Drosophila germline transformation

(Nicolas Gompel, Eva Ayla Schröder, October 2015)

This protocol is for the genetic transformation of *Drosophila melanogaster* and other *Drosophila* species, using transposable elements. The difference to classical protocols (*e.g.*, Spradling & Rubin, 1982) is that embryos are not dechorionated. It can be adapted to the injection of CRISPR RNA or DNA, and repair matrices for transgene integration.

Material

Injection mix

This protocol allows the injection of various types of nucleic acid mixes. These include transformation vectors (P-elements, Piggybac, Hermes, Phi-C31) and their helper plasmids or RNA, CRISPR sgRNA, CRISPR plasmids and Cas9 mRNA (or even Cas9 protein).

The transposable elements used for transformation are modified such that they can no longer mobilize autonomously. The recombinase (integrase, transposase) mediating their integration is provided in *trans*. It can be produced endogenously in the embryonic germline cells under the control of a specific promoter (best option; *e.g.*, *nanos* promoter), or provided along with the vector to be transformed, in the form of a helper plasmid with the recombinase gene (reasonable option), or in the form of the recombinase mRNA or protein (stay-away-from option).

The transformation of fragments of various sizes, from plasmids to fosmids and to BACs is possible. The overall principle is identical, the handling of the injection mix changes. Depending on the type of integration system, construct size and the source of integrase, the throughput of transformation varies.

Mix preparation

1. Mix ingredients in a 0.5 ml eppendorf tube. Typically prepare 10 to 20 µl of mix. In our hands, the precise concentration of each reagent was not very important, and we found that very low (0,15 µg/µl) concentrations of the vector were sufficient to grant integration at a reasonable rate. What is very helpful, is to keep the overall concentration low (0.5 µg/µl or lower), to keep the mix fluidity high and facilitate the injection.

A typical mix for P element or ϕC31/attP injection would be:

10 µl of construct vector (miniprep, ≃300 ng/µl); 5 µl of helper plasmid if needed (miniprep, ≃300 ng/µl); optional: 1-2 µl of filtered food dye (*e.g.*, McCormick green food coloring; 0.2 µm filter)

2. All mixes are diluted in ddH_2O (other protocols use phosphate buffers)

3. Vortex mix (for small plasmids) or gently mix with a pipette (for bigger constructs such as fosmids and BACs)

4. Centrifuge mix for 10 minutes at 14 000 rpm (bench-top centrifuge)

5. Carefully harvest most of the supernatant (*e.g.,* 17 µl out of 20 µl) with a pipette and transfer it to a clean eppendorf tube. Use this centrifuged mix to fill needles. The mix can be kept at 4°C for weeks.

[Alternative to 4-5, run your mix through a Millex-GV column (Millipore)]

Flies

Fly stocks should be amplified in advance, for two reasons: (i) to get enough eggs for injection and (ii) to collect virgin females for subsequent crosses with the injected flies (G0).

The specific stocks vary with what needs to be injected, and what transformation marker is used.

Transfer ~300 mature flies to an egg-lay cage (see picture below; *e.g.*, Flystuff Cat #59-100), 1-3 days prior to the injection to accustom the flies. Placing the cage under a desk lamp controlled with a programmable timer switch helps to have regular egg collections. Change the egg-lay cap (*e.g.*, agar-molasses: see picture below, recipe to the right) at least once per day; spread some yeast paste in the center.

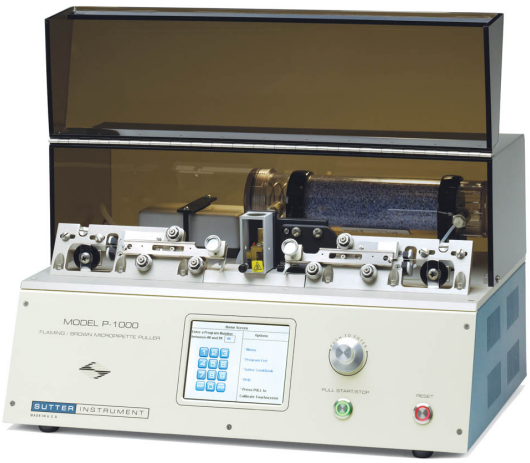
Egg lay caps
-500 ml tap water, boil
-25 g agar, dissolve completely while mixing from time to time
-200 ml molasses or 200 ml grape juice
-[optional: When cooled below 70°C: 6 ml Tegosept/Nipagin solution (10 g in 100 ml 95% ethanol)]
-Pour plates (ca. 50)
-Store solidified plates with lid at 4°C.

The conditions for embryo collection should be such that at least 200 freshly laid embryos are available every 20-30 min when injecting (see picture below).

