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The Use of P-Element Transposons to Generate Transgenic Flies

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Summary

The development of a technique to stably integrate exogenous DNA into the germline of *Drosophila melanogaster* marked a milestone in the ability to study gene function in the fly.

On the molecular level germline transformation mainly relies on a particular transposable element, the *D. melanogaster* P-element. Based on certain features of the P-element, vectors have been designed for diverse applications like gene disruption, chromosome engineering, gene tagging, and inducible gene expression/repression. Despite the fact that an increasing number of other transposons have been utilized for germline transformation of *Drosophila* most transformation vectors are still P-element based.

Technically, microinjection serves as the method of choice to physically introduce transgenes into preblastoderm *Drosophila* embryos. Besides an appropriate technical equipment including suitable microcapillaries in conjunction with a micromanipulator, a microinjector, and a microscope, proper handling of the *Drosophila* embryos before and after microinjection is the key step to the generation of transgenic flies. Pioneer work in *Drosophila* also served as a general guideline for the transformation of other insect species including those with medical and agricultural importance.

Key Words: *Drosophila melanogaster*; germline transformation; microinjection; P-element; transformation vector; transgenic fly; transposable element.

1. Introduction

The discovery of P-elements in *Drosophila melanogaster* is tightly connected to a syndrome called hybrid dysgenesis. When females of so-called M strains were mated to males of a P stock the offspring displayed a number of defects including sterility, mutation, chromosome breakage, and male recombination. The molecular basis for hybrid dysgenesis turned out to be the presence of a particular type of mobile genetic elements, P-elements, in P stocks but not in M strains. Depending on their capability to transpose, P-elements are classified as autonomous or

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nonautonomous. Autonomous P-elements encode a functional transposase that enables them to move by a cut-and-paste mechanism inside a genome, whereas nonautonomous ones lost this ability owing to internal deletions. The functional P-element is 2907 bp in length with 31 bp inverted terminal repeats and 11 bp inverted subterminal repeats as the major *cis*-acting target sequences for excision by the transposase. Reintegration of an intact P-element at a random chromosomal location generates a flanking direct 8 bp duplication at the target site ***(1)***.

In order to serve as a tool for germline transformation, P-elements have been integrated into plasmid vectors and undergone further modifications ***(2,3)***. To inhibit their autonomous movement the transposase gene has been transferred to another vector named "helper-plasmid", which provides the transposase *in trans*, but is itself unable to integrate into the genome ***(4,5)***. In exchange for the transposase gene a multiple cloning site and a visible marker gene to identify transformants are inserted inside the inverted terminal repeats ***(6)***. The multiple cloning site facilitates the insertion of desired DNA sequences into the transformation vector. In most cases the visible marker gene is a *white* mini-gene, which in a *white* mutant background allows detection of an integration event ***(7,8)***. Besides *white* other marker genes like *rosy* ***(6)***, *vermilion* ***(9)***, or *yellow* ***(10)*** have been applied, which for their detection require injection of the transformation vector into embryos of corresponding mutant recipient strains. However, *white* has turned out to be the most convenient and frequently used marker gene. Nowadays a number of non-P-element transformation vectors use fluorescent proteins like GFP as a visible marker to uncover transformants because of its higher sensitivity ***(11–13)***. The transposase gene on the "helper-plasmid" has been modified, too. Normally the mobility of wild-type autonomous P-elements is restricted to the germline. This is owing to gene regulation at the level of RNA-splicing, which involves the 2-3 intron of the transposase gene. Artificial removal of this particular intron resulted in a transposase, termed Δ2-3, that functions in germline and somatic cells ***(5)***.

Besides the general modifications of a P-element-based transformation vector aforementioned, adaptation of the vector to different tasks requires special alterations. Numerous P-element vectors have been created that are suited for gene disruption, gene-, protein- and enhancer-trapping, transgene misexpression, promotor studies, genome manipulation, gene targeting, and RNA interference ***(14)***. A large collection of diverse transformation vectors is available from the *Drosophila* Genomics Resource Center at http://dgrc.cgb.indiana.edu/.

Germline transformation requires the stable integration of the DNA of interest into the germ cells of the recipient embryo. This is accomplished by the physical delivery of the desired DNA at the posterior pole of syncytial blastoderm *Drosophila* embryos where the precursors of the germ cells form. On cellularization the DNA will then, in theory, be incorporated into the so-called pole

cells and integrated into their genome. Deposition of the DNA of interest is fullfilled by penetrating the preblastoderm embryos with a suitable injection capillary and application of an injection mix including transformation vector and "helper-plasmid" ***(15)***. Coinjection of the transformation vector with purified P-element transposase has also been described, but appears to be less convenient ***(16)***. Thirdly, injection of only the transformation vector into embryos with a stably integrated source of transposase is possible, too ***(17)***.

Microinjection requires a couple of technical devices like a micropipet puller to produce the injection capillaries, a micromanipulator, a microinjector, and a microscope. Whereas the microscope is needed to optically control the microinjection procedure, the micromanipulator helps to gently align the injection capillary with the embryos. Finally, the microinjector is used to control the delivery of a reproducible amount of injection mixture to the embryos.

Apart from technical prerequisites correct treatment of the *Drosophila* embryos is critical for successful germline transformation. This includes collection of properly staged embryos, careful handling before and after microinjection, and selection and characterization of the transformants.

