrifuge at 12,000 x g for 5 min to remove any insoluble particles. Collect the supernatant after centrifugation, avoiding any particulate matter at the bottom of the tube, aliquot and freeze at -20°C. Stock aliquots in use can be kept at 4°C for one to two weeks. Avoid freeze-thawing aliquots multiple times. If needles are clogging due to dye, the dye can also be filtered through Whatman #2 filter paper.



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Fluorescent dissecting microscope	Leica	M165 FC	Stereomicroscope with fluorescence
External light source for fluorescence	Leica	EL 6000	
Microinjector	Narishige	IM-300	Accessories may include Injection Needles Holder, Input Hose (with a hose connector), AC Power Cord, Foot Switch, Silicone Rubber Gasket
mCherry filter cube	Leica	M205FA/M165FC	Filter cube for mCherry or similar red dye will work
Micromanipulator	World Precision Instruments, Inc.	M3301R	Used with Magnetic Stand (Narishige, Type GJ-8)
Magnetic stand	Narishige	MMO-202ND	
Pipette Holder (Needle holder)	Narishige	HD-21	
Tubing to connect air source to microinjector			
Egg well stamp	3D printed	custom	3D printed on a Lulzbot Taz 5 using Poly Lactic Acid thermoplastic
Microwave	various		
Incubator or temperature controlled room	various		Temperatures of 23.5-26°C are needed.
cricket food	various		cat food or fish flakes are appropriate food.
cricket wter	vairous		Water can be held in vials and presented to crickets through cotton balls
cricket shelter	arious		Shelter materials can include crumpled paper towels or egg cartons
Glass capillary tubes	World Precision Instruments, Inc.	Item no. 1B100F-4	Kwik-Fil™ Borosilicate Glass Capillaries, 100mm length, 0.58 mm ID, 1.0 mm OD, with filament
Micropipette puller	Flaming/Brown	Model P-97	Distributed by Sutter Instrument Co.
Beveller/Micro grinder	Narishige	Model EG-45/EG-400	EG-400 includes a microscope head
Petri dishes	CellTreat	Product code 229693	90mm diameter
Play Sand	Sandtastik Products Ltd.	B003U6QLVS	White play sand
Agarose	American Bioanalytical	AB000972	Agarose GPG/LE ultrapure
Egg Strainer: Extra Fine Twill Mesh	US Kitchen Supply	Model SS-C123	Pore size should be between 0.5 - 1.0 mm
Stainless Steel Conical Strainers	Gibco by Life Technologies	Ref 15070-063	Pen Strep
Penicillin Streptomycin	Sipel Electronic SA	P3C-STD	Black Static Dissipative, 118mm
Plastic tweezers			
syringe filters, 25mm diameter, 0.45 µm	Nalgene	725-2545	Use with 1 ml syringe
1 mL syringe, with Tuberculin Slip Tip	Becton Dickinson	309602	Use with syring filter to filter Injection Buffer , Luer-Lok tip syringes would also work
Air tank (optional)	Midwest Products	Air Works®	Portable air tank
Rhodamine dye	Thermofisher	D-1817	dextran, tetramethylrhodamine 10,000MW,
20 µL loading tips	Eppendorf	Order no. 5242 956.003	epT.I.P.S. 20uL Microloader
Compound microscope	Zeiss	Axioskope 2 plus	
20X objective	Ziess	Plan-Apochromat 20x/0.75	
camera	Leica	M27	
Leica Application Suite software	Leica	DMC 5400	
		LAS	Version 4.6.2 used here

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Author(s):

Samantha Barry, Taro Nakamura, Yuji Matsuoka, Christoph Straub,  
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Dear Dr. Bajaj,

We have submitted our requested revisions to our JoVE article (JoVE59726) now titled: “Injecting *Gryllus bimaculatus* eggs.” All edits have been captured using Track Changes, as requested. Below are notes explaining how we responded to all editorial and reviewer comments.

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We have carefully checked the spelling throughout the document.

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All abbreviations have been defined the first time with two exceptions: RNA and HEPES. If these need to be defined, we can add “ribonucleic acid” and (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid” to the text. Please advise.

**3. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points**

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Changed to: “Here we present a protocol to inject cricket eggs, a technique which serves as a foundational method in many experiments in the cricket, including, but not limited to, RNA interference and genomic manipulation.”

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**7. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).**

All in-text citations have been changed to superscripts.

**8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.**

All parts of the protocol were edited to be in the imperative tense.

