

Protocol

The Making of Transgenic *Drosophila guttifer*

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Abstract: The complex color patterns on the wings and body of *Drosophila guttifer* (*D. guttifer*) are emerging as model systems for studying evolutionary and developmental processes. Studies regarding these processes depend on overexpression and down-regulation of developmental genes, which ultimately rely upon an effective transgenic system. Methods describing transgenesis in *Drosophila melanogaster* (*D. melanogaster*) have been extensively reported in several studies. However, these methods cannot be directly applied to *D. guttifer* due to the low egg production rate and very delicate embryos' pre-injection treatment requirements. In this protocol, we describe extensively a comprehensive method used for generating transgenic *D. guttifer*. Using the protocol described here, we are able to establish transgenic lines, identifiable by the expression of Enhanced Green Fluorescent Protein (EGFP) in the eye disks of *D. guttifer* larvae. The entire procedure, from injection to screening for transgenic larvae, can be completed in approximately 30 days and should be relatively easy to adapt to other non-model *Drosophila* species, for which no white-eyed mutants exist.

Keywords: microinjection; *Drosophila guttifer*; transgenesis, *piggyBac*

1. Introduction

Our ability to genetically manipulate an organism of choice has become a powerful tool for studying gene function. The introduction of a foreign DNA into the genome of an organism to produce germline transformations is known as transgenesis. Until recently, genetic manipulation in *Drosophila* has been largely achieved by transposase-mediated transgenesis [1,2]. In this technique, transposases catalyze the integration of a transposon into the genome of an organism in an unpredictable manner [3]. However, in recent years, significant improvements in fly genetic techniques have been reported, particularly the integration of transgenes into the genome in a site-specific manner, through the use of different integrases and recombinases [4-7]. These techniques have been used to manipulate a variety of species [7-9], but arguably most of these studies have been conducted in *D. melanogaster*.

D. guttifer belongs to the *quinaria* species group. It is a very rare mycophagous species, which is native to the Midwest and Southeast of the USA [10]. *D. guttifer* has recently become a useful model for pigmentation studies [11]. Most studies carried out on this species are focused on unraveling the functional mechanism for the formation of polka-dotted patterns on the wings of this species. For example, Werner et al. (2010) reported that the wing spots are induced by the *Wingless* morphogen, and the unique polka-dotted expression pattern of *wingless* is due to the evolution in *cis*-regulatory elements of the *wingless* gene [12-14]. Recently, a study that focuses on the pupal development and the onset of pigmentation was reported [15]. All these studies depend on

overexpression and down-regulation of developmental genes, which ultimately rely on an effective and efficient transgenesis system.

A protocol highlighting the strategies for making transgenic *D. melanogaster* has been previously published [16]. However, this protocol cannot be directly applied to *D. guttifer* transgenesis for the following reasons: 1) the egg production rate of *D. guttifer* is one to two orders of magnitude lower than that of *D. melanogaster*, and thus, a large fly population is needed; 2) *D. guttifer* flies die quickly from the ethanol fumes produced by the yeast required for egg-laying, requiring proper ventilation during the egg collection process; 3) *D. guttifer* females stick their eggs into the substrate, making them virtually impossible to collect from an agar surface; 4) *D. guttifer*'s embryos are covered by a thin proteinaceous chorion, which causes rapid embryonic desiccation in this species; 5) there is no white-eyed mutant available for *D. guttifer*, which is why larvae have to be screened for fluorescent markers; and 6) besides the *piggyBac* transposon, most other tested transposons do not result in transgenic *D. guttifer*. For these reasons, critical steps must be deployed to successfully transform *D. guttifer*.

Here we provide a detailed approach for creating transgenic *D. guttifer*. This protocol is accompanied by a video and troubleshooting steps, which makes it easy for researchers in the field to follow. To the best of our knowledge, this protocol is the first to describe how to make transgenic flies in a non-model *Drosophila* species. It is our expectation that this protocol can be adopted by insect enthusiasts, who might be interested in experiments involving transgenesis in other non-model insect species.

2. Experimental Design

2.1. Required Materials and Equipment

2.1.1. Materials

- Plexiglas egg laying cage measuring (300 mm x 200 mm x 200 mm)
- Lamp
- Instant yeast (Instaferm)
- Sponges
- Medium-sized Petri dish (100 mm x 15 mm)
- 2-L plastic jar
- Embryo collection filter with Corning Netwell insert (fine filter)
- Embryo collection filter with Corning Netwell insert (coarse filter)
- Squeeze bottle
- Micro cover glass (18 mm x 18 mm)
- Micro slide (25 mm x 75 mm, 1 mm thick)
- Small brush (to move egg masses)
- Very fine brush (to line up individual embryos)
- Halocarbon oil 700 (Sigma-Aldrich, cat. no. H8898)
- Halocarbon oil 27 (Sigma-Aldrich, cat. no. H8773)
- 10-mL syringe
- 50-mL Falcon tube
- FHC capillary tube (Borosil 1.0 mm x 0.75 mm ID/Fiber with Omega dot fiber)
- Nitrogen gas

