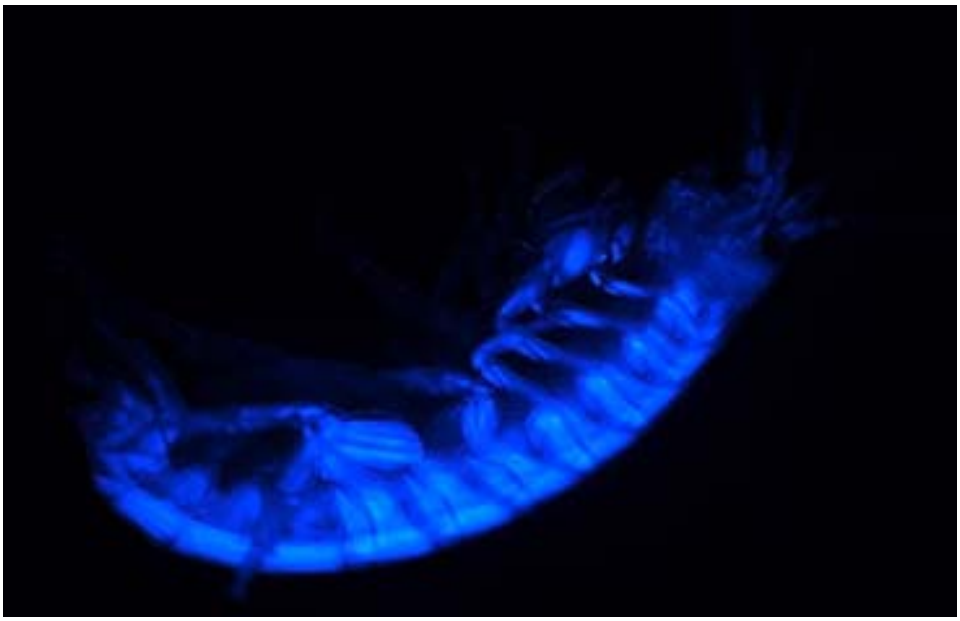
University of Crete, Department of Biology
Graduate program ‘Molecular Biology and Biomedicine’

Master’s thesis

‘Targeted cell ablation towards the identification of cell populations that participate in regeneration’



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Περίληψη:

Η επαγωγή κυτταρικού θανάτου αποτελεί ένα χρήσιμο εργαλείο για το καθορισμό του ρόλου διαφορετικών κυτταρικών τύπων στην ανάπτυξη και αναγέννηση. Ένα χημικό-γενετικό σύστημα βασίζεται στη χρήση του βακτηριακού ενζύμου, νιτροαναγωγάσης (nitroreductase, NTR). Η νιτραναγωγάση ανάγει το υπόστρωμα Μετρονιδαζόλη (Metronidazole, Mtz) και το μετατρέπει σε κυτταροτοξικό μεταβολίτη προκαλώντας κυτταρικό θάνατο. Το σύστημα NTR/Mtz έχει εφαρμοστεί επιτυχώς στο *Danio rerio* και στο *Mus musculus*, υποδεικνύοντας το ως αποτελεσματικό εργαλείο σε διαφορετικούς οργανισμούς. Εφαρμόσαμε το NTR/Mtz σύστημα στο αμφίποδο καρκινοειδές *Parhyale hawaiensis*, το οποίο αποτελεί αναδυόμενο μοντέλο-οργανισμός για τη μελέτη της αναγέννησης των άκρων με πληθώρα γενετικών εργαλείων.

Διαγονίδια που έφεραν ιστοειδικούς υποκινητές ανοδικά του *cfp-ntr* γονιδίου, όπου η νουκλεοτιδική αλληλουχία του ενζύμου NTR έχει συζευχθεί με τη νουκλεοτιδική αλληλουχία της κυανής φθορίζουσας πρωτεΐνης (Cyan fluorescent protein, CFP), εντέθηκαν στο γονιδίωμα του *Parhyale* με τη χρήση του Minos μεταθετού στοιχείου, με αποτέλεσμα την έκφραση της συζευγμένης πρωτεΐνης σε κύτταρα συγκεκριμένου κυτταρικού τύπου. Η παρουσία της φθορίζουσας πρωτεΐνης CFP (Cyan Fluorescent Protein) επιβεβαίωνε την έκφραση του διαγονιδίου και τη παρουσία της πρωτεΐνης στα υπό-μελέτη κύτταρα. Επιπλέον, τα μωσαϊκά ζώα που έφεραν τα διαγονίδια παρουσίαζαν φυσιολογική ανάπτυξη. Προσθέτοντας το υπόστρωμα της νιτροαναγωγάσης, Mtz, προκαλέσαμε κυτταρικό θάνατο σε μυϊκά κύτταρα και παρατηρήσαμε τη διαδικασία μέσω των αλλαγών του κυανού φθορισμού. Με σκοπό να περιορίσουμε την έκφραση του διαγονιδίου *cfp-ntr* επάγοντας NTR^+ κυτταρικών κλώνων στο υπο-μελέτη ιστό, ελέγξαμε δύο συστήματα ανασυνδυασμού (Cre/loxN και Flp/FRT) στο *Parhyale*. Το σύστημα ανασυνδυασμού FRT/Flp επιλέγηκε και ακολούθησαν πειράματα μοριακής βιολογίας για τη κατασκευή πλασμιδιακών κατασκευών με απώτερο σκοπό τη δημιουργία διαγονιδιακών σειρών. Εφαρμόζοντας το σύστημα κυτταρικού θανάτου σε NTR^+ κυτταρικούς πληθυσμούς και με τη χρήση μικροσκοπίας φθορισμού (time-lapse imaging), θα μελετήσουμε την διαδικασία της αναγέννησης των άκρων μετά από θανάτωση του εκάστοτε κυτταρικού τύπου με στόχο το προσδιορισμό του ρόλου διαφορετικών κυτταρικών τύπων στην αναγέννηση.

Abstract:

Conditional targeted cell ablation is a powerful tool for determining the role of specific cell types in development and regeneration. A recently established chemical-genetic system based on the bacterial *E.coli* enzyme nitroreductase (NTR) is efficient for conditional cell ablation. NTR reduces the drug-substrate Metronidazole (Mtz) and converts it into a cytotoxic DNA-cross linking metabolite resulting in cell death. The NTR/Mtz cell ablation system was previously shown to function in zebrafish and mice, indicating that it is likely to be an effective tool in a wide range of animals. I established this system in the amphipod crustacean *Parhyale hawaiensis,* which is an attractive model for studying regeneration, with a range of genetic tools based on transgenesis.

Using a cell type specific promoter upstream of *cfp-ntr* gene (NTR fused to the Cyan Fluorescent Protein) and inserting it into *Parhyale* genome via Minos transposable element, I achieved to express the fused protein in specific cells and visualize its expression in the transgenic animals by the presence of CFP fluorescent marker. At low concentrations of Mtz substrate (which are otherwise harmless), I could ablate muscle cells and monitor the process through changes in CFP fluorescence.

I tested FRT/Flipase (Flp) and Cre/lox recombination system in *Parhyale* by an excision assay. Such recombinase system will enable us to restrict NTR expression within clones of cells in specific cell types or tissues. By combining nitroreductase-mediated ablation and time-lapse imaging, we will be able to monitor regenerating legs after the ablation of specific cell types (e.g. muscle or neural cells) to determine their role in limb regeneration.

