Transgenesis in *Drosophila*

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Main Products and Reagents:

-10X injection buffer: 1mM Sodium Phosphate Buffer pH 6.8, 50mM KCl. Prepare in sterile bi-distillated water, filter through 0.22µm Millipore filters. Store aliquots at minus 20°C.
-FemtoJet 5247 (Eppendorf)
-Needles (Femtotips , Eppendorf)
-Fly media :

Standard Drosophila medium (83.3g fresh yeast, 83.3g corn meal, 11.25g agar, 50ml Moldex)

Egg lay medium (1000g fresh live baker's yeast, 650ml vinegar, 20g Sucrose, Water 350ml).

Fresh yeast.

-Embryo coverslips are obtained by cutting in 5 slices a 22x22mm coverslip with a diamond tip pen. Embryos are stuck on that coverslip using a lab made embryo glue.
-Embryo Glue: Put 20 small pieces of 3M adhesive, insulating tape into a glass scintillation vial. Add 10 ml of heptane. Incubate at least 24 hours on a rotating wheel at room temperature in order to dissolve enough glue into heptane.
- Injection oil Voltalef 10S (VWR International, Cat No. 24627188)
- Oil Voltalef 3S (VWR International, Cat No. 24626185)

Introduction :

Classical transgenesis efficiency is often around 3%, or lower (i.e. 3 independent transformant lines from 100 injected embryos).
The following protocol gave us much better efficiencies since we obtained no less than 10%, and often 12% efficiency. This protocol is not a complete rewriting of previous ones since some critical steps have been empirically optimized. For a complete overview of the original method, see (Spradling, 1986).

1-DNA mix preparation:

1.1-Injection mix purification:

1-Purify the transgenic vector using columns from 'Qiagen' (Maxi- or Midi- DNA preps and columns, following manufacturer's instructions) and accurately quantify DNA by OD_{260} reading AND gel quantification. Also perform a Maxi/Midi prep of the pΔ2-3 vector,

usually called the helper plasmid because this vector provides the transposase protein, which is necessary for P-element integration in germline cells.

2-Take 5 µg of pΔ2-3 (10kb) and add to this transposase plasmid, 2 times more (molar ratio) of the P transgene of interest. For instance, if the construct is approximately 14-17 kb, one should mix 5 µg of pΔ2-3 and 15-20 µg of constuct.

3-Adjust the volume up to 100 µl with double-distilled water.

4-Add 100 µl of (1:1) Phenol/Chloroform and mix by finger tapping. This step is absolutely required as Qiagen columns may not remove bacterial components such as endotoxins. Not performing this step can lead to a considerable loss of the survival rate of injected embryos.

5-Microfuge for 5 minutes, 14000 rpm at 4°C.

6-Keep supernatant (avoid any contact of the pipette tip with the organic phase, it's safer to withdraw no more than 90 µl) and add 9 µl of Sodium Acetate 3M pH5.

7-Add 1 ml of 100% ethanol and allow to precipitate 60 minutes or more at minus 80°C.

8-Spin for 30 minutes, 14000 rpm at 4°C.

9-Wash with 500 µl of 70% ethanol.

10-Spin for 5 minutes, 14000 rpm at 4°C.

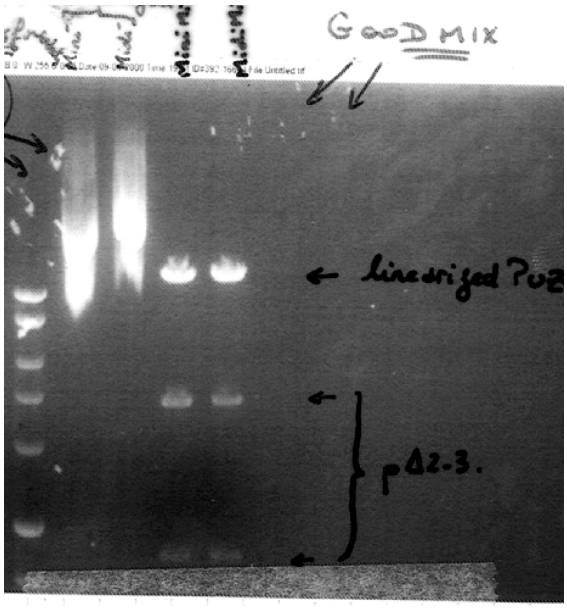
11-Carefully remove ethanol (we use home-made glass capillaries, pulled from Pasteur pipettes) and allow the pellet to dry under the chemical fume hood (avoid speed-vacuum).