- Fly food vials
- Cornmeal-sucrose-yeast medium (fly food) [10]
- Aluminum foil
- Two pairs of forceps
- Cotton plugs
- Moist chamber
- Anti-fungus paper

2.1.2. Equipment

- Flaming/Brown micropipette puller Model P-97 (Sutter instruments)
- Micromanipulator for the needle (Narishige IM 300)
- Needle holder (Narishige)
- Dissecting microscope
- Water bath (Thermo Scientific, HAAK S3)
- Mercury burner (Olympus U-RFL-T)
- Imaging microscope (Olympus SZX16) with fluorescence filters for GFP and DsRed

3. Methods

3.1. The egg-laying cage

The egg-laying cage is made of Plexiglas that allows good aeration through the nylon entrance at the front and the plastic mesh at the back of the cage. Aeration is necessary to prevent the accumulation of toxic ethanol fumes produced by the yeast. The cage measures (300 mm x 200 mm x 200 mm) and holds thousands of flies. Before a clean cage can be populated, six small (35 mm x 10 mm) plastic Falcon Petri dish bottom halves are taped upside-down (using double-sided tape) onto the bottom of the cage. They will serve as “tables” for the bigger cornmeal-sucrose-yeast medium plates and must be spaced out evenly. Medium-sized empty dishes can be used to see if the spacing is good. Without these “tables”, the cornmeal-sucrose-yeast medium plates would crush lots of flies on the ground with every food exchange (Figure 1A and 1B).

Feed the flies in the cage every 2-3 days with six cornmeal-sucrose-yeast medium plates at a time, which are medium-sized (100 mm x 15 mm) Petri dish bottom halves, filled to the top with cornmeal-sucrose-yeast medium. Right before feeding the flies, add some dry Baker’s yeast to the top of the food and wipe off any excess water from the plates with a paper towel. When replacing old food plates with new plates, follow the three rules below to minimize the killing of flies:

1. Knock the plate gently against the round Petri dish “table” to remove any flies before taking out a plate. Use the thumb and middle finger to hold the dish, while holding the index finger in place to prevent the food from falling out.
 - a. If the gap between the food and the Petri dish is large due to excessive drying of the food, then bump towards the large gap. The food will not move much downwards, and no fly will be trapped.
 - b. If the gap is narrow, bump towards the side where the food still sticks to the plastic. This prevents most of the flies in the gap from being squeezed to death.

2. One out, one in! Always take only one plate out at a time and replace immediately with a fresh plate. If there are flies on the food “table” in the cage, they can be gently pushed away with the front edge of the fresh food plate when putting it down. Then repeat this step with the remaining plates.
3. Freeze and discard the used food as the flies developing from it will be of poor quality and a source of fungus infestations.

An egg-laying cage can be in operation for up to 10 weeks before the flies must be transferred to a new cage. If fungus grows (pay attention to places where the food comes in contact with the wall), change the cage earlier. Perform the following steps to move the flies to a new cage:

1. Take out all cornmeal-sucrose-yeast medium plates, close them, and continue working at a fly bench with CO₂ gas.
2. Cover an area with unfolded paper towels to your right and put a 5 L beaker on it.
3. Have a new cage with a fresh entrance ring plus nylon ready.
4. Put the populated cage to the left of the new cage (not on the towels), the entrance facing upwards.
5. Open the CO₂ gas, lead it through the nylon to anesthetize the flies, and then close the CO₂ gas supply.
6. Remove the ring with the nylon of the old cage and pour the flies into the 5-L beaker.
7. Flies that fall on the towels can now be thrown into the beaker as well.
8. Gas the old cage again to blow loose the remaining living flies and shake the cage over the beaker.
9. Pour the flies from the beaker into the new cage.
10. Close the freshly filled cage, bring it to its designed place in the lab and supply the flies with food.
11. Clean the old cage and the nylon with tap water. Do not use soap or bleach. Rinse with distilled water.

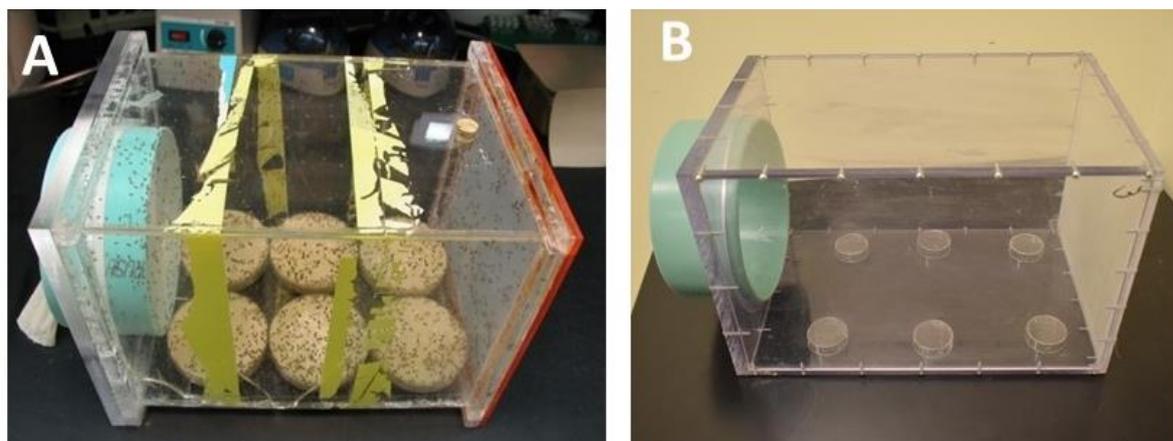


Figure 1. The egg-laying cage measuring (300 mm x 200 mm x 200 mm). **(A).** An egg-laying cage holding thousands of flies, which contains six plates of cornmeal-sucrose-yeast medium. **(B).** An empty egg-laying cage showing the six small (35 mm x 10 mm) plastic Petri dish bottom halves taped upside-down onto the bottom of the cage, using double-sided tape.

