Chapter 8

Simple and Efficient Transgenesis with Meganuclease Constructs in Zebrafish

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Summary

In the past, microinjection of plasmid DNA into early embryos represented the state of the art to generate transgenic zebrafish. However, this approach suffers significant drawbacks (mosaic distribution of the injected transgene, late transgene integration at high copy numbers, low transgenesis frequency), making the generation of transgenic lines a laborious task. Coinjection of *I-SceI* meganuclease with a reporter construct flanked by *I-SceI* sites overcomes these problems by earlier transgene integration into the host genome. Here, we provide an optimized protocol for *I-SceI* meganuclease-mediated transgenesis in zebrafish. This simple protocol provides a reliable method to transiently test tissue-specific reporter expression of meganuclease constructs in injected embryos (F0). Furthermore, it substantially facilitates the generation of multiple stable transgenic lines increasing transgenesis frequencies up to 45%, compared with 5% without *I-SceI*. The reliable reporter activity in F0 and the improved transgenesis frequency make this protocol a powerful tool for use in gain- and loss-of-function, cell tracing, and cell labeling experiments.

Key words: *I-SceI*, Meganuclease, Transgenesis, Transient transgenesis, Zebrafish, Transgenesis frequency, Germline transmission.

1. Introduction

Historically, transgenic zebrafish strains have been produced by the direct microinjection of circular or linearized plasmid DNA into early embryos *(1–3)*. Commonly, this traditional approach results in the formation of long DNA concatamers, which are replicated during initial cleavages, maintained in an episomal state, and eventually degraded during gastrulation *(4, 5)*. Because of its extrachromosomal location, the injected DNA is distributed

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unevenly, leading to highly mosaic and transient reporter expression in injected embryos (F0) *(4–7)*. Stable transgene integration into the host genome occurs during late cleavages. Therefore, injected embryos are highly mosaic and the chance of germline integration (transgenesis frequency) is relatively low (5% or less) *(4)*. Nevertheless, due to its ease, DNA microinjection has been used extensively to assess reporter activity in F0 animals (transient transgenesis) and to establish transgenic lines (stable transgenesis). Both the mosaic reporter expression and the low germline integration in F0 made transgenesis a laborious task in the past. Hundreds of embryos had to be injected and screened in order to transiently test reporter constructs or to identify multiple independent founder fish, which is desirable during stable transgenesis experiments in order to exclude positional effects *(4, 8, 9)*.

The recent development of transgenesis approaches mediated by coinjection of transposase or *I-SceI* meganuclease substantially improves transient and stable transgenesis in zebrafish *(10–13)*. These approaches promote earlier integration of transgenes into the host genome improving both the extent and specificity of transient expression in F0 and increasing the transgenesis frequency *(14, 15)*. Transposase allows a large number of single-copy integrations in host genomes, while *I-SceI* meganuclease mediates a single integration event at low copy number *(12, 15–18)*. Single insertions of transgenes have advantages such as better long-term maintenance (no loss of insertions by outcrosses), easy determination of the insertion site, and selection of specific lines with no positional effects.

The mechanism by which *I-SceI* meganuclease mediates enhanced transgenesis is not yet fully understood. *I-SceI* is a homing endonuclease isolated from yeast *S. cerevisiae (19)* with no known restriction sites (18 bp in length) in any sequenced vertebrate genome. When coinjected with a plasmid containing the transgene cassette flanked by *I-SceI* sites, the meganuclease counteracts concatamerization, probably by remaining attached to its recognition sites and inhibiting endogenous ligases present in the early embryo *(2, 10, 15, 20)*. This activity of *I-SceI* might be responsible for decreasing mosaicism of transient expression and enhancing transgenesis frequency *(15)*.