12-After drying (5-10 minutes), resuspend the DNA pellet in 37 µl of sterile double distilled water or milli-Q water (Sigma n° W4502).

1.2-Quality/Ratio controls:

1-Save 1 µl for an EcoRI digestion of the DNA mix.

2-Store the other 36 µl at -20°C. Follow manufacturer's instructions for complete digestion of the mix (usually 2 h at 37°C with 10 U EcoRI, we use enzymes from New England Biolabs in our lab) in a reaction volume of 20 µl. Load 10 µl of the solution on agarose gel of appropriate concentration. If complete, the EcoRI digestion yields 2 bands of approximately 3.5 and 6.8 kb corresponding to 2 fragments from the pΔ2-3 vector. Each of these bands should be approx 50-70 ng. Additional bands from the P-transgene vector should be detected. From these bands, the ratio between transgene vector and pΔ2-3 vector should be estimated. An example is given from a vector approx 14-17kb containing one EcoRI site is shown in the figure below. The vector band MUST BE NO LESS than 3 times more intense than the 2 pΔ2-3 vector signals:



If the ratio does not approach the one described above (i.e. 2-3 times more construct than helper), this means that the DNA quantification step was not accurate. A new DNA mix should be prepared since other mix conditions might not be suitable for efficient transgenesis.

1.3-Injection mix preparation:

1-Add 4 µl of 10X injection buffer to the stored 36 µl DNA mix.

2-Make 4 aliquots of the resulting 'Injection Mix' (4x10 µl) and store at -20°C.

3-Prepare 100 µl of pure and sterile 1X Injection Buffer with double-distilled or milli-Q water (Sigma). Store at -20°C.

2-Injections :

If flies are submitted to a normal photoperiod, eggs are usually laid from the beginning of the afternoon, until late in the evening. Thus, injections should not be programmed in the morning, as very few embryos may be collected during this time. Old females should not be used, since they often retain fertilized eggs in the abdomen.

2.1-Amplification of flies

If the transgene contains a reporter gene like *white*, or *yellow*, or both, one should start to amplify flies mutant for this reporter. Since we commonly use *white* as reporter, we usually inject into embryo of the w^{1118} Canton S strain, carrying a deletion of the whole endogenous *white* gene in the Canton S genetic background.

1-Raise 5 full bottles of 4-5 days old flies kept at 25°C. Put them into the egg lay chamber at least 18h before the time of injection (usually the evening of the day before).

2-Keep these empty bottles for later collections of virgin females.

3-Replace the egg lay slide early in the morning and add to the egg lay medium a drop of liquid live baker yeast (freshly made).

4-Leave the egg lay chamber on the bench, near the injector, at 22°C. There are enough flies in the egg lay chamber IF approx. 100 eggs are laid on the egg lay slide every 25-30 minutes.

5-Start to amplify the line commonly used for transgene mapping. In general, we use the strain: w^{1118} ;Ap^{Xa}/(*Cy* ;TM3*Sb*). This strain is rather difficult to amplify (very weak flies) in bottles. We raise this line in tubes at 22°C (This strains grows better at 22°C than at 25°C).

2.2- Injection needle loading and mounting:

The DNA injection mix is not very stable in injection buffer. Always keep it on ice during injections and if not used, store it at -20°C.

1-Prior to loading the DNA mix into the needle, centrifuge one aliquot at 14000 rpm during 5 minutes at 4°C. This allows any non dissolved DNA to be pelleted at the bottom of the tube.

2-Using a sequencing gel loading tip, load the needle with 2 µl of DNA mix. During this process, the pipette tip should be held close to the surface of the liquid, since there is a risk of taking up non-dissolved DNA. Non-dissolved DNA often blocks the injection needle. The liquid should be visible at the tip of the needle.

3-Turn the transjector on (FemtoJet5247, Eppendorf).

4-Carefully remove the plastic cap from the needle.

5-Mount the needle and open the corresponding transjector channel. This allows you to toggle between V1, or V2 or X channels (X channel corresponds to the closed position).