It should be noted that besides the P-element a couple of other transposons like *Minos* ***(18)***, *hobo* ***(19)***, *mariner* ***(20)***, *Hermes* ***(21),*** and *piggyBac* ***(22)*** have been introduced as tools for germline transformation in *D. melanogaster*. The germline transformation technique described in **Subheading 3**. should be applicable to these transposons, too. However, with the exception of the *piggyBac* transposon their usage for germline transformation in *D. melanogaster* is limited owing to the lack of a broad range of transformation vectors for the diverse applications listed above. On the other hand and in contrast to the P-element some of them display a broad host range allowing their usage as vectors for germline transformation in a wide variety of non-drosophilid insect species ***(23)***.

2. Materials

Depending on the equipment of your institute you might use other devices (micropipet puller, micromanipulator, and so on) than described here to generate transgenic flies. Ask coworkers familiar with a particular device for appropriate handling. We will refer mainly to the tools that we use and can recommend from our experience with P-element-mediated germline transformation. Therefore, some details on the set up and performance of germline transformation might not be useful for everyone, but could nevertheless serve as a general guideline.

2.1. Embryo Collection/Handling

1. A w^{1118} stock (Bloomington stock 5905) as the source of embryos for injection when using the *white* mini-gene or a fluorescent marker for the selection of transformants.

2. As egg laying cages we use self-made cylindric acrylic glass vials (1000 mL) that fit into standard Petri dishes (94×16 mm^2) filled with apple juice agar and are covered on their top side with a commercially available fly screen.
3. For apple juice plates take 40 g of agar, 340 mL of filtered apple juice (100%), 17 g of table sugar or sucrose and scale up to 1000 mL with sterile water. Boil until the agar and the sugar have dissolved completely and cool down to 60°C. Add Nipagin, a preservative and fungicide, to a final concentration of 0.3%, mix and immediately pour it into Petri dishes (94×16 mm^2). Avoid bubbles and make sure that the surface of the apple juice plates is smooth. After they have hardened and cooled down plates can be stored at 4°C for approx 2 wk.
4. Nipagin/Methylparaben: prepare a 10% stock solution in ethanol and store it at 4°C.
5. Yeast paste: dissolve Baker's yeast in sterile water until it has a paste-like consistency. Fill it into a plastic syringe to facilitate the dosage and store it at 4°C.
6. 10% Acetic acid.
7. To prepare steel mesh baskets cut a plastic tube (15 mm diameter) into pieces of 10 mm height, heat the plastic briefly on one side under a Bunsen burner and press it with the melted side onto a steel mesh whose mesh size will retain embryos (≤0.1 mm). Let it cool down and cut out the basket exactly from the steel mesh.
8. 7% Bleach/sodium hypochlorite.
9. A fine paintbrush.
10. Microscope slides ($76 \times 26 \times 1$ mm^3).
11. A blunt straight teasing needle.
12. For glue-coated cover slides incubate 10 pieces of adhesive tape (5×1 cm^2) in 10 mL of heptane for several days at room temperature. The heptane will dissolve the glue from the tape. Centrifuge the liquid part to precipitate indissoluble components and store the supernatant/liquid glue in a glass bottle at 4°C (*see* **Note 1**). Distribute 1–2 drops of the liquid glue on one half of a cover slide (20×20 mm^2), place it on a planar surface and let the heptane evaporate. The glue will remain on the cover slide. Test adhesiveness with your fingertip (the cover slip should stick to it tightly). Do not prepare more glue-coated cover slides than you will need for 1 d of injection.
13. A stereo dissecting microscope, for example, a Stemi 2000 from Zeiss, Göttingen, Germany.
14. A small spatula.
15. Medium-sized vials of fresh fly-food (~15 mL).

2.2. DNA Mix Preparation

1. A commercially available midi plasmid purification kit (e.g., NucleoBond PC100 from Macherey-Nagel, Düren, Germany).
2. 10X injection buffer: 1 m*M* sodium phosphate buffer, pH 6.8, 50 m*M* KCl. Filter-sterilize and store aliquots at –20°C.
3. Phenol red: dissolve in sterile water at 20 mg/mL, filter-sterilize, and store aliquots at –20°C.

2.3. Injection Needle Preparation

1. Micropipet puller: we obtained good results with the horizontal Flaming/Brown Micropipet Puller P-97 from Sutter Instrument, Novato, CA. We also successfully used the PUL-1 micropipet puller from World Precision Instruments, Berlin, Germany.
2. Microcapillaries: the choice of microcapillaries depends on your micropipet puller. We take borosilicate glass capillaries (100 mm length, 1.2 mm outside diameter, 0.69 mm inside diameter), for example, GB120F-10 from Science Services, Hofheim, Germany.
3. Tip grinder/beveler: We use a tip grinder from Saur, Reutlingen, Germany.
4. Petri dishes (94×16 mm^2) with a bar of plasticine for storage of the injection needles.