Here, we provide an optimized protocol for *I-SceI* meganuclease transgenesis in zebrafish. We introduce a reliable method to determine bolus size in order to calibrate the volume injected. We used this approach to compare different parameters such as DNA concentration, composition of the injection mix, and time point of injection side by side. We suggest keeping the bolus size (100 μm or less) and concentration of the buffer (1×), $MgCl_2$ (5 mM), and *I-SceI* (20 U) constant and optimizing the DNA concentration for individual constructs. This simple protocol greatly reduces mosaicism and enhances tissue-specificity of

reporter expression in injected embryos. These features enable researchers to quickly and reliably test various meganuclease constructs for reporter activity in the F0 generation. The improved transgenesis frequency (up to 45%) for our *I-SceI* meganuclease protocol means that this protocol reliably delivers multiple transgenic insertions from one injection session for any given construct, reducing time- and space-consuming screening for founder fish. Additionally, we report a quick in vitro assay that allows the testing of *I-SceI* activity in individual experimental injection mixes. Neither the *in vitro* results nor the reporter activity *in vivo* support earlier observations that preincubation of the injection mix enhances transgenesis mediated by *I-SceI* meganuclease *(10, 12)*.

2. Materials

2.1. Zebrafish

1. Zebrafish AB strain (*see* **Note 1**).

2.2. Instrumentation

1. Needle puller (e.g., P-97 Flaming/Brown Micropipette Puller, Sutter Instruments).
2. Microinjection apparatus (e.g., Pneumatic Picopump PV820, World Precision Instruments).
3. Micromanipulator (e.g., MN153, Narishige).
4. Stereomicroscope (e.g., Olympus SZ40).
5. Fluorescence stereomicroscope (e.g., Olympus SZX12) with suitable filter sets (e.g., GFP, RFP2).
6. Agarose gel electrophoresis equipment.

2.3. Small Equipment

1. Injection tray mold (*see* **Note 2**).
2. Borosilicate filamented injection needles: 1.0-mm OD, 0.78-mm ID, 75-mm length (Harvard Apparatus).
3. Standard metric hemocytometer or scaled microscope slide: Hemocytometer; depth 0.1 mm, 1/400 mm^2 (Hawksley, Lancing, UK). Scaled microscope slide; 0.01-mm scaled microscope slide with reservoir **(Fig. 1)**.
4. Fine dissecting forceps: NeoLab-Dumont (Number 5).
5. Sequencing gel pipette tips (Eppendorf microloader tips; 20 μL).
6. Petri dish or plastic box with Plasticine/modeling clay.
7. Micropipette (P20, Gilson).
8. Plastic Pasteur pipettes.