6-A properly mounted needle should result in a compensation pressure reading varying between 40 and 60 Hpa. No continuous noise coming from the transjector pump should be heard; if this happens, this might be due to the fact that the needle is not screwed tightly enough on the needle holder. If the pressure still leaks, this may indicate breakage of the needle during the mounting process (this happens very easily and is not detectable until the mounting has been completed).

7-Put 5-10 µl of 10S oil in the middle of a microscope slide.

8-Stick an embryo coverslip (see products and reagents) on the 10S droplet and cover with a large drop of 10S oil.

9-On the microscope (phase transmission 10X), focus on the edge of the embryo coverslip.

10-Allow the needle to go down until the tip touches the oil surface.

11-Carefully (with the micrometric command on the needle holder) let the needle go further into the oil (going faster with the normal command is possible but often results in needle breakage, this operation should be reserved to experienced operators).

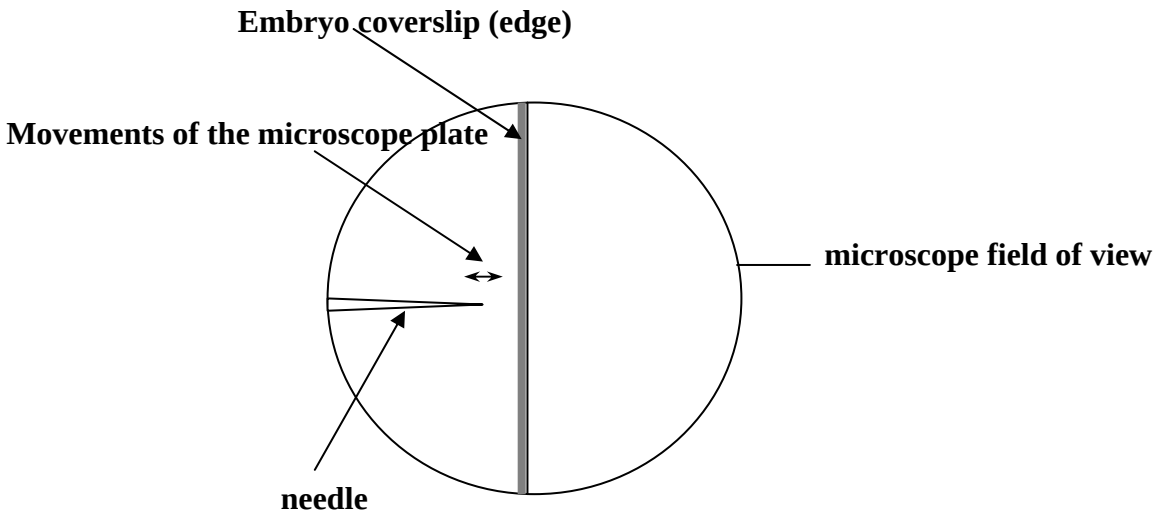
12-Focus on the tip of the needle. Position the needle tip in the left region of the vision field (needle holder should be on the left side of the microscope).

13-Check for suitable DNA flow through the needle tip by holding down either the injection button (see scheme) or the ‘clean’ button. Holding down the injection button results in an injection pressure of no more than 1000 HPa (usually 500) while one can obtain an injection pressure of up to 7000 HPa by using the ‘clean’ function.

14-At this point, it rarely happens that a liquid bubble ($1/10^{th}$ of embryo’s size) comes out instantly from the needle into the oil. If this happens, this means that the mounted needle is a «perfect needle» (minimal injury for injected embryos means huge survival rates). Usually, nothing or a little bubble comes slowly out from the needle. In this case, the DNA mix has to be "primed" (the following steps are the most difficult to perform of the whole protocol).

