Creating transgenic *Drosophila* by microinjecting the site-specific ϕC31 integrase mRNA and a transgene-containing donor plasmid

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We describe a microinjection-based ϕC31 integrase mRNA-mediated method for creating transgenic *Drosophila* strains. This approach is more efficient than traditional methods and ensures that the transgene is targeted to a precise genomic position. The method involves targeting integration of an exogenous plasmid (containing the transgene and sequences to facilitate integration) to a preplaced recipient site in the *Drosophila* genome. The plasmid is coinjected into embryos with mRNA encoding the ϕC31 integrase, the enzyme that catalyzes the integration reaction. Using the protocol described here, transgenic lines can be established from, on average, 46% of fertile adults obtained after injection, and all integrations should be targeted to the chosen genomic insertion site. The whole procedure, from injection to established transgenic stocks, can be completed in three generations (approximately 1 month) and can be adapted for other types of transgenesis and mRNA injections in *Drosophila*.

INTRODUCTION

Transposase-mediated transgenesis in *Drosophila*

The ability to introduce and control the expression of transgenes in a model organism is a powerful tool for understanding how genes function. This type of genetic manipulation has been described for a number of species, but arguably most of the work has been carried out in the fruit fly *Drosophila melanogaster*. Introduced in the early 1980s[1], the P-element technique for making transgenic flies allowed researchers to examine the consequences of global overexpression of a gene in a multicellular organism. The technique was based on naturally occurring P-element transposons. The element-encoded transposase enzyme facilitated movement of elements throughout the *Drosophila* genome. Subsequent refinements and adaptations of the technique allowed greater control of transgene expression using the Gal-4/UAS binary system[2]. With this system, expression of a transgene can be directed to a specific set of tissues or cells. The Flipase recombination enzyme/Flipase Recognition Target (FLP/FRT) system[3] provided the ability to direct overexpression in clones of cells within the same tissue, enabling the importance of the study of borders or compartments.

While these different methods for bringing about transgene expression have been important, each of them is based on the use of the P-element transposase, which catalyzes the integration of P-elements into the genome in an unpredictable manner. The sites where P-elements integrate are numerous and scattered throughout the genome, making it highly unlikely that two different transgenes would ever integrate into the same genomic site. Position effects, depending on the integration site, are known to influence the expression of transgenes in *Drosophila*, in particular when the transgenes insert into heterochromatic areas. Therefore, the level of expression of a specific transgene often cannot be compared to that of another in a different location. In addition, the frequency of integration events is relatively low, ranging from 5% to 20% in fertile adults.

ϕC31-mediated site-specific transgenesis in *Drosophila*

The *Streptomyces* phage ϕC31 has biological characteristics that resemble the movement of transposons in *Drosophila*. This phage can insert its own genomic DNA into that of bacterial genomes using an enzyme called integrase, which catalyzes the insertion process. However, unlike a transposase, ϕC31 integrase operates in a very sequence-specific and unidirectional manner. A preplaced sequence (attP) acts as the recipient site in the *Drosophila* genome. A pUASTB plasmid containing both a transgene and donor sequence (attB) is coinjected with ϕC31 integrase mRNA into attP-containing recipient embryos, resulting in the site-specific insertion of the transgene into the attP site. Hybrid sites (attL and attR) are formed during this process, which prevents further integrase-catalyzed movement of the integrated transgene (**Fig. 1**). ϕC31 integrase has also been shown to function in mammalian cells[4], the frog *Xenopus laevis* embryos[5], the mosquito *Aedes aegypti*[6] and *Drosophila* cell culture[7].

Here, we provide a detailed protocol for creating transgenic *Drosophila* using the ϕC31 integrase system, based on our previously published method[7]. The method requires a fly line carrying a defined genomic ϕC31 *attP* integration site, enabling the subsequent use of that site to target insertion of incoming transgenes (http://www.frontiers-in-genetics.org/flyc31/why_phic31.php).

Advantages and limitations of this protocol

The ϕC31 integrase method allows researchers to quickly and efficiently insert transgenes into specific sites of the *Drosophila* genome. The fact that the insertion site is known decreases the time between injection and establishing a stable stock, as chromosome mapping is not required. It also reduces the concern regarding position effects.

In addition, the injection method described here can easily be adapted to introduce mRNAs, double-stranded RNAs, proteins or other biologically active molecules directly into *Drosophila* embryos.