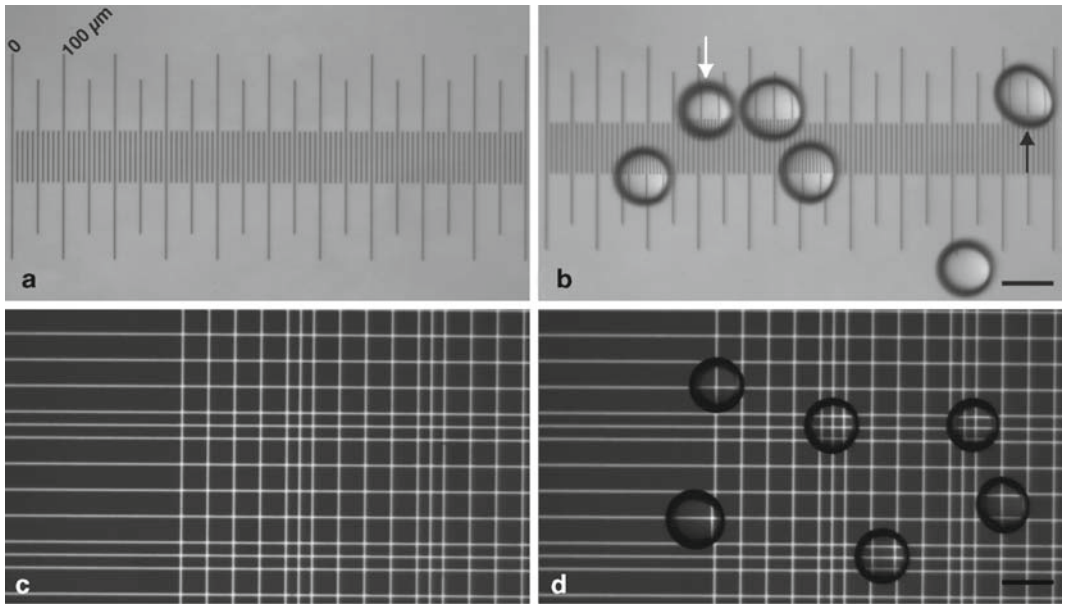

Fig. 1. Bolus size calibration: In order to guarantee reproducibility and minimize toxicity the bolus size should be calibrated prior to injection. In our hands, a bolus of approximately 100 µm diameter gives best results. The optimal DNA concentration for each construct is determined by injection of various dilutions at the given bolus size. (**a**) We use a scaled microscope slide for accurate calibration. (**b**) The scale bar is covered with a droplet of oil (*see* **Subheading 2.4**). Once the needle is broken and the back pressure is adjusted (*see* **Note 11**) several boli of constant size are injected into the oil. Calibration of floating (*white arrow*) or flattened (*black arrow*) boluses can influence the calibration procedure and enhance toxicity. Therefore, these should not be considered during the calibration of the needle. (**c, d**) Alternatively, a standard metric hemocytometer can be used for calibration. Scale bar = 100 µm.

2.4. Reagents, Media, and Solutions

1. Injection mix:
   (a) Reporter plasmid: Transgene cassette in *I-SceI*-pBSII-SK + vector backbone (Genbank accession number DQ836146) (15), e.g., pαact-GFPI2 (7.9 kb): αact promoter driving muscle-specific GFP expression; αact-GFPI2 cassette is flanked by two *I-SceI* sites.
   (b) Plasmid DNA stock solution: DNA of high quality (midiprep or maxiprep) stored at a concentration of 100 ng/µL (*see* **Note 3**).
   (c) Injection solution premix on ice (without *I-SceI*):

DNA	10–50 ng/µL
Buffer without $MgCl_2$ (10× Roche)	2 µL (*see* **Note 4**)
$MgCl_2$ (50 mM)	2 µL
Phenol red 0.5% (Sigma)	1 µL
H_2O	Up to 18 µL

(d) *I-SceI* meganuclease (Roche): *I-SceI* enzyme should be stored at −80°C in individual aliquots and added to the premix (2 μL to earlier mix) immediately prior to injection (*see* **Note 5**).

2. Paraffin oil (relative density 0.865, viscosity 171 mPa s).
3. Egg water: Stock Sea Salts (Aquasonic), final concentration 60 μg/mL.
4. E3 embryo medium (without methylene blue): 5 mM NaCl; 0.17 mM KCl; 0.33 mM $MgSO_4$; 0.33 mM $CaCl_2$; pH 7.5.
5. Proteinase K 10 mg/mL.

3. Methods

3.1. Preparation of Needles for Injection

1. Load filamented capillaries into a commercially supplied micropipette puller.
2. Pull capillaries using settings that result in needles that are long and thin with a tapered tip (*see* **Note 6**).
3. Multiple needles can be produced using the desired settings and stored in rows on Plasticine or modeling clay in a Petri dish.

3.2. Preparation of Injection Tray

1. Prepare a 1–2% agarose solution with embryo medium; dissolve it by heating and pour into a Petri dish.
2. Carefully place a prewet standard plastic injection mold (*see* **Note 7**) face down onto the cooling agarose so that the mold floats.
3. Allow the agarose to set and then remove the plastic injection mold.
4. Store injection tray at 4°C submersed in E3 medium or water.
5. Prewarm the injection tray to 28.5°C prior to use.

3.3. Preparation of Injection Mix and Mounting of Embryos for Injection

1. Prepare injection premix containing no *I-SceI* meganuclease and store it on ice.
2. Collect embryos from pairwise matings (*see* **Note 8**) in E3 medium immediately upon spawning (*see* **Note 9**). Inspect clutch quality and ensure that the embryos are of the desired stage **(Fig. 2)**.
3. Remove *I-SceI* meganuclease aliquot from storage and add directly to the premix on ice immediately prior to injection (*see* **Note 5**). Mix without vortexing.