3.2. Egg collection and preparation for microinjections

D. guttifer females lay comparably few eggs within a given period. For this reason, the cage setup described above is used to obtain at least 200-300 freshly fertilized eggs per hour. Keep the cage on the lab bench with a 40-W lamp placed ~30 cm above it, which is timed to turn on at 6 AM and off at 6 PM. In the cage, females need to be reared on fresh food with dry yeast at all times in order for their ovaries to develop and to achieve a continuous egg production. If the food is in poor condition, females will retain their fertilized eggs inside of their bodies and lay them when the embryos are too old for micro-injections. Supply the cage with fresh cornmeal-sucrose-yeast medium 3-4 times a week and especially one day before injections are carried out.

Eggs that are laid in the morning are often already cellularized and tend to have a thicker chorion, which is disadvantageous for injections. The egg quality increases later during the day. The best time to start the egg-laying is 11 AM. Harvest the eggs in 1-hour cycles, starting at noon. It is advisable that one person lines up two slides (~200 embryos) during a 1-hour cycle, while another person injects them. All wash steps of the embryos are done with distilled water.

▲ CRITICAL STEPS: Never wash *D. guttifer* eggs with ethanol because it seems to harden the chorion and prevents the injection needles from penetrating it, and never leave the cage without food.

Make a starter yeast paste from instant Baker's yeast grains one day before using it, and on the morning of injection, mix it again with additional dry yeast and water. Keep the yeast paste at room temperature during the injections; it should smell a bit like fermented fruit. At 11 AM, replace all cornmeal-sucrose-yeast medium plates in the cage with four moist sponges, which are to be placed into medium-sized (100 mm x 15 mm) Petri dish bottom halves and covered with a thin layer of fresh, active yeast paste (Figure 2). A second set of four sponges will be needed for the next round.

The best way to prepare the sponges is to first make them too wet and then to carefully squeeze off the excess water with two thumbs pressing and moving from the top to the bottom throughout the surface of the sponges, until no drops are running out easily. The sponges should be dry enough that no water runs out when flies are bumped off from them during removal from the cage but wet enough to supply the flies with enough liquid. Sponges that are too dry seem to result in thicker coronas of the eggs (the thickening near the entry site of the injection needle), while sponges that are too wet cause the accumulation of liquid in the cage. The yeast paste is then supplied evenly over the sponge surface.

Care should be taken when yeast is handled because contaminating flies will be quickly attracted. Allow the females to lay eggs on the 4 sponges for one hour. The flies prefer to sit on the sponge part that is closest to the cage wall. Sponges that are placed too far away from the wall are often ignored by the flies.



Figure 2. The yeast paste is evenly applied with a flat spatula over the sponge surface.

To collect the eggs, remove the sponges one by one from the cage and immediately replace them with sponges covered with fresh yeast paste. Gently squeeze out the eggs-containing sponges one by one in a 2-L jar filled with 1 L of distilled water (repeatedly bend the sponges from the edge outward to avoid crushing the eggs, and the eggs will drop out of the pores), then allow the eggs to sink to the bottom of the 2-L jar for 2 minutes. While waiting, wash the sponges and Petri dishes with distilled water. The sponges should be squeezed hard while washing to kill any possible remaining eggs. After the four used sponges are washed, put fresh yeast paste on them and store them in a drawer (away from contaminating flies) until they are used again. At the end of the injection day, wash the sponges thoroughly and place them on a few paper towels on the bench for drying.

Pour the first half of the egg collection water right into the sink. The second half is swirled and poured through a double filter system, consisting of two embryo collection baskets, both are stuck to the bottom end of a funnel (Figure 3A). The finest filter basket is sitting underneath and retaining the eggs, while the coarse filter above is trapping sponge fibers and flies (Figure 3B). Rinse the 2-L jar once with 100-200 mL of distilled water, then pour it through the filter system to collect the remaining eggs. With a squeeze bottle of distilled water, wash the eggs of the upper filter and allow them to slip through the coarse mesh into the fine filter below. After obtaining a sufficient number of eggs in the lower filter, remove the baskets from the funnel and wash the eggs thoroughly with distilled water from a squeeze bottle. Then put the basket with the washed eggs into a bottom half of a small Petri dish, which is halfway filled with distilled water. Line up the embryos under a dissection scope.