This method has significant advantages over previously described methods. First, every integration event we have examined occurs precisely in the chosen attP location[7]. This feature reduces complications imposed by position effects. Because each transgene is in the same genomic context, expression levels between constructs can be

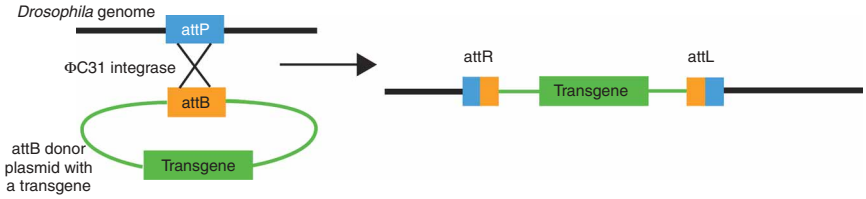

Figure 1 | A schematic outlining ϕC31-mediated integration. A preplaced sequence (attP) acts as the recipient site in the *Drosophila* genome. A pUASTattB plasmid containing both a transgene and donor sequence (attB) is coinjected with ϕC31 integrase mRNA into attP-containing recipient embryos, resulting in the site-specific insertion of the transgene into the attP site. Hybrid sites (attL and attR) are formed during this process, which prevents further integrase-catalyzed movement of the integrated transgene.

considered equal, allowing direct comparison of effects between transgenes that have been modified in various ways. Second, as the rate of integration is higher than that of the P-element transposase method, fewer embryos need to be injected to achieve a transgenic animal (**Table 1**). For these reasons, the ϕC31 integrase method is being widely adopted in the *Drosophila* community, and variations of the method have been published[8–10] (see below). It must be noted that between the works of Groth *et al.*[7], Venken *et al.*[9] and Bischof *et al.*[10], over one hundred known attP lines have been generated. This represents a wide variety of mapped attP insertion sites, each of which may be valuable for a specific set of experiments (http://www.frontiers-in-genetics.org/flyc31/landing_platforms.php).

There are, however, limitations to this protocol. *In vitro*-made integrase mRNA is subject to degradation if not handled properly. Therefore, RNase-free pipettes and reagents should be used when making the ϕC31 integrase mRNA to ensure the best possible integration frequency. This limitation has been reduced by the creation of stocks that produce ϕC31 integrase mRNA *in vivo*[10]. Another limitation to this protocol is the fragility of the embryos injected. Although a high rate of transgenesis can be obtained, it is dependent on a reasonable number of embryos surviving injection: approximately 50%. This requires proper handling and care of the embryos during their development to the adult stage; these steps will be explained in the PROCEDURE section.

Variations of the ϕC31 integrase method

In Bateman *et al.*[8], it was shown that recombinase-mediated cassette exchange could be used to screen for transgenic flies. There, the attP-recipient stock was marked with w^+ (white, red eyes) and the donor plasmid was marked with y^+ (yellow; yellow body) or GFP (green fluorescent protein). The activity of the recombinase results in the loss of w^+ in the recipient F1 generation and incorporation of only the cassette, or transgene element of the donor plasmid. In addition, this method allows both marked and unmarked donor plasmids to be used for screening of transgenics.

Venken *et al.*[9] showed that the ϕC31 integrase method could overcome the problems associated with obtaining transgenics when trying to insert large bacterial artificial chromosomes. A transgenic bacterial artificial chromosome as large as 133 kb was successfully inserted into the proper recipient site and could rescue an associated phenotype. The usefulness of this strategy is that large DNA fragments can be inserted into areas of the genome devoid of annotated genes and will not remobilize, whereas P-element inserts often land in regulatory elements of genes and can move about.

Finally, Bischof *et al.*[10] introduced a method by which the transformation enzyme ϕC31 integrase is produced endogenously in germ cells via the *vasa* promoter. This is a great advantage in that one of the problems of coinjecting *in vitro*-made mRNA is that it can degrade before injection and thus lose efficacy. If there are any drawbacks to this method, it would only be the need to remove the *vasa*-driven ϕC31 integrase transgene from an established stock by subsequent genetic crosses.

Applications

This method can be applied to the generation of many different kinds of transgenic *Drosophila* strains. It can be used to generate lines with the transgene reproducibly integrated at the same site in the genome, reducing position-dependent gene expression. It also provides a method to test the activity of mRNA directly.

