Ligand-controlled site-specific recombination in zebrafish

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Summary

Cre-mediated site-specific recombination has emerged as an indispensable tool for the precise manipulation of genomes allowing lineage tracing studies, temporal and spatial misexpressions and in particular the generation of conditional knockout alleles. Previously, we and others showed that Cre and its ligand inducible variant CreER^{T2} are also highly efficient in the developing and adult zebrafish. The number of Cre driver and effector lines is currently still limited in zebrafish. However, the recent advent of novel genome editing tools such as TALEN and CRISPR/Cas will significantly increase interest in the conditional Cre/lox-technology in this organism. The considerations of basic transgene design and subsequent transgenesis have been addressed elsewhere. Here we outline practical experimental steps for transient functionality tests of CreER^{T2} driver and effector constructs. In addition, we introduce detailed protocols to elicit CreER^{T2}-mediated recombination *in vivo* at embryonic as well as adult stages.

1. Introduction

Site-specific recombinases promote strand exchanges between two sequence-specific target sites without any additional cofactors [1]. The most commonly used recombinase is the bacteriophage P1-derived cyclic recombinase Cre, which catalyzes site-specific recombination between two *loxP* target sites. Each *loxP* site consists of two 13-bp repeats flanking an 8-bp asymmetric spacer sequence that confers directionality (Fig. 1A). Head-to-head orientation causes inversion of the DNA between the two target sites, whereas head-to-tail orientation results in the excision of the intervening DNA sequence, an irreversible reaction due to the rapid loss of the excised product (Fig. 1A). Temporal control of recombination can be achieved by using recombinases fused to a ligand-binding domain of a steroid hormone receptor [2]. Only administration of the ligand results in the translocation of the recombinase to the nucleus, where it catalyzes the recombination event (Fig. 1B). Currently, Cre fused to the mutated human ligand-binding domain of the estrogen receptor (CreER^{T2}) has the best properties for ligand sensitivity and inducible recombination efficiency [3].

Previous attempts to establish the Cre/lox system in zebrafish showed its general functionality [4,5] but recombination frequencies were very low [6,7]. The reason of this inefficiency is still unknown but the transgenesis approach might be crucial. Initially, transgenic zebrafish were generated by microinjection of naked plasmid DNA into the cytoplasm of one cell-stage embryos resulting in tandem arrays or concatemeric DNA integration of up to 2000 copies [8]. In contrast, single integration events using transposon-mediated transgenesis resolved these issues [9,10] and has triggered a strong increase in Cre/lox applications in zebrafish [11-15]. The methodology of transposon-mediated transgenesis in general and considerations of basic transgene design for Cre/lox recombination experiments in particular have been addressed previously [16,17]. Here in this chapter we aim to provide detailed practical experimental steps for transient functionality tests of CreER^{T2} driver and effector constructs and step by step protocols for ligand-mediated CreER^{T2} activation *in vivo* at embryonic as well as adult stages.

2. Material

1. Fish tanks and systems for maintenance and breeding of fish (Aqua Schwarz GmbH).
2. Fish food: paramecia.
3. Fish food: brine shrimp.
4. Fish food: Tetramin flakes.

2.1 Transient functionality tests of CreER^{T2} driver and effector constructs by microinjection

1. Needle puller (Flaming/Brown P-97 Micropipette Puller, Sutter Instrument Co.).
2. Glass capillaries (WPI, TW100-3 for injection).
3. Microinjector (WPI, PV820 pneumatic Pico Pump with foot pedal).
4. Micromanipulator (Narishige M-152).
5. Ball-joint-holder (WPI).
6. Magnet holder MB-B (Kanetec).
7. Petri dish, diameter: 60 mm.
8. Watchmaker forceps.
9. Dissecting scope (Olympus SZX16).
10. E3 embryo medium: 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, 0.0002 % methylene blue (pH adjusted to 7.2 with NaOH).
11. 2 % phenol red (RNase-free).
12. Microloader tips (Eppendorf).
13. Fluorescent stereoscope (Olympus MVX10).

2.1.1 Transient functionality test of CreER^{T2} driver constructs

Injection of a novel CreER^{T2} driver construct into the established Cre effector line *Tg(hsp70l:LOXP-DsRed2-LOXP-EGFP)^{tud107}*

1. Novel CreER^{T2} driver construct (endotoxin-free).
2. Wild-type fish (AB).

3. *Tg(hsp70l:LOXP-DsRed2-LOXP-EGFP)^{tud107}* (a Cre-dependent reporter line which expresses DsRed2 in the absence of Cre activity but changes to EGFP after a successful recombination event [12]).