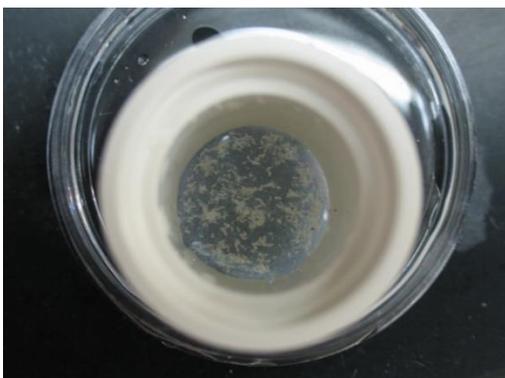


Figure 3. Eggs collected into the lower fine filter.

3.3. Lining up the eggs for microinjections

Place a micro cover glass (18 mm x 18 mm) onto a micro slide (25 mm x 75 mm, 1 mm thick) with a small droplet of water so that it sticks. Dry off the edges with a piece of tissue. The washed embryos are now transferred from the basket onto the cover glass as a clump, using a small brush. It is essential that the eggs are kept wet at all times. During lining up, always pick only the white and structureless appearing embryos. It is advisable to put a clump of more than 100 embryos into the upper middle of the cover glass. Then, use a very fine brown-haired brush, make it wet, briefly dry it on a tissue paper, pull the first embryo into position, dry the brush, pull the next embryo to extend the line, and so on.

Align the first 10 embryos with a relatively dry (very fine) brush, or otherwise the surface tension will make it impossible to position them correctly. Then, gently move the clump of ~100 embryos down while extending the line of selected embryos downwards, otherwise you lose sight of your egg clump, and the eggs may dry out and die by accident. In the end, the row consists of about 100 embryos, which shall have their posterior end towards the right edge of the cover glass. The row of embryos should be positioned about 5 mm away from that edge. Keep the embryos moist at all times by supplying water with the brush to the clump of unaligned eggs, as well as the top, middle, and end of the row of aligned embryos.

When the row is completed, remove all unused eggs and let the row dry, while carefully monitoring the eggs under the microscope. The row can now be rearranged slightly U-shaped to make them fit better to the cornmeal-sucrose-yeast medium surface later. When the embryo line appears completely dry, wait about 3-5 more seconds (depending on the humidity of the room) and then quickly cover them with the Halocarbon oil mixture (7 parts of type 700 + 1 part of type 27), supplied by a syringe (Figure 4). The Halocarbon oil stops further desiccation but allows breathing. The halocarbon oil mix must be prepared at least a day before you can use it, so plan ahead.

- ▲ **CRITICAL STEPS:** If the embryos become too desiccated (oil added too late), they die and become deformed when the needle pokes against them. If, however, the oil is put on too early, the embryos will not stick to the glass and move away when the injection needle is poked against them. Only a few seconds lay between both extremes. Do not try to inject a slide with swimming or dead embryos. Instead, save your needles for a better slide.

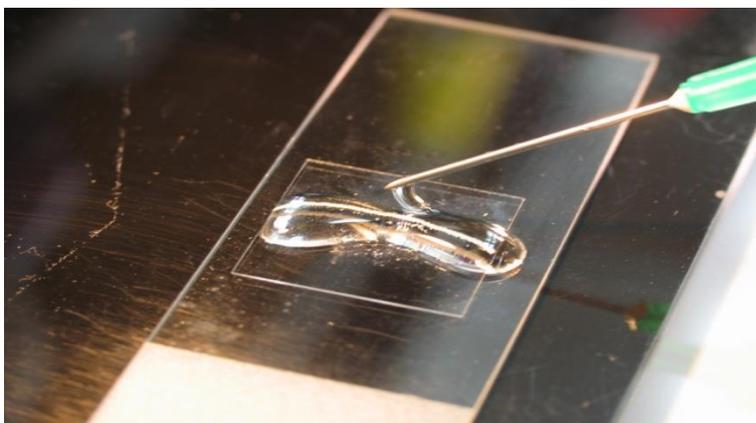


Figure 4. Putting the halocarbon oil mixture on a line of embryos.

- ▲ **CRITICAL STEPS:** Note the following important timelines: egg laying in the cage = 1 hour, egg collection and lining up = 20 minutes, injecting one slide containing about 100 embryos = 15 minutes. It is important to ensure that the embryos are in the syncytial blastoderm stage during injection, which lasts for about 2 hours or less at room temperature.

3.4. Preparation of transgene DNA

1. Follow the manufacturer's instructions on the isolation of the plasmid DNA containing the transgene of interest from an overnight *E. coli* culture. In our laboratory, we use the Invitrogen HiPure Plasmid Midi-prep Kit by Thermo Fisher Scientific.
2. Add 3.5 mL of isopropanol to the eluted DNA in a Corex glass vial.
3. Close the Corex vial with Parafilm, invert 10 times, take away the Parafilm, and wipe off any drops with Kimwipes.
4. Centrifuge at 11,000 rpm for 30 minutes at 4°C.
5. Mark the area of the DNA pellet with a marker, decant, and invert the vial on a paper towel.
6. Add 350 μL of sterile MQ water and dissolve the DNA pellet by vortexing.
7. Collect the plasmid DNA in a 1.5-mL Eppendorf tube.
8. Add 35 μL of 3 M of sodium acetate (pH 5.5).
9. Add 875 μL of 200-proof ethanol and invert 10 times.
10. Spin the DNA at 14,000 rpm for 20 minutes at 4°C on a bench-top centrifuge.
11. Remove supernatant and wash with 300 μL of 70% ethanol by inverting the tube 10 times.
12. Centrifuge again at 14,000 rpm for 10 minutes at room temperature.
13. Remove supernatant and centrifuge for 1 minute.
14. Remove all ethanol and dry the pellet for 5 minutes.
15. Dissolve the DNA in 50-100 μL of elution buffer, measure the DNA concentration, and add more elution buffer if necessary, to a final concentration of 1 $\mu\text{g}/\mu\text{L}$.

