Expressing exogenous genes in newts by transgenesis

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The great regenerative abilities of newts provide the impetus for studies at the molecular level. However, efficient methods for gene regulation have historically been quite limited. Here, we describe a protocol for transgenically expressing exogenous genes in the newt *Cynops pyrrhogaster*. This method is simple: a reaction mixture of I-SceI meganuclease and a plasmid DNA carrying a transgene cassette flanked by the enzyme recognition sites is directly injected into fertilized eggs. The protocol achieves a high efficiency of transgenesis, comparable to protocols used in other animal systems, and it provides a practical number of transgenic newts (~20% of injected embryos) that survive beyond metamorphosis and that can be applied to regenerative studies. The entire protocol for obtaining transgenic adult newts takes 4–5 months.

INTRODUCTION

Undoubtedly, the newt is the master of regeneration in the animal kingdom. No other animal can parallel its regenerative abilities in tissues and body parts such as the limbs, the tail and spinal cord, parts of the eye (such as the retina and the lens), the brain, the heart and the jaw[1,2]. Another important aspect is that regeneration is mediated by dedifferentiation of the somatic cells at the site of the injury or even (in the case of retina and lens regeneration) by transdifferentiation[3–5]. The relevance of these events in basic biology is obvious. The newt can thus address issues of development, differentiation, repair and stem cells. Until now, functional experiments met with difficulty mainly because of a lack of efficient techniques allowing gene expression manipulation. In a companion protocol, we provide methods for controlling gene loss of function during regeneration in newts[6]; here, we describe a protocol for generating transgenic newts expressing exogenous genes with sufficient efficiency to allow further experimentation[7]. An example showing a regenerating limb expressing GFP is also presented.

This protocol was developed by adapting an I-SceI-mediated method for use in the newt *Cynops pyrrhogaster*[7]. I-SceI is a meganuclease isolated from the yeast *Saccharomyces cerevisiae*[8]. The method was originally developed in a fish, medaka (*Oryzias latipes*)[9], and then applied successfully to several other fish species (stickleback (*Gasterosteus aculeatus*)[10] and zebrafish (*Danio rerio*)[11]), amphibians (axolotl (*Ambystoma mexicanum*)[12]; *Xenopus laevis*[13]; and *Xenopus tropicalis*[14]) and ascidians (*Ciona savignyi*)[15].

Advantages, applications and limitations of the method

This new procedure for studying the newt is very simple: a plasmid DNA carrying a transgene cassette flanked by 18-bp I-SceI recognition sites is incubated with I-SceI, and the resulting reaction mixture is directly delivered into naturally fertilized eggs by conventional microinjection techniques. Furthermore, following the protocol, ~20% of the injected embryos express transgenes with non-mosaic widespread expression patterns and survive beyond metamorphosis. This success rate is about tenfold higher than that (0.9–3.4%) achieved in previously described newt transgenic protocols[16,17], reaching near the values obtained in I-SceI transgenesis in *Xenopus* (*laevis*, 20%; *tropicalis*, 30%)[14] and axolotl (32.7%)[12].

Another transgenic protocol for use in the newt has been reported by Ueda *et al.*[16], which entails coinjection of inositol 1,4,5-triphosphate with DNA-sperm nucleus complex into unfertilized eggs. This protocol achieves non-mosaic widespread expression of transgenes in metamorphosed newts, and ectopic expression of transgenes has been examined by the use of the lens-specific crystalline promoter. Therefore, this protocol can be an alternative for newt transgenesis. However, the procedure is far more complicated than the protocol described here, and the success rate is yet to be improved as mentioned above. In addition, the protocol from Ueda *et al.* requires higher numbers of animals for obtaining unfertilized eggs and sperm.

The new protocol described here could be applied practically to various studies on regeneration. For example, in conjunction with tissue transplantation[3], it would be possible to analyze the tissue origin of regenerates by tracing the grafted tissues with reporter-gene expression. Furthermore, if the transgene construct is designed appropriately, it would be possible to analyze gene functions in regeneration of various body parts; for example, for gain of function, we can design constructs to overexpress a gene of interest or a constitutively active form of protein, or for loss of function, constructs can be designed to express short hairpin RNAs or a dominant-negative form of protein, in either intact (i.e., before injury) or regenerating tissues. To minimize unexpected effects (e.g., embryonic lethal abnormality) of these manipulations until analysis, we can use conditional (e.g., heat shock promoter) or tissue-specific promoters that can restrict the time and place of gene expression.

Ectopic expression of transgenes has been argued to be a possible limitation of the protocol with I-SceI. This problem might arise from undesirable *cis*-element interactions between the integrated DNA (assumed as the transgene cassette alone or with a plasmid sequence) and the host genome[14]. Therefore, to prevent this

problem, it might be favorable to insert insulator sequences (DNA sequence elements, similar to those in the β-globin locus[18], which can block interactions between a promoter and a distal enhancer) at both ends of the cassette, as well as to modify the DNA-I-SceI concentrations. The conditions described here have been optimized to achieve high efficiencies of integration of exogenous genes in the host genome. Therefore, in future studies with tissue-specific promoters, this problem needs to be addressed individually.