Introduction

1. Animal Regeneration

Regeneration is the process of regrowth or repair of cells, tissues and organs. Animal regeneration is observed in a plethora of animal phyla and their regenerative strategies are diverse. Rearrangement of pre-existing tissue, use of adult somatic cells and the de-differentiation and/or transdifferentiation of cells reflect different regeneration mechanisms.

Animals with regenerative capacity are found in most animal phyla. The cnidarian *Hydra vulgaris* and planarians *Schmidtea mediterranea* and *Dugesia japonica* have been extensively studied. *Hydra* has ectodermal and endodermal epithelial cells and interstitial stem cells. The latter ones are able to self-renew and produce neurons, nematocytes, secretory cells and gametes (Tanaka and Reddien, 2011). The triploblast planarians (*S. mediterranea and D. japonica*) have multipotent stem cells, the c-neoblasts that exist throughout the body and produce all differentiated cell types (Wagner et al, 2011).

Among vertebrates, newts, salamanders and fish have remarkable capacities for regeneration. *Xenopus laevis* and mammals have more limited regenerative capacities. Newts are able to regenerate many organs such as their limbs, tail, brain, spinal cord, hair cells, lens, retina, jaws and heart. Salamanders can replace their amputated limbs, tail and spinal cord. Limb regeneration is also observed during pre-metamorphic stages in *Xenopus*, and is also able to regenerate its retina, lens and hair cells of the inner ear. In zebrafish, *Danio rerio,* regeneration capacity is observed for fins, tail, heart, liver, spinal cord, hair cells of inner ear and lateral line. Among mammals, mice and humans have the capacity to regenerate their digit-tips and liver.

In general, amputation is followed by the wound healing and the formation of regeneration blastema within the wound followed by proliferation, differentiation and tissue patterning. Blastema is composed by a mass of undifferentiated cells underlying the wound from which the new tissues will be formed. In principle, blastema cells can be stem cells that are multipotent like planarian neoblasts or cell lineage-restricted stem cells. Moreover, blastemal cells could originate from differentiated cells which de-differentiate. In addition, except from the blastemal cells, different cell types are participating through extrinsic signals from the surrounding during regeneration process. For example, in salamander limb regeneration, Schwann cells release nAG protein which is the ligand of Prod1 receptor of blastemal cells; nAG also induces its own expression at glands of specialized wound epidermis (Kumar et al, 2007). However upon denervation nAG protein is abrogated from Schwann cell and the wound epidermis (Kumar et al, 2007) which affects regeneration process, suggesting nerve dependent regeneration. Nerve-dependence is also observed in *Xenopus* tail regeneration (Brockes and Kumar, 2008).

2. *Parhyale hawaiensis: an emerging model-organism*

The field of development has advanced dramatically within the last years. Mostly, this is due to the use of genetic approaches in well-established genetic models such as *Drosophila melanogaster, Caenorhabditis elegans* and *Mus musculus.* Although important insights for developmental mechanisms were gathered in these organisms, there are still questions about the diversity or universality of these mechanisms between different taxa and species. In order to address these questions, new model organisms are needed.

The amphipod crustacean *Parhyale hawaiensis* is such an emerging model-organism for regenerative studies and has a plenty of genetic tools. *Parhyale* is able to replace its amputated limbs within seven days (Fig 1). On-going work in our lab is addressing questions about limb regeneration in this small crustacean.

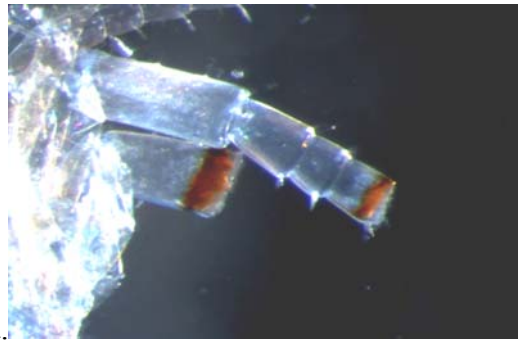
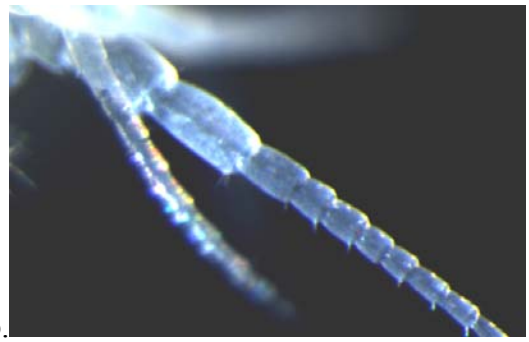
a.  b. 

Fig 1: The amphipod *Parhyale* regenerates its amputated limb (a) in 7days (b).

Its small size and its relative rapid life cycle of 2 months, allow its culture under typical laboratory conditions. After reaching sexual maturation, females brood young in a ventral pouch.

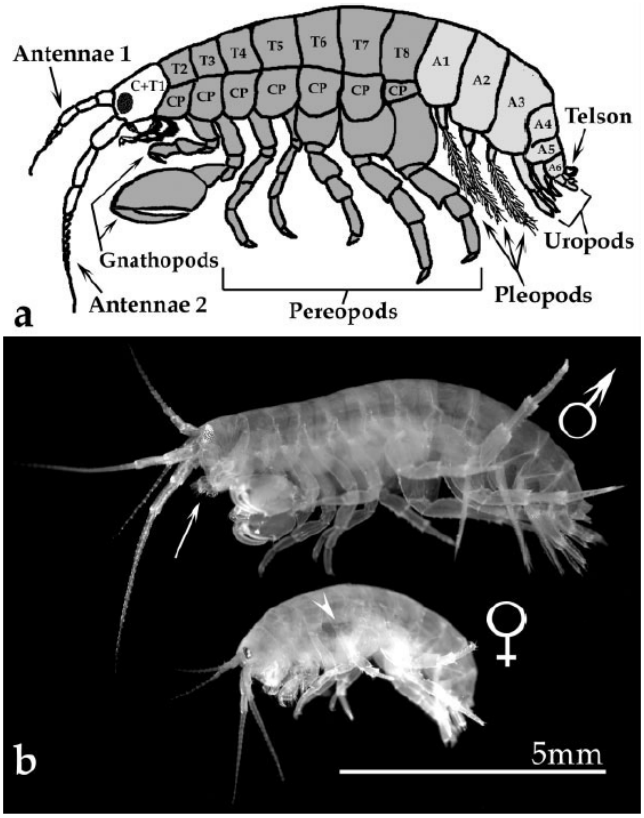


Fig 2: The *Parhyale hawaiensis* body. (a) Schematic representation of the adult body plan. (b) Imges of adult *Parhyale* male and female (Browne et al, 2005).

Embryonic development of *Parhyale* completes within 12 to 14 days at 25^{o}C. Three holoblastic unequal cleavages result in 8-cell embryo with four micromeres and four macromeres. Lineage studies (Gerberding et al, 2002) have revealed a restriction of cell fates at the 8-cell stage (Fig 3A, 3B). The three macromeres, El, Er, Ep, give the left, right and posterior ectoderm, respectively. The forth macromere, Mv, generates the anterior and visceral mesoderm. The four micromeres, named 'g', 'ml', 'mr' and 'en', generate the germline, left mesoderm, right mesoderm and endoderm, respectively. Injection at specific blastomeres of the 8-cell stage embryo gives rise to mosaic animals, where transgene expression is restricted to a specific germ layer.