3.5. Preparation of the injection cocktail

1. On ice, add 20 μL of transgene-containing *piggyBac* construct [1 $\mu\text{g}/\mu\text{L}$] to a 1.5-mL test tube, 5 μL of the *piggyBac* helper plasmid (phspBac [1 $\mu\text{g}/\mu\text{L}$]), and 15 μL of sterile distilled water. Note: We do not add food color because it is not necessary for the visualization of the injected material.
2. Centrifuge the DNA mixture for 20 minutes at maximum speed and transfer the upper 39 μL of it to a fresh tube (do not touch the bottom of the tube with the pipette tip). Spin the DNA again for 20 minutes and transfer 38 μL of the injection mix into another fresh tube. This DNA is now clear of debris and can be used to load the needles. The *piggyBac* system pBac (backbone)/phspBac (*piggyBac* helper) results in 1 transformation event every 50 *D. guttifera* embryos with empty vector and 1 every 500 embryos with an 8 kb insert [17].

3.6. Needle preparation and microinjections

Use a Flaming/Brown micropipette puller Model P-97 and FHC capillary tubing (Borosil 1.0 x 0.75 mm ID/Fiber with Omega dot fiber) to obtain standard needles. We currently use the following parameters, but these parameters can change based on the filament, machine, and humidity of the room: P = 500, Heat = 496, Pull = 125, Vel = 10, Time = 186 [18]. Do not use gloves when pulling needles

but wash your hands before touching the capillaries! Extreme care should be taken to avoid touching the platinum heating filament of the machine with the glass capillaries. Load the needles at least one hour before using them with approximately 0.5 μ L of the DNA mix and store them in a moist chamber at 4°C for up to a few days. Use an automated injection system based on nitrogen pressure, a micro-manipulator for holding the needle, and an inverted microscope for viewing.

The very tip of every needle must first gently be broken off before the first injection can happen. To do this, carefully touch the chorion of an embryo with the needle tip, while continuously pushing the "BALN" or "INJ" button, until the DNA flows out. Ensure that the needle is not badly broken, as this may cause the granular cytoplasmic content to ooze out of the embryo (Figure 5A). Inject the embryos with a very fine needle tip into the posterior-most part (Figure 5B) by hitting either the "INJ" button or the foot pedal (it is a matter of preference).

In order to prevent the needle from getting clogged, the "BALN" or "INJ" button or the foot pedal should be hit frequently, while the needle tip is outside of the embryos. If the needle is clogged, carefully rub the tip of the needle against an embryo while holding the BALN button pressed. Also, moving the embryos away from the needle at high speed can help make the DNA flowing again.

- ▲ **CRITICAL STEP:** The needles must be changed when the embryos start leaking out a lot of granular cytoplasm, whereas small clear droplets are acceptable and normal.

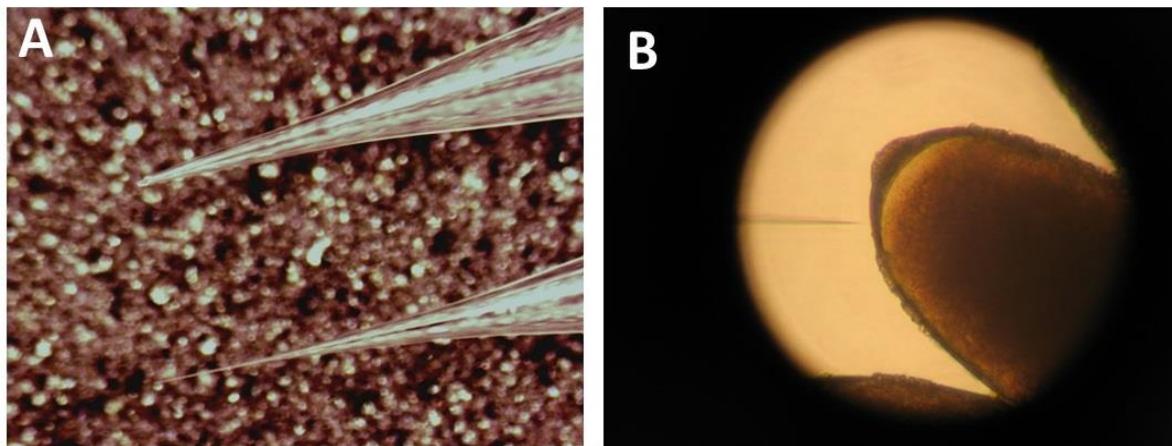


Figure 5. Needle qualities and correct injection site (A). The top needle is a badly broken needle, while the second needle is the standard needle. Note that the best needle is the one that has a beveled tip with a small opening. (B). The transgene can be injected through the posterior part of the embryo.