Experimental design

Choice of attP-containing fly line. Many fly lines containing a recipient attP site have been made in several different labs[7,9,10]. The genomic position of the attP sequence will be different in each line, which may have an impact on the experiment. Thus, the choice of appropriate fly line should be considered carefully.

Donor plasmid. The plasmid pUASTB or pUASTattB (**Fig. 2**) carries the donor site (attB) and the dominant marker w^+, which allows transgenic lines to be identified by the change in eye color from white (w^-, injected stock) to red after the first cross to a w^- genetic background. Specific transgenes of interest need to be introduced into the pUASTB vector by standard subcloning methods. The presence of the UAS site allows Gal4-driven gene expression[2].

MATERIALS

REAGENTS

- attP-containing fly stock (e.g., P{CARY.attP}1 (Bloomington Drosophila Stock Center, stock no. 8621) or P{CARY.attP}2 (Bloomington Drosophila Stock Center, stock no. 8622)). Other stocks may be obtained from the above-mentioned references
- pUASTattB (donor plasmid containing the w^+ marker and attB site; **Fig. 2**). Available upon request—contact calos@stanford.edu; see also http://www.frontiers-in-genetics.org/flyc31/sequences_and_vectors.php
- pET11ϕC31polyA (plasmid used for making ϕC31 integrase mRNA[11]). Available upon request—contact calos@stanford.edu
- Water, double distilled or molecular biology grade
- Absolute ethanol **! CAUTION** Flammable.
- Halocarbon 700 oil (Sigma, cat. no. H8898)

TABLE 1 | Expected results.

	P-element and transposase	ϕC31 integrase
Number of embryos injected[a]	269	297
Hatched larvae[b], % (number)	58 (157)	42 (126)
Adults[b], % (number)	44 (28)	60 (73)
Fertile crosses[b], % (number)	32 (72)	50 (37)
Transgenics[b,c], % (number[d])	15 (5)	46 (17)
Projected minimum number of injections needed for one transgenic	54	17

[a]Average of three experiments. [b]Average values. [c]% transgenics = (number of transgenics/number of embryos injected) × 100. [d]Number of independent crosses producing F1 adults with red eyes.

- LiCl
- Drierite ($CaSO_4$; Sigma, cat. no. 7778-18-9)
- NaOAc
- Isopropanol ! CAUTION Flammable.
- Instant *Drosophila* medium (Carolina Biological, cat. no. 17-3200)
- Fly food

EQUIPMENT

- Capillary tubes (World Precision Instruments, cat. no. 1B120-3)
- Microscope slides (Superfrost plus; Fisher, cat. no. 12-550-15)
- Razor blades
- 3M double-coated tape 415 clear, 0.25 inch 'Embryo Tape' (R.S. Hughes, cat. no. 021200-05051)
- Collection cage
- Embryo collection caps, 10 and 15 cm diameter filled with standard grape juice/agar-based media
- Micropipette loaders (Brinkman Inc., cat. no. 930001007)
- 18 mm × 18 mm coverslips
- Fly food bottles
- Fly food vials
- Humid chambers
- 60 mm × 15 mm plastic Petri dishes
- 150 mm × 15 mm plastic Petri dishes
- Dissecting microscope
- Upright compound microscope with ×10 objective and ×10 oculars
- Needle holder (Leica)
- Micromanipulator (Leica)
- P-97 micropipette puller (Sutter Instruments)
- Desiccation chamber, desiccator (VWR)
- 50 ml glass syringe
- Tygon tubing (VWR, cat. no. 63010-015)
- Three-way stop cock
- Bench-top centrifuge
- Vacuum centrifuge
- Embryo collection basket

REAGENT SETUP

Preparing attP fly stocks for embryo collection Place a total of approximately 500 male and female flies, in a 1:1 ratio, in a food bottle seeded with activated dry yeast, at least 2 d before embryos are to be collected. This ensures that adult females will be laying well on the day of embryo collection and injection. On the day of injection, transfer the flies into collection cages containing embryo collecting caps that have 0.5 ml activated dry yeast paste on them. Yeast paste is made by mixing activated dry yeast with water until the paste has a semisolid, but not runny, consistency. Caps should be changed hourly on the day of collection.