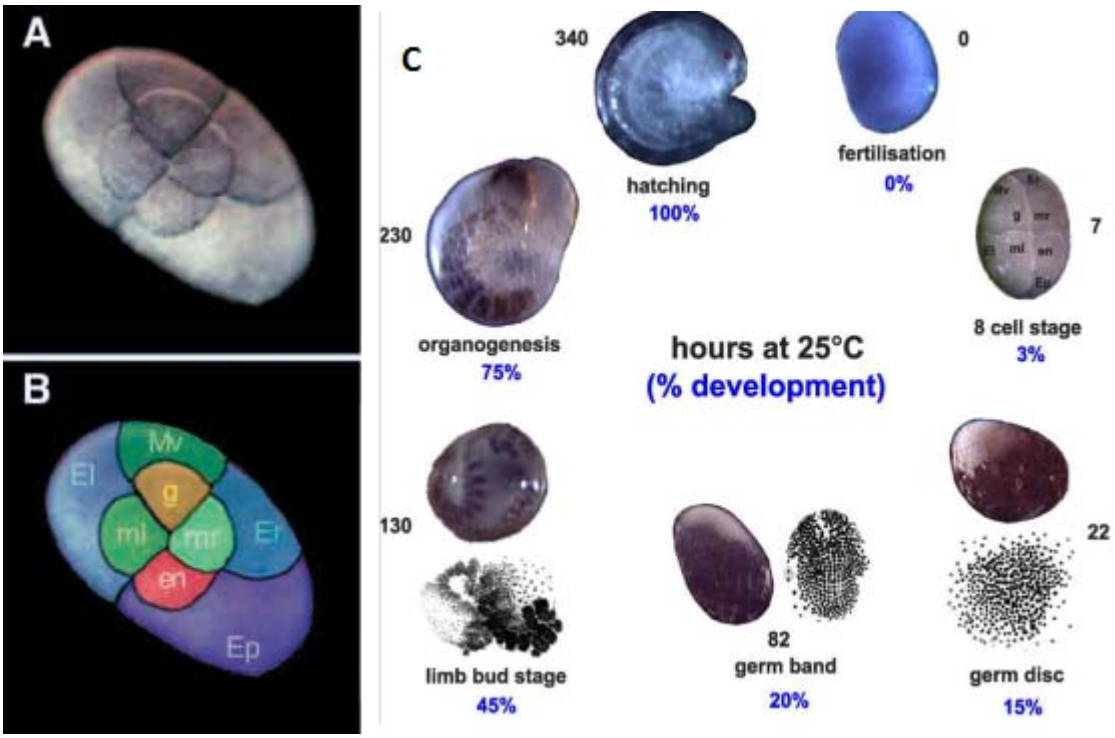


Fig 3: *Parhyale* embryogeneisis. (A-B) Eight-cell stage *Parhyale* embryo at a dorsal view. (Gerberding et al, 2002). (C) Embryos at various developmental stages from fertilization to hatching at 25^{o}C and the percentage of total development (Extravour CG, 2005).

3. *Genetic Tools and cis-regulatory elements in Parhyale*

The existence of genetic tools is a prerequisite for genetic manipulation and functional genetics studies. In *Parhyale hawaiensis*, the use of the Minos transposable element has allowed us to establish transgenesis and genetic tools. Pavlopoulos and Averof (2005) established random insertions of a transgene in *Parhyale*, by co-injecting Minos with *in vitro* synthesized *Minos transposase* mRNA in 1- or 2-cell stage embryos. The Minos transposable element is unable to transpose in the absence of Minos transposase, enabling the establishment of transgenic lines carrying stable Minos TE insertions (Palvopoulos and Averof, 2005).

Recently, site-specific integration of transgenes was established in *Parhyale* by the use of φC31 intergrase system (Kontarakis et al, 2011). This system in combination with lines generated by Minos TE-based exon-trap is a useful tool to integrate new constructs into the trapped loci and study them.

Genetic resources (EST datasets and BAC libraries and on-going *Parhyale* genome sequencing project) in combination with the above genetic tools make *Parhyale* an attractive model-organism for developmental and regenerative studies.

For the purpose of this project, three cis-regulatory elements were used:

i. 3xP3 regulatory element

3xP3 is an artificial promoter (Sheng et al, 1997), which contains three Pax6-homodimers binding sites. Pax-6 is a transcription factor containing a homeodomain (HD) and a Paired domain (PD). Sheng et al (1997) showed that *Drosophila* homologue of Pax6, *eyeless* (*ey*), binds and activates the *rhodopsin 1* (*rh1*) expression in the *Drosophila* photoreceptor cells through HD of Ey binding to the palindromic HD binding P3 site in *rh1* promoter and a multimerized P3 site upstream of a basal promoter drives the expression of a reporter gene in all *Drosophila* photoreceptors. The 3xP3 element drives also the expression of a fluorescent protein in the eyes of *Tribolium castaneum* and other insects (Berghammer 1999).
Pavlopoulos and Averof (2005) showed that 3xP3-driven DsRed expression can be used as an effective transformation marker in *Parhyale hawaiensis.* Unlike reporter expression in many insects, the 3xP3 element is not active in the photoreceptors of *Parhyale* compound eyes (Fig 4). DsRed fluorescence is detected as a pair of dots in the head region of the transformant embryos (Fig 4). In some lines, it is possible to observe the red fluorescent cells sending projections into the brain (Pavlopoulos and Averof, 2005, Fig 4).

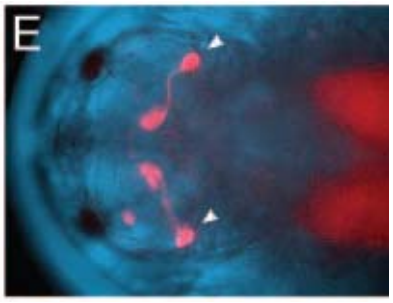


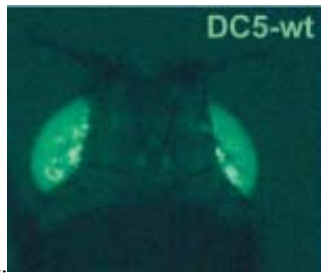
Fig 4: Localization of 3xP3-DsRed fluorescence in *Parhyale* transgenic embryo (Pavlopoulos and Averof, 2005).

ii. DC5 regulatory element

The DC5 element is a small DNA fragment which is derived from the chicken *δ1-crystallin* gene. The chicken *δ1-crystallin* gene is one of the best characterized Crystallin genes. Its lens-specificity is regulated by the 30bp DC5 sequence which is located in the the 3rd intron. The DC5 fragment contains two binding sites, a PAX6 binding site and a SOX2 binding site, and the cooperative binding and activity of PAX6 and SOX2 to the DC5 fragment enables its activity. SOX2 protein is a member of the Group B1 subfamily of Sox transcription factors that bind to DNA by a high-mobility group (HMG) domain with different partner proteins, and may activate or repress target genes.