4. 50 mM Tamoxifen (TAM; Sigma) in DMSO or 25 mM 4-Hydroxytamoxifen (4-OHT; Sigma) in EtOH. (Prolonged storage decreases potency of 4-OHT. Use of fresh or recently dissolved 4-OHT is advised [16].)

5. Water bath (GFL 1002).

Injection of a novel CreER^{T2} driver construct along with *pTol(hsp70l:LOXP-DsRed2-LOXP-EGFP)* into wild-type embryos

1. *pTol(hsp70l:LOXP-DsRed2-LOXP-EGFP)* (an established effector construct) (endotoxin-free).

2.1.2 Transient functionality test of CreER^{T2} effector constructs

Injection of a novel Cre effector construct into the established CreER^{T2} driver line *Tg(her4.1:mCherry-CreERT2)^{tud106}*

1. Novel CreER^{T2} effector construct (endotoxin-free).

2. *Tg(her4.1:mCherry-CreERT2)^{tud106}* (an established CreER^{T2} driver line which expresses CreER^{T2} under the control of a *her4.1* promoter fragment) [12].

Injection of a novel Cre effector construct along with Cre mRNA into wild-type embryos

1. *pCS2+ Cre* (a plasmid to synthesize Cre mRNA) [5].

2. Notl.

3. Gel and PCR clean up kit (NucleoSpin Extract II kit, Machery-Nagel).

4. mMESSAGE mMACHINE SP6 kit (Ambion).

2.2 CreER^{T2} mediated site-specific recombination at embryonic stages

1. Dimethyl sulfoxide (DMSO).

2. Embryo lysis buffer: 10 mM Tris-HCl (pH 8.2), 10 mM EDTA, 200 mM NaCl, 0,5 % SDS, 200 µg/mL Proteinase K.

3. 100% and 70% ice cold EtOH.
4. Table-top centrifuge (Pico 21, Thermo).
5. PCR machine (Eppendorf Mastercycler ep Gradient S).
6. Primer for amplification: hsp70l-for 5'-CCCCGACGAGGTGTTTATTCGCTC- 3'; GFP-rev 5'-CCGTCCAGCTCGACCAGGATG-3'.
7. PCR tubes.
8. PCR reagents: 10 mM dNTP mix, DreamTaq (Fermentas).
9. Electrophoreses chamber and power supply.
10. DNA Ladder (GeneRuler 1 kb Plus DNA Ladder, Fermentas).

2.3 CreER^{T2} mediated site-specific recombination in the adult central nervous system

1. Heat shock unit (Immersion Thermostat LAUDA Ecoline Staredition E 200).
2. Tricaine methane sulfonate solution: 200-300 mg/l.
3. 0,5 M phosphate buffer: 94 mM NaH₂PO₄, 0.4 M Na₂HPO₄, pH 7.5.
4. 4 % paraformaldehyde / 0.1 M phosphate buffer, pH 7.5.
5. 20 % sucrose / 0.5 M EDTA in 0.1 M phosphate buffer, pH 7.5.
6. 7.5 % gelatin / 20 % sucrose in 0.1 M phosphate buffer, pH 7.5.
7. Cryostat (Kryostat HM 560", Microm).
8. PBSTX: 1x PBS, 0.3% TritonX (w/v).
9. Humid chamber: rectangular petri dish with wet paper towel.
10. Anti-GFP antibody (ABcam, Ab13970).
11. Alexa Fluor® 488 Goat Anti-Chicken (Molecular Probes).
12. 1 µg/ml DAPI in PBSTX.
13. 70 % glycerol/PBS.
14. Fluorescent microscope (Zeiss Axio Imager Z1).

3. Methods

The Cre/lox system is a genetic two component transgene system with Cre driver lines expressing Cre under the control of a specific promoter and Cre effector lines driving a *loxP*-flanked cassette upstream of a second cassette containing the gene of interest (Fig. 2). Successful recombination events can be proven using polymerase chain reaction (PCR), immunohistochemistry or native fluorescence depending on the nature of the Cre effector line (see note 1). Subsequently, we describe the execution of the before-mentioned methods in detail for transient functionality tests of CreER^{T2} driver and effector constructs and CreER^{T2} mediated site-specific recombination at embryonic as well as adult stages. However, the described methods are not restricted for the individual purpose and can be applied for the others as well.