2.4. Microinjection

1. Microscope: almost any microscope equipped with ×10 eyepieces, a ×10 objective, and a microscope stage that allows a fine two-dimensional micrometer movement will do, so choice may depend on availability and personal preferences. We use a BH-2 upright microscope from Olympus, Tokyo, Japan.
2. Micromanipulator: a manual micromanipulator, which allows fine adjustment in all three axes (x, y, and z), is sufficient. We use a MM33 micromanipulator from Märzhäuser, Wetzlar, Germany.
3. Ball-joint/pipet holder: ball joints attach pipet holders to micromanipulators and enable that the pipet holder angles can be set freely and easily. We use ball-joint B-8 and pipet holder H-7 from Narishige, Tokyo, Japan.
4. Microinjector: a microinjector is a syringe driver for pneumatic or hydraulic control of injection needles. We use a manual microinjector: a 1-mL glass syringe (Fortuna Optima, Poulten & Graf, Wertheim, Germany) is inserted into a self-made syringe driver. A motor-driven microinjector is not necessary for the generation of transgenic flies in *D. melanogaster*. Microinjectors can also be purchased from Narishige, Tokyo, Japan, from Sutter Instrument, or from World Precision Instruments, Berlin, Germany.
5. A self-made heavy metal plate with rubber bumpers to mount the microscope and the micromanipulator and minimize vibrations.
6. Silicone tube (3 mm outside diameter, 0.5 mm inside diameter) (e.g., Rotilabo Silicone tube from Roth, Karlsruhe, Germany).
7. 3S Voltalef oil (VWR International, Darmstadt, Germany, cat. no. 24626.185).
8. 10S Voltalef oil (VWR International, Darmstadt, Germany, cat. no. 24627.188).
9. In order to coat cover slides immerse them briefly in Repel-Silane, let them air-dry for a few minutes, rinse them with water, and let them air-dry again.
10. Petri dishes (35×10 mm^2) for incubation of the injected embryos.
11. Petri dishes (145×20 mm^2) for preparation of a moist chamber.

3. Methods

3.1. Flies

1. If the construct of interest contains a mini-*white* reporter gene or a fluorescent marker a *white* mutant strain (e.g., w^{1118}) is suited best for this protocol (*see* **Note 2**).

Begin to amplify the *white* mutant strain a couple of weeks before the injections are scheduled.

2. Three days before injections start transfer an appropriate number of *white* mutant flies into egg-laying cages to allow them to get used to the new environment (with our egg-laying cages we take up to 800 flies per cage; we do not especially balance the number of females and males). Cover the cages with apple juice plates and add a drop of yeast paste to the plates to make sure that the flies are well nourished **(Fig. 1A)**. Until the day of injection, incubate flies at 25°C in a quiet place and change the plates at least once a day (*see* **Note 3**).

3.2. Injection Mix Preparation

1. Make midi preparations of your construct of interest and of the "helper–plasmid," which serves as the transposase source by using a commercially available midi plasmid purification kit and quantify DNA by OD_{260} measurement (*see* **Note 4**).
2. Mix approx 4 μg of your plasmid of interest with approx 1 μg of the helper plasmid, add 1 μL of 10X injection buffer, 1 μL of 2% phenol red and scale up to 10 μL with sterile water (*see* **Note 5**).
3. Spin the injection mix for 15 min at maximum speed (~16,000*g*) in a desk centrifuge to precipitate junk. Carefully take 1 μL of the supernatant to fill the injection needle. The rest of the injection mix can be stored at –20°C and should be centrifuged again before each usage.

3.3. Preparation of the Injection Needle

1. Preparation of the injection needle requires glass capillaries and a micropipet puller. In order to produce appropriate needles the settings of the puller have to be adjusted according to the capillaries and the puller used. Several parameters influence the shape of the needle and have to be tested individually. In general, length and tapering of the needle depends on the temperature of the glass and the velocity of the pulling process. Needles that are too long will bend and break and those that are too short will damage the embryos and reduce the survival rate (*see* **Note 6**). Injection needles can be stored in a Petri dish on a bar of plasticine before and after they are sharpened **(Fig. 1B)**.
2. To sharpen the injection needle to an appropriate inner diameter (~5 μm) a tip grinder is used. The injection needle is inserted into the tip grinder under an angle of 30° and sharpened for about 5 min on a wet rotating grinding stone (*see* **Note 7**).

3.4. Filling of the Injection Needle

1. Insert the sharpened injection needle into the pipet holder of the micromanipulator. We use a manual micromanipulator with a ball joint, which attaches the pipet holder to the headstage of the micromanipulator. The ball joint enables the adjustment of the angle height and distance of the pipet holder. A rather simple microscope is sufficient for microinjection. To ensure a vibration-free surface it is recommended to place the micromanipulator and the microscope on a heavy metal plate with rubber bumpers **(Fig. 1C)** (*see* **Note 8**).