Needles

Pulling

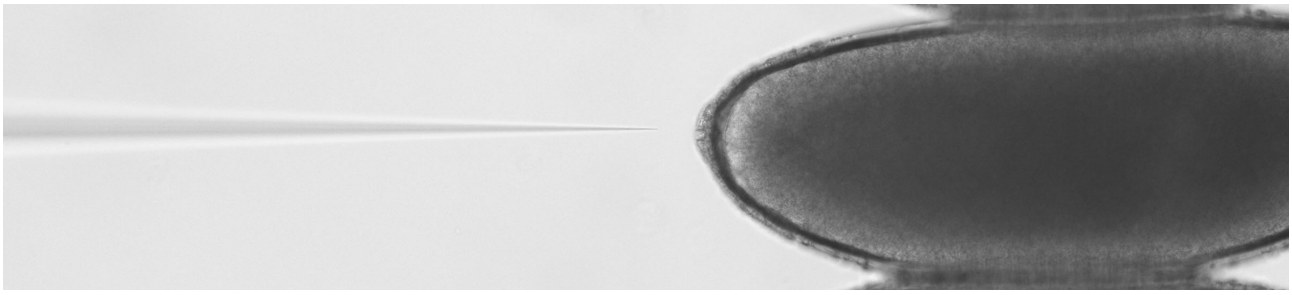
– Do not operate the needle puller without adequate training! –



The quality of needles is critical for the smoothness of the injection process, the embryo survival and the transformation efficiency. Needles should be pulled on any Sutter instruments brand horizontal puller (see picture on the right; *e.g.*, P-1000 Flaming/Brown Micropipette Puller). Needles are pulled from 1.0 mm OD borosilicate capillaries with omega dot fiber (*e.g.*, Sutter instruments #BF100-50-10). The settings for the design of optimal needles are linked to the capillary batch and the shape/type/position of the heating filament. These parameters define a *ramp value* –the melting point of the capillaries under these conditions. A specific function of the puller calculates this ramp value. Refer to the manual of your puller for details.

The ideal needle to inject an embryo in its chorion should have a short taper, a thin tip and no discontinuity or step. A needle with a long taper will bend against the embryo and will not go through the

chorion. A needle with a blunt tip will cause damages and cell leakage, thereby increasing embryonic lethality. Different parameters influence the shape and properties of the needle and the effect produced by changing any of them (heat, velocity of pull, pressure of gas flow, number of steps) is not intuitive. The most useful documentation to design proper needles is the pipette cookbook provided by Sutter Instruments. In addition, Miller et al. (2002) published a very useful guideline for designing suitable needles specifically for Drosophila eggs in their chorion.



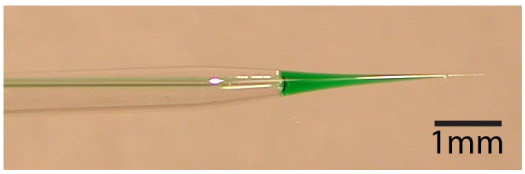
For instance, a successful needle type that we have been using for a long time, was obtained using a box filament (Sutter Instrument FB230B) with thick walled glass capillaries (Sutter instruments #BF100-50-10, ramp = 625) and the following parameters:

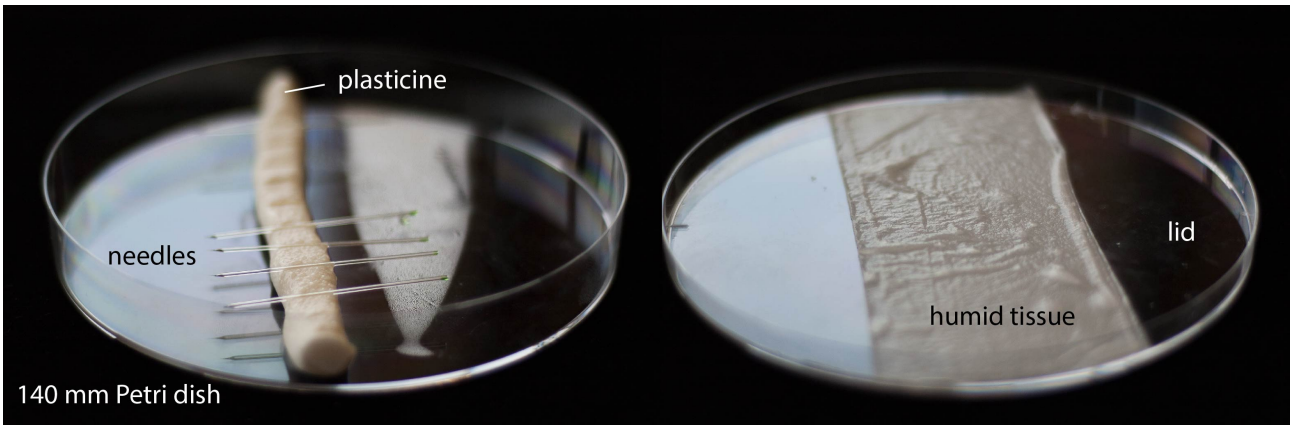
Pressure: 300 Heat: 505 Pull: 20 Velocity: 60 Delay: n/a Time: 250 Looping: none

The needles can be prepared in larger sets and stored for months in 140 mm Petri dishes, on a strip of plasticine, in a dry location at room temperature (as on picture below, but without humid tissue).