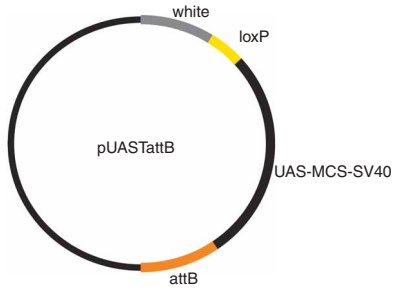

Figure 2 | Physical map of the plasmid pUASTattB used in this protocol. The vector contains a multiple cloning site (MCS) and the white gene for selection of transgenic flies[10]. The loxP site can be used to excise the white marker and other unnecessary sequences after the integration[10]. UAS sequences allow for spatio/temporal expression and SV40 sequences terminate transcription.

Larvae food setup Add instant *Drosophila* medium to a standard food vial that already contains an agar-based medium. Add water to the instant food and leave for 5 min. If the instant food has absorbed most or all of the water, add more water. Mix the instant food to break up any large pieces and remove any excess water with a tissue wiper. The consistency of the instant food should be moist but not runny and should be 5 mm thick on top of the agar food.

EQUIPMENT SETUP

Injection needles Using the P-97 micropipette puller, perform a ramp test to determine the heat setting to be used for the capillary tubes, as described by the manufacturer. Adjust pull, velocity, time and pressure settings so that the pulled needles have a slender taper, but are not very flexible[12].

Injection slides Place a 5 cm length double-coated tape lengthwise in the center of a microscope slide and remove the paper backing. Large numbers of injection slides can be made before injection and stored in a dust-free environment indefinitely.

Humid chamber setup Fold three 11.4 cm × 21.3 cm light-duty tissue wipers in half and place them in the bottom of a 150 cm × 15 mm Petri dish. Cut 2 ml plastic pipettes to a length of 12 cm. Tape two of the lengths together and place two sets on top of the tissue wipers. These pipettes act as stands for the injection slides after injecting. Soak the tissue wipers in water and cover.

Injection rig Place the micromanipulator in a position that will not interfere with stage controls of the upright microscope. Connect a length of Tygon tubing from the needle holder to the 50 ml syringe, with a three-way stop cock in between.

PROCEDURE

Preparation of transgene DNA

1| Isolate the plasmid DNA containing the transgene of interest from an overnight culture of *Escherichia coli* using a Qiagen midi or maxi prep kit, following the manufacturer's instructions.

2| After isolation, re-precipitate the DNA: add one-tenth volume of 3 M NaOAc and 1 volume of isopropanol, mix well and leave for 15 min at −20 °C.

▲ CRITICAL STEP Although Qiagen-isolated DNA is clean, it is eluted in a buffer that is not suitable for injecting and can be toxic to embryos. The DNA prep must therefore be re-precipitated and resuspended in water. Checking the concentration of eluted DNA at this point will be helpful in determining the volume of water required for resuspension in subsequent steps.

3| Spin the DNA at 20,000*g* for 20 min at 4 °C on a bench-top centrifuge.

4| Remove the supernatant and wash the pellet with 200 µl of 70% (vol/vol) ethanol.

5| Spin again for 20 min at room temperature (25 °C) at 20,000*g* on a bench-top centrifuge.

6| Remove the supernatant and dry the pellet in a vacuum centrifuge; this will take approximately 7 min in a standard vacuum centrifuge.

7| Resuspend the dried pellet in molecular biology grade water or double-distilled water to a concentration of at least 1 µg µl^{-1}. If desired, check the concentration of the DNA and confirm that the DNA is intact by agarose gel electrophoresis, although we usually find this unnecessary.

■ **PAUSE POINT** Plasmid DNA can be stored at −20 °C for an indefinite period of time; it does not need to be made fresh unless results of Step 14 indicate otherwise.

Integrase mRNA preparation

8| Set up a standard restriction digest to linearize approximately 1 μg of the pET11ϕC31polyA plasmid[11] with *Bam*HI. Follow the recommended procedure of the enzyme supplier.

9| Amplify the integrase mRNA using the MEGAscript kit and T7 polymerase following the manufacturer's instructions (Ambion).

10| After amplification, purify the mRNA either by LiCl precipitation or phenol/chloroform extraction followed by isopropanol precipitation (see Steps 2 and 3) using RNase-free reagents.