Experimental design

Animal housing conditions. The protocol described here has been optimized for the newt *C. pyrrhogaster*. Therefore, to adopt this protocol for experiments, it is necessary to obtain this animal and keep it in the laboratory.

For *C. pyrrhogaster* newts, the quality and quantity of eggs/sperm are highly dependent on the animals that are collected from the field. Moreover, according to the Ministry of Environment of Japan, this species is seriously decreasing in number (2006 press release (in Japanese); http://www.env.go.jp/press/press.php?serial=7849). To preserve this species and its habitat environments, as well as to construct a stock center ('Imori-no-Sato', Toride city, Ibaraki, Japan), the Japan Newt Research Community (JNRC) was established by the author (C.C.) in 2008 (http://imori-net.org/). Currently, *C. pyrrhogaster* can be obtained through JNRC for research purposes.

Mature females of this species store a spermatophore in their abdomen; this spermatophore has been transferred from a male in the field during the mating season (November to April). To obtain fertilized eggs, the female newts are injected with a hormone, gonadotropin, every other day, and kept in a container/aquarium tank at 18–24 °C. A few days after the first injection, the female newts start to lay fertilized eggs, mostly early in the morning, on strips of plastic sheets/ribbons placed in water (as an imitation of leaves of water plants). A total of 100–200 eggs can be obtained from the same females over a span of 2 weeks, although the number of eggs per day is drastically decreased thereafter. Thus, using this system, males are not necessary, and a total of > 3,000 fertilized eggs can be obtained from the same 30 females in 3 weeks. However, the female newts are limited in number, as mentioned above, and available only from November to June. Alternatively, to obtain fertilized eggs constantly in the laboratory, a two-aquarium-tank (TAT) system is favorable (see ref. 7). In this system, both sexually mature females and males are reared in semi-natural conditions. Under these conditions, animals show a characteristic courtship behavior that is followed by delivery of a spermatophore from male to female. The female newts that have exhausted their eggs and sperm stock are allowed to recover in order to be able to yield fertilized eggs again. Using this system, with 60 female newts (30 per tank), we can obtain ~100 fertilized eggs per day (2–3 d per week) from the same batch of animals from October to July (10 months) every year (the average life span of this species is estimated as >15 years).

For obtaining transgenic newts, surroundings and facilities that are used for standard transgenic experiments can be applied. However, rearing temperature should be controlled rigidly (14–24 °C; see PROCEDURE for details), because this animal is highly sensitive to temperature changes (lethal at >27 °C).

Controls. To establish the effects of gene expression by transgenesis, appropriate control constructs with no such effects should be compared in an identical protocol.

MATERIALS

REAGENTS

- *C. pyrrhogaster* (available from JNRC (http://imori-net.org/; imori@imori-net.org) or contact C.C.) ▲ **CRITICAL** Sexually mature adult newts (total body length: male, ~9 cm; female, 11–12 cm; see ref. 7) must be used
  ! CAUTION Experiments must comply with the national regulations concerning animals and their use.
- Human chorionic gonadotropin (HCG, 3,000 U; Asuka Seiyaku or equivalent)
- A plasmid carrying a transgene construct flanked at both ends by I-SceI recognition sites. pCAGGs-EGFP (Sce)[12], which allows the enhanced GFP (*EGFP*) gene to be expressed strongly in the whole body of a transgenic *C. pyrrhogaster* with non-mosaic patterns beyond metamorphosis[7], is available from C.C. or from E.M. Tanaka at the Max Planck Institute of Molecular Cell Biology and Genetics, Germany (tanaka@mpi-cbg.de)
  ▲ **CRITICAL** The plasmid must be purified and stored appropriately (for details, see Steps 1–3 in PROCEDURE).
- I-SceI enzyme (5,000 U ml^{-1}) and 10× I-SceI buffer (New England Biolabs, cat. no. R06945) ▲ **CRITICAL** Both reagents must be aliquotted on arrival and stored at −80 °C to avoid loss of their activities caused by higher temperature and repeated freeze-thaw cycles.
- Phenol red (Wako, cat. no. 165-01121 or equivalent)
- Penicillin-streptomycin (Invitrogen, cat. no. 15140) ▲ **CRITICAL** This should be dissolved in distilled water, aliquotted and stored at −20 °C
- Ethanol (70% (vol/vol); Wako, cat. no. 057-00456 or equivalent)
- Sodium thioglycolate (Wako, cat. no. 194-03551 or equivalent)
- Ficoll PM 400 (Sigma-Aldrich, cat. no. F4375)
- Agarose (0.8% (wt/vol) standard 01; Solana, cat. no. 10110)
- Living brine shrimp larvae (A&A Marine)
- Frozen mosquito larvae (Akamushi; Kyorin)
- NaCl (Wako, cat. no. 191-01665 or equivalent)
- KCl (Wako, cat. no. 163-03545 or equivalent)
- $CaCl_2$ (Wako, cat. no. 039-00475 or equivalent)
- $MgCl_2 \cdot 6H_2O$ (Wako, cat. no. 135-00165 or equivalent)
- $NaHCO_3$ (Wako, cat. no. 191-01305 or equivalent)
- Tris (2-amino-2-hydroxymethyl-1,3-propanediol; Wako, cat. no. 207-06275 or equivalent)
- EDTA (EDTA·2Na·$2H_2O$; Wako, cat. no. 345-01865 or equivalent)
- Milli-Q ultrapurified water (from Millipore water system)