The DC5 element is also active in invertebrates such as *Drosophila melanogaster* (Blanco et al, 2005) and *Parhyale hawaiensis* (unpublished data, Nikolaos Konstantinides and Michalis Averof). Blanco et al (2005) showed that an octamerized version of the DC5 element with a minimal promoter is able to induce the expression of the reporter gene EGFP in the cone cells of the *Drosophila* adult compound eye. Enhancer activity was also detected in the eye imaginal disc during the 3rd instar larva and in the larval visual system (Bolwig' organ). The cooperative binding of PAX6/2 and SOX2 homologues and activation of DC5 element were confirmed in vivo and in vitro.

In *Parhyale*, the DC5 element drives the expression of reporter genes in the central and peripheral nervous system (Fig 5b). Early after hatching, the reporter expression is observed segmentally at the ventral cord, which gradually fades and becomes restricted to cell projections leading to the limbs (Fig 5b).

a. 

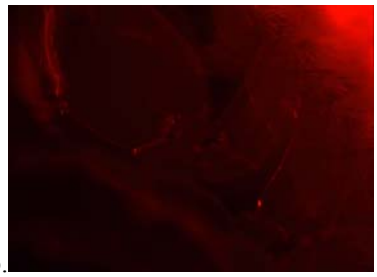
b. 

Fig 5: The DC5-driven reporter expression pattern in (a) Drosophila (Blanco et al, 2005) and in (b) *Parhyale*.

iii. Parhyale hawaiensis Muscle-Specific (PhMS) regulatory element

*Ph*MS is a *Parhyale* cis-regulatory fragment identified by cloning genomic fragments upstream of *Parhyale* hsp70 family genes and constructing reporter constructs (Pavlopoulos and Averof, 2005). It contains 11 tandem repeats of a 16bp sequence (Fig 5a), with a core sequence (CAGTTG) which may serve as binding site for myogenic b-HLH transcription factors. The *Ph*MS fragment drives the expression of a fluorescent reporter gene in all muscles (Fig 5b), from late embryogenesis to adulthood.

a."CAGTCAGTTGACTTAACAGTCAGTGACTTA
ACAGTCAGTTGACTTAACAGTCAGTTGACTTA
ACAGTCTGTTGACTTAAGTCAGTTGACTTAAC
AGTCAGTTGACTTAACAGTCTGTTGACTTAAC
AATCTGTTGACTTAACAATCTGTTCACTTAGCA
GTCAGTTGACTTAA"

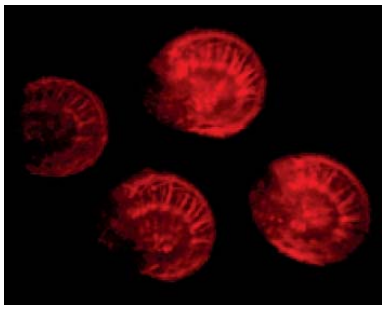


Fig 5: (a) The 11-repeat of 16bp sequence and (b) the expression pattern of *Ph*MS-driven DsRed reporter in *Parhyale* embryos (Pavlopoulos and Averof, 2005).

4. The inducible nitroreductase/metronidazole cell ablation system

In order to study the requirement of different cell-types in *Parhyale hawaiensis* regeneration, we need a cell ablation system to specifically kill cells and examine their requirement in regeneration process. A spatially and temporally inducible ablation system, utilizing the Nitroreductase (NTR) enzyme and its substrate Metronidazole (Mtz), was recently established in zebrafish (Curado et al, 2007; Curado et al, 2008). It relies on cell type-specific expression of *E. coli* nitroreductase enzyme and its non-toxic substrate Mtz. NTR converts the drug to a DNA inter-strand cross-linking agent, which subsequently causes the death of NTR-expressing cells. Because the cytotoxic agent (toxic form of Mtz) remains only in the NTR-expressing cell, no neighboring cells are affected and the 'bystander effect' observed with another NTR substrate (CB1954) (Bridgewater et al, 1997) has not be detected. Also, the use of the *ntr* gene fused with the Cyan Fluorescent Protein coding sequence allows monitoring of NTR+ cells during the process. The spatially and temporally controllable aspects of the system by the use of cell type- or tissue-specific promoters and Mtz substrate are the most important benefits of this system. The NTR-Mtz cell ablation system is independent of cell cycle and proliferation so it is also effective against quiescent and non-dividing cells (Isles et al, 2001).

In *Danio rerio,* using the NTR/Mtz system Curado et al (2007) ablated successfully three different cell types: cardiomyocytes, pancreatic β-cells and hepatocytes. Confocal images confirmed no 'by-stander' effect during pancreatic β-cell ablation. TUNEL and anti-caspase 3 staining suggest apoptosis as the process that is induced by the NTR/Mtz ablation system. Pisharath et al (2007) had also used this system to ablate pancreatic β-cells in zebrafish. NTR/Mtz system was also used to conduct skin ablation (Chen et al, 2011) in zebrafish. In agreement with the above mentioned data, when the epidermal-specific NTR+ lines were incubated with Mtz, immunostaining against apoptotic markers like caspase 3 confirmed apoptosis (Chen et al, 2011). Additionally, combining these NTR+ lines with the expression of constitutive active anti-apoptotic elements like Akt1 kinase, Stat3 and HPV16 E6 resulted in apoptosis-resistant phenotypes to the cytotoxic Mtz substrate and NTR+ fluorescent signaling was retained even after Mtz treatment (Chen et al, 2011). Another interesting work (Hsu et al, 2010) in zebrafish has used the NTR/Mtz system to induce male infertility after Mtz treatment.

In addition to its use in zebrafish, the NTR ablation system has also been used effectively in mice. Initial work in mice has shown the T-cell specific ablation after CB1954 treatment (Drabek et al, 1997). Using CB1954 drug, Isles et al (2001) ablated

olfactory and vomeronasal receptor neurons in transgenic mice, while Felmer et al (2002) ablated adipocytes in adult transgenic mice expressing the *ntr* gene. However, both Isles et al (2001) and Felmer et al (2002) did not detect the by-stander killing effect of CB1954 treatment at the neighbor of NTR+ cells in contrast to previous reports (Bridgewater et al, 1997).

The aim of this project was to check NTR/Mtz system in *Parhyale*. We decided to use the DC5 and *PhMS* cis-regulatory elements to drive the expression of the fused gene (*cfp-ntr*) in muscles and nervous system. By changing different parameters (drug concentration, duration of treatment), we established this cell ablation system and we tried to optimize the exposure conditions according to the tissue-specific context. The ultimate aim of these experiments will be to test the contribution of different cell types during limb regeneration in *Parhyale.*

Materials and Methods:

1. *Bacteria Medium, Antibiotics and Bacterial Strains*

Bacterial media used were Luria Bertani (LB) for liquid cultures and Luria Agar (LA) for bacterial plates. LB medium contained 1% w/v bacto-tryptone, 0.5% w/v yeast extract, 1% w/v NaCl and pH 7.5. The LA gel contained the same ingredients as LB medium and 1.5% w/v agar.

To select the transformed bacteria, antibiotics were added to the bacterial media. All the constructs used carried ampicillin resistance which was added at a final concentration of 100µg/ml.

For plasmid amplification competent E.coli DH5alpha bacteria *(F^- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK^-, mK^+) phoA supE44 λ^- thi-1 gyrA96 relA1)* were used. Frozen chemically competent *E.coli* cells were produced according to standard calcium chloride protocol and were stored at -80°C.