11| Wash the precipitated RNA pellet with 200 μl of 70% ethanol.

12| Spin at 20,000*g* for 20 min at RT in a bench-top centrifuge.

13| Remove the supernatant and dry the pellet as described in Step 6.

14| Resuspend in diethylpyrocarbonate (DEPC)-treated water or milliQ water to a concentration of 1.5 μg μl^{-1}. If desired, check that the RNA is intact by agarose gel electrophoresis; we find this unnecessary.

■ **PAUSE POINT** Prepared RNA can be stored at −20 °C for an extended period of time. However, the quality of the RNA should be checked by agarose gel electrophoresis if it is not used immediately after Step 14.

Preparation of injection cocktail

15| Mix the previously prepared plasmid DNA (Step 7) and integrase RNA (Step 14) such that the final concentration of the DNA is 200 ng μl^{-1} and the RNA is between 800 and 1,000 ng μl^{-1}.

16| Mix the injection cocktail by flicking and spin in a microcentrifuge at 25 °C at 20,000*g* for 5 min to sediment any particulate matter that may clog the injection needle.

17| Remove a 10 μl aliquot from the top and place in a clean tube. If desired, the quality of the injection cocktail can be determined both before and after injection by agarose gel electrophoresis.

Preparation of injection needles

18| Back load a needle using a micropipette and place in the needle holder[12].

19| Break an 18 mm × 18 mm coverslip into half and place it on a microscope slide.

20| Cover the broken edge of the coverslip with halocarbon oil and place the slide on the microscope.

21| Bring the unbroken needle tip and the broken edge of the coverslip into the same plane of focus.

22| Break the tip of the needle by moving the broken coverslip (stage) against the needle and applying pressure on the syringe (**Fig. 3**).

▲ **CRITICAL STEP** We have found that needles that are broken to produce a sharp beveled tip are better than those with blunt tips; they tend to cause less damage to the embryo and therefore result in higher survival rates.

Preparation of embryos for injection

23| Add a fresh collection cap to the fly cage (see REAGENT SETUP) and collect embryos for a 1 h period. Note that when flies are set up as described, most embryos collected within a 1 h period will be still young enough to inject at Step 32.

24| With a paint brush, transfer the collected embryos into the bottom of a 60 mm × 15 mm Petri dish.

25| Add 10 ml of 60% bleach and gently agitate the embryos with a paint brush for 1.5 min to remove the chorion.

26| Pour the dechorionated embryos into an embryo collection basket and wash with water for 30 s.

27| Transfer the embryos into an injection room where the temperature is set at 18 °C. This temperature will slow down the development of the embryos, helping to ensure that they are still young enough at Step 32.

28| Place the embryos on a 15 cm collection cap and align them side by side one or two embryo widths apart and in the same anterior/posterior orientation.

29| Transfer the embryos from the cap onto an injection slide (see EQUIPMENT SETUP) by gently pressing the tape onto the aligned embryos. Try to ensure that the embryos are in the center of the tape.

30| Place the slide into a desiccation chamber containing 500 ml of drierite for approximately 15–20 min; use a three-way stop clock to keep track of up to three slides at the same time.
▲ CRITICAL STEP Proper desiccation time is critical for the survival of injected embryos and may need to be determined in advance.

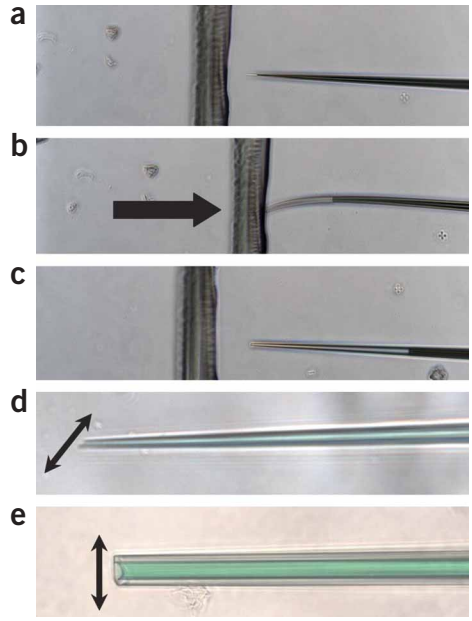

Figure 3 | Breaking an injection needle. A previously pulled needle[12] is loaded with an injection cocktail and placed in the needle holder. A broken coverslip is covered with halocarbon oil and brought into focus. (**a**) The injection needle is positioned into the same plane of focus. (**b**) The coverslip is moved toward the needle until it begins to bend (arrow) and then pressure is applied to the syringe to break the needle. (**c**) Needles that perform the best have a beveled tip and are not blunt. (**d**) A needle that is a preferred sample is shown; the arrow indicates the beveled point. (**e**) A less desirable needle is shown; the needle is too blunt.