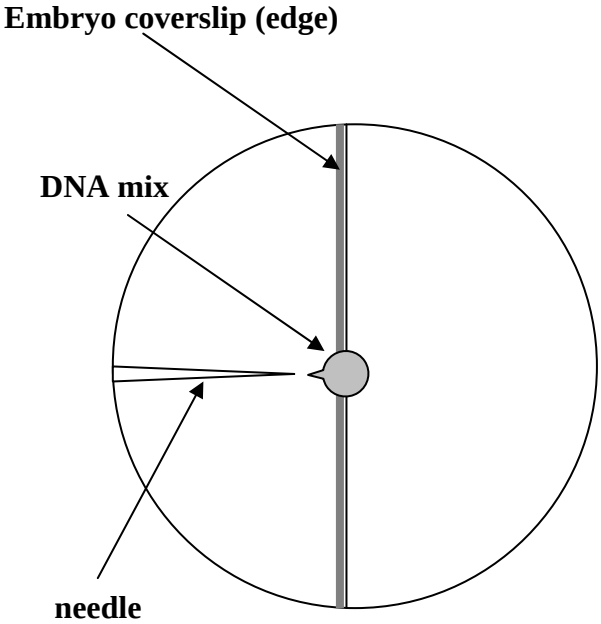
15-To prime**,** focus on the edge of the embryo coverslip, and by moving the microscope plate, position the coverslip left edge in the right region of the vision field. Allow the needle to go down with the micrometric command until the needle tip is clearly in focus.

This means that one should clearly see the needle tip AND the coverslip left border as shown below:



This step is critical and beginners often crash the needle on the slide by allowing it to go much too deep.

16-Carefully touch the needle tip with the coverslip border by moving the microscope plate laterally. This simple action should be sufficient to prime the transjector pump. Try to eject some DNA mix by pushing injection button or «clean» button. A bubble of approximately $1/10^{th}$ of embryo size should come out instantly as shown below:



17-Repeat DNA mix ejection 4 times and take the needle out of the oil.

18-If the «tip touching step» is not sufficient to obtain appropriate water bubbles, this means that the DNA mix in the needle is too viscous. One should dilute this mix two to five times with 1X Injection Buffer (prepared in section **1.3.3** following the recipe in products and reagents) and use the resulting lighter mix in a new needle.

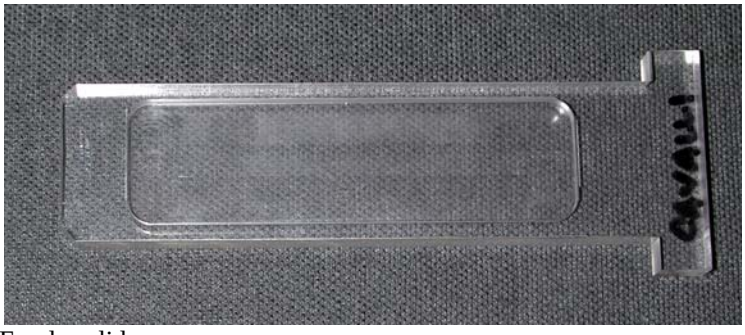
2.3- Embryo collection :

Embryos must be injected before blastoderm cellularization, a developmental stage that begins 45-50 minutes after eggs are laid at 22°C. Cellularization is easily visible at the microscope, and such old embryos should not be injected. They should be killed by piercing them with the injection needle. Injections should be performed during the first 45 minutes after egg laying.
Injection through the chorion (an extra-embryonic enveloppe) is not possible with the type of needle we use. That is why each embryo to inject should be dechorionated. We perform manual dechorionation as described in step 7 below.
Sufficient amounts of flies in the egg lay chamber should yield 50-100 embryos/ egg lay slide/20-30 minutes.

Egg lay chamber.

1-Let the flies lay eggs during 20-25 minutes (we use this time to mount the needle).

2-Get the egg lay slide with embryos to dechorionate and replace it by a new one (it will constitute the second egg lay).



Egg lay slide.

3-Put double sided adhesive tape (see products and reagents) on your finger tip.

4-Tap the tape very gently on the surface of the medium where embryos are laid. This steps results in collection of embryos on one side of the adhesive tape. If this does not work, it

is may be because the medium is too wet. In this case, one should collect each embryo using a paintbrush on the double sided adhesive tape.

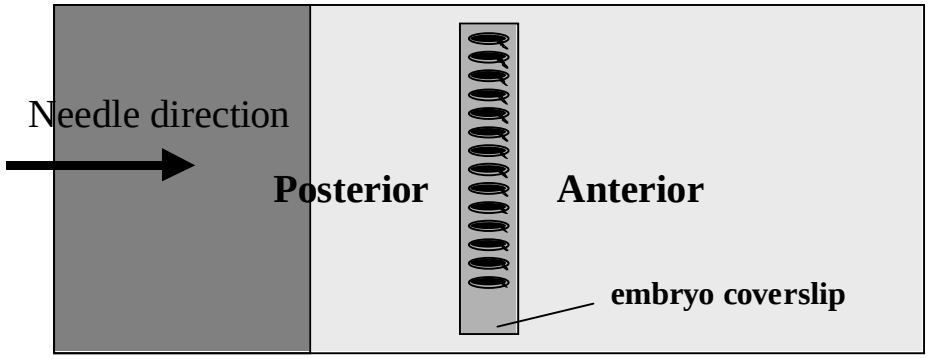
5-Load 5-10 µl of 10S oil in the center of a microscope slide.