EQUIPMENT

- Plasmid maxi kit (Qiagen, cat. no. 12162 or equivalent)
- Plastic cups (bottom diameter: 6–8 cm; lid diameter: 8–10 cm; height: 4 cm; 100–200 ml, or equivalent)
- Terasaki plate (96-wells, sterile; Watson, cat. no. 4846-TPS)
- Sterile wide-bore pipettes
- Parafilm sheet (Pechiney Plastic Packaging)
- Syringe filter of 0.2-μm pore size (DISMIC-25 cs, Cellulose Acetate; Advantec)
- Glass Petri dish (diameter: 3 cm; height: 1.5 cm; see EQUIPMENT SETUP)
- Plates (6-well; Falcon 3502, Becton Dickinson or equivalent)
- Tanks or containers for keeping adult newts that have been administered a HCG injection
- Air incubator (adjustable between 12 and 25 °C)
- Dry-bath incubator (adjustable to 37 °C)
- Borosilicate glass capillaries (model G-1, outer diameter: 1 mm; inner diameter: 0.6 mm; length: 90 mm; no internal filament; Narishige or equivalent; see EQUIPMENT SETUP)
- Pressure microinjector (PV820 Pneumatic Picopump; WP Instruments or equivalent)
- Needle vertical puller (PC-10; Narishige or equivalent)
- Needle microgrinder (EG-400; Narishige)
- Needle micromanipulator (MP-330; Narishige or equivalent)
- Fluorescence stereomicroscope (Leica M165 FC, Leica or equivalent; see EQUIPMENT SETUP)
- Digital video camera (C-5060; Olympus) or CCD camera systems

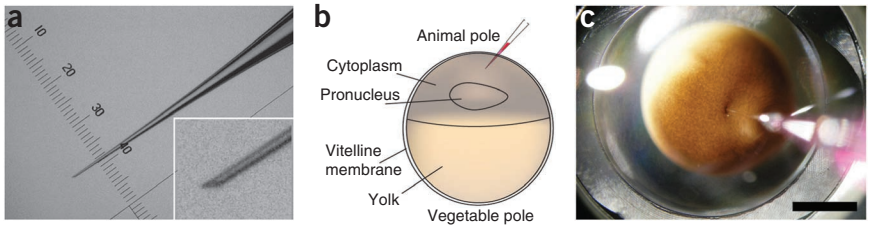

Figure 1 | Microinjection into a *C. pyrrhogaster* egg. (**a**) The tip shape of a micropipette for injection. Inset is a magnified view of the tip. The scale on the ruler is 30 µm. (**b**) Schematic diagram showing the injection site. (**c**) A view of microinjection. Scale bar, 1 mm. Permission was obtained from the University of Tsukuba Animal Use and Care Committee (AUCC) for these experiments. This figure was reproduced, with permission, from the data shown in reference 7.

REAGENT SETUP

Modified Holtfreter's solution (MHS) stock solution (20×) MHS stock solution is prepared with70 g per liter NaCl, 1 g per liter KCl, 2 g per liter $CaCl_2$ and 4 g per liter $MgCl_2{\cdot}6H_2O$. It can be stored at room temperature (22–24 °C) before use (within 6 months). Shake the solution well before use.

MHS (0.5×) Dilute 20× MHS stock solution to 0.5× MHS with distilled water and autoclave. After the solution is cooled to room temperature, add penicillin-streptomycin to obtain a final concentration of 1× . Adjust the pH to 7.4 with $NaHCO_3$. It can be stored at 4 °C for up to 1 month.

MHS (0.1×) Dilute 20× MHS stock solution to 0.1× MHS with distilled water and autoclave. After the solution is cooled to room temperature, add penicillin-streptomycin to obtain a final concentration of 1×. Adjust the pH to 7.4 with $NaHCO_3$. It can be stored at 4 °C for up to 1 month.

Dejellying solution (500 ml) Dejellying solution is prepared by mixing 0.5× MHS with 2% (wt/vol) sodium thioglycolate. Adjust the pH to 9–10 with $NaHCO_3$. ▲ **CRITICAL** Store the solution at 4 °C. It should be used as soon as possible (within 1 month) because the activity gradually declines. Shake the solution well before use.

Microinjection medium (250 ml) Microinjection medium is prepared by mixing 0.5× MHS with 6% (wt/vol) Ficoll PM 400. Warm (~60 °C) and stir the solution well. Allow the solution to cool to room temperature and add penicillin-streptomycin to obtain a final concentration of 1×. Adjust the pH to 7.4 with $NaHCO_3$. It can be stored at 4 °C for up to 4 months.

Phenol red stock solution (0.1% (wt/vol)) Mix 0.05 g phenol red in 45 ml Milli-Q water, dissolve by adding 800 µl of 0.3 M NaOH, adjust the pH to 7–8 with NaOH by checking coloration (purple) and fill up to 50 ml with Milli-Q water. It can be stored at 4 °C for up to 6 months.