3.7. Post-injection treatment and heat shock of the embryos

After injecting all embryos on a slide, remove the slide from the microscope stage, remove the cover glass from the slide, and briefly let the oil drop off. Quickly wipe off some excess oil against the microscope slide. Stick the cover glass twice into a fresh cornmeal-sucrose-yeast medium vial, thereby carving out a small trench of food. Then place the posterior ends of the embryos with the cover slip into the trench without pushing the eggs too hard against the food. Close the vial with a cotton ball and squeeze enough distilled water into the cotton ball to provide immediate moisture (do not make them dripping-wet, but sufficiently moist). Place the vial vertically into a wet chamber

(a beaker with 5-mm-high water on the ground and 2 wet paper towels stuck against the inner wall). Hold up to 8 vials with injected embryos together with a rubber band (Figure 6). Avoid water flowing into the vials by keeping the cotton balls low in the vials, so that they do not touch the wet paper on the side of the wet chamber. Close the chamber with aluminum foil, label the lid, and store it at room temperature.

On the next morning (16 – 18 hours after injection), heat shock the embryos at 40°C for 90 minutes in the water bath (use a rubber band to hold the vials together and submerge the vials as deep as needed to get the food level under water; do not let any water get into the vials!). After the heat shock, fill a wet chamber with some cold water and put the vials back into it (now remove the wet paper towels that lined the wall). Place the foil lid back on and store the embryos at room temperature for roughly a week. Check daily for problems (mold, flooded vials), and the appearance of the first pupae.

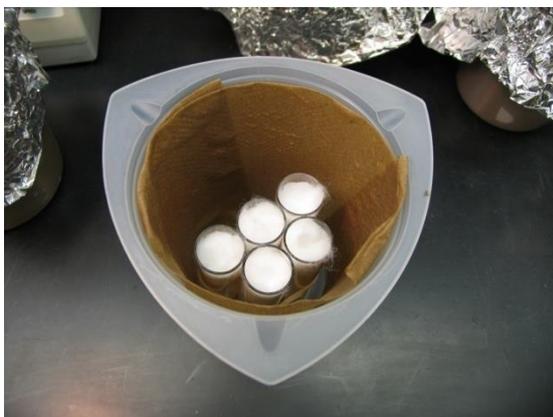


Figure 6. A wet chamber for injected embryos.

3.8. After-care and fly crosses

Look at the moist chamber every day to ensure it remains moistened. When most pupae have formed and no more wandering larvae are seen, collect the pupae from the cotton plugs with a brush and two pairs of forceps (Figure 7A). Making the cotton very wet helps dissolve the glue, with which the pupae stick to it. Do not leave much cotton left on the pupae; however, a few fibers can remain attached. Collect the pupae temporarily on a wet tissue paper. Take a clean and empty glass vial ("hatching vial"), place a long piece of anti-fungus paper along one side of the wall and moisten the paper with a squirt bottle (remove excess water with a paper towel afterwards so that no free water is in the vial - or else the pupae will drown). Transfer the collected pupae with a moist brush from the wet tissue paper onto the anti-fungus paper in the vial. Then collect the remaining pupae of the same construct as described above and combine them with the others in the same hatching vial. Close the hatching vial with a fresh cotton ball (push the ball deep enough down to prevent it from picking up water) and store it angled upwards (on a small plastic lid from a pipette box) in a moist chamber (Figure 7B).

- ▲ **CRITICAL STEP:** Moisten the anti-fungus paper inside the glass vials whenever needed and never let the anti-fungal paper dry out. Check the chamber twice daily.

Start collecting wild type virgin flies of both sexes for backcrosses as soon as you see the first pupae forming from the injection experiment vials. The collected virgins are stored in cornmeal-sucrose-yeast medium vials with a few grains of dry Baker's yeast and must be bumped to fresh vials every 3-4 days (or they will get stuck in slimy food). They will be sexually mature when your first injection survivors hatch. The hatched flies from the injections (the injection survivors) are collected once a day in the late afternoon on a CO₂ pad and immediately crossed with wild type virgins of the opposite sex. Use cornmeal-sucrose-yeast medium vials with a few grains of Baker's yeast and combine 3 males or 10 females of the injection survivors with 15 wild type virgins of the opposite sex in one vial. The lumping of injection survivors into the same cross is done to produce enough larvae per vial (to keep microorganisms in the culture down). Bump the crosses to fresh food every 3-4 days. Look at your crosses and their offspring every day. Screen for transgenic larvae 3 days after the parents have been removed from the vials.