6-Stick an embryo coverslip (see products and reagents) on the 10S droplet and cover with embryo glue (obtained by macerating fragments of insulating adhesive tape into heptane into a glass scintillation vial for at least 24 hours). Air-dry the slide (5-10 seconds).

7-With the help of stainless-steel tweezers (Dumont n°5 or equivalent) dechorionate embryos under binocular (we commonly use 2,5X magnification, and the weakest light possible in order to avoid drying of dechorionated embryos) by gently pushing them on the adhesive surface. Since the chorion is stuck by the adhesive, pushing the embryo will result in chorion disruption. Alternatively, an old-fashioned metal nibbed quill pen (with the point split in half) can be an efficient tool for rolling and transferring embryos. Some practice beforehand is recommended for manual dechorionation.

8-Dechorionated embryos will stick to the tweezers by capillarity. Transfer to the embryo coverslip, posterior pole oriented to the left (where the needle will penetrate). During this process, dechorionated embryos should not be in contact with the adhesive tape, and should be manipulated as little as possible.

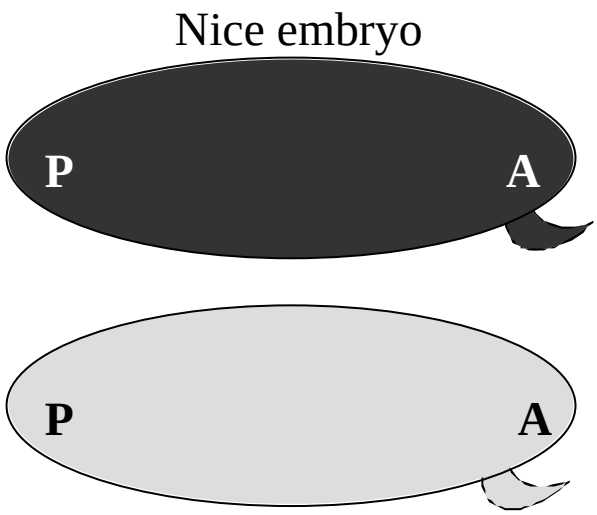
9-Align 20-60 embryos on the coverslip as shown below:



10-Do not stick embryos together on the coverslip, it is better to leave some space between each of them (of one embryo width as shown in the scheme above).

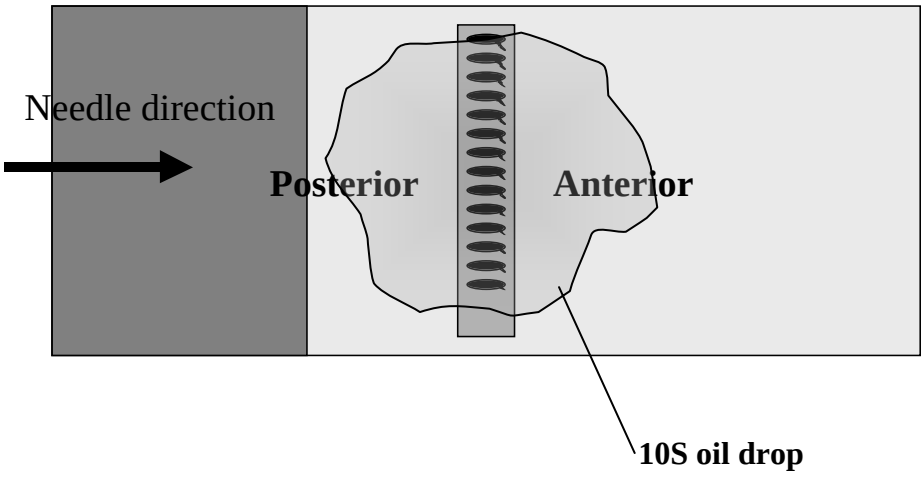
11-The whole collection/alignment process should not take more than 15 minutes.