TE buffer TE buffer is prepared by combining 10 mM Tris and 1 mM EDTA. Mix 1 ml of 1 M Tris solution (pH 8.0, adjusted with HCl) and 200 µl of 0.5 M EDTA solution (pH 8.0, adjusted with NaOH) and adjust the volume up to 100 ml with Milli-Q water. It can be stored at room temperature for up to 6 months.

EQUIPMENT SETUP

Injection needles These are made from borosilicate glass capillaries (outer diameter: 1.0 mm; inner diameter: 0.6 mm). Conventional needle pullers can be applied (for example, the Narishige PC-10). Needle tips should be ground at an ~35° angle using a microgrinder (for example, the Narishige EG-400 (see **Fig. 1**, panel **a**)). The outer and inner tip diameters should be 10–15 µm and 2–3.5 µm, respectively. Such a tip shape is required to prevent physical damage to the injected embryos. We use needles such that it takes 10–20 ms to inject 1 nl of solution under a nitrogen gas pressure of 4 to 6 psi when controlled by a microinjector.

Glass Petri dish This should be sterilized by autoclaving. Coat the inner surface of the dish with ~0.25 ml of 0.8% (wt/vol) agarose to prevent embryos from sticking to the glass.

Fluorescence microscopy Conventional dissecting microscope and camera combinations would be applied. We use a fluorescence stereomicroscope (Leica M165 FC) with a narrow bandwidth-filter set for GFP (Leica GFP-Plant; 470/40 nm excitation filter; 495 nm dichromatic beam splitter; and 525/50 nm barrier filter). Photos and movies are taken with a digital video camera (C-5060; Olympus) attached onto the microscope.

PROCEDURE

Preparation of an I-SceI plasmid carrying a transgene ● TIMING 5–7 d

1| Clone a transgene of interest into a suitable vector, as described in other animals[9–15].

▲ **CRITICAL STEP** The plasmid must carry I-SceI sites flanking a transgene cassette. An example, pCAGGs-EGFP (Sce), is shown in **Figure 2a** (a gift from E.M. Tanaka; see ref. 12).

2| Purify the plasmid DNA using plasmid maxi kit or equivalent protocols.

▲ **CRITICAL STEP** The purified DNA sample must be free of toxic contaminants.

3| Dissolve the plasmid DNA in TE buffer at a concentration of 10 µg μl^{-1} and store it at −20 °C.

Collection of fertilized eggs (one-cell-stage embryos) ● TIMING 4–5 d

4| Inject 30 *C. pyrrhogaster* females with 30 U (50 µl) of HCG in the abdominal region every other day.

▲ **CRITICAL STEP** HCG injection allows eggs to mature. A subcutaneous injection may be better than an injection of the same dose into the abdominal cavity. A few days after the first injection, female newts become capable of releasing fertilized eggs.

5| The night before microinjection, place strips of plastic sheets (imitation of leaves of water plants) into the tanks/containers to allow the animals to lay eggs on them.