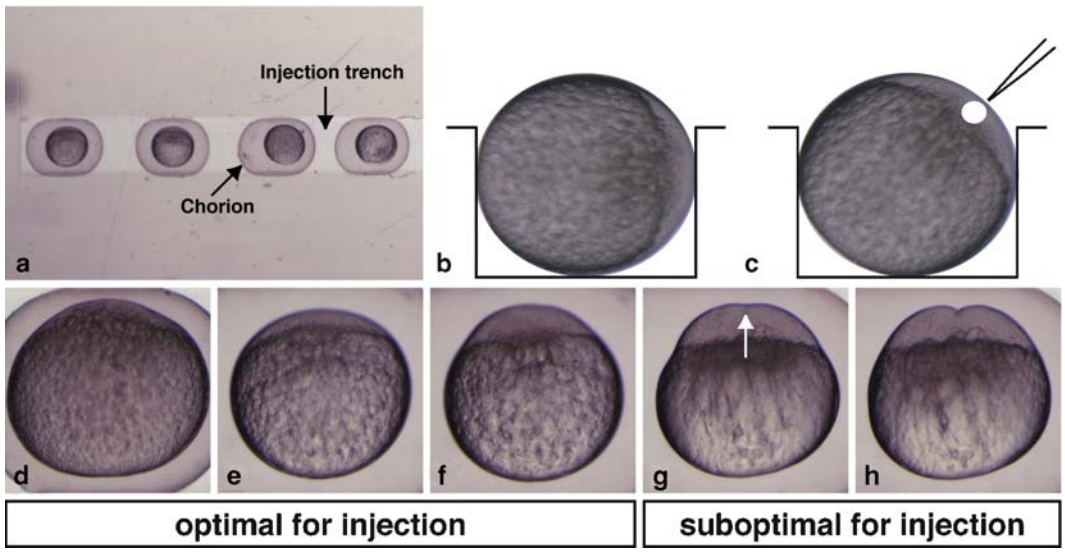

Fig. 2. Staging of one-cell embryos. The stage of injected embryos has a strong influence on the mosaicism of the germline. For the generation of transgenic lines it is optimal to harvest and inject embryos as early as possible to ensure early transgene integration at the one- or two-cell stage. (**a**) Immediately after spawning, clutches are harvested and embryos are aligned individually in the injection tray. The depicted injection trench is 0.95-mm wide and 0.8-mm high. (**b**) Prior to injection single embryos are oriented so that the animal pole faces the injection needle. (**c**) Subsequently embryos are tilted approximately 45° upward and the DNA is delivered at the indicated position (white point). Never inject more than 50–80 embryos or for a period of more than 15 min using the same needle (*see* **Note 15**). (**d**–**f**) For the generation of transgenic lines only early one-cell embryos should be considered. (**g**) As soon as a cleavage furrow is visible (*white arrow*) the embryos should be discarded. Injection at late one-cell stage (**g**) or early two-cell (**h**) stages results in late transgene integration and in a more mosaic animal and germline.

4. Array selected early one-cell embryos side by side in each trench in an injection tray with the animal pole (single cell) facing the needle for injection (*see* **Fig. 2** and **Note 10**).
5. Take a few microliters of injection mix and fill premade injection needles with microloaders.
6. Proceed immediately to calibration of bolus size.