2. *Transformation of competent DH5alpha E. coli cells*

After thawing of competent *E. coli* cells on ice, plasmid DNA was added and incubated for 20min at 4°C. Heat shock (42°C, 1min 30sec) of the cells allowed the incorporation of the DNA. After heat shock, LB medium (300µl) was added and the cells were incubated for 20min at 37°C. The cells were centrifuged and plated on LB plates with antibiotic followed by overnight incubation at 37°C.

3. *Small- and large-scale extraction and purification of plasmid DNA*

For small-scale plasmid DNA extraction 'mini preps' the Merlin protocol was used (http://130.15.90.245/methods/merlin_miniprep.html). The overnight culture (2ml) was harvested by centrifugation (maximum speed, RT, 10min) and the cell pellet was resuspended in Merlin I solution (100µl) followed by cell lysis with addition of Merlin II solution (200µl) and incubation for 5min at RT. Neutralization was achieved by adding the Merlin III solution (150µl) and mixing gently. Following the addition of chloroform (250µl), the lysate was centrifuged and the aqual phase (400µl) was selected in a new tube. DNA precipitation was achieved by adding 2 volumes of 100% Ethanol and centrifugation (maximum speed, 4°C, 10min). After DNA pellet was washed with 70% Ethanol, the DNA was dissolved with sterile H_2O (20-30µl) and 1/10 of the volume was used for digestions.

For plasmid DNA extraction of high amounts (midi preps), the NucleoBond Xtra Midi kit from Macherey-Nagel was used. Overnight bacterial culture (150-200ml) was harvested, followed by cell lysis, lysate filtration by an anion-exchange column, DNA elution and precipitation, DNA pellet washes and dissolving (to 80-100µl H_2O).

4. ***Whole-embryos plasmid DNA extraction (Holmes-Bonner protocol)***

Embryos were selected and 100µl of Holmes Bonner (HB) solution were added. After 1min embryos' homogenization, we added 100µl HB solution and 200µl Phenol/Chloroform (pH 7). The homogenate was mixed gently for 10min and centrifuged at 13,000rpms at RT for 5min. The supernatant was selected in a new tube followed by 2 times Phenol/Chloroform (pH 7) extraction and one time Chloroform. DNA was precipitated with 100% ethanol and the pellet was washed with 70% ethanol. After pellet drying, DNA was dissolved in 20µl H_2O.

HB buffer
100mM Tris·HCl pH7.5
10mM EDTA
300mM NaCl
2% SDS (last added)
7M Urea

5. ***Molecular cloning techniques:***

- ***Reagents and Buffers Used in Molecular Cloning***

The restriction enzymes and DNA polymerases by Minotech and New England Biolabs (NEB) were used according to instructions. The rAPid alkaline phosphatase by Roche was used for vector dephosphorylation. The DNA marker was λ/StyI and TAE buffer (0.04M Tris-acetate, 0.001M EDTA) was used for gel electrophoresis.

i. Restriction enzyme reaction

To perform restriction enzyme reaction for isolating an insert or linearizing a vector, a typical reaction was the following:

DNA (from midi prep)	(5µg)
Buffer 10X	5µl
BSA 10X	5µl
Restriction enzyme	2µl
H_2O	*to 50µl*
Final volume	**50µl**

After small scale plasmid DNA preparation, analytical digestions were performed following the listed typical reaction:

DNA (from midi prep)	2µl
Buffer 10X	2µl
BSA 10X	2µl
RNase A 20X	1µl

Restriction enzyme	0.5µl
H_2O	*to 20µl*
Final volume	**20µl**

ii. Klenow reaction

The Klenow (*E.coli* DNA polymerase I, Large fragment, NEB) enzyme has a 5'→3' polymerase and 3'→5' exonuclease activity. During cloning, Klenow reaction was performed to convert the sticky ends from restriction enzymes digestions to blunt ends according to which, the 5' extended ends were filled in with the 5'→3' polymerase activity and the 3' extended ends were removed by the exonuclease. For a typical reaction, after the restriction enzyme reaction and heat inactivation (at 65^{o}C for 20 min), 1µl Klenow enzyme (NEB) and dNTPs at a final concentration of 33µM were added, incubated for 15min at RT and heat inactivated at 75^{o}C for 20min, followed by gel extraction (Nucleobond) from 1-1.2% agarose gel.

iii. Mung Bean (MB) reaction

Mung Bean enzyme (NEB) is a single-strand DNA and RNA endonuclease which degrades single-stranded extensions from the ends of DNA and RNA molecules resulting in blunt ends for ligation. After NcoI digestion of pSL(PhMS.DsRed.SV40) and gel extraction, we used the MB enzyme to eliminate a ATG site which was next to the NcoI sticky ends in order to avoid the presence of two ATG sites and the incorrect transcription of the downstream DsRed coding sequence. The reaction was performed with 0.2µl enzyme (5units) to a final volume reaction of 20µl, at RT for 30min. Reaction inactivation and DNA extraction was performed by using the Nucleobond kit (like PCR clean-up protocol) and proceeded to the next digestion. The final construct was confirmed by sequencing for ATG deletion.

iv. Ligation reaction

For a typical ligation reaction, a 1:3 ratio (vector to insert) was used with 50ng of vector. The reaction was performed with 1µl of T4 ligase enzyme (Minotech) and ATP (final concentration 100µM) in 15µl final volume. The reaction was performed at 16^{o}C overnight followed by transformation and plating on LB ampicillin plates.

v. PCR reaction and primers sequences

The PCR reactions were performed according to the following:

plasmid DNA	5µl (5ng)
Cre FOR / FlpFOR primer (100nM)	1µl
Cre REV / FlpREV2 primer (100nM)	1µl
dNTPs	2.5µl
Buffer 10X	2.5µl
Taq Polymerase enzyme	0.5µl

H_2O	*To 20µl*
Final volume	**20µl**

With the following program:

1st step	94°C, 5min
2nd step *(annealing)*	94°C, 30sec
3rd step *(Primers hybridization)*	50°C, 30sec
4th step *(Extension)*	72°C, 1min 30sec
5th step	Go to 2nd step, 30 times
6th step	72°C, 10min
7th step	HOLD

Then the KAPPA polymerase (KAPPABIOSYSTEMS) was used (PCR for Flp/FRT system, Results and Discussion, Fig 14), we followed the instructions, protocol and the following program:

1st step	95°C, 5min
2nd step *(annealing)*	98°C, 20sec
3rd step *(Primers hybridization)*	50°C, 15sec
4th step *(Extension)*	72°C, 1min 30sec
5th step	Go to 2nd step, 35 times
6th step	72°C, 5min
7th step	HOLD

The primers sequences are:

Primer name	***Sequence***
CreAssFOR	5' CAGGGTTTTCCCAGTCACG 3'
CreAssREV	5' CAGCGAGTCAGTGAGCGAG 3'
FlpAssFOR	5' GTGTAATGGCTTTCGGCAGT 3'
FlpAssREV2	5' TTGGAAAGATTCGGAGTTCG 3'

6. Construction of the plasmids:

i. pMi (3xP3.DsRed.SV40; DC5.CFP-NTR.SV40)

To construct the pMi(3xP3.DsRed.SV40; DC5.CFP-NTR.SV40) plasmid two cloning steps were performed: (i) the insertion of the CFP-NTR.SV40 sequence downstream of the DC5 regulatory element and, (ii) the transfer of the DC5.CFP-NTR.SV40 sequence to the pMi vector which carries a transformation marker (3xP3.DsRed.SV40).