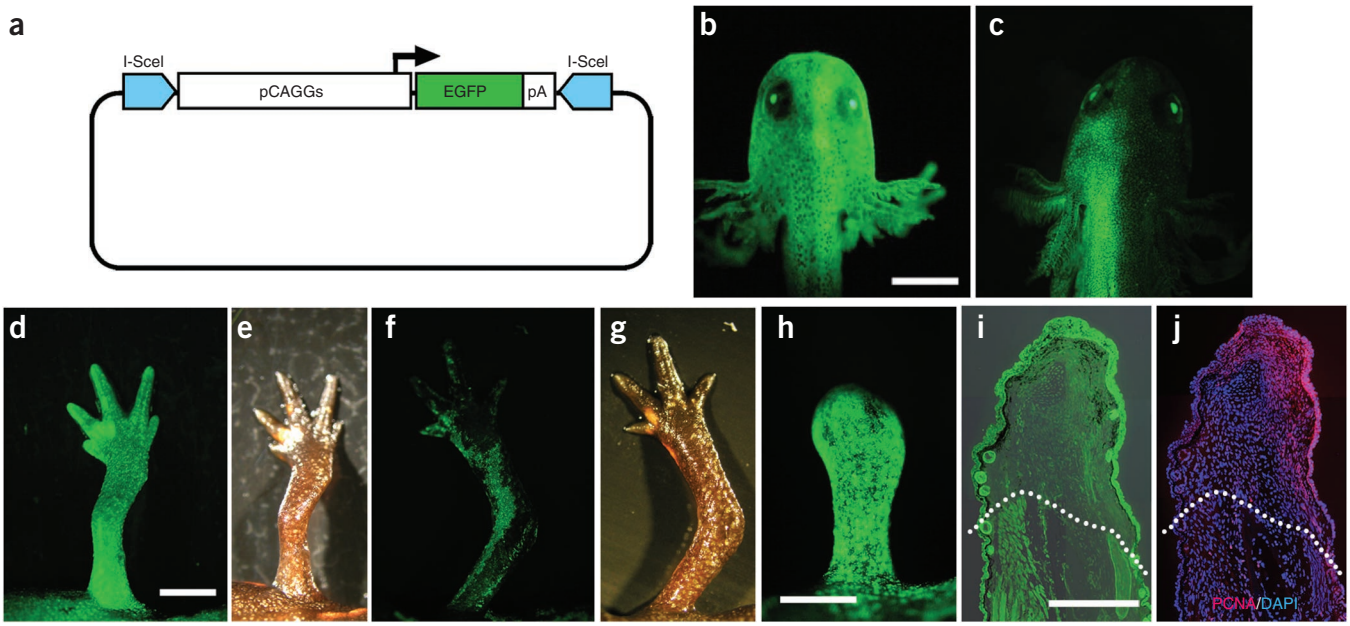

Figure 2 | Examples of transgenic *C. pyrrhogaster* newts generated by the I-SceI method. (**a**) An I-SceI transgene construct, pCAGGs-EGFP (Sce). The pCAGGs-EGFP cassette is flanked at both ends by I-SceI recognition sites (black arrow: initiation site and direction of transcription; pA: SV40 polyadenylation signal). (**b**–**i**) This was used for generating the transgenic larvae (**b**,**c**) and metamorphosed juveniles (**d**–**i**). (**b**,**c**) Swimming larvae showing EGFP expression in the whole body evenly (**b**, classified as 'uniform'; stage 50) and only in one side of the body (**c**, classified as 'mosaic'; stage 56). A dorsal view of the head region is shown. (**d**–**g**) Limbs of metamorphosed juveniles (4–5 months) classified as 'uniform' and 'mosaic' at swimming larvae stage. (**d**–**g**) Fluorescence and bright-light images of a hind limb of the 'uniform' juvenile (**d**,**e**); those of a forelimb of the 'mosaic' juvenile (**f**,**g**). (**h**) A regenerating limb (34 d after amputation) of a metamorphosed juvenile showing 'uniform' expression pattern. (**i**) A dorsoventral section of the regenerating limb in **h**. The right-hand side of the panel is the ventral side. The dotted line shows the site of amputation. (**j**) Proliferating cell nuclear antigen (PCNA)-immunohistochemistry with the same section, visualizing many proliferating cells (red) in the regenerating tip of the limb. DAPI (4,6-diamidino-2-phenylindole) counterstaining of nuclei is shown in blue. Scale bars in **b** (applied to **c**), **d** (applied to **e**–**g**), **h**, 2 mm; scale bar in **i**, 200 μm (applied to **j**). Permission was obtained from the University of Tsukuba AUCC for these experiments. A part of this figure was reproduced, with permission, from the data shown in reference 7.

6| On the morning (0600 to 0800 hours) of the day of microinjection, collect eggs enclosed in a jelly capsule (jellied eggs) together with the plastic sheets.

▲ **CRITICAL STEP** This timing is critical to obtain one-cell-stage embryos for microinjection, because female newts lay eggs early in the morning. A total of 100–200 eggs can be obtained from the same female newts every morning for over 2 weeks. When the TAT system, including 60 female newts (30 per tank), is adopted, ~100 eggs can be obtained per day of microinjection (2–3 d per week)[7]. Data in **Figure 2** and **Table 1** were obtained with the TAT system (see Experimental design for further information on animal housing conditions).

7| Isolate the jellied eggs carefully from the plastic sheets with forceps and transfer them to a plastic cup containing 50 ml of chilled 0.5× MHS.

▲ **CRITICAL STEP** Keep 0.5× MHS (used for holding or washing eggs), 70% (vol/vol) ethanol, dejellying solution and injection medium at 4 °C or on ice. This makes the progress of early embryonic development slow, thus helping to increase the transgenesis efficiency.

Preparation of dejellied eggs for microinjection ● TIMING 30 min

8| Carefully decant 0.5× MHS from the cup containing the jellied eggs. Sterilize the eggs with 30–50 ml of 70% (vol/vol) ethanol for 1 min and then rinse with the same volume of 0.5× MHS three times to remove the ethanol.

▲ **CRITICAL STEP** Sterilization of jellied eggs is necessary to increase the survival rate of embryos by minimizing bacterial contamination in rearing solutions.

9| To remove the jelly capsule from the eggs, carefully replace the 0.5× MHS in the cup with 20–30 ml of dejellying solution. Immediately after the egg comes out of the jelly capsule (10–15 min), transfer them quickly and carefully to another clean cup containing 0.5× MHS one by one with a sterile wide-bore pipette.

TABLE 1 | Optimization of I-SceI transgenesis in *C. pyrrhogaster*.