3.4. Calibration of Injection Bolus and Volume

1. Attach loaded injection needle to the injection arm of the microinjection apparatus.
2. Break the tip of the needle using forceps so that the needle is sharp but not overly flexible (*see* **Note 11**).
3. Apply paraffin oil to the surface of a scaled slide or hemocytometer and inject several boli (3–5 is sufficient) immediately above the gridded surface **(Fig. 1**).
4. Adjust bolus size to deliver the desired volume (*see* **Note 12**) based on the following calculations used to estimate amount of DNA and injection mix delivered:

- Estimate bolus diameter based on the number of hemocytometers or scaled slide grids spanned (*see* **Fig. 1** and **Note 13**).
- Calculate the volume of the injection bolus, assuming that the bolus is a sphere, using the equation for the volume of a sphere (volume in $\mu m^3 = 1/6\pi d^3$, where d = bolus diameter).
- Calculate the amount in nanograms to be delivered based on the volume of the bolus and the concentration of the DNA injection mix.

5. For rapid use, the bolus size can be precalibrated on a hemocytometer/gridded slide and then referenced against an eyepiece with reticule. The required bolus can thus be rapidly set at a given volume (e.g., 0.5–1 nL) based on the number of scaled eyepiece bars spanned at a predetermined magnification.

3.5. Injection of Embryos

1. Inject DNA directly into the cytoplasm of carefully staged embryos (*see* **Fig. 2** and **Note 14**).
2. Inject approximately 50–80 embryos over a period of 15 min using the first injection needle.
3. Change needles, refreshing with injection mix that has been stored on ice for every consecutive batch of embryos to be injected (*see* **Note 15**).
4. Discard and do not inject any embryos that are at late one-cell or early two-cell stages throughout the injection process **(Fig. 2)**.
5. Incubate embryos in an embryo medium at 28.5°C until the desired stage.
6. Retain uninjected embryos from every individual clutch as an injection control.

3.6. Assay Reporter Activity in Injected Embryos (F0)

1. Screen injected embryos for reporter activity at the desired stage using a fluorescence stereomicroscope.
2. Record strength, extent, and tissue specificity of obtained signals (*see* **Note 16**).
3. Record the number of phenotypes and dead embryos and compare directly to uninjected control embryos (*see* **Note 12**).

3.7. Assays to Control for Meganuclease Efficiency

In order to control for the activity of the *I-SceI* enzyme we have provided the following methods. The first is a simple gel electrophoresis approach to assay *I-SceI* activity in vitro, which can be performed at the bench rapidly after injection. The second is a longer term but simple genetic approach, which can be used to determine from the transmission rate both the integration efficiency and the insertion frequency achieved in injection experiments for scenarios where such readouts are desirable.

3.7.1. A Simple Assay for Meganuclease Activity

1. After injecting, aliquot the remaining injection solution into three prechilled and labeled Eppendorf tubes.
2. Incubate one aliquot at room temperature for 30 min.
3. Add 1 μL of Proteinase K (10 mg/mL) to a second aliquot and leave it together with the third and final untreated aliquot on ice.
4. Prepare an agarose gel suitable for separation of the expected fragments.