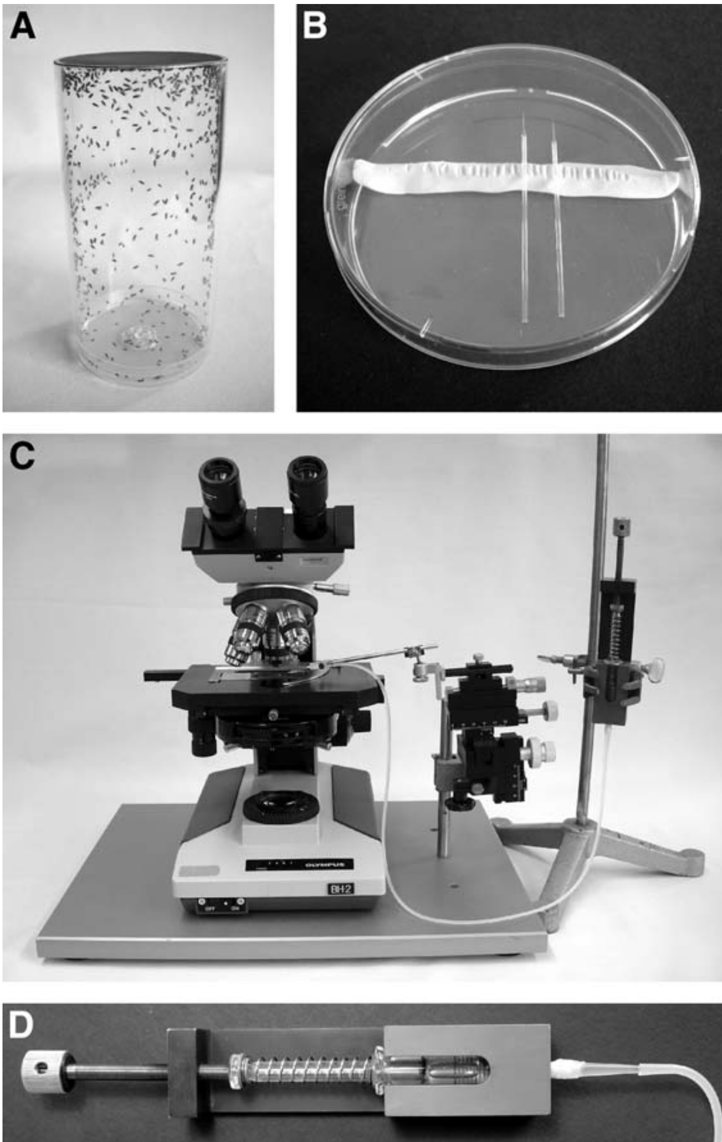

Fig. 1. Tools required for P-element-mediated germline transformation I. **(A)** A self-made 1000 mL acrylic glass egg-laying cage covered on the top side with a fly screen and mounted onto an apple juice agar plate with yeast paste. **(B)** Injection needles stored on a bar of plasticine in a Petri dish to protect them from dust. **(C)** The manual microinjection apparatus used by the authors. Microscope and micromanipulator are mounted on a heavy metal plate with rubber bumpers to reduce vibrations. **(D)** Higher magnification of the self-made manual microinjector. An oil-filled 1-mL glass syringe is inserted into the self-made syringe driver and connected through a silicone tube to an injection capillary (not shown).

2. Fill the 1-mL glass syringe of the microinjector, which is a manual syringe driver for hydraulic control of injection needles **(Fig. 1D)**, and the attached silicone tube completely with 3S Voltalef oil. Connect the injection needle through the silicone tube to the prepared microinjector.
3. Fill the injection needle with 3S Voltalef oil by gently turning the driver of the injector until oil droplets appear on the tip of the needle (*see* **Note 9**).
4. Put 1 μL of the centrifuged injection mixture on a cover slide (20×20 mm^2) that has been coated with Repel-Silane. This treatment will facilitate the uptake of the injection mixture into the injection needle (the injection mixture forms a rounded drop). Fix the cover slide on a microscope slide by placing it in a small drop of 10S Voltalef oil and gently pressing both slides together and place the microscope/cover slide sandwich on the microscope stage.
5. Under optical control bring the oil-filled injection needle and the injection mixture into the field of vision, lower the injection needle into the injection mix, and gently turn the driver of the injector so that the injection mixture is sucked constantly and slowly into the tip of the needle.
6. Once a sufficient amount of injection mixture has been taken up by the needle (uptake of half of the injection mixture is normally sufficient for 1 d of injection) turn the driver of the injector so that an equilibrium is formed between influx and efflux. Place a drop of 3S Voltalef oil next to the injection mixture so that both fluids contact each other and move the filled needle into the 3S Voltalef oil. Adjust the pressure in the injector so that neither injection mixture leaves the needle nor oil enters it (*see* **Note 10**).
7. If flies are kept under a normal day/night cycle they usually start laying eggs from the early afternoon on until late in the evening. Plan your microinjections accordingly, i.e., do the setup of the system described in **Subheadings 3.2–3.4.** preferentially in the morning and schedule microinjections for the afternoon.

3.5. Embryo Collection

1. On the day of injection change the apple juice plates every hour to empty females from old embryos and synchronize the egg laying (*see* **Note 11**). For injections harvest embryos from a 30 min egg-laying period with a fine paintbrush, transfer them into a small steel mesh basket **(Fig. 2A)** and wash them thoroughly with water (*see* **Note 12**).
2. Place the steel mesh basket with the cleaned embryos into a Petri dish with 7% sodium hypochlorite solution for 2 min to chemically remove the chorion (*see* **Note 13**). Wash dechorionated embryos thoroughly with water and place the steel mesh basket in a Petri dish with water. Dechorionated embryos will appear glossy and float on the surface.
3. Pick up the dechorionated embryos with a brush, remove excessive water by briefly touching a paper towel, and transfer them to a rectangular piece of apple juice plate (20×20 mm^2) that has been placed on a microscope slide. This will protect embryos from drying out.
4. Under a stereo dissecting microscope orient the embryos with a blunt straight teasing needle in a straight row (do this near the edge of the apple juice plate and leave