DNA[a] (×10^{-2} ng)[b]	I-SceI (×10^{-3} U)[b]	Injected embryos	Expression in normal survivors (%)[c]				Dead or abnormal (%)
			Uniform		Mosaic	None	
			Strong	Weak or moderate			
40	0	126	0	1	18	13	68
8	1	133	0	8	5	5	82
40	1	129	3	9	7	5	76
80	1	116	5	14	9	3	69
40	4	86	7	13	7	1	72
80	4	94	5[d]	11	2	0	82

[a]pCAGGs-EGFP (Sce) was used. [b]The amount of DNA and I-SceI injected per embryo. [c]The expression was scored at swimming larval stage (stages 40–42). Injection volume was 4 nl per embryo. 'Uniform': fluorescence is observed throughout the body, and the fluorescence intensity of cells in the body is almost even, except for pigment cells. 'Mosaic': fluorescence is observed only in parts or regions of the body, or in larvae showing widespread expression patterns, the fluorescence intensity of cells in the body is obviously different. [d]Every larva showed very strong fluorescence. The data for different conditions were collected in parallel using the same batch of eggs and accumulated from three independent experiments because the tendency of the results in each experiment was consistent, i.e., I-SceI increases 'Uniform' while decreasing 'Mosaic/None', and 4 × 10^{-3} U I-SceI and 40–80 × 10^{-2} ng DNA are better for obtaining 'Uniform' with strong expression. This table was reproduced, with permission, from the data shown in ref.7.

10| Gently wash the dejellied eggs ten times with 50 ml of 0.5× MHS to remove excess dejellying solution and jelly debris.
! CAUTION The vitelline membrane should be preserved. For further details on dejellying eggs, follow standard protocols[12,16].
▲ CRITICAL STEP Place the cup containing the dejellied eggs on ice beside a stereomicroscope.

11| Fill the wells of a Terasaki plate with the microinjection medium by pouring it on the plate through a syringe filter (0.2-μm pore size). Keep the plate on ice beside the stereomicroscope.

12| Separate one-cell-stage embryos with no apparent damage (i.e., uncleaved eggs with no leakage of yolk; see **Fig. 1c**) from the dejellied eggs in the cup under the stereomicroscope and transfer and submerge them into the wells of the plate, one embryo per well. Keep the plate containing the embryos on ice or at 4 °C.
▲ CRITICAL STEP To maintain the concentration of the microinjection medium, 0.5× MHS surrounding the embryo must be removed before they are transferred to the plate. Therefore, the embryos can be soaked in a small volume of microinjection medium in a dish before transferring them on to the plates. The number of one-cell-stage embryos suitable for microinjection is 85–170 (~85% of all eggs). If the TAT system is adopted, it is 60–80 (see ref. 7).
■ PAUSE POINT The one-cell-stage embryos on Terasaki plate may be stored on ice or at 4 °C for 1–2 h.

Preparation of microinjection solution ● TIMING 45 min
13| Set up the microinjection solution reaction mixture as follows and incubate at 37 °C for 40 min.

Reagents	Volume (μl)
Phenol red (0.1%)	1
I-SceI plasmid (1 μg μl^{-1}) diluted from Step 3	2
I-SceI buffer (10×)	1
I-SceI (5 U μl^{-1})	2
Milli-Q water	to 10

▲ CRITICAL STEP Prepare the I-SceI plasmid solution (1 μg μl^{-1}) immediately before use, by diluting the plasmid stock (10 μg μl^{-1} in TE buffer; see Step 3) with Milli-Q water. This dilution with water reduces toxic effects of Tris/EDTA in the TE buffer on embryos. This 40-min reaction is critical for successful transgenesis, with non-mosaic widespread expression of transgene (see ref. 7). The amount of the plasmid DNA may be modified depending on the transgene of interest. For pCAGGs-EGFP (Sce), 2 μl apparently increases the ratio of transgenic *C. pyrrhogaster*, exhibiting even and very strong fluorescence (see ANTICIPATED RESULTS). The volume of the microinjection solution per embryo should be kept at 4 nl, as described in Step 17.

Preparation of injection needles ● TIMING 5 min

14| After the start of incubation in Step 13, prepare microinjection needles as described in EQUIPMENT SETUP (**Fig. 1a**).
▲ **CRITICAL STEP** This Step must be completed before Step 13 ends. After 40 min of incubation, the reaction mixture must be placed on ice immediately and used for microinjection as soon as possible.

Injection of embryos ● TIMING 30 min for 85–170 embryos

15| Place a drop of the reaction mixture on a parafilm sheet and load 2 μl of this solution into an injection needle from its front tip by suction.

16| Place the one-cell-stage embryos on Terasaki plate under the stereomicroscope.

17| Inject 4 nl of the solution into the cytoplasm below the cortex (~50 μm in depth) adjacent to the animal pole (**Fig. 1b**,**c**).
▲ **CRITICAL STEP** Injection can be carried out at room temperature. Injection near the equator is lethal to embryos, and that into the vegetal pole is difficult because the yolk plugs up the needle tip[7]. Larger amounts of the injection solution cause a decrease of the success rate of transgenesis (see ref. 7). The time window in which the injected DNA is successfully integrated into the host genome is restricted to the early period of the one-cell stage. The one-cell stage of *C. pyrrhogaster* embryos typically lasts 2–4 h at room temperature after they are collected in early morning (Step 6). Therefore, we may take 1 h for injection even at room temperature.
! **CAUTION** The reaction mixture may not be stable for a long period, as suggested in ref.14. Therefore, we usually complete all injections within 30 min.