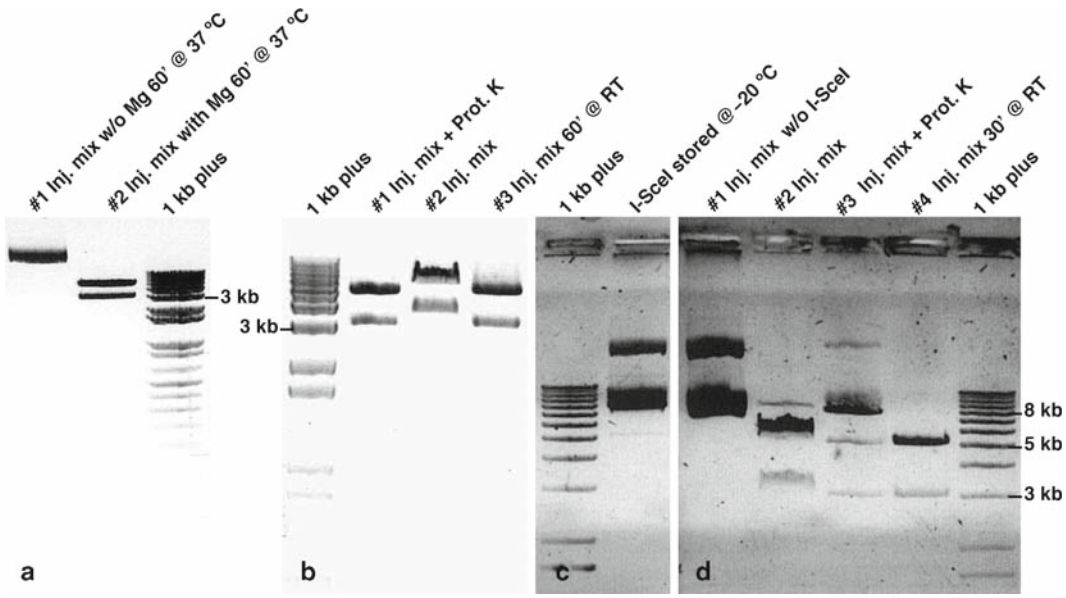

Fig. 3. In vitro assay for *I-SceI* activity. (**a**) Side-by-side comparison of injection mixes containing pαact-GFPI2 (7.9 kb; *see* **Subheading 2**) with and without (w/o) $MgCl_2$ shows that *I-SceI* meganuclease activity is Mg^{2+}-dependent. Therefore, rather than rely on endogenous Mg^{2+} levels, we recommend adding $MgCl_2$ to the injection mix. (**b**) In our hands, preincubation of the injection mix at room temperature (RT, 23°C) does not produce optimal reporter expression in F0 embryos. In order to compare *I-SceI* meganuclease activity under incubated and unincubated conditions, we loaded different injection mixes on an agarose gel. Plasmid DNA was digested in all samples. However, fragments from the mix incubated on ice (lane #2), rather than at RT (lane #3) showed a clear band shift, running at a higher molecular weight. Importantly, the addition of Proteinase K to the injection mix inhibited this band shift, demonstrating that this shift is *I-SceI*-dependent (lane #1). Therefore, *I-SceI* meganuclease probably retains a higher activity when incubated on ice because it stays attached to its recognition sites (leading to DNA running at a higher molecular weight), which is believed to enhance transgene integration. (**c**) *I-SceI* meganuclease stored incorrectly dramatically loses activity and does not digest plasmid DNA when incubated on ice, while enzyme stored at –80°C consistently digests the plasmid DNA even when stored on ice (lane #2 in **b**, **d**). (**d**) *I-SceI* meganuclease activity can be tested in vitro. Side-by-side comparison of identical injection mixes incubated under different conditions confirms that the *I-SceI*-dependent band shift is temperature-dependent. On ice, *I-SceI* retains its ability to both digest and attach to the DNA (lane #2). If Proteinase K is added both digestion and band shifting are inhibited (lane #3). Preincubation at RT results in complete digestion of the plasmid DNA; however, the *I-SceI* enzyme loses its ability to bind to the DNA and no longer causes band shift (lane #4). Considering these data, we suggest that cutting of the plasmid DNA alone is not a suitable readout for *I-SceI* meganuclease activity, but that cutting and band shifting is. All samples in (**c**) and (**d**) were incubated for 30 min. Samples in (**d**) originated from the same master mix and were incubated on ice (lane #2, #3) or RT (lane #4).

5. Load sample after incubation and run high-resolution gel (*see* **Note 17**).
6. Document and interpret fragment pattern **(Fig. 3)**.

3.7.2. Genetic Determination of Germline Mosaicism and Integration Number

1. After the analysis of transient expression in injected embryos (F0), maintain injected embryos and grow to adulthood (approximately 2–3 months).
2. Outcross F0 animals to nontransgenic wild-type fish in pairs.
3. Collect clutches of embryos (F1) from outcrosses and score the transgenesis frequency from the number of positive clutches. Within these clutches, score the percentage that carries the transgene (e.g., fluorescent embryos) to estimate the mosaicism of the F0 germline **(***see* **Table 1** and **Note 18**).
4. Retain fluorescent outcrossed F1 embryos and grow to adulthood (approximately 2–3 months).
5. Outcross F1 animals to nontransgenic wild-type fish in pairs.
6. Collect clutches of embryos (F2) from outcrosses and score the percentage that carries the transgene (e.g., fluorescent embryos) to determine the number of genomic insertions achieved per positive founder (*see* **Table 1** and **Note 19**).