Filling

Fill needles with the smallest amount of injection mix (1 µl or less) using an Eppendorf GELoader tip (eppendorf) (although filling in a drop of mix with a regular tip at the end of the needle works as well - the mix migrates to the needle tip by capillarity, thanks to the omega dot fiber). Filled needles are stored in the same Petri dish as before, turned into a humid chamber by the addition of a humid, folded tissue coating the inner side of the lid (see picture below).





Needles are ideally filled hours before the injection, to allow small air bubbles, trapped at the needle tip, to disperse. One needle is generally sufficient for the injection of over 100-200 embryos. However, needles can break or get clogged and it's a good idea to fill 5-6 needles for the injection of a standard construct (400-600 embryos).

Filled needles can be stored at 4°C and be used for a few days after filling.

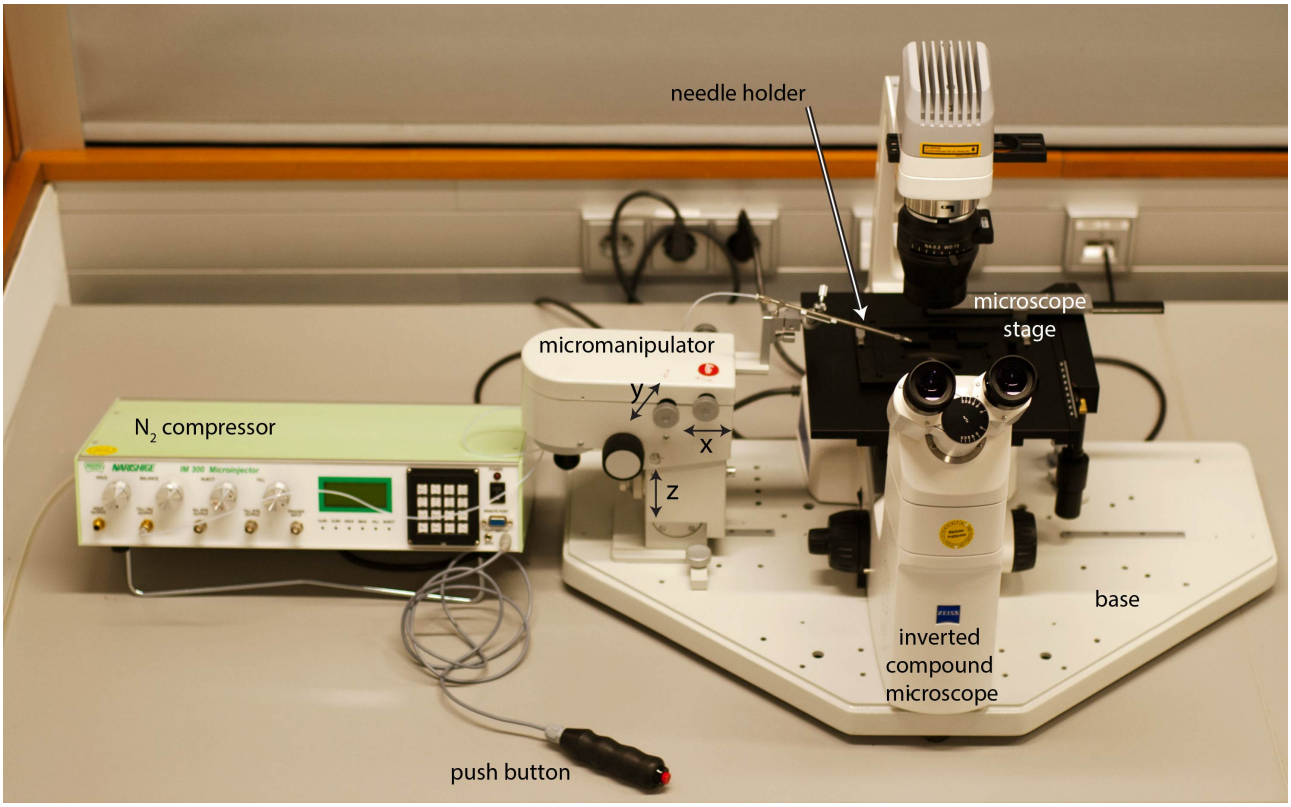
Injection setup

The injections are monitored under an inverted compound microscope equipped with a 20x objective (*e.g.,* Zeiss LD A-Plan 20x/0,35 Ph1, ZeissObjectives), bright field optics and a xy-movable stage (*e.g.*, Zeiss Axio Vert.A1).