3.1 Transient functionality tests of CreER^{T2} driver and effector constructs by microinjection

It is pivotal to verify the functionality of newly cloned CreER^{T2} driver or effector constructs prior to the establishment of the respective transgenic line. This can be easily achieved by microinjection of the newly generated CreER^{T2} driver or effector construct into an existing effector or CreER^{T2} driver line, respectively. Alternatively, simultaneous microinjection of the newly generated CreER^{T2} driver construct along with an established effector construct or the newly generated effector construct along with Cre mRNA can be employed.

3.1.1 Transient functionality test of CreER^{T2} driver constructs

Injection of a novel CreER^{T2} driver construct into the established Cre effector line *Tg(hsp70l:LOXP-DsRed2-LOXP-EGFP)^{tud107}*

1. Pull needles using needle puller and glass capillaries to obtain rigid, non-flexible needles which are sealed at the tip. (Our parameters: Heat: 535; Pressure: 250; Velocity: 250; Time: 150).
2. Place female and male zebrafish of wild-type and *Tg(hsp70l:LOXP-DsRed2-LOXP-EGFP)^{tud107}* background in mating boxes separated by a divider in the evening prior to the injection day. Next morning combine fish to elicit mating. Microinjection should be carried out at the 1-cell stage (0-30 min post fertilization). Several fish couples can be used subsequently to ensure a constant supply of freshly laid eggs.
3. Prepare injection solution: 35 µg/µl novel CreER^{T2} driver construct, 0,2 % phenol red.
4. Load needle with 2 µl injection solution using a microloader tip.

5. Place 20-25 embryos in a petri dish with E3 medium into the field of view of the dissecting scope. Attach needle to the holder of the micromanipulator and adjust it to a 45° angle. Lower the needle tip into the focus plane of the embryos.
6. Open the needle with a pair of sharp watchmaker forceps and adjust the hold pressure. Inject 1-4 nl of the injection solution into the cytoplasm. A drop of mineral oil on a micrometer can be used to calculate the volume of each injection. When injected into the oil, a bead with a diameter of 0.1 mm contains 500 pL of injection material [18]. Carry out injections using the up and down control of the micromanipulator only. Injected embryos can stick to the needle but are released if the needle is pulled out of the E3 medium.
7. Transfer injected embryos into a fresh petri dish containing E3 medium. Continue injections, collect injected embryos in the same petri dish and transfer them into a 28°C incubator when finished.
8. Thaw 50 mM TAM or 25 mM 4-OHT stock 8 hours post injection and prepare E3 medium containing 5 µM TAM or 5 µM 4-OHT, respectively (see note 2).
9. Remove dead and malformed embryos from the injected clutch. Replace E3 medium with working E3/TAM or E3/4-OHT solution and incubate at 28°C overnight.
10. Preheat E3 medium in a 42°C water bath in the evening prior to the heat shock.
11. Next morning, remove working E3/TAM or E3/4-OHT solution entirely, pour in preheated E3 medium and incubate in a 37°C incubator for 60 min (see note 3).
12. Transfer petri dish back to 28°C and observe GFP expression under a fluorescent stereoscope 3 hours post heat shock. Presence of GFP indicates functionality of the novel CreER^{T2} driver construct. If no GFP signal was observed, check for DsRed fluorescence 24h post heat shock to validate the efficiency of the heat shock (see note 4).

Injection of a novel CreER^{T2} driver construct along with *pTol(hsp70l:LOXP-DsRed2-LOXP-EGFP)* into wild-type embryos

1. Place wild-type female and male zebrafish in mating boxes separated by a divider in the evening prior to the injection day.
2. Prepare injection solution: 20 µg/µl novel CreER^{T2} driver construct, 20 µg/µl *pTol(hsp70l:LOXP-DsRed2-LOXP-EGFP)*, 0,2 % phenol red.

3. Perform microinjection, pharmacological treatment, heat shock and fluorescent analysis as described above. GFP expression might be present even without a heat shock because the *hsp70l* promoter is transiently active when not integrated into the genome. However, expression levels are significantly increased after heat shock.

3.1.2 Transient functionality test of fluorescent CreER^{T2} effector constructs

Injection of a novel fluorescent CreER^{T2} effector construct into an existing driver line

1. Place female (wild-type) and male (*Tg(her4.1:mCherry-CreER^{T2})^{tud106}*) zebrafish in mating boxes separated by a divider in the evening prior to the injection day.
2. Prepare injection solution: 35 µg/µl novel CreER^{T2} effector construct, 0,2 % phenol red.
3. Perform microinjection, pharmacological treatment, heat shock and fluorescent analysis as described above.