Table 1
Transmission rates determined from F1 and F2 offspring (*see* Notes 18 and 19)

Transmission rates determined from F1 offspring			
Founder fish (F0)	*n*	T rates (%)	Integration at
#10	89	20	2-cell stage
#11	130	10	4-cell stage
#15	55	45	1-cell stage
Transmission rates determined from F2 offspring			
Clutches F1 #10	*n*	T rates (%)	
1	84	33	
2	171	46	
3	372	51	
4	248	44	
5	125	47	
Clutches F1 #11	*n*	T rates (%)	
1	114	57	
2	228	48	
3	75	47	
4	310	49	
5	142	48	

(continued)

Table 1 (continued)

Clutches F1 #15	*n*	T rates (%)
15	157	55
16	154	56
17	225	50
18	271	43
19	171	50

Injected construct: pher1-UbCherry-3′her1, *n* Total number, *T rates (%)* transmission rates in percent

4. Notes

1. Although the specific strain used appears not to be critical and a number of strains are available (www.zfin.org), the assays used to optimize methods described here were performed primarily using the AB strain.
2. The use of standard plastic injection tray molds is a routine approach in zebrafish research. One common example of these molds with full specifications can be found in *The Zebrafish Book* (http://www.zfin.org/zf_info/zfbook/zfbk.html). Recently, Rembold et al. provided another useful mold *(12)*.
3. Dilution of high-concentration midiprep DNA directly into the final injection mix will increase concentration variation that can occur due to small differences when pipetting. The storage and reuse in multiple experiments of a more diluted DNA stock solution is suggested here to reduce variations in DNA concentration introduced when pipetting and to improve the reproducibility observed between different experiments.
4. It is important to be aware of whether or not the buffer used contains $MgCl_2$. Injections performed in the absence of $MgCl_2$ rely on the presence of embryonic Mg^{2+} ions for the enzyme to function in vivo. This is evidenced by the inability of *I-SceI* (Roche) to digest DNA upon in vitro incubation in the absence of $MgCl_2$ (*see* **Fig. 3**).
5. *I-SceI* is commercially available from a number of companies. We have experienced surprising variation in the activity of enzymes supplied from different sources. This variation appears to be, at least in part, due to the method of shipment used. *I-SceI* enzymes should be shipped on dry ice. An observable reduction in the activity of *I-SceI* meganuclease can be seen when the enzyme is stored at –20°C when compared with the enzyme stored at –80°C **(Fig. 3)**.

Hence, we suggest the storage of small individual aliquots (2 μL) of enzyme for single use to reduce freeze-thawing damage. The methodological optimizations described here are based on the use of *I-SceI* enzyme sourced from Roche.

6. Needles that are long and thin are ideal for injection into early one-cell stage embryos. Settings on a needle puller will vary dependent on the age of the filament. As a guide, we commonly use the following or similar settings: *P* = 150; heat = 635– (check by ramp test); pull = 100; vel = 170; time = 120. The heat can be determined first by a ramp test (see manufacturer's instructions for the needle puller). As a rule of thumb the indicated value from the ramp test should not be exceeded by more than 20. Subsequently, the other parameters can be adapted.
7. In order to avoid bubbles forming between the injection mold and agarose as the agarose is setting, we suggest wetting the injection tray mold with water before lowering it slowly one side first onto the hot agarose.
8. Although mass matings can be (and are commonly) used for microinjection, we recommend pairwise mating. This approach has an advantage when it comes to timing and staging early one-cell embryos. Once an injection premix has been prepared on ice, mating can be induced by combining male and female fish. *I-SceI* can then be added, allowing for bolus size calibration immediately upon the induction of spawning. Using this approach, embryo injections can be performed with fresh *I-SceI* meganuclease into the earliest possible one-cell stage embryos.
9. Although egg water and E3 medium are both used interchangeably by many laboratories for incubation of developing embryos, E3 medium is a more carefully buffered medium and provides more controlled laboratory conditions. Methylene blue is best omitted as it can complicate the detection of weak fluorescence.
10. The staging of early versus late one-cell stage embryos for injection (*see* **Fig. 2**) influences the distribution of transgene expression in injected embryos. It is intuitive that the earlier the genomic integration, the more ubiquitous the transgene expression will be in the target tissue in transiently expressing F0 embryos. Injection into early one-cell stage embryos gives less mosaic expression and the injection of late one-cell stage embryos gives a more mosaic pattern of expression in injected embryos. It should be noted that when a more mosaic expression pattern is desired (e.g., for cell tracing or fate mapping experiments), injection into single cells in late 1-, 2-, or 4-cell stage embryos can also be performed.
11. Needles with a very small opening at the tip will not cause damage to the injected embryo but will bend easily when