The glass needles are fitted into a needle holder (*e.g.*, Narishige HI-7), which is controlled by a micromanipulator. The needle is also linked to an N_2 compressor that facilitates injection. We use a Narishige IM-300 injector, fitted with a hand trigger push button.

The manipulator is used for the fine-scale approach of the needle toward the embryos. We are using a Leica mechanical micromanipulator. Both the micromanipulator and the compound microscope are fixed to a heavy base.

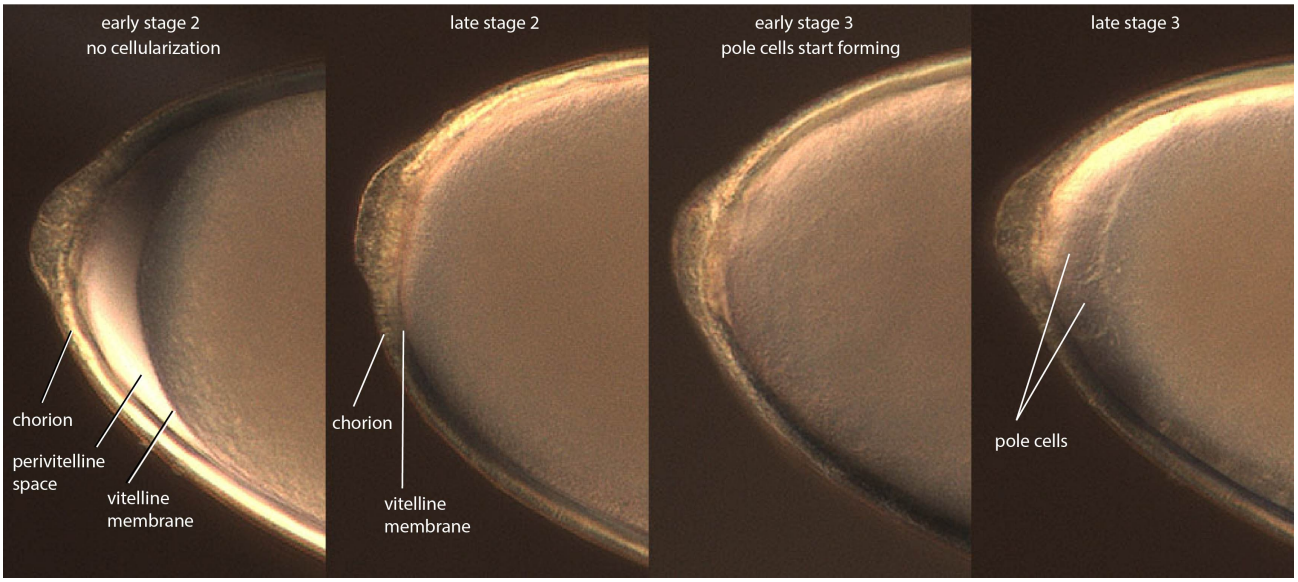
The temperature of the room, where our setup is installed varies from 20˚C to 25˚C without affecting the injection process.



Methods

Embryo collection

-2 to 3 hours before injection, change egg-lay cap every 30-60 min to flush older eggs withheld by females. The proportion of stage 2 embryos should be above 90%. The figure below shows the posterior end of embryos with increasing age between stage 2 and 3. Embryos suitable for injection should look like those at stage 2 on the images below (pole cells not formed). As soon as the pole cells are formed (stage 3), the embryos are too old for injection.



-For injection, collect a new batch of embryos every 20 minutes.

-Harvest embryos, using a moist, medium-sized brush (type 1 or 0), preferentially around the yeast paste. Transfer them to a clean mesh basket (home-made or Costar Netwell™, Corning 3477, 74 µm mesh).

-Wash the embryos thoroughly with distilled water, using a squirt bottle. Target the water spurt directly to the embryo clumps to separate the embryos.

-Briefly wash the embryos with 95% non-denatured ethanol. The ethanol penetrates between the chorion and the vitelline membrane, making the embryos translucent. This later allows a rough staging under the stereoscope.

-Wash-off the ethanol with distilled water.

-Keep the mesh basket in a bit of water in a small, clean Petri dish.

Embryo alignment

-Fix an 18x18 mm coverslip onto a microscope slide by capillarity with a droplet of water. Transfer a clutch of clean embryos onto the coverslip, using a fine, pointy, clean brush (type 000, also named 3/0).

-Start aligning the embryos with the moist brush, close to one edge of the coverslip (see pictures below). The posterior pole of the embryos must point toward the nearest coverslip edge. If possible, arrange the embryos in the same dorsal-ventral orientation, ideally with their back against the coverslip (see below). This limits the need to refocus the needle during the injection.