18| Keep the injected embryos at 14 °C in the injection medium until the next morning (for 20–24 h).
▲ **CRITICAL STEP** Temperature for rearing embryos after injection must be lower than room temperature. A temperature of 14 °C is the best for their survival (see ref. 7).

Rearing of transgenic *C. pyrrhogaster* beyond metamorphosis ● TIMING 4–5 months

19| Using a sterile wide-bore pipette, carefully transfer embryos from the Terasaki plate to Petri dishes (<20 embryos per dish) filled with 0.5× MHS that has been sterilized using a syringe filter (0.2-μm pore size). Incubate the embryos at 14 °C until they reach stage 22 (for developmental stages, see refs. 16,17). Exchange the solution every other day. Check the embryos daily. If dying or abnormal embryos (for standard development, see refs. 16,17) are found, remove them and immediately exchange the solution for fresh solution using sterile pipettes.
▲ **CRITICAL STEP** The bottom of the Petri dish must be coated with ~0.25 ml of 0.8% (wt/vol) agarose before MHS is poured; otherwise, the injected embryos stick to the bottom of the dish and mostly fail to develop normally. Too many embryos should not be placed in the same dish (<20 embryos per dish (diameter: 30 mm; height: 15 mm; 5 ml)). Embryos reared at higher densities can become unhealthy or die, particularly because of the presence of necrotic embryos.

20| At stage 22 (~1 week after microinjection; neurula to tail-bud stage), exchange the rearing solution for 0.1× MHS. Incubate the embryos at 14 °C until stage 35 is attained. Exchange the solution every other day. Check the embryos daily. If dying or abnormal embryos are observed, remove them and immediately exchange the solution for fresh solution using sterile pipettes.
▲ **CRITICAL STEP** Decreasing the osmolarity of the rearing solution at this stage is critical. Otherwise, the embryos cannot develop normally.

21| At stage 35 (~2 weeks after microinjection), transfer embryos to multiwell plates with no agarose coating, one embryo per well (diameter: 35 mm; height: 20 mm; 10 ml), and thereafter rear them at room temperature. Exchange the solution every other day. Check the embryos daily and remove dying or abnormal embryos.

22| At stages 45–47 (~3 weeks after microinjection; swimming larvae at this stage still have the yolk in the digestive tract), start feeding the larvae with live brine shrimp larvae a few times a week.
▲ **CRITICAL STEP** The multiwell plates should be exchanged for clean ones 12 h after feeding. Keep the larva alone in a chamber or cup; otherwise they will feed on each other.

23| At stage 59 (6–7 weeks after microinjection), transfer grown swimming larvae (~3 cm) to plastic cups (bottom diameter: 6 cm; lid diameter: 8 cm; height: 4 cm) filled with 50 ml of 0.1× MHS, one embryo per cup.
▲ **CRITICAL STEP** The cups should have lids with holes punctured by a needle to allow aeration. From this stage, antibiotics are not necessary and 0.1–0.2× MHS can be used as rearing solution. Feed excess brine shrimp a few times a week. When the

swimming larvae grow larger than 3 cm, foods can be switched to a small mosquito larvae (Akamushi) or earthworms. Ensure that the rearing solution is clean. The cups should be exchanged for clean ones 12 h after feeding.
! CAUTION The larvae stop feeding as they approach metamorphosis.

24| Decrease the level of 0.1× MHS in the cup gradually when metamorphosis starts (4–5 months after microinjection). After metamorphosis, rear juveniles in 0.1× MHS, shallow enough to wet their abdomen and feet.
▲ CRITICAL STEP Do not submerge juveniles in water because they would drown. Alternatively, a rest place (such as a piece of sponge) can be placed in the cup. Continue to feed small mosquito larvae or earthworms a few times a week. Ensure that the cups are clean at all times.
? TROUBLESHOOTING

25| Monitor the expression of transgenes using an appropriate method, for example, through a digital video camera (C-5060; Olympus) attached onto a fluorescence stereomicroscope (Leica M165 FC).
▲ CRITICAL STEP For living animals, bright-light and fluorescence images/movies should be acquired. In the case of pCAGGs-EGFP (Sce), strong EGFP fluorescence is observed in the whole body from late blastula stage beyond metamorphosis (**Fig. 2**; also see ref. 7). To monitor the EGFP expression, we use the above-mentioned system with a narrow bandwidth-filter set for GFP (Leica GFP-Plant; for details, see EQUIPMENT SETUP). To analyze regenerating body parts of metamorphosed animals, transgene expression should be examined using tissue sections (**Fig. 2i**). Immunohistochemistry can be a powerful tool to determine whether the transgene is expressed in the cells of interest. For example, proliferating/stem cells in regenerating limbs can be stained with a proliferating cell nuclear antigen antibody (**Fig. 2j**), according to the method described in reference 19.
? TROUBLESHOOTING