attempting to pierce the chorion and will become blocked regularly. Needles with a large opening will cause more damage but will bend less and pierce the chorion more readily. A balance can be found when breaking the tip of the needle. When using needles produced as described earlier (*see* **Note 6**) a correctly broken needle should produce boli of approximately 100 μm diameter at the following microinjector settings: 40 psi, 200–500 ms, with a back (hold) pressure that avoids backflow and free flow.

12. Toxicity and mortality of injected embryos depends on bolus size and DNA concentration. If either significantly exceeds more than 30% in an injection clutch, confirm that the bolus size is 100 μm or less and reduce DNA concentration in the injection mix. As a guide, we recommend the use of a standard bolus size of approximately 0.5–1 nL (1nL = 120 μm) to minimize developmental abnormalities.
13. In order to guarantee accurate estimations of bolus size between injections one should estimate bolus diameter at a consistent depth in the paraffin oil. We suggest focusing the microscope on the scale bar and making sure that the bolus is in the same focal plane when estimating diameter **(Fig. 1)**.
14. We use a very steep angle (~45%) for injection into the cell. This helps to avoid injection into the yolk or the injection tray, which represent the most common errors for beginners. Furthermore, the angle ensures that the embryo does not move when injected right into the middle of the cell **(Fig. 2)**.
15. *I-SceI* has been proposed to enhance transient expression levels and stable integration efficiency by remaining attached to the digested recognition sites on the injected transgene and thereby inhibiting the formation of concatamers *(10, 15, 20)*. We have found that once taken off ice during incubation, *I-SceI* digests and detaches from the injected DNA **(Fig. 3)**. Hence, while the needle stands at room temperature during injection, the *I-SceI* will be progressively detaching from the injection DNA. To circumvent this, we recommend using the injection needle for only a limited time and changing to a fresh needle containing unincubated injection mix at regular intervals. We observe significant improvement in transient expression levels and the extent of transient expression using this approach.
16. We suggest scoring positive embryos according to extent and specificity of the signal. Maintaining a record of these data from multiple experiments provides a quality readout for individual injections. If expression intensity is consistently low for a given construct the GAL4.UAS system can be used to optimize signal intensity from weak promoters *(21)*.
17. To detect band shifts as shown in **Fig. 3**, run standard 1% agarose gels at a low voltage.

18. The transmission rate scores the percentage of transgenic F1 offspring that arise from an outcross between a founder fish (F0) and a wild-type fish. Transgene insertion at the one-cell stage ensures that the transgene is distributed equally during subsequent cleavages and results in a transmission rate of 50%. Integration events that occur later lower this percentage (Two-cell = 25%; Four-cell = 12.5%). Therefore, the transmission rate reflects the mosaicism of the germline of a given founder fish.
19. The transmission rate determined from transgenic F2 offspring reflects the transgene insertion number for a given transgenic line. Outcrossing of F1 transgenics should result in 50% of transgenic F2 offspring. If this percentage reaches 75%, the F1 carries more than one insertion.

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