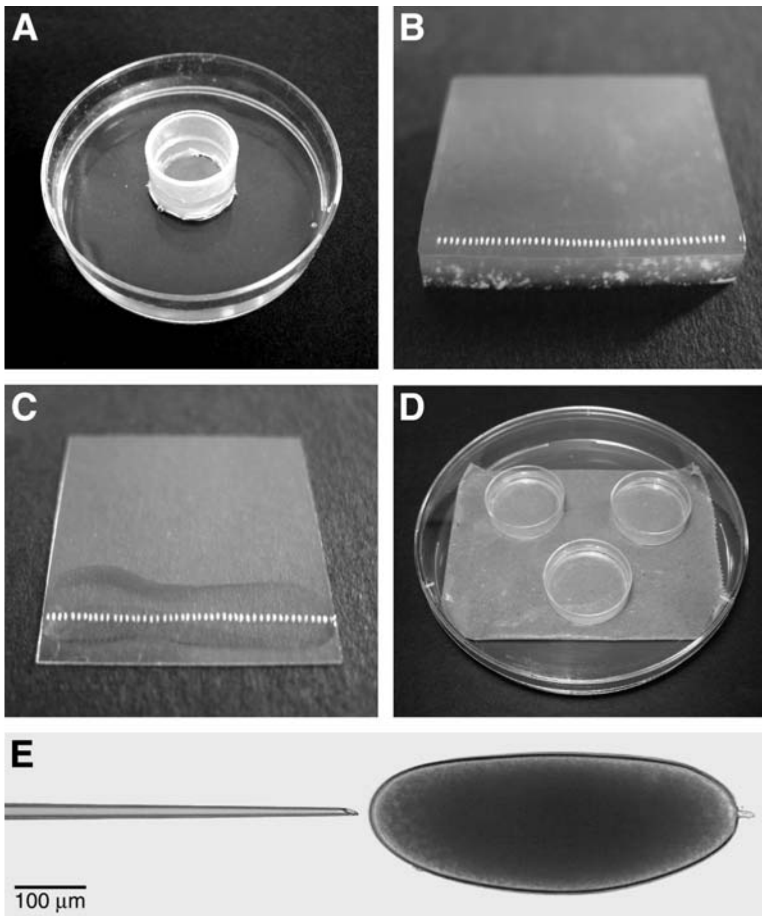

Fig. 2. Tools required for P-element-mediated germline transformation II. **(A)** A self-made steel mesh basket for dechorionating and washing of *Drosophila* embryos placed in a Petri dish (35 × 10 mm^2). **(B)** Alignment of dechorionated and oriented embryos on a rectangular piece of apple juice plate. **(C)** Embryos on a glue-coated cover slide under 10S Voltalef oil after transfer from a rectangular piece of apple juice plate and drying. **(D)** A water-moist chamber for incubation of injected embryos. **(E)** A preblastoderm embryo and a suitable injection needle shortly before injection into the posterior pole. Notice the relative size of the beveled tip of the injection needle in comparison with the embryo.

some space between them to prevent oxygen depletion) so that their anterior poles marked by the small outgrowth of the micropyle all point to the edge of the apple juice plate **(Fig. 2B)** (*see* **Note 14**).

5. By gently pressing a glue-coated cover slide (20 × 20 mm^2) onto the lined-up embryos transfer them to the cover slide. Their posterior ends should point to the nearby edge of the cover slide.

6. Before injecting them embryos have to be dried correctly. The time necessary for drying depends on the surrounding temperature and air humidity and has to be tested individually (*see* **Note 15**). To determine the correct drying grade gently touch an embryo with a blunt straight teasing needle and move the needle carefully over its surface. A furrow should form that has to disappear once the needle has been retracted. If you have not yet reached the correct drying grade you will only observe a small indentation but no furrow at the point of contact. In the case of an overdried embryo the furrow will not disappear but remain after needle retraction. Overlay properly dried embryos immediately with a drop of 10S Voltalef oil. This prevents them from drying out any further **(Fig. 2C)**.

3.6. Microinjection

1. Fix the cover slide with the embryos on a microscope slide by placing it in a small drop of 10S Voltalef oil and gently pressing both slides together.
2. Exchange the microscope/cover slide sandwich with the 3S Voltalef oil, where the injection needle has been stored in so far, for the microscope/cover slide sandwich with the dried embryos submerged in 10S Voltalef oil. Orient it so that the posterior ends of the embryos point into the direction of the injection needle.
3. Bring the first embryo in the row and the injection needle into the field of vision and into the same focal plane **(Fig. 2E)**. The tip of the injection needle should be in the center of the field of vision. During one injection session there should be no further need to move the injection needle beside minor corrections in the z-axis. Instead embryos are shifted by gently moving the microscope stage.
4. Touch the tip of the injection needle with the center of the posterior back of the first embryo. A small indentation forms. By retracting the back of the embryo from the needle tip the indentation should vanish proving correct desiccation of the embryo.
5. Penetrate the embryo with the tip of the injection needle and avoid entering it to more than 1/4 of its overall length (*see* **Note 16**). Retract the embryo until the needle is barely inside of it. Only minimal leakage of cytoplasm is tolerable. If the pressure from the microinjector is balanced correctly the injection mixture will leak automatically and slowly out of the injection needle. If this is not the case gently adjust the pressure by turning the driver of the microinjector in small steps. The influx of the injection mixture is visualized by the phenol red dye. A sufficient amount of injection mixture is injected once you faintly see the red dye. Overcome the tendency to inject too much, because this will not help but harm the embryo.
6. Retract the embryo from the injection needle, move over to the next one by moving the microscope stage and repeat the injection procedure.
7. You may encounter embryos that are too old for injection and therefore, are no longer suited for germline transformation. They can be killed by penetrating them completely (*see* **Note 17**).
8. Embryo preparation and microinjection should take no longer than 25 min to be ready before the next 30 min egg-laying period is finished.