12-Put the slide with dechorionated embryos in a dry box containing pre-dried silica gel (if dry, it should be of dark blue colour). This step is necessary and critical for the whole transgenesis. In particular, the drying time should be adjusted as a function of the injection room humidity and the drying rate/hydration state of the silica gel. For humidity ranging from 30 to 45%, drying time is usually between 1.5 and 4 minutes and is adjusted empirically by the user after viewing embryonic opacity under the microscope. The darker the embryo looks, the better. As time passes, the silica gel becomes wet (crystals turn to pink colour) and the drying time should be increased. An insufficient drying will result in so called «embryonic explosion» during penetration of the needle.



Overdried embryo= already dead

13-After drying, cover embryos with a large drop of 10S oil and put the slide on the microscope plate, as shown below.

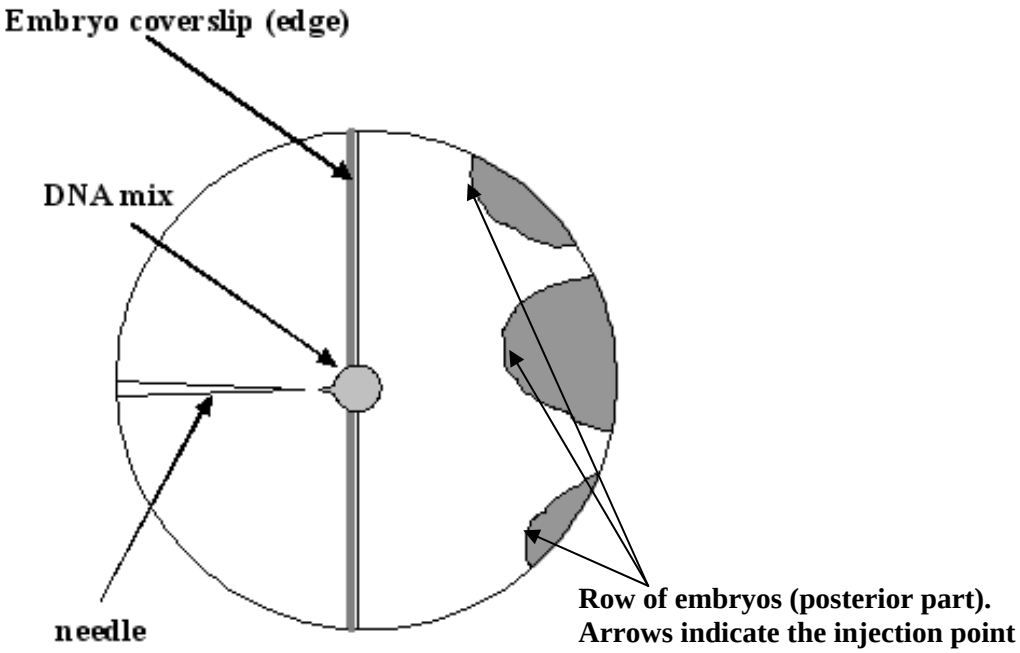


2.4- Injections :

1-Focus on the middle (in Z axis) of one embryo.

2-Put the needle down in order to touch the oil surface with the tip

3-With the needle holder's micrometric command, put the needle at the level of embryos (see the scheme below).



This step is critical for beginners, since it often results in needle breakage. If this represents a difficulty, here is a step by step procedure :

a-the needle tip has to be in contact with the oil surface
b-focus on one embryo
c-turn the micrometric command clockwise one turn
d-move embryos laterally (by moving the microscope stage command) in order to see if the needle touches the embryo. This should not be the case for the first attempt but it gives an idea of how far (in Z axis) the needle is relative to the embryo. Contact between embryo and needle is easy to detect as there is a visible deformation of the embryo.
e-turn the micrometric command clockwise for half a turn.
f-with the same procedure as above, check if this is sufficient for a contact between embryo and needle (by moving the microscope plate command).
g-Repeat these steps until the needle touches the embryo.