Injection of a novel fluorescent CreER^{T2} effector construct along with Cre mRNA into wild-type embryos

1. Linearize *pCS2+ Cre* using NotI. Purify with Gel and PCR clean up kit and elute with nuclease-free water.
2. Use 1 µg of linearized plasmid for *in vitro* transcription with mMESSAGE mMACHINE SP6 kit according to the manufacturer's instructions.
3. Place wild-type female and male zebrafish in mating boxes separated by a divider in the evening prior to the injection day.
4. Prepare injection solution: 35 µg/µl novel CreER^{T2} effector construct, 25 ng/µl Cre mRNA, 0,2 % phenol red.
5. Perform microinjection, pharmacological treatment, heat shock and fluorescent analysis as described above.

3.2 CreER^{T2} mediated site-specific recombination at embryonic stages

1. Breed *Tg(hsp70l:LOXP-DsRed2-LOXP-EGFP)^{tud107}* females with *Tg(her4.1:mCherry-CreER^{T2})^{tud106}* males. It is highly recommended to use male CreER^{T2} driver lines in order to avoid unwanted maternal recombinase contribution [16].
2. Collect progeny, remove unfertilized embryos and transfer into a 28°C incubator.
3. Perform pharmacological treatment as described above. Retain DMSO control sample.
4. At 24 hours post fertilization transfer embryos into 1,5 ml tube. Remove E3 medium, add 500 µl embryo lysis buffer and incubate at 55°C in shaker until clear.
5. Add 1000 µl ice-cold 100% EtOH and centrifuge for 10 min at full speed.
6. Discard supernatant, add 200 µl ice-cold 70% EtOH and centrifuge for 2 min at full speed.
7. Discard supernatant, dry pellet and add H₂O with 4µl per embryo.
8. Mix 4 µl of the sample with 2 µl 10x reaction buffer, 0,8 µl dNTP mix (10mM each), 0,5 µl hsp70l-forward primer (20µM), 0,5 µl GFP-reverse primer, 0,2 µl DreamTaq polymerase and 12 µl H₂O. PCR reaction was run in a thermocycler with the following program: 94°C for 30 sec; 32 cycles of 94°C for 30 sec, 62°C for 30 sec, 72°C for 120 sec; followed by 72°C for 8 min.
9. Check PCR samples by agarose gel electrophoresis (1,2 %). Presence of a 525 base pair fragment reveals successful recombination whereas the 1744 base pair amplicon is the un-recombined control.

3.3 CreER^{T2} mediated site-specific recombination in the adult central nervous system

1. Breed *Tg(hsp70l:LOXP-DsRed2-LOXP-EGFP)^{tud107}* females with *Tg(her4.1:mCherry-CreER^{T2})^{tud106}* males. Collect progeny, remove unfertilized embryos and transfer into a 28°C incubator.
2. At 24 hours post fertilization, sort mCherry-positive embryos under a fluorescent stereoscope. Discard mCherry-negative siblings. Perform heat shock as described above. Check for GFP expression under a fluorescent stereoscope 3 hours post heat shock. Presence of GFP indicates unconditional recombination (leakiness) of the CreER^{T2} driver.
3. Check for DsRed fluorescence 24h post heat shock. Select DsRed-positive embryos which represent double transgenic animals carrying CreER^{T2} driver and effector constructs. Incubate double transgenic fish at 28°C (see note 5).