Table 1
Transformation Results From Microinjection of a P-Element-Based Transformation Vector

Injected embryos	Hatched larvae	Eclosed flies	Fertile flies	Transgenic flies
1013	514	217	175	23

Exemplary transformation frequency of a P-element transformation vector for enhancer detection, $pCAB_{70}$, with an overall vector-insert size of 22 kb ***(29)***. From 175 fertile G_0 flies 23 independent transgenic lines could be established, which corresponds to a transformation rate of 13.1%. Related to the number of injected embryos this equals an overall transformation frequency of 2.3%. In the described experiment three transgenic lines could be mapped to the 1st, six to the 2nd, and fourteen to the 3rd chromosome. Four of them were derived from double-insertion events that could be separated during the mapping procedure.

3.7. Postinjection Care

1. After injection place the cover slide with the injected embryos in a small Petri dish (35×10 mm^2) and overlay it with 3S Voltalef oil. This on the one hand allows gas exchange as embryos need oxygen for development and on the other hand prevents their desiccation.
2. Put the small Petri dish with the embryos into a water-moisted chamber (e.g., Petri dish, 145×20 mm^2; **Fig. 2D**) and incubate them at 18°C.
3. On the day after tomorrow those larvae that survived the injection procedure will hatch (*see* **Note 18**). They will either crawl on the cover slide, the walls of the Petri dish, or float in the oil. Under optical control take a blunt straight teasing needle, carefully reach under the larvae and lift them with the needle. Larvae tend to adhere to it. Do not use tweezers! You could squeeze the larvae to death! Transfer the larvae to a medium-sized vial with fresh food (~15 mL), which in advance has been prepared as follows: stir the food with a small spatula, add some sterile water to make it more fluid (this will facilitate the larvae to invade the food), and add a small drop of yeast paste. Always check the needle under a stereo dissecting microscope for successfull transfer of the larvae into the vial. Collect approx 50 larvae into one vial and incubate them at 18°C. Larvae derived from the injection of different constructs should of course be transferred to separate vials. After about 14 d flies will eclose. Shortly before hatching, lay the vial on the side to avoid newly eclosed flies from falling into the food (*see* **Note 19**).

3.8. Establishment and Mapping of Transgenic Lines

1. Collect hatched flies (G_0) and cross males and virgins individually with three w^-; *Gla/CyO* flies of the opposite sex (*see* **Note 20**). Incubate the crossings at 25°C.
2. Screen each single crossing for offsprings with pigmented eyes (F_1) (*see* **Note 21**). The eye color of these transgenic flies can vary from bright yellow to dark red depending on the insertion site of the transgene (for simplicity we will generally refer to transgenic flies as "red-eyed") **(Table 1)**. From one vial you may also encounter

Table 2
A Simple Table to Determine the Chromosomal Insertion of a Transgene

w^-;*Gla/CyO* virgins x	F_2:w^+; *CyO*		F_2:w^+, *Gla*		F_2:w^-; *Gla/ CyO*		F_2:w^+; *Gla/ CyO*		F_2:w^-, *CyO*		F_2:w^-, *Gla*		Insertion on chromosome
	m	f	m	f	m	f	m	f	m	f	m	f	
F_1 male:	–	+	–	+	+	–	–	+	+	–	+		1st
w^+; *Gla*	+	+	+	+	+	+	–	–	–	–	–	–	2nd
or *CyO*	+	+	+	+	+	+	+	+	+	+	+	+	3rd

According to the table, write down the occuring phenotypes of the F_2 generation and directly determine the chromosomal insertion site of your transgene. Note that the table is only valid for a distinction between a 1st and a 3rd chromosomal insertion when transgenic F_1 males are used.

F_1-flies with different eye colors. This is indicative of multiple insertions (*see* **Note 22**). In this case choose those with brighter eye colors (putative single insertions) for further crosses. "Red-eyed" *CyO* males should be preferred for further mapping the localization of the transgene, although in general "red-eyed" *Gla* or *CyO* flies of both sexes can be used (*see* **Note 23**). If all transgenic flies from one vial display the same eye color it is likely that they are derived from one single transgene-insertion event. In this case cross two transgenic F_1-flies individually for mapping the chromosomal insertion site. Otherwise take two flies of each individual eye color (*see* **Note 24**).

3. Cross each transgenic F_1-fly individually with three w^-; *Gla/CyO* flies of the opposite sex and incubate the crossings at 25°C.
4. Collect virgins and males from the offspring of each individual crossing (F_2), determine their genotypes, and identify the chromosomal insertion site of the transgene with the help of **Table 2** (*see* **Note 25**).
5. In case of a second chromosome insertion cross "red-eyed" *CyO* males with "red-eyed" *CyO* virgins. If 25% of their descendants (F_3) have straight wings this means that the transgene insertion is homozygous viable. Establish a stock by crossing straight-winged flies (*see* **Note 26**). Otherwise keep the homozygous lethal stock balanced over *CyO*.