(i) The CFP-NTR.SV40 sequence was cut with EcoRI (followed by Klenow fill-in) and MfeI from the 14xUAS.CFP-NTR.SV40 plasmid and inserted into pSL (DC5.DsRed.SV40) (provided by Nikolaos Konstantinides) which was linearized by BstXI (followed by Klenow treatment) and MfeI digestions; thus the DsRed.SV40 sequence was replaced by CFP.NTR.SV40 (Fig 6a)

(ii) To insert the DC5.CFP-NTR.SV40 sequence in the pMi(3xP3.DsRed.SV40) vector, I digested the pSL(DC5.CFP-NTR.SV40) vector with AscI and ScaI, recovered the DC5.CFP-NTR.SV40 sequence as an AscI fragment, and inserted it into the AscI site of the pMi(3xP3.DsRed.SV40) vector, resulting in pMi(3xP3.DsRed.SV40; DC5.CFP-NTR.SV40) (Fig 6b).

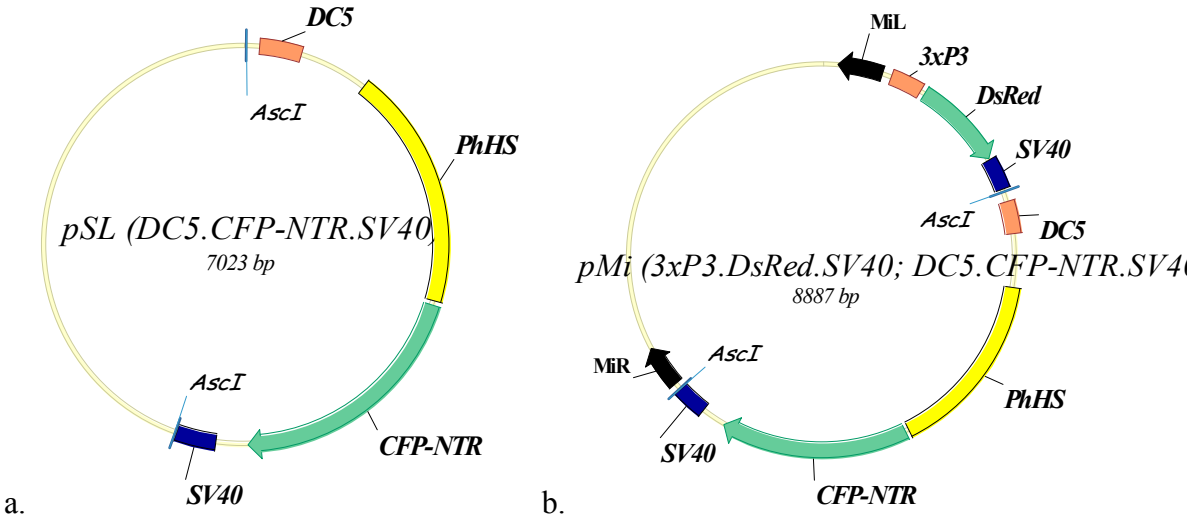


Fig 6.: The pSL(DC5.CFP-NTR.SV40) and pMi(3xP3.DsRed.SV40; DC5.CFP-NTR.SV40) plasmids.

(ii) pMi (3xP3.DsRed.SV40; MS.CFP-NTR.SV40)

To construct the pMi(3xP3.DsRed.SV40; MS.CFP-NTR.SV40) plasmid two cloning steps were performed: (i) the insertion of the CFP-NTR.SV40 sequence downstream of the Muscle Specific (MS) regulatory element, (ii) the transfer of the MS.CFP-NTR.SV40 sequence to the pMi vector.

(i) The CFP-NTR.SV40 sequence was cut with NcoI and HindIII from the pSL(DC5.CFP-NTR.SV40) plasmid and inserted to pSL (MS.DsRed.SV40) which was linearized by NcoI and HindIII digestions (the DsRed.SV40 sequence was abolished with these digestions).

(ii) To insert the MS.CFP-NTR.SV40 sequence to pMi(3xP3.DsRed.SV40) vector, we digested the pSL(MS.CFP-NTR.SV40) vector with AscI, since the MS.CFP-NTR.SV40 sequence is between two AscI restriction sites, and it

was inserted at the only AscI site of the pMi(3xP3.DsRed.SV40) vector resulting in pMi(3xP3.DsRed.SV40; MS.CFP-NTR.SV40) plasmid.

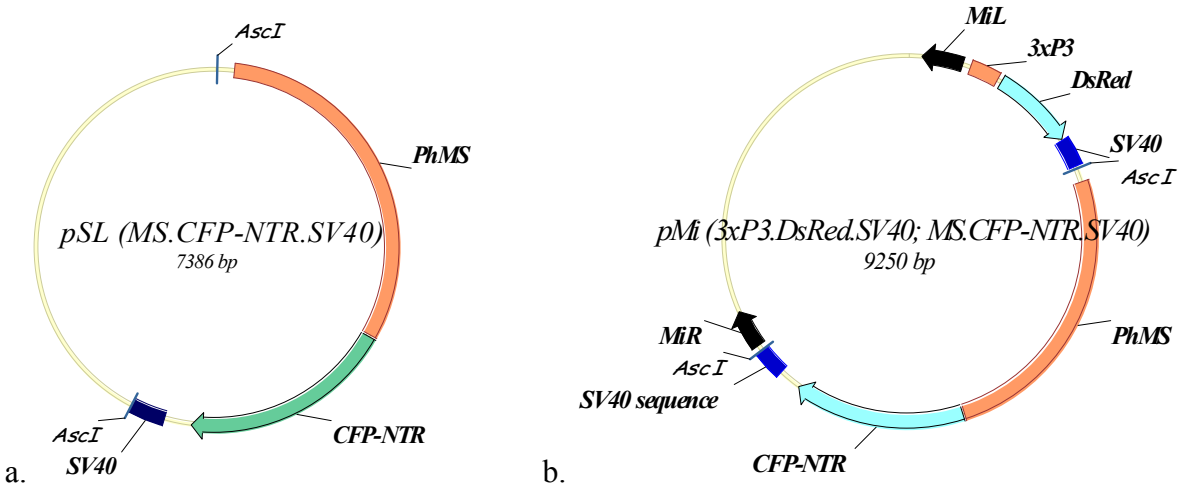


Fig 7: The pSL (MS.CFP-NTR.SV40) (a) and pMi (3xP3.DsRed.SV40; PhMS.CFP-NTR.SV40) (b) plasmids.