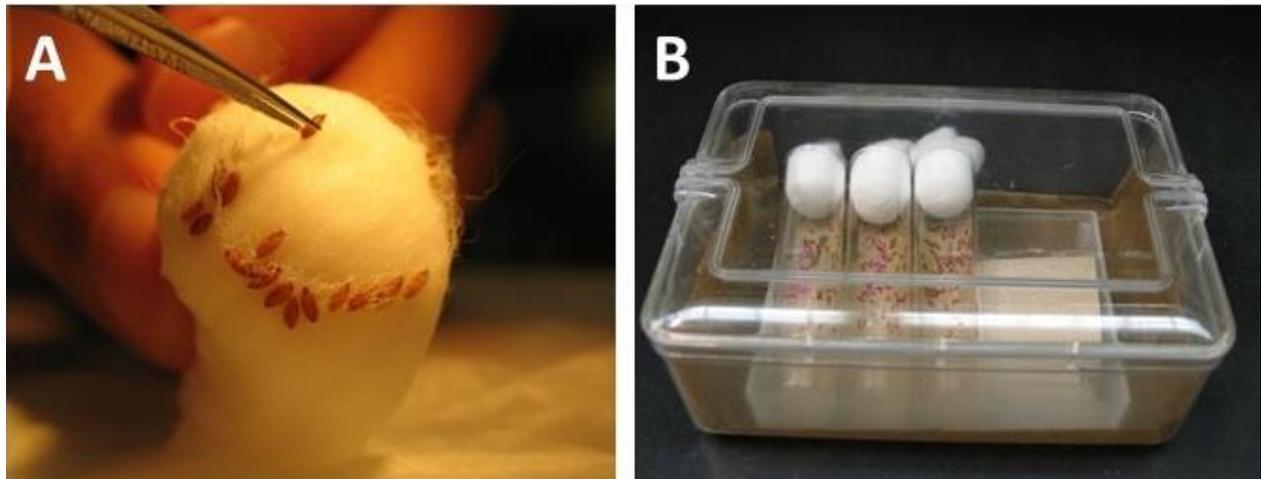


Figure 7. Collection of pupae and after-care. **(A).** Pupae are collected from the cotton plugs with fine forceps. **(B).** Pupae collected on moist anti-fungus paper in a hatching vial and stored angled upwards in a moist chamber.

3.9. Screening for transgenic larvae

The larval EGFP screen is carried out in the fly room with the main lights switched off as follows:

1. Switch on the UV lamp 10 minutes prior to screening.
2. Microscope settings: light filter = "GFP"; bottom filter = "Oblique"; objective = 1x; shutter open; camera tract closed; all visible microscope lights off (check that the bottom light is off as well!).
3. Repeat the following steps until all vials have been screened:
 - a. Remove the larvae from a food vial by squirting distilled water (squeeze bottle) and stirring with a rough brush, then pour the larva/food soup into a medium-sized Petri dish bottom half.
 - b. Collect positive larvae (Figure 8) with a pair of forceps into 2-mL tubes filled with 1 mL cornmeal-sucrose-yeast medium (briefly angle the food by centrifugation, allowing the larvae to be removed more easily from the collection forceps). Wash and screen each vial 3 times for transgenic larvae.
 - c. Poke two holes into each lid (not too big that the larvae can escape) after putting larvae into the 2-mL food tubes (only add up to 10 larvae per vial). Label each vial with the construct and line

information.

d. Discard screening soup with negative larvae and food debris into an empty 5-L beaker. Rinse the Petri dish with distilled water after screening each vial.

4. Expected Results

The perfect timepoint for screening for transgenic larvae is 3 days after the parents have been removed from the vials as all larvae would have hatched, but the pupae would not have formed yet. EGFP can only be detected in larvae, but not in eggs or pupae. Look for EGFP in the larval eye disks, Bolwig organs, brain, and/or in the anal plates (Figure 8) [19]. EGFP expression is usually strong and clearly visible in all larval instars. Finding multiple positive larvae per vial is not uncommon. Note that many transgenic lines are obtained in later screening sessions, so it is worth the time and patience bumping the crosses for 2 or 3 weeks before declaring them negative.