   If the crossing scheme suggests a third chromosomal insertion, cross "red-eyed" *CyO* males with virgins from a third chromosomal balancer stock (e.g., w^-; *TM3/TM6B*). Interbreed transgenic *TM3* males with "red-eyed" *TM3* virgins. If 25% of their descendants (F_3) lack the dominant *TM3* marker gene *Stubble* (short, thick bristles) the transgene insertion is homozygous viable. Establish a stock by crossing non-*Stubble* flies. If all the offsprings carry the *TM3* balancer chromosome keep the transgene balanced over *TM3*. Depending on your personal preferences establishment of a third chromosomal insertion can of course also be done using the *TM6B* balancer chromosome.

   For a first chromosome insertion mate "red-eyed" *CyO* virgins with *FM7/Y* males. If "red-eyed" males show up in the offsprings (F_3) this demonstrates that the transgene is homozygous viable. Mate them with "red-eyed" *FM7* virgins from the

same crossing and from their descendants interbreed "red-eyed" males with "red-eyed" non-*FM7* virgins to establish a stock. If no "red-eyed" males appear in the F_3 generation "red-eyed" *FM7* virgins have to be crossed against *FM7/Y* males again to balance the homozygous lethal transgene (*see* **Note 27**).

6. It is desirable to obtain at least two independent transgenic lines with P-elements located on the 1st, the 2nd, and the 3rd chromosomes. Insertions on different chromosomes facilitate bringing your transgene into certain genetic backgrounds without the need to recombine it. Additionally, the activity of the same transgene can vary considerably depending on its integration site. If your transgene will be used for misexpression experiments (e.g., UAS-effector lines) ***(24)***, it is always advantageous to have strong- and weak-expressing strains. In many instances there seems to be a correlation between the activity of the transgene and the marker gene, for example, the *white* mini-gene. In this case the intensity of the eye color can be used to roughly estimate the probable expression level of the transgene.

4. Notes

1. Depending on the kind of adhesive tape you are using the amount of heptane may have to be adjusted to obtain a suitable liquid glue. Ingredients of some adhesive tapes are toxic to the embryos. We obtained best results when using Packaging Tape (tesapack) or Extra Power Perfect Tape from tesa, Hamburg, Germany.
2. As an alternative to the w^{1118} stock a *y w* stock can be used for injection. The *y* phenotype provides an extra security to ensure that pigmented flies in the F_1 generation are not contaminants. Besides the w^{1118} or a *y w* stock it is also possible to use flies with a stable source of transposase for P-element-mediated germline transformation (Δ2-3[99B], Bloomington stock 3629). Its potential advantage is that the presence of the transposase is ensured in any germ cell that takes up the plasmid vector ***(17)***. On the other hand, in following crosses one has to get rid of the Δ2-3(99B) chromosome to avoid further transposition events.
3. The size of the egg-laying cages has to be coordinated with the number of flies. Overcrowding stresses the flies and reduces the egg-laying rate. The flies should also not be too old and between 4 and 8 d of age.
4. Different "helper-plasmids" can be used and appear to be equally suited, for example, the "wings-clipped" pπ25.7wc construct ***(4)*** or the pUChsπΔ2-3 plasmid ***(5)***. Check the purity of your construct of interest and of the "helper-plasmid" by measuring the OD_{260}:OD_{280} ratio. Contaminated DNA may harm injected embryos and reduce the survival rate.
5. The transformation efficiency of a construct also depends on its size. Large constructs (>12 kb) show a significantly lower integration rate ***(25)***. This might be partially compensated by scaling up their concentration in the injection mixture. However, too highly concentrated injection mixtures are more viscous and may clog the injection needle.
6. Reproducible production of suitable injection needles is critical for successful performance of germline transformation and requires a high-quality micropipet puller. We obtained best results with the Flaming/Brown-Type Micropipet Puller P-97 (Sutter Instrument) using the following settings: 1. cycle: heat = 460, pull = 0, vel = 30,

del = 50; 2. cycle: heat = 460, pull = 100, vel = 60, del = 5. However, these settings depend on the current heating filament and are only valid when used in conjunction with appropriate glass capillaries (in our case GB120F-10 from Science Services, Hofheim, Germany). These injection needles are sealed at their tip and have to be opened and beveled by grinding.