**9. Please write the steps in the order of it being performed.**

Minor edits were completed to make sure all steps were listed in the order performed.

**10. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).**

All personal pronouns have been removed from the protocol section of the document.

**11. The Protocol should contain only action items that direct the reader to do something.**

All steps have been edited to be action items, and non-action item material has been moved to Notes, Results or to the Discussion

**12. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.**

The Protocol has been edited to fit these guidelines.

**13. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.**

We added more information to the sections on setting up a cricket container (1.7-1.9) and pulling needles (2.1-2.4).

**14. 1.6: Please include a short detail for how to prepare each of the experimental solution to be injected.**

The preparation of each of these experimental solutions is quite involved, which is why we cited sources on how to synthesize and prepare instead. It is not clear to us how we would include details for the preparation of these solutions without adding significant length to the paper. Instead, this section has been edited to list these examples and sources in a more compact manner. Is this now acceptable?

**15. 2.2: What is the size of the injection needle? How is the needle pulling performed? Please also make substeps for 2.3 and 2.4.**

This section has been edited to include more information about pulling the needle (section 2.1 through 2.4). The instructions for beveling have been expanded (now 2.5-2.8). The question about the “size” of the injection needle confuses us. The details on the glass used are in the Methods Table, and we explain how to pull, bevel, and measure the needle tips, as well as what size tips are acceptable. Can you clarify the question about size, or do the details we added address your concerns?

**16. 3.1: How do you prepare the container. How do you visually identify post-imaginal age?**

More detail on preparing the container has been added, and these steps have been moved earlier in the protocol, now steps 1.7-1.9. Text about identifying adults (which have wings) has been added.

**17. 3.3: How did you get to the embryo from the eggs. Please bring out clarity in step. So first you are putting the embryo then removing it?**

On occasion, we used the terms “embryo” and “egg” interchangeably, which we acknowledge is quite confusing. The manuscript has been edited to use “egg” and “embryo” more clearly: use of

these words is now consistent with the idea that eggs are injected, which can influence the development of the embryo inside the egg. Please note that this led us to change the title of the manuscript to “Injecting *Gryllus bimaculatus* eggs.”

**18. Citations for line 341-343.**

In the original submitted document, lines 341-343 were:

341 *An example of a simple and safe way to store needles is shown in Figure 2D.*

342

343 *Setting the balance and injection pressure appropriately on the microinjector should allow one*

What information needs to be cited here? Can you please give us the lines again?

**19. Lines 306- 312: Please move critical steps to the discussion section.**

The text: “keeping track of the time elapsed during injection and the progression of embryonic development, to ensure that the proper stage eggs are being injected” has been edited into the discussion. We have also expanded here on several steps that are critical to the protocol.

**20. The Representative Results should be written in paragraph format describing in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.**

This section has been edited as follows. First, subsection headings were removed so that it is just in paragraph format. Next, a new first paragraph was added that refers to Figure 1 and gives more detail about collecting eggs, identifying the posterior end, and injecting. All figures are now referred to in the Results section.

**21. Please obtain explicit copyright permission to reuse any figures from a previous publication. 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].”**

All the images used in our figures have not been published elsewhere, so we are not reusing any figures. To be clear, the results described here have been published elsewhere, but the particular images used in these figures have not. We cite the original papers in our results paragraph, and we have added a sentence to the legend of Figure 4 explaining that the experimental results were previously published.

**22. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the**

**Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.**

The figure legends were edited to remove any discussion-type material or methodology.

**23. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:**

- a) Critical steps within the protocol**
- b) Any modifications and troubleshooting of the technique**
- c) Any limitations of the technique**
- d) The significance with respect to existing methods**
- e) Any future applications of the technique**

The discussion section was significantly reorganized and edited to make sure the points listed above were addressed.

**24. Please make a separate table for solutions composition and upload it as .xlsx file. Please do not embed these in the manuscript.**

The solution recipes have been added to an .xlsx file and have been uploaded.

**25. Figure 3: Please include a scale bar.**

A scale bar that applies to figure 3A and B was added to 3B. Details are now noted in the Figure legend for this figure.

**26. Please change hours to h, second to s, minutes to min, both in the manuscript**

This has been done throughout.

**Addressing Reviewer #1 Comments:**

**In section 5.1.1: It would be good to refer to Fig. 2 for the recommended controls. You might also suggest that the researcher practice injecting buffer before starting actual experiments (this could also be noted in the discussion).**

I assume this reviewer is referring to Figure 3 (not 2). We have added a note after 5.2 advising that people design a number of controls for their injections. We have also added more information to our discussion about the controls.