-The amount of water in the brush is key to move embryos around and align them with ease. Too much water will make the embryos float around, too little water, and they will be hard to move and will start to get damages. To adjust the degree of moisture, dip the brush tip into clean water (keep a Petri dish with water nearby), and then lightly touch a dry Kimwipes tissue with the brush tip. The level of moisture should be about right for aligning embryos.

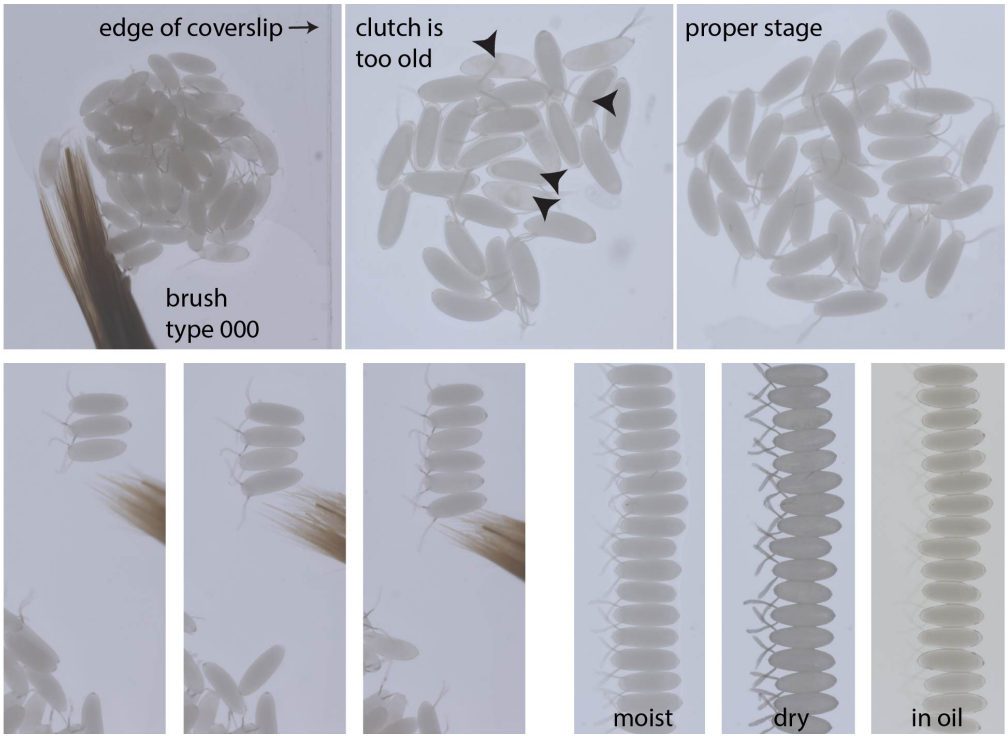
-Keep the non-aligned embryos moist during the procedure, by regularly adding water with the brush.

-Reject embryos older than stage 2 (*i.e.*, make sure, pole cells have not formed; see figure above and Campos-Ortega and Hartenstein, 1997).

-Let the embryo chorions dry completely (1-2 min after finishing the alignment) to ensure a firm attachment to the coverslip. The embryos are dry enough when the respiratory filaments are white and not translucent anymore.

-Cover the line of embryos with olive oil[1] (prefer extra virgin organic from Crete ☺). After some seconds, the oil will have entered the space between the chorion and the vitelline membrane, thereby clearing the embryos.

-Move the slide to the microscope stage for injection.



[1] Rationale for using olive oil: Drosophila embryos are classically covered with a mix of halocarbon oil (Spradling & Rubin, 1982) for injection. We found that this affects survival of all stocks, mildly so for *D. melanogaster*, dramatically for other species. Unfortunately the viscosity of halocarbon oil makes it very difficult to take it off the embryos immediately after injection. We therefore resorted to another kind of oil that could easily be removed, and olive oil came handy.

Injection

Prepositioning the needle

-Insert a filled needle into the needle-holder of the micromanipulator and tighten the screw.