4-At this point:
a-if the needle seems to penetrate the embryo without problems, proceed to step **2.4.5**
b-if the embryo «explodes» when the needle enters: EITHER
-the embryos are not adequately dried (refer to step **2.3.12**), OR
-the needle has been broken during the pump priming process and is not sharp enough to penetrate the embryo without destroying it (refer to steps **2.2.14** & **2.2.15**). To check this, try to inject other embryos in the alignment to check if a majority explodes, OR finally
-the angle of the needle holder has been modified (refer to technical section to fix this problem).

c-if embryos detach from the coverslip when trying to inject :
-the embryo glue is not good and has to be replaced, or
-the angle of the needle holder has been modified (refer to technical section to fix this problem), or

-the embryos are not sufficiently dried (a typical ‘phenotype’ of this happens when the first embryos of the row can be injected, while the last embryos unstick from the coverslip when touched by the needle. Increasing the drying time by 30 sec/1min should be sufficient to solve this issue).

5-Allow the needle to penetrate in posterior part of embryo (don’t exceed the posterior one-third length of the embryonic body, by moving the microscope plate lateraly. Push the injection or the «clean» button. Injected liquid should be visible, as a halo of less dense color in cytoplasm. Exit the embryo by moving the microscope plate laterally.
The whole process (entry, injection, exit) should not take more than 2 seconds.

6-Proceed with the same method for the whole row.

7-Eliminate cellularized embryos by transpiercing them with the needle

8-At the end of the row, put the needle up (with the help of micrometric and normal commands) and count injected embryos.

9-Do not take in the final account:

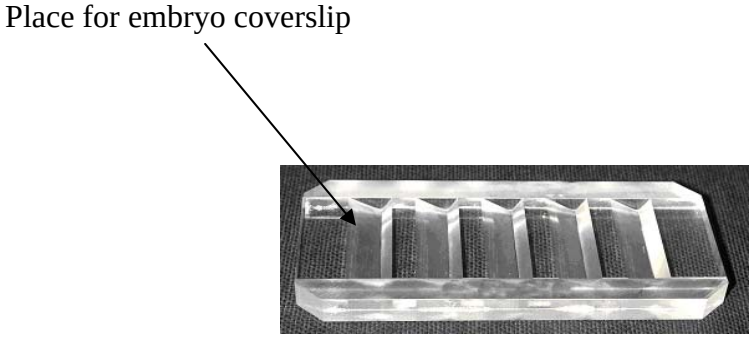
a-old (and, by this time, killed) embryos

b-embryos that have not exploded but have lost some cytoplasmic material.

c-uninjected embryos (those that detached from the coverslip and you don’t know why). It is advised to remove these embryos from the slide (physically) because if they survive you will be screening non-injected individuals.

The injection process, everything going well, should not take more than five minutes for 60 embryos. Between each embryo, one usually has to move the needle slightly up or down, because different embryos are not positioned at the same level in the Z-axis. This may constitute a cause of needle breakage for beginners.

10-Place the injected embryo coverslip in a cavity slide and cover with 3S mineral oil (embryo coverslip is depicted below).



Cavity slide

11-Place the cavity slide into a Petri dish filled with normal *Drosophila* food.

12-Injected embryos should be kept at 25°C in humid chamber.

13-Never put Petri dishes containing embryos injected with different constructs into the same chamber, as larvae often get out of the petri dish to pupariate.

If every step of this protocol has been followed carefully, the survival rate after injection should vary between 50 and 70% (at L1 stage, lethality can moderately occur during later stages). Usually, injected embryos hatch 24 to 48 hours after injection at 25°C. It is strongly recommended to help them find their way out of the oil into the food.

3- Establishment and mapping of transgenic lines:

(valid for transgenes containing the mini-*white* reporter)

3.1- Overview:

Each pupae obtained from injection should be placed in individual tube and allowed to develop until hatching. This (F0) generation will be white eyed, since the transgenes are inserted in germ-line precursor cells. Occasionally one may detect pigmented spots or areas in the eye of few injected embryos. This reflects insertion of the transgene in eye precursor cells, but these individuals are normally not of interest since they do not carry the transgene in the germ line and therefore their progeny will not be transformed.
Each F0 female should be crossed with three males of the w^{1118} strain, while each male should be crossed with three w^{1118} virgin females. Transformants are screened in the progeny of these single mates. Usually, three kinds of configuration may be observed in progenies:

-All F1 individuals in the tube are white eyed: no transformants (may vary between 3/4 and 9/10 of the tube).

-Some F1 individuals (less than 10%) display pigment in the eye. If every female display the same eye colour, that is lighter than pigmented males, it is likely that this tube will give a single transgenic line. This is the most desirable situation.