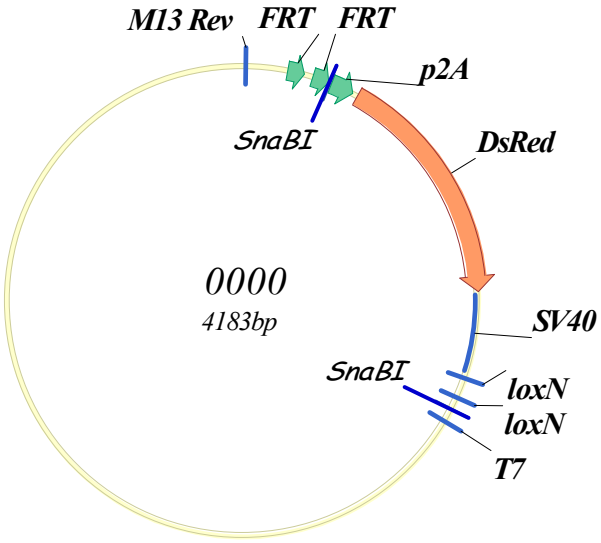
(iii)pBME (FRT/ DsRed.SV40 /FRT/ CFP-NTR.SV40)

To make pBME (FRT/ DsRed.SV40 /FRT/ CFP-NTR.SV40), three cloning steps were done: (i) the construction of the pBME vector carrying the two FRT sites, (ii) the insertion of DsRed.SV40 sequence between the two FRT sites, and (iii) the addition of the CFP-NTR.SV40 sequence downstream of the second FRT site.

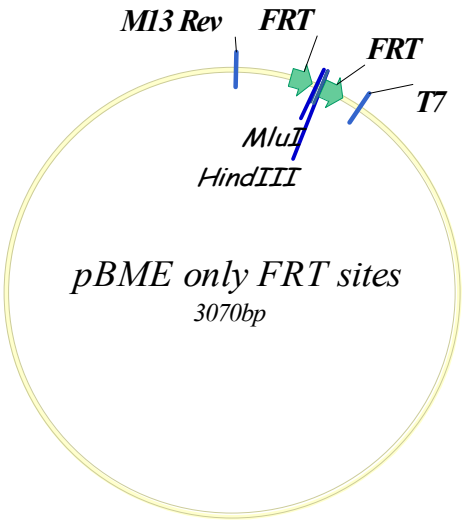
(i) The pBME(FRT-p2A-DsRed-LoxN) (short name "0000" or pBMBE) plasmid (provided by Johannes Schinko) (Fig 8a), was cut with SnaBI and self-ligated resulting in the pBME vector which only had two FRT sites (Fig 8b).

(ii) Insertion of DsRed.SV40 sequence was achieved by recovering a DsRed-SV40 fragment from pSL(MS.DsRed.SV40) with NcoI (followed by Klenow fill-in) and HindIII, and ligation into pBME(FRT/FRT) which had been digested with MluI (followed by Klenow treatment) and HindIII (Fig 8c).

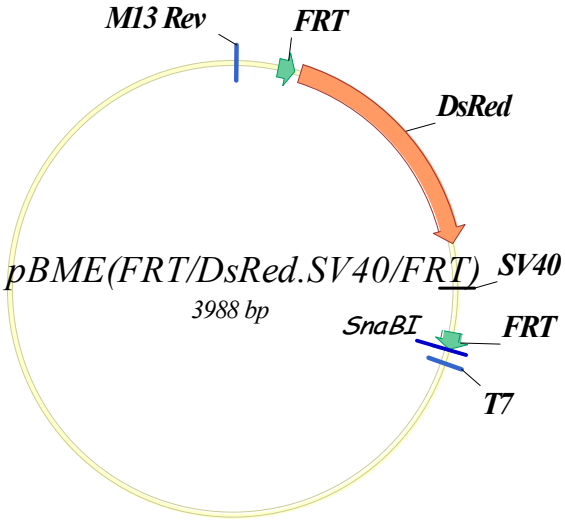
(iii) The CFP-NTR.SV40 sequence was isolated with EcoRI (followed by Klenow treatment) and SspI digestions from the 14xUAS.CFP-NTR.SV40 vector and ligated to the pBME(FRT/ DsRed.SV40/FRT) vector which was digested with SnaBI, resulting in the pBME(FRT/ DsRed.SV40 /FRT/ CFP-NTR.SV40 (Fig 8d).


M13 Rev
FRT
FRT
p2A
SnaBI
DsRed
0000
4183bp
SV40
SnaBI
loxN
loxN
T7

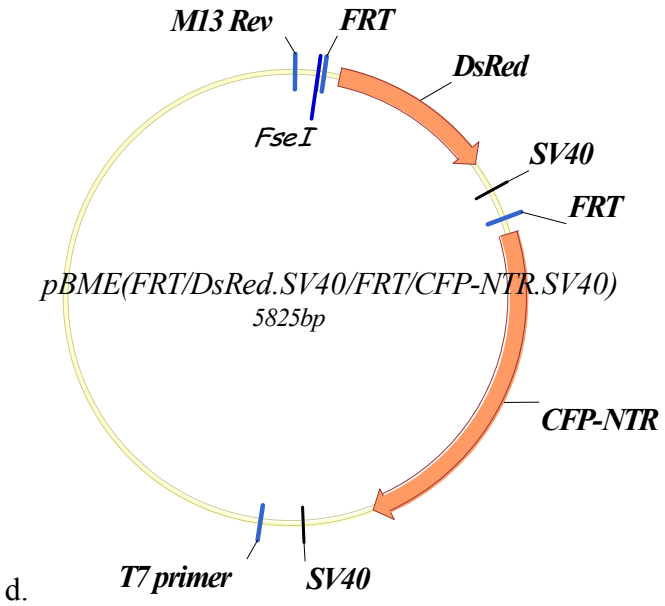
a.


M13 Rev
FRT
FRT
T7
MluI
HindIII
pBME only FRT sites
3070bp

b.


M13 Rev
FRT
DsRed
pBME(FRT/DsRed.SV40/FRT)
3988 bp
SV40
SnaBI
FRT
T7

c.



d.

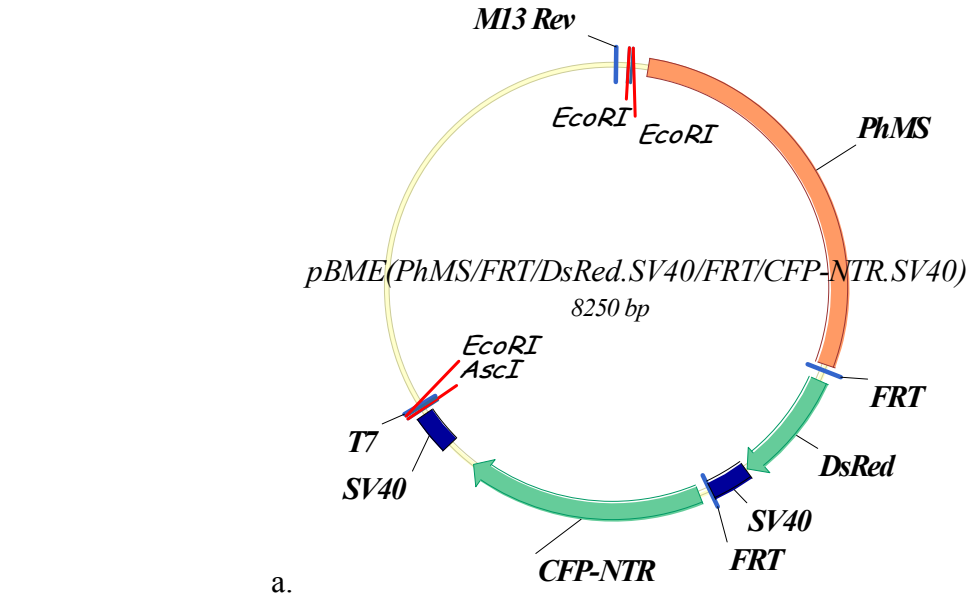
Fig 8: The "OOOO", pBME(only FRT sites), pBME(FRT/DsRed.SV40/FRT) and pBME(FRT/DsRed.SV40/FRT/CFP-NTR) plasmids.