**Section 5.14: It would be good to recommend approximately how many eggs can be injected per round of experiments. I realize this may vary, but a ballpark figure is useful.**

The range of eggs one can inject at first and after practice has been noted (now in 5.28).

**Also, please note the time point at which the syncytial blastoderm ends and cellularization initiates.**

The time (starting at 14 hours after egg laying) has been noted and cited in the Protocol (as a note after 3.11) and in the Discussion.

**Line 292: Is there any particular humidity level recommended for the incubator during this incubation?**

No particular % relative humidity is necessary, but the incubator should be slightly humid so that samples don't dry out too quickly. We have explained this in 5.30 and added the word "humid" to 5.30 and 5.32.

**Fig. 3: You should label the y axis. Are there any error bars, or is this from a single representative experiment? For the latter, you can just indicate this.**

A label (% Survival) has been added for the Y axis. Because this data is from multiple experiments, error bars have been added and the number of individual experiments contributing data for each condition have been added to the figure legend for Figure 3.

**Addressing Reviewer #2 Comments:**

**1. The set up for collecting embryos could use some more detail. Perhaps there is enough information for those who have used this species before but more detail would be needed for those new to this species. For example, how old should eggs be for injection?**

More information about the cricket container has been added, to the Protocol (1.7-1.9) and a note after 3.11 has been added about the relative timing for injections.

**What is the optimal temperature for egg collection and staging?**

Temperature/housing information was added to the note after 1.9

**Can crickets be housed together? I understood that there is a problem with cannibalism for this species.**

In the same note (just after 1.9), we added more detailed information about the density of crickets. It is true that with increasing densities of crickets, one will find an increasing amount of cannibalism. The two dozen crickets we have recommended for a container of 40x60 cm should limit this problem.

**Please provide more detail on how the A-P and D-V sides of the egg are identified. The figure could also be improved to show more of the steps.**

We have added more information about discerning the anterior from the posterior end to the results. We feel the existing figures are adequate to describe the steps in the protocol, especially because there will be a video accompanying this manuscript,

**2. For the injection, it sounds like the embryos are floating in the small wells. Is this the case? How tight a fit are the wells for the embryos?**

In fact, the eggs sink. We have noted this in the protocol (3.15 and 3.17).

**3. The one worry I have about this paper is that the techniques described use highly sophisticated and specific instrumentation that may not be accessible to small or less well-funded labs. Can the authors suggest some alternatives, for example, for those lacking funds to purchase a beveller? Similarly, can injections be done without microinjector? As in the "old days" of Drosophila injections using constant air pressure from a 50-cc syringe?**

This is an excellent point. We have added a paragraph to the discussion outlining some ideas for cheaper options.

**A 3D printer is likely to be available on more campuses than the laser-engraver for generating the container in which eggs are injected but I tried to track down the program for producing the 3D stamp and was not able to find it. Would the authors clarify this and/or include the program in this manuscript? Overall, these types of additions would allow many more researchers to use the techniques described here.**

We are not sure exactly what the reviewer is referring to here, but the information about the 3D templates are in the referenced paper (Donough et al 2018). The size information given in the note after 1.3 is enough information to design and print one's own stamp, assuming one has access to a 3D printer.

**Line 331: assessing after 4-6 days corresponds to what stage of development?**

It is equivalent to stages 8-15, and we have cited the appropriate resource for this (now line 1008).

**Line 436 "before cellularization" - when is this?**

It is before 14 hours, and this has been added to the protocol (line 593) and discussion (now line 1320)

**Lines 436-438: Time window should not depend that exactly on the targeted genes, as dsRNA and its effects should last for some time (e.g., pRNAi). Please clarify what is meant by this sentence.**

We added the following sentence to clarify the timing issues (starting at line 1322):  
“While the effects of RNAi likely last for some time, dsRNA needs to be introduced in advance of the time at which one plans to assess any changes in phenotype. Keeping track of the time

elapsed during injection and the progression of embryonic development may be important to ensure that the proper stage eggs are being injected.”

**I assume there is no overlap with the recent book "The Cricket as a Model Organism"**

Chp 21 of the Cricket book is a methods chapter that does mention injection protocols. It presents several different options for injecting crickets of various ages and stages. One of these methods is similar to the one presented here, though far less detail about the actual injection protocol is included in the chapter. Instead, Chapter 21 focuses more on the synthesis of injectants, which is why it is referenced in our paper. In line 127 and 128 of the introduction, we note that some limited information on injections has already been published.