-Many F1 individuals (more than 50%) display various eye colours: this is indicative of a multiple insertion. Depending on the situation, individuals displaying the lighter eye colour should be selected for further crosses. Discard individuals displaying stronger eye colour, as they often bear multiple insertions.

3.2- Crosses:

F1 Individuals may bear one transgene insertion on any of the chromosomes: X, II or III. Transgenes inserted on the fourth chromosome are very rare as this chromosome is rather small and essentially heterochromatic (out of more than 500 transgenic lines done in our lab, we never observed an insertion on the fourth chromosome).
The transgene should be immediately placed in front of a balancer chromosome, to avoid its loss. As mentioned earlier, we use the Ap^{Xa}:*Cy*;TM3*Sb* which carries a translocation of a part of the third chromosome on the second. CurlyO and TM3Sb always segregate together from this cross as the Xa mutation holds second and third chromosomes together. Thus, every individual in the progeny of a cross between this strain and transgenic w^{1118} will carry either the Ap^{Xa} second and third chromosomes, or the CurlyO and TM3Sb balancers.
By using single mates, cross every F1 transformant with 3 individuals of the Ap^{Xa};*Cy*,TM3*Sb*. In the F2 progeny of these crosses, select individuals bearing the transgene and *Cy*,TM3*Sb* balancers. Avoid using F2 tranformants bearing Ap^{Xa}, as these chromosomes are not balancers. Check that every F2 tranformant female displays homogenous eye colour from each single mate. Usually, F2 transformant males from the same insertion display a stronger eye colour.

Let's suppose that the insertion lays on the second chromosome, scheme of the further cross is as follows:

F2 Males T/Cy;+/TM3Sb **X** Females T/Cy ;+/TM3Sb

F3

Select F3 Males and Females that are neither [Cy] or [Sb], this will constitute the stock, where the transgene is at the homozygous state. Note that the eye colour of these flies often differs from their heterozygous siblings (that still show [Cy] and [Sb])

If the insertion is on the second chromosome, it should be possible to observe individuals bearing Sb and the same eye colour as flies in the stock. If flies can be homozygous for the transgene and also Sb, this strongly indicates that the transgene lays on the second chromosome.

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Conversely, if the transgene is inserted on the third chromosome:

F2 Males +/Cy;T/TM3Sb **X** Females +/Cy ;T/TM3Sb

F3

Select F3 Males and Females that are neither [Cy] or [Sb], this will constitute the stock, where the transgene is at the homozygous state. Note that the eye colour of these flies often differs from their heterozygous siblings (that still show [Cy] and [Sb]).

If the insertion is on the third chromosome, it should be possible to observe individuals bearing Cy and the same eye colour as flies in the stock. If flies can be homozygous for the transgene and also Cy, this is strongly indicating that the transgene must lay on the third chromosome.

A more complex situation arises if the transgene is inserted on the X chromosome: this can be readily determined after the single mate between a F1 transgenic male and ApXa;Cy,TM3Sb. ALL females in the progeny should be eye pigmented, whereas ALL males should be white eyed. If a F1 transgenic female was used for the same cross, it is not possible to predict whether the transgene is inserted on the X, as transgenic males and females are obtained. Thus, individuals bearing the transgene and Cy and TM3Sb balancers should be crossed together as above. The detail of the cross is described below:

F2 Males **T**/Y ;+/Cy;+/TM3Sb **X** Females **T**/+ ;+/Cy ;+/TM3Sb

F3

Several parameters can help determining whether the transgene is on the X chromosome at the F3 stage, and they should be taken into account:

If the transgene is on the X, one eye colour should be observed in F3 males (and not two, as observed for autosomal insertions), whereas two kinds of eye colour are observed in females. F3 Males or females that can be supposed to be homozygous for the insertion as judged by the eye colour may either harbour CyO alone, TM3Sb alone, both balancers together or none of them.

In order to establish whether the transgene is on the X chromosome, perform single matings between one male and one female that you suppose to be homozygous and observe the F4 progeny of this cross. If all males display the same eye colour, the X chromosome transgenic line is obtained.

References:

Spradling, A. C. (1986). P element-mediated transformation. In *Drosophila*: A practical approach, D. B. Roberts, ed. (Oxford, IRL Press), pp. 175-197.