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

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
24	Injected embryos die or fail to become metamorphosed juveniles	The amount of injected DNA is too large	Decrease the DNA amount to 0.4 ng per embryo (Step 13)
		The rearing temperature for injected embryos is too high	Keep the rearing temperature after microinjection at 14 °C (Step 18)
		Embryos stick onto the bottom of the Petri dish	Coat the Petri dish with agarose (Step 19)
		The osomolarity of the rearing solution for embryos after neurula stage is too high	Exchange the rearing solution to 0.1× MHS at a transitional stage (stage 22) from the neurula to the tail-bud stage (Step 20)
		The level of a rearing solution for metamorphosing larvae is too high	Keep the solution level shallow enough to wet the abdomen and feet of larvae (Step 24)
		Rearing solutions are dirty	Keep rearing solutions and dishes/cups clean at all times
		The quality of fertilized egg is not good	Improve rearing conditions of female newts or request higher-quality females from JNRC
25	Injected embryos develop normally, but show little or only mosaic transgene expression	I-SceI enzyme stock may be losing activity	Use newly purchased I-SceI
		Injection is carried out during the transition from one- to two-cell stage (Step 17)	Keep embryos on ice or at 4 °C to slow down early development (Steps 7 and 12), and inject them as soon as possible (Step 17)

(continued)

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
		The I-SceI activity in the reaction mixture has stopped by the time of injection (Steps 14 and 17)	Use an I-SceI reaction as soon as possible after the end of its 37 °C, 40-min incubation
		The amount of the injected DNA is insufficient to be integrated into the host genome	Increase the DNA amount up to 0.8 ng per embryo (Step 13)
		The amount of I-SceI is insufficient to mediate transgene integration into the host genome	Increase the amount of I-SceI to up to 0.004 U per embryo (Step 13)

● TIMING

Steps 1–3: 5–7 d
Steps 4–7: 4–5 d
Steps 8–12: 30 min
Step 13: 45 min
Step 14: 5 min
Steps 15–18: 30 min to inject 85–170 embryos, plus 20–24 h of incubation
Steps 19–25: 4–5 months

ANTICIPATED RESULTS

One person can inject 85–170 embryos per day, which results in 17–34 transgenic swimming larvae that express a transgene throughout the body evenly. Under our rearing conditions, one can obtain almost the same number of metamorphosed juveniles (~20% of the injected embryos). Therefore, by a 3-week microinjection experiment with 30 female newts, one can obtain a total of > 3,000 injected embryos and > 500 transgenic juveniles. If the TAT system, including 60 female newts (30 per tank), is adopted[7], one can obtain 12–16 transgenic juveniles per microinjection experiment (two to three times per week) constantly from October to July (10 months) in the laboratory.

For *C. pyrrhogaster* newt transgenesis, the optimum amount of injection volume is 4 nl per embryo[7], and the optimum amounts of DNA and I-SceI are 40×10^{-2} ng and 4×10^{-3} U per embryo, respectively. **Table 1** shows the results of our experiments to determine these parameter values using pCAGGs-EGFP (Sce). The transgenesis efficiency can be determined at the swimming larvae stage (stage 40–42), because the result is a good indicator to estimate the number of survivors beyond metamorphosis (swimming larvae mostly survive beyond metamorphosis). As can be seen in **Table 1**, I-SceI obviously improves the expression patterns from 'none/mosaic' to 'uniform'; in the absence of I-SceI, almost all transgenic larvae show mosaic expression patterns, as observed in the injection of DNA alone[17]; however, incubation of DNA with I-SceI at 37 °C for 40 min (Step 13 in PROCEDURE) apparently increases the ratio of 'uniform' larvae while decreasing that of 'none/mosaic' ones. In the presence of 1×10^{-3} U I-SceI in the 4-nl injection solution, the ratio of 'uniform' larvae and the fluorescence intensity increase with increasing amount of DNA in the injection solution. The increase of I-SceI up to 4×10^{-3} U further improves both the expression pattern and fluorescence intensity. However, under these conditions, one must ensure that optimum amount of DNA is injected, because increasing the amount of DNA up to 80×10^{-2} ng leads to an increase in dead or abnormal embryos, although leading to a significant increase in the fluorescence intensity (see asterisk in **Table 1**). Therefore, in this study, we recommend 40×10^{-2} ng per embryo as the optimum amount of DNA for injection. However, one can examine from 40×10^{-2} to 80×10^{-2} ng of DNA, depending on the nature of transgenes (see **Table 2** for TROUBLESHOOTING).

Under these optimized conditions, ~20% of swimming larvae express transgenes throughout the body evenly (see 'uniform' in **Table 1** and **Fig. 2b**; and see Supplementary Video 1), whereas ~7% exhibit mosaic patterns of expression (see 'mosaic' in **Table 1** and **Fig. 2c**). **Figure 2d–g** shows limbs of metamorphosed juveniles (4–5 months after microinjection) that have been classified as 'uniform' (panels d and e) and 'mosaic' (panels f and g) at the swimming larval stage.

Metamorphosed juveniles (later than 4–5 months after microinjection) can be used for the study of regeneration. For example, **Figure 2h** shows a regenerating limb of a 9-month-old transgenic juvenile. Histological analyses demonstrate that transgenes can be expressed in the blastema (containing proliferating/stem cells) as well as in the amputated limb (**Fig. 2i**,**j**).

Note: Supplementary information is available via the HTML version of this article.

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