4. At 5 days post fertilization transfer animals to a nursery of a fish facility providing paramecia, brine shrimp and TetraMin flakes according to established guidelines [19].
5. At 6 months post fertilization, place 4 adult zebrafish in a 1000 ml beaker with 750 ml system water and 2.5 μ M TAM. Cover with aluminum foil, provide air supply and incubate overnight. Perform three consecutive overnight treatments with a 12 hour resting period in between. Wash fish several times in system water prior to transfer back to fish facility.
6. After a one month chase period, transfer TAM-treated fish to the heat shock unit and apply a 2 hour heat shock on two consecutive days. Using the Immersion Thermostat LAUDA Ecoline Staredition E 200 we employ the heat shock profile presented in Table 1. Briefly, water temperature is slowly increased from 28°C to 37.5°C over the course of 30 min, maintained at 37.5°C for 90 min and steadily decreased to 28°C over 60 min.
7. Euthanize fish 5 hours post second heat shock by overdosing with tricaine methane sulfonate. Carefully open the skull with watchmaker forceps and fix brains within the skull at 4°C overnight in 4 % paraformaldehyde / 0.1 M phosphate buffer, pH 7.5.
8. Wash twice for 15 min with 0.1 M phosphate buffer, pH 7.5 and transfer for decalcification and cryoprotection to 20 % sucrose / 0.5 M EDTA in 0.1 M phosphate buffer, pH 7.5. Incubate at 4°C for 24 hours.
9. Place the heads/brains in 7.5 % gelatin / 20 % sucrose in 0.1 M phosphate buffer, pH 7.5 at 37°C for 10min. Freeze brains in 7.5 % gelatin / 20 % sucrose in 0.1 M phosphate buffer, pH 7.5 and prepare 10-16 μ m cryosections using a cryostat.
10. Dry sections for 30 min at room temperature and store at -20°C.
11. Thaw sections for 10-30 min at room temperature. Incubate for an hour in 100 % ice-cold Methanol at -20°C and rehydrate for 15 min in PBSTX on a shaker.
12. Incubate sections with anti-GFP antibody diluted 1:2500 in PBSTX in a humid chamber at 4°C overnight.
13. Wash sections 3 times for 10 min in PBSTX on a shaker and incubate with Alexa Fluor® 488 Goat Anti-Chicken diluted 1:500 in PBSTX in a humid chamber for 1-2 hours.
14. Incubate sections in DAPI solution for 3 min.
15. Wash sections 3 times for 10 min in PBSTX on a shaker and mount in 70 % glycerol/PBS.
16. Analyze GFP expression using a fluorescent microscope.

Table1: Heat shock profile

segment	1	2	3	4	5	6	7	8	9	10	11	12	13	14
°C	28	29	30	31	32	33	34	35	36	37	37.5	34	31	28
min	3	3	3	3	3	3	3	3	3	3	90	20	20	20

4. Notes

1. Validation of successful recombination is easily determined by native fluorescence. It is therefore advisable to include a fluorescent protein in the second cassette containing the gene of interest. This can be either a direct fusion or via the viral T2A peptide sequence allowing equimolar production of two proteins from a single open reading frame [20].
2. Time point of TAM/4-OHT application depends on CreER^{T2} expression and the corresponding experiment.
3. When using *hsp70l* promoter driven effector lines, a heat shock can be applied immediately after TAM removal at embryonic stages. In contrast, larval and adult animals are more susceptible and require time – in our experience at least 24h - to recover prior to heat shock. Treatment with 4-OHT (up to 10µM) followed by an immediate heat shock however does not result in fatal consequences.
4. Use of *hsp70l* promoter driven effector lines requires heat shocking to activate the effector construct. In contrast, *ubiquitin B (ubb)* driven effector lines like *ubi:Switch* [14] and *ubi:Zebrawow* [15] are constitutively active at all stages of zebrafish development and in adulthood. In our lab, we have used both approaches and based on our experience the optimal Cre effector line requires individual elucidation.
5. Alternatively, double transgenic animals can be identified using allele-specific PCRs on genomic DNA extracted from fin clips of adult fish.

5. Figure legends

Figure 1: (A) Basic principles of Cre/lox. The Cre/lox system comprises the site-specific recombinase Cre and its target site *loxP* which consist of an 8 bp spacer, flanked by two 13 bp inverted repeats. Orientation of two *loxP* target sites determines the outcome of the recombination

event. Head-to-head orientation causes inversion of the DNA between the two sites, whereas head-to-tail orientation results in the excision of the intervening DNA sequence. (B) Scheme of the ligand-dependent recombination in cells of a red-to green reporter. Due to binding to heat shock protein 90 (Hsp90), the chimeric CreER^{T2} recombinase is retained in the cytoplasm in the absence of the ligand. Upon administration of Tamoxifen which is converted to the active ligand 4-Hydroxytamoxifen (4-OHT), CreER^{T2} translocates to the nucleus catalyzing the recombination event.

Figure 2: The Cre/lox system is a binary system consisting of a Cre driver and an effector line. Most commonly, Cre is driven by a cell-or tissue specific promoter (promoter A) providing spatial control whereas the effector is regulated by a ubiquitous promoter (promoter B) driving expression of a *loxP*-flanked marker or STOP cassette. This cassette is followed by a second cassette or gene of interest (GOI). In cells of double transgenic animals carrying Cre driver and effector transgenes, Cre elicits recombination once promoter A is activated, putting the second cassette or GOI under the control of promoter B.

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