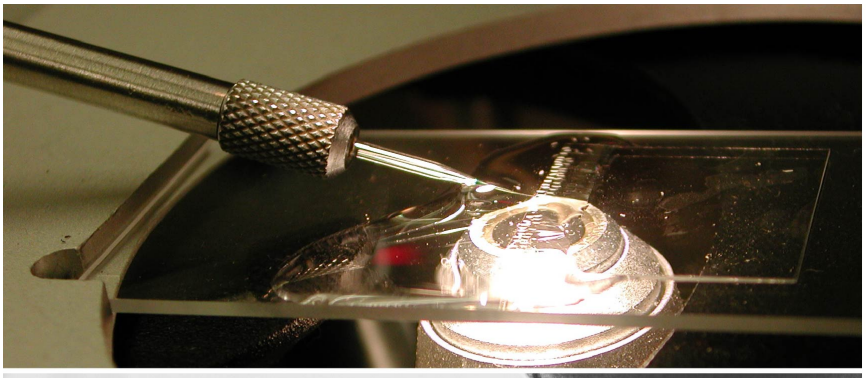
-Orient the slide on the microscope stage with the posterior pole of the embryos toward the needle. Position the embryos in the light beam. While monitoring through the eyepiece, bring the first embryo to be injected to the right side of the field of view. Bring the focus to the embryo outline. This focal plane can be recognized by the sharp outline at the interface of the vitelline membrane and the chorion (see below).

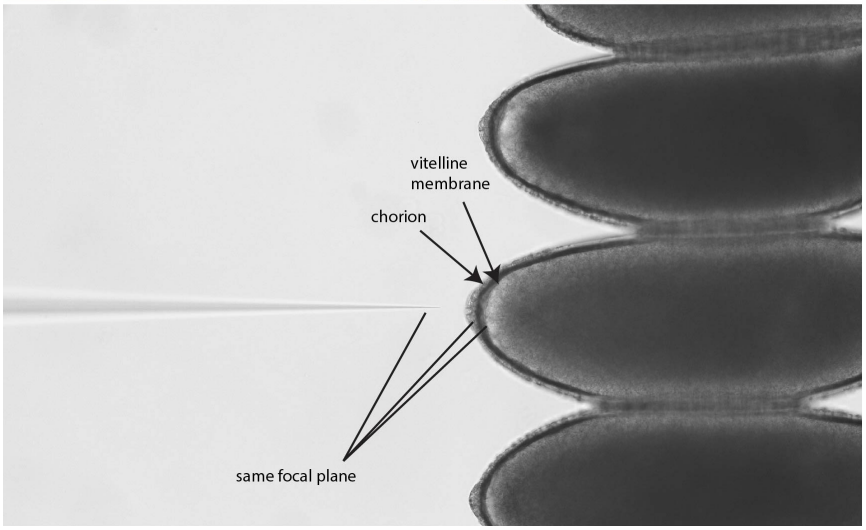
-Manually bring the needle tip close to the embryo line with the naked eye (grab and move the distal end of the needle holder).

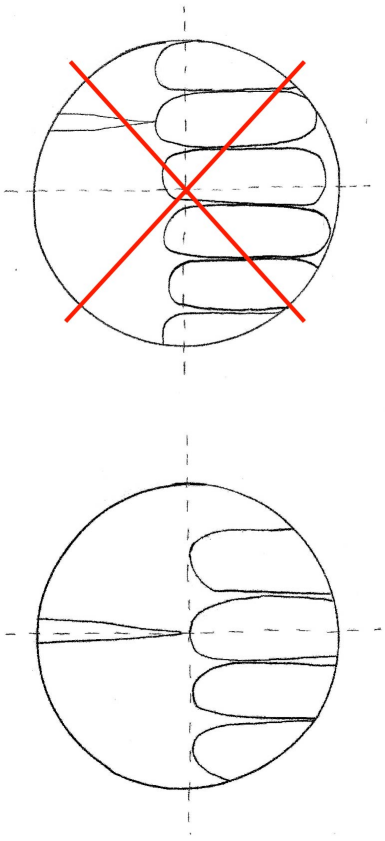
-Still monitoring with the naked eye, use the x and y knobs of the micromanipulator to bring the needle tip even closer to the first embryo of the row.

-Monitor the next steps through the eyepiece. Use the x and y knobs to hover the needle back and forth over the embryos, until you see its shadow across the field of view. Center the shadow in the field of view. Lower the needle tip with the z knob, until its tip enters the oil.

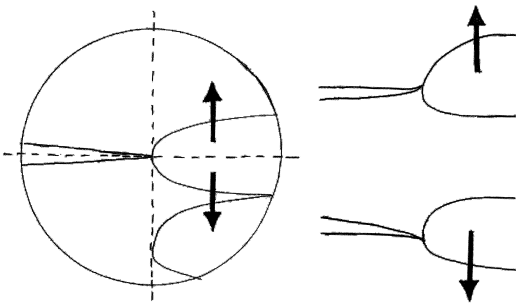
-Fine-tune the position of the needle tip with all 3 knobs to reach the final configuration (see schematic below). The needle tip must be in the same focal plane as the posterior-most end of the embryo.