Injection

31| Place a drop of halocarbon oil over the desiccated embryos and place the slide on the microscope.
▲ CRITICAL STEP If more than one slide of embryos is prepared, it is important to immediately cover the embryos with halocarbon oil when they have completed desiccation. Halocarbon oil allows the exchange of air but not water to the embryos. Therefore, the halocarbon oil at this step has two functions that increase the number of embryos that will survive the injection procedure: it prevents the embryos from desiccating further and provides adequate oxygen exchange.

32| Bring the posterior of the embryo into sharp focus; then bring the previously prepared needle into the same focal plane. Pierce the embryo with the needle by moving the stage to the needle. Once the embryo is pierced, move the embryo so that the tip of the needle is as close to the posterior as possible (**Fig. 4**).

33| Apply enough pressure to the syringe attached to the needle to inject the cocktail. Repeat for each embryo on the slide.
▲ CRITICAL STEP Embryos that are either under- or over-desiccated may not survive injection. Embryos that are over-desiccated will deform past 40% of the egg length when being injected. Embryos that are under-desiccated may leak after injection.

Post injection

34| Remove excess halocarbon oil with a razor blade, such that only a drop of oil three to four embryo widths covers the embryos and clean tape is exposed on the anterior and posterior sides. Place slides into a humid chamber.
▲ CRITICAL STEP Failure to remove excess oil as described can lead to increased embryo lethality: excess oil can inhibit the exchange of oxygen to the embryos leaving them susceptible to asphyxia; excess oil also tends to flow off the embryos onto the slide on which they are mounted, leaving them susceptible to further, unwanted, desiccation. Note that unwanted desiccation may also occur if too much oil is removed.

35| Incubate the embryos in the injection room (18 °C) for 18 h and then move them to RT until the embryos hatch, about 18 h later.

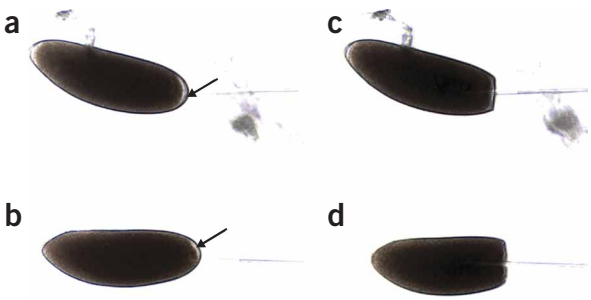

Figure 4 | Injecting embryos. (**a**,**b**) Two properly desiccated embryos are shown. (**a**) The embryo shown is less desiccated as indicated by the presence of perivitelline fluid (arrow). (**b**) The embryo shown is more desiccated and has lost the perivitelline fluid (arrow). (**c**) The needle deforms the embryo to approximately 10% of egg length before penetration. (**d**) The embryo deforms to approximately 20% of egg length before the needle penetrates. The maximum tolerable deformation before needle penetration is 30% of egg length; above this, embryos will not survive. Penetration at a deformation of less than 10% may cause leaking or may preclude a proper dose of the injection cocktail. (**d**) An optimally desiccated embryo, leading to sufficient uptake of injection cocktail, is shown.

36| Collect hatching or hatched embryos using an L-shaped wire. A total of 10–20 larvae can be gathered in a ball of the halocarbon oil and then placed into the instant media in the previously made food vial (see REAGENT SETUP). The optimal number of larvae per vial is approximately 50. Less than ~50 larvae per vial can prohibit cooperative working of the food, making it harder for the group to feed. More than ~50 larvae per vial can lead to overcrowding and lessen the percentage of larvae that survive to adulthood.
? TROUBLESHOOTING

37| Rear larvae to adulthood at 25 °C (approximately 10 d).