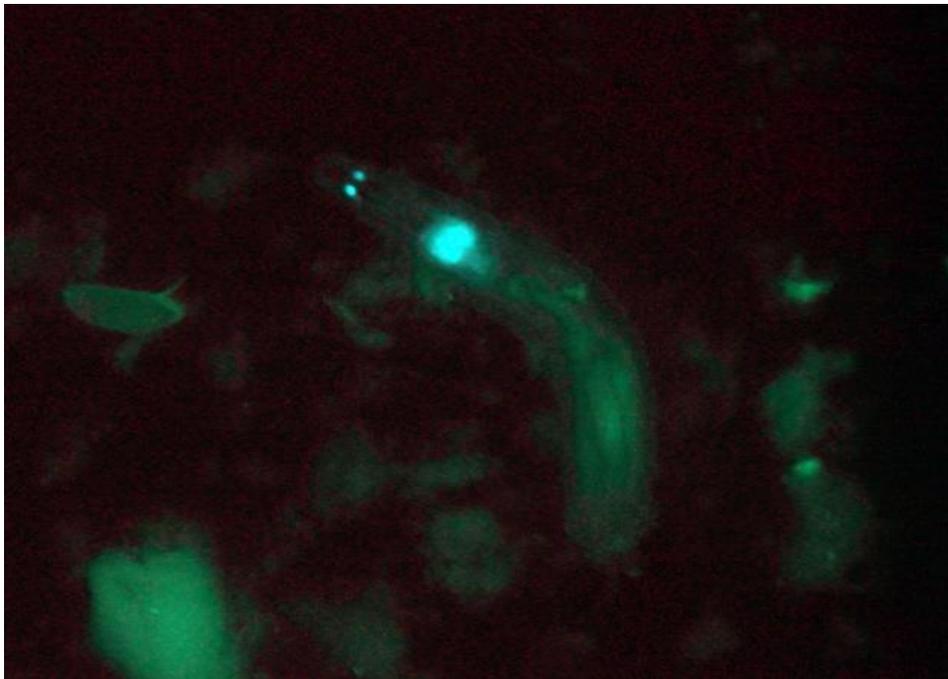


Figure 8. A transgenic *D. guttifer* larva showing EGFP expression in the Bolwig organs (two small glowing dots on the left) and the eye disks/brain (larger blob to the lower right).

5. Troubleshooting

Problem	Reason	Solution
Few larvae hatch.	<p data-bbox="608 255 866 286">Bad injection needles.</p> <p data-bbox="608 439 978 515">Over-desiccation of embryos during lining up.</p> <p data-bbox="608 663 751 694">Toxic DNA.</p> <p data-bbox="608 889 847 965">Too high heat shock temperature.</p> <p data-bbox="608 1294 978 1505">If the Halocarbon oil mixture is suffocating and killing the embryos. This does not normally happen, but we have seen this problem in our lab.</p>	<p data-bbox="1003 255 1374 376">Make sure you use standard needles. Strictly follow the instructions under section 3.6.</p> <p data-bbox="1003 439 1374 604">Add injection oil within 3-5 seconds after embryos appear completely dry under the microscope.</p> <p data-bbox="1003 663 1374 828">Use highly pure DNA (no mini preps). Additional isopropanol precipitation after the first extraction of DNA is necessary.</p> <p data-bbox="1003 889 1374 1234">The heat shock temperature for <i>D. guttifer</i> transformation is 40°C; however, if this temperature is causing strong reduction in survival rate, reduce the temperature by 1-2 degrees but never go below 37°C.</p> <p data-bbox="1003 1294 1374 1460">Use Halocarbon oil 27 only or GEM extra virgin olive oil. We are using extra virgin olive oil in our lab with high success.</p>
Few surviving adults.	<p data-bbox="608 1576 978 1742">Having only few injection-surviving larvae causes bacterial growth and/or mold formation in the food.</p> <p data-bbox="608 1800 978 1921">Desiccation of pupae due to dry chamber and/or dry cotton plugs.</p> <p data-bbox="608 2024 831 2056">Poor DNA quality.</p>	<p data-bbox="1003 1576 1374 1742">Ensure a high injection survival rate. At least 10-20 larvae can keep the food mold-free.</p> <p data-bbox="1003 1800 1374 1877">Always keep the chamber and the cotton plugs moist.</p>

No transgenic larvae	Insufficient heat shock temperature.	<p>Always clean the DNA by isopropanol precipitation or prepare new DNA samples.</p> <p>Always ensure the heat shock temperature is not below 37°C. Check the temperature of your water bath with a calibrated thermometer.</p>
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6.0 Reagents

6.1. Anti-fungus paper

Dissolve 2 g of sorbic acid and 0.6 g of methyl paraben in 200 mL of 95% ethanol. Roll ~20 paper towels and stick them into a 1-L glass beaker. Slowly pour the anti-fungus solution over the towels to soak them evenly. Unfold the towels and let them dry completely. Anti-fungus paper does not go bad and can be stored for years.

6.2. Halocarbon oil mixture

Use a 50-mL Falcon tube, add 35 mL of Halocarbon oil 700 and 5 mL of Halocarbon oil 27. Homogenize the mixture on a nutator overnight.

Author Contributions: All authors have read and agree to the published version of the manuscript. Conceptualization, Shigeyuki Koshikawa and Thomas Werner; Data curation, Mujeeb Shittu and Thomas Werner; Formal analysis, Shigeyuki Koshikawa and Thomas Werner; Funding acquisition, Shigeyuki Koshikawa and Thomas Werner; Investigation, Mujeeb Shittu, Tessa Steenwinkel, Shigeyuki Koshikawa and Thomas Werner; Methodology, Mujeeb Shittu, Tessa Steenwinkel, Shigeyuki Koshikawa and Thomas Werner; Project administration, Thomas Werner; Resources, Shigeyuki Koshikawa and Thomas Werner; Supervision, Thomas Werner; Validation, Mujeeb Shittu, Shigeyuki Koshikawa and Thomas Werner; Visualization, Mujeeb Shittu, Tessa Steenwinkel and Thomas Werner; Writing – original draft, Mujeeb Shittu and Thomas Werner; Writing – review & editing, Mujeeb Shittu, Tessa Steenwinkel, Shigeyuki Koshikawa and Thomas Werner.

Funding: “This research was funded by JSPS KAKENHI, grant number 18H02486 to S.K. and the NIH, grant number 1R15GM107801-01A1 to T.W.

Acknowledgments: We thank Lucinda Hall, David Trine, Daniel Noren, and Prajakta Kokate for technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

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