Opening of the needle

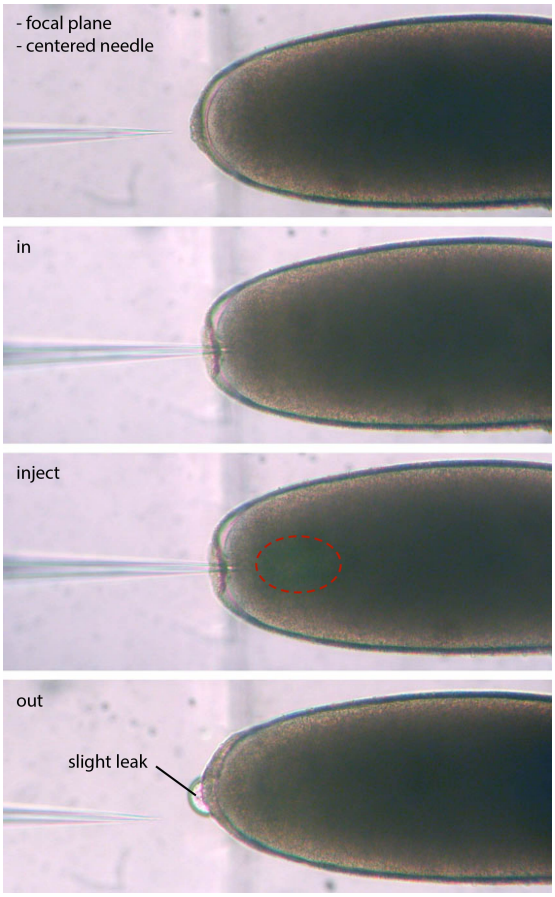


-Set the pressure, such that after a short push on the manual pressure trigger (our setup works well with 45 psi), a droplet of liquid is released at the needle tip (plunged in oil).

-If no droplet is released, carefully bring the needle tip in contact with the chorion of an embryo (by moving the microscope stage, not the needle) and move the embryo up and down along the y-axis to rub the needle tip. Do this, while repeatedly pushing the pressure button, to see if the needle opens.

-Should the previous step prove insufficient, attempt to break the needle tip open against the edge of the coverslip. Move the stage of the microscope along the x-direction, until the edge of the coverslip is at the center in the field of view (make sure the edge is covered by oil). Focalize on the coverslip edge and bring the needle into that focal plane by using the z knob of the micromanipulator. Carefully approach the coverslip edge (using the microscope stage controls) against the needle tip, while repeatedly pushing the pressure button. This is a delicate operation that takes some practice; the first attempts will probably result in a tip, too largely opened and no longer suitable for injection. Keep trying with new needles! The opening is hardly visible, but the criterion is a suitable flow of liquid at the needle tip.

Injection proper



As the needle is now open and in the same focal plane as the posterior-most end of the embryo, injection can begin. The following movements are performed, using the stage of the microscope:

-Insert the needle tip into the posterior fifth of the embryo (close to the germline nuclei) in a gentle motion. Push the pressure button and inject a drop of the mix. You will notice a small cloud of dye or the turbidity (you may occasionally see the embryo inflating). If you see none of this, inject again. Back the embryo off the needle. A small amount of liquid may leak out. If there is always a milky leak, this is the sign of a harmful injection and you may want to replace your needle.

-It is difficult to estimate the appropriate amount of mix to be injected. The dashed line in the figure shows the size of the drop in a typical injection.

-Center the next embryo in front of the needle. It may be necessary to refocalize to the outline of the new embryo, and to adjust the needle position.

-Proceed with the injection, skip embryos that have formed the pole cells (this occurs approximately 80 minutes after fertilization, Raff & Glover, 1989), as these will not integrate any DNA in their germline and may unnecessarily damage the needle.

Post-injection processing

-Use a metallic pin to poke embryos older than stage 2 under the stereoscope (to not have to cross them as adults).

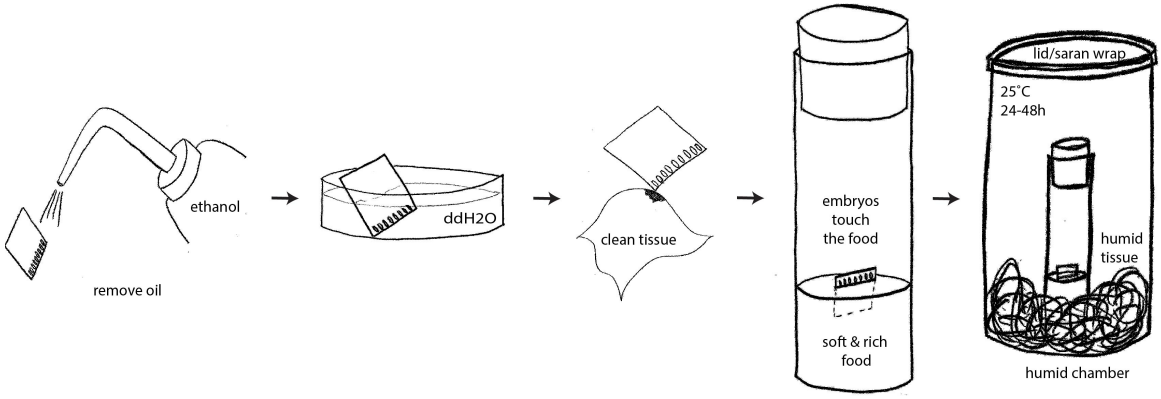
-Thoroughly wash off the olive oil, by pouring 95% non-denatured ethanol directly onto the embryos. Wash-off the ethanol with distilled water.