38| Collect the surviving adults and cross each individually with flies with a w^- genetic background; a specific w^- stock is not necessary unless a certain genetic background is required. Individual surviving adult males should be crossed with 3–4 w^- virgin females. Individual surviving females should be crossed with 2–3 w^- males.
? TROUBLESHOOTING

39| Screen the progeny from each cross for red-eyed (transgenic) flies and set up sib-crosses between red-eyed progeny to establish a homozygous stock.
? TROUBLESHOOTING

● **TIMING**
Preparation of transgene DNA, Steps 1–7: 1 h
Preparation of integrase mRNA, Steps 8–14: 2–3 h
Preparation of injection cocktail, Steps 15–17: 5 min
Preparation of injection needles, Steps 18–22: 15 min
Preparation of embryos for injection, Steps 23–30: 30–40 min
Injection, Steps 31–33: 30–40 min
Post injection: 12–13 d (Steps 34–38); 11–12 d (Step 39)

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Reason	Solution
36	Few embryos hatch	DNA is toxic	Clean DNA by isopropanol precipitation or prepare a new sample
		Embryos are damaged during injection	Prepare better needles (**Fig. 3**)
		Over-desiccation of embryos	Adjust desiccation time
38	Few adults survive	Too few larvae are added to food vial	Add more larvae (50 per vial)
		Instant food dries out	Ensure that the food is moist by adding small amounts of water as required
39	No red-eyed progeny	Quality of DNA/RNA is poor	Clean by isopropanol precipitation or prepare new samples

ANTICIPATED RESULTS
As can be seen in **Table 1**, the frequency of integration averaged 46% of fertile adults being recovered. This is three times higher than the frequency of P-element transposition that we see under similar circumstances. As the integration event occurs in the same place every time[7], there is no need to establish multiple transgenic lines. Based on our survival rates of injected embryos and the frequency of integration, we calculated the projected minimum number of injected embryos required to obtain one transgenic to be 54 (see **Table 1**).

ACKNOWLEDGMENTS This work is dedicated to the memory of my son Lance Raymond Fish (July 30, 2004 to April 30, 2007).

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1. Rubin, G.M. & Spradling, A.C. Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348–353 (1982).
2. Brand, A.H. & Perrimon, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401–415 (1993).
3. Xu, T. & Rubin, G.M. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223–1237 (1993).
4. Groth, A.C., Olivares, E.C., Thyagarajan, B. & Calos, M.P. A phage integrase directs efficient site-specific integration in human cells. *Proc. Natl. Acad. Sci. USA* **97**, 5995–6000 (2000).

5. Allen, B.G. & Weeks, D.L. Transgenic *Xenopus laevis* embryos can be generated using ϕC31 integrase. *Nat. Methods* **2**, 975–979 (2005).
6. Nimmo, D.D., Alphey, L., Meredith, J.M. & Eggleston, P. High efficiency site-specific genetic engineering of the mosquito genome. *Insect Mol. Biol.* **15**, 129–136 (2006).
7. Groth, A.C., Fish, M., Nusse, R. & Calos, M.P. Construction of transgenic *Drosophila* by using the site-specific integrase from phage ϕC31. *Genetics* **166**, 1775–1782 (2004).
8. Bateman, J.R., Lee, A.M. & Wu, C.T. Site-specific transformation of *Drosophila* via ϕC31 integrase-mediated cassette exchange. *Genetics* **173**, 769–777 (2006).
9. Venken, K.J.T., He, Y., Hoskins, R.A. & Bellen, H.J. P[acman]: a BAC transgenic platform for targeted insertion of large DNA fragments in *D. melanogaster*. *Science* **314**, 1747–1751 (2006).
10. Bischof, J., Maeda, R.K., Hediger, M., Karch, F. & Basler, K. An optimized transgenesis system for *Drosophila* using germ-line-specific ϕC31 integrases. *Proc. Natl. Acad. Sci. USA* **104**, 3312–3317 (2007).
11. Hollis, R.P. *et al.* Phage integrases for the construction and manipulation of transgenic animals. *Reprod. Biol. Endocrinol.* **1**, 79 (2003).
12. Miller, D.F., Holtzman, S.L. & Kaufman, T.C. Customized microinjection glass capillary needles for P-element transformations in *Drosophila melanogaster*. *Biotechniques* **33**, 366–372 (2002).