7. Instead of sharpening the injection needle on a tip grinder it can also be adapted to a suitable inner diameter by breaking it. This can be done under a stereo dissecting microscope with a tweezer or a sharp scalpel. However, in our hand usage of a tip grinder gave more reproducible results and injection needles of constant quality.
8. It is recommended but not absolutely required to do the microinjections in an air-conditioned room at about 18°C. This gives you more time flexibility as embryonic development is slowed down and the time for the injections is prolonged. Furthermore, it has been suggested that the survival rate of embryos injected at 18°C is higher owing to reduced leakage of cytoplasm.
9. Make sure that there are no air bubbles in the injection needle and the connected silicone tube. This often leads to occlusion of the needle. Use the Voltalef 3S and Voltalef 10S oil sparingly, because it is very expensive.
10. Whenever you are not injecting store the injection needle in 3S Voltalef oil to prevent the injection mixture from drying. Long air-exposure will clog the needle and normally requires preparation and filling of a new one.
11. Warm the apple juice plates to room temperature before usage. Flies do not like to lay eggs onto cold plates. Egg laying can further be stimulated by putting a drop of 10% acetic acid on the apple juice plates and distributing it evenly. However, avoid moistening the plates too much because otherwise flies will stick to the surface. To save apple juice plates they can be reused: remove all remaining eggs with water, briefly dry the apple juice plate, and put fresh yeast paste onto it. At 1 d of injection two apple juice plates can be used in rotation for one egg-laying cage.
12. Embryos must be injected before pole cell formation (stage 2), which at 22°C starts approx 80 min after egg laying ***(26)***.
13. Do not incubate the embryos for more than 2 min in sodium hypochlorite because this will harm them. Alternatively, the chorion can also be removed manually by hand peeling: collect and wash the embryos as described, dry them briefly on a paper towel and transfer them to a microscope slide that is partially covered with a piece of double-sided adhesive tape. Under a stereo dissecting microscope carefully move the adherent embryos with a blunt straight teasing needle. The chorion will break open and stick to the adhesive tape whereas the dechorionated embryos will adhere to the needle. Transfer them with the needle to a rectangular piece of apple juice plate and proceed with orienting them.

    It is also possible to inject *Drosophila* embryos without removing the chorion. Although the general procedure of germline transformation is the same, this requires preparation of an injection needle with different properties ***(25)***.
14. Dim the light source of your stereo dissecting microscope. Full light may produce too much heat and quicken the drying of the embryos. Sort out embryos that are too old for injection (beyond stage 2). Suitable embryos appear cloudy. The number of embryos to line up for injection mainly depends on your experience. As a beginner

start with about 20–40. If you are more experienced you may manage to inject up to 100 embryos (arranged in two rows) during one round of injection.

15. Proper drying of the embryos is a critical step. Insufficient dessication results in leakage of cytoplasm from the embryo when it is penetrated by the injection needle. Excessive drying leads to embryonic death, too. Doing injections in a humidity- and temperature-controlled room helps estimating the correct drying point.
16. Entering the embryo too deeply may disturb morphogen gradients and lead to developmental defects and death.
17. Killing over-aged embryo with the injection needle is risky. Embryonic material can attach to the needle and finally occlude it. Alternatively, number lined-up embryos and note down those that are over-aged. Kill them after finishing the whole injection procedure with a blunt straight teasing needle.
18. If you are familiar with germline transformation expect 30–70% of the injected embryos to hatch.
19. The G_0 flies will be white-eyed, because the insertion of the transgene is restricted to the germ cells. Do not expect all hatched larvae to survive until adulthood. The survival rate will be around 50%. Moreover up to 50% of the G_0 adults can be sterile owing to disruption of pole cell formation or damage of other posterior structures.
20. Crossing the G_0 flies directly to a balanced *w*⁻-stock will save you one generation during the mapping process. In general any well-reproducing 2nd or 3rd chromosomes balancer stock can be used. We use a *w*⁻*; Gla/CyO* balancer stock, because it can be kept at all standard temperatures (18–25°C) and reproduces quite well. Usage of three *w*⁻*; Gla/CyO* flies for the G_0-crossing turned out to be a good compromise between the effort of virgin collection and successful reproduction of the G_0 flies. Note that the *Cy* phenotype (curled wings) is temperature-sensitive and at 18°C may only barely be visible. After approx 7 d transfer each G_0 cross once to a new vial to increase the number of offspring and minimize the loss of transformants.
21. The transformation efficiency is measured by the ratio between fertile G_0 flies and established transgenic stocks and in our hands ranges between 10 and 25%. Based on the number of injected embryos this corresponds to an estimated transformation efficiency between 1 and 3% **(Table 1)**. Remember that besides your personal experience and technical equipment the size of your transgene considerably influences the transformation rate.
22. Owing to a certain degree of dosis compensation, transgenic males have a slightly darker eye color than the corresponding females (even autosomal insertions). Do not confuse this with a separate insertion of the transgene.
23. Whenever possible avoid using the *Gla* chromosome, as this is not a balancer. It is just marked with the *Gla* mutation (smaller eyes with a glass-like texture) as a dominant, homozygous lethal marker.
24. From every separate transgene-insertion event cross two F_1-flies individually to *w*⁻*; Gla/CyO* balancer flies. This reduces the probability to lose a whole transgenic line if one transformant dies.
25. **Table 2** helps you to map the transgene insertion to a particular chromosome. However, the scheme is only valid without limitation when “red-eyed” males are

used. Otherwise discrimination between an insertion on the 1st or on the 3rd chromosome is not possible.

26. In comparison with the heterozygous situation, homozygosity of the transgene leads to a darker eye color as now two copies of the *white* mini-gene are present. Therefore, homozygotes may also be selected just on the basis of their eye color.
27. In general transgene insertion can occur randomly on any of the four chromosomes with the probability being relative to the length of the corresponding chromosome. However, transgene insertions on the 4th chromosome are very rare as it is very small and mainly heterochromatic. P-elements appear to favor euchromatic regions on a chromosome for integration ***(27)*** and have a tendency to integrate at the 5′-end of genes ***(28)***.

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