-Rehydrate the embryos, by plunging them into distilled water in a small Petri dish for 10 minutes (different species have different demands; a simple H_2O wash suffices for *D. melanogaster*).

-Drain the water from the coverslip, by applying its edge onto a clean tissue.

-Transfer the coverslip into a food vial (soft and rich food) and push it, embryos up, into the food, until the embryos slightly touch it. When the larvae hatch, they are forced to crawl into the food.

-Place the marked food vial into a humid chamber at 25°C, until the larvae hatch (24-48 h).



G0 crosses & F1 screening

Note: to transform a small construct (*e.g.*, an UAS construct) injecting 300-600 embryos is a good starting point. Expect 50-70% of the embryos to hatch into larvae (fewer will make it to adulthood). If the survival is lower, consider re-injecting immediately.

-Collect the hatching G0 adults and separate the sexes.

Note: Upon hatching from the pupa, the injected animals (G0) tend to get stuck in the food and die. Transferring pupae to a fresh food vial increases the number of adults to be crossed by 10-20%. Therefore spray water on the G0 pupae, to loosen them from the tube wall; collect them with a brush and stick them to the wall of a clean tube.

-Separately cross each G0 male to 3 virgin females and each G0 female (even if not a virgin) to 2 males of a stock, allowing to see the selection marker in the progeny. Each cross gets its own vial[2].

-For many Drosophila species, other than *D. melanogaster*, pair-matings yield very little progeny, probably because there is a group effect, enhancing egg-laying. This reduces the throughput of transformant identification considerably. Therefore, we pool G0 females by groups of 5-20, crossed to 5-10 males. Reciprocally, each G0 male is crossed to 10-20 virgin females.

-For several species, larvae pupate in the food and get buried. To prevent this, stick a piece of Whatman filter paper into the food for L3 larvae to pupate in.

-Efficient selection marker screening of F1 transformants is best done when 20-50 adult F1 have hatched in each vial.

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[2] G0 females can be pooled when transforming a construct with the ϕC31 system, as all the resulting insertion should be identical, and mixing them does not matter.

Troubleshooting

Problem	**Possible reason(s)**	**Solution(s)**
needles filled with the same injection mix get clogged after a few injections	-injection mix is too viscous -injection mix contains debris	-dilute the mix with water -spin the mix, transfer a fraction from the top in a new tube → fill new needles
no drop comes out of a particular needle	-needle is not open	-mechanically open the tip as explained above. Not too much!
needle gets clogged repeatedly (from poor or irregular flow to complete absence of liquid coming out)	-embryos are not clean enough (covered with yeast) -embryo flesh enters the needle tip	-use cleaner embryos, wash brush -rub the needle tip against the chorion of an embryo -replace needle -skip embryos that are too old
A good way to diagnose a consistent problem with liquid flow, is to fill needles with a mix of water and dye and see how the injection of this solution behaves. The mix should come out of the needle very easily.		
flies do not lay enough eggs	-they are not accustomed to the cage -yeast paste is not fresh enough -cage is dirty -too few flies in the cage, too old, or too young	-wait another day, change the caps more often -check yeast paste texture (should be like toothpaste) and odor (should not smell like cheese) -clean the cage with water, replace flies
embryos are too old upon 30 min collection	-females withhold their eggs	-change the caps more often before starting to harvest, in order to flush all withheld eggs
embryos stick together during alignment	-yeast is not properly removed	-only harvest embryos around the yeast -wash longer and stronger with water
Embryo is not firmly attached to the coverslip	-the embryos are not dry enough when oil is added	-increase drying time before adding oil
low survival rate of the G0 embryos	-not enough rehydration -not enough oil removed (choke) -food is not rich enough -they dry out -injection harmed them too much	Run controls to identify, at what stage the lethality mostly happens. Consider aligning embryos that you do not inject, or inject with water only, and process them otherwise as the injected ones.
Many sterile flies in G0	-germline is damaged	-work on the shape of the needles -be more delicate during injection

References

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Spradling, A. C. and Rubin, G. M. (1982). Transposition of cloned P elements into Drosophila germ line chromosomes. *Science* **218**, 341-7.