(iv) pMi (3xP3.DsRed.SV40; MS /FRT/ DsRed.SV40 /FRT/ CFP-NTR.SV40)

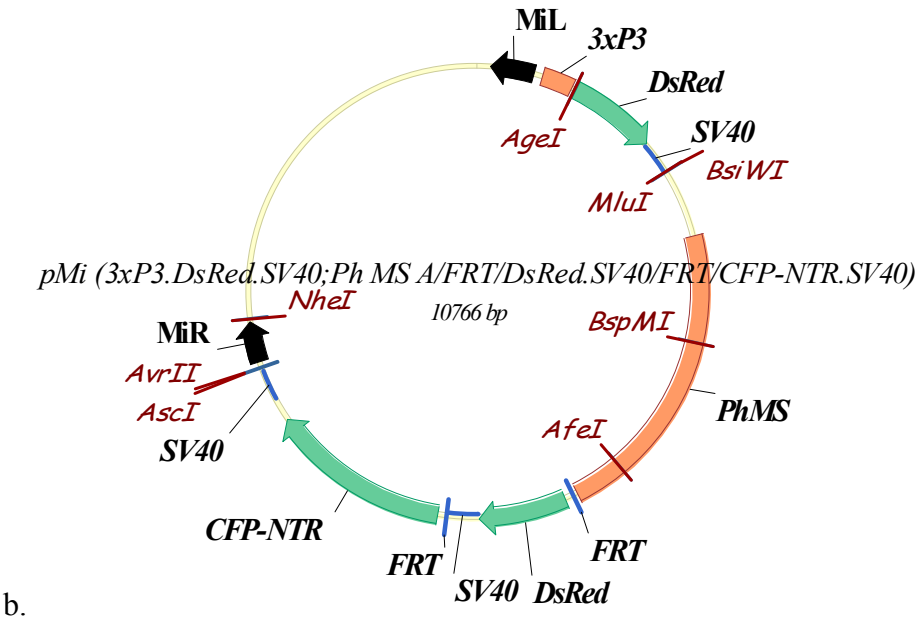
The construction of pMi (3xP3.DsRed.SV40; MS /FRT/ DsRed.SV40 /FRT/ CFP-NTR.SV40) plasmid was performed in two cloning steps: (i) the insertion of Muscle Specific regulatory element to the pBME(FRT/ DsRed.SV40 /FRT/ CFP-NTR.SV40) plasmid upstream of the first FRT site, and (ii) the insertion of the "MS/FRT/DsRed.SV40/FRT/CFP-NTR.SV40" sequence to the pMi(3xP3.DsRed.SV40) transformation vector.

(i) The MS regulatory element was isolated with NcoI (followed by mung bean treatment) and EcoRI (followed by Klenow fill-in) digestions from pSL(PhMS.DsRed.SV40) and inserted to the pBME(FRT/DsRed.SV40/FRT/CFP-NTR.SV40) which was digested with FseI (followed by Klenow treatment), resulting to pBME (MS/FRT/DsRed.SV40/ FRT/CFP-NTR.SV40). (Fig 9a)

(ii) To add the "MS/FRT/DsRed.SV40/FRT/CFP-NTR.SV40" sequence to the pMi(3xP3.DsRed.SV40), the pBME (MS/FRT/DsRed.SV40/FRT/CFP-NTR.SV40) plasmid was cut with EcoRI (followed by Klenow fill-in) and AscI (followed by Klenow treatment) and ligated with pMi(3xP3.MS.DsRed.SV40) which was digested with AscI (followed by Klenow fill-in), resulting in pMi (3xP3.DsRed.SV40; MS /FRT/ DsRed.SV40 /FRT/ CFP-NTR.SV40). (Fig 9b)



a.



b.

Fig 9: (a) The pBME (PhMS/FRT/DsRed.SV40/FRT/CFP-NTR.SV40) and (b) pMi(3xP3.DsRed.SV40;PhMS/FRT/DsRed.SV40/FRT/CFP-NTR.SV40) plasmids' maps are shown.(At Fig 9b, the unique RE sites are being shown.)

7. *In vitro synthesis of Minos transposase mRNA*

The T7mMESSAGEmMACHINE (Ambion) kit was used to synthesize the Minos transposase mRNA. For each reaction (20μl) about 30μg mRNA were produced. Template DNA was pBlueSKMimRNA plasmid (Pavlopoulos et al, 2004) digested with

NotI. At the end of each in vitro mRNA synthesis reaction, a treatment with TURBO DNaseI was performed to digest the template DNA, which was followed by phenol/chloroform extraction and mRNA precipitation with isopropanol. The *Minos transposase mRNA* was stored at -20^{o}C in isopropanol. For mRNA precipitation, 13-15µl of the mixture centrifuged at 4^{o}C at 13,000rpms for 30 min. The pellet was washed with 70% RNase–free ethanol and dissolved in H_2O.

8. *Parhyale hawaiensis cultures and injections*

Parhyale hawaiensis animals were maintained in artificial sea water of 3% salinity (specific gravity of 1.021). Wild-type (wt) animals were kept in large plastic boxes on the bottoms covered with small gravels, at 25^{o}C and continuous water circulation through a pump. The water was changed every 7 days and the animals were fed with fish food.

For microinjections couples were selected from wt pools one day earlier and placed in large Petri dishes. The next day, the fertilized females (distinguishable because couples separate after fertilization) were selected and anaesthetized with clove oil (diluted in artificial sea water, 1/2500). The fertilized eggs were visible as they are placed in an external pouch at the ventral side of the animal. The fertilized eggs were selected and separated using forceps under stereoscope and put in filtered artificial sea water. Eggs of preferred developmental stage were selected and used for injections.

For injections, aluminosilicate glass capillary needles were prepared with a needle puller (program: heat 587, pull 60, velocity 90, time 200, and pressure 700), and were beveled in a 30^{0} degrees angle (Narishige EG-40).

The embryos were placed on stages (2.5% w/v agaroze in filtered artificial sea water), and injected with a mix containing the *Minos transposase mRNA* (150ng/µl), pMi vector carrying the transgene (300ng/µl) and Phenol Red (1µl to 10µl mix). The injected embryos were recovered to filtered artificial sea water in Petri dishes, which were changed every 4 days until they reached adulthood.

9. *Imaging*

Embryos were screened and selected for transformants through presence of DsRed fluorescence at the end of embryogenesis using a LEICA MZ 16F epifluorescence stereoscope with DsRed filter set. Transformants were CFP positive and were imaged with a CFP filter set.

In order to induce cell type-specific ablation, CFP+ animals with tissue-specific CFP-NTR expression, were exposed at adjusted concentrations of Metronidazole. By using the Leica MZ 16F epifluorescence stereoscope equipped with CFP filter set, we monitored the changes of CFP fluorescence before and after the treatment with the same settings per animal. The images were processed by using ImageJ software and the calculated integrated density values were subjected to Student t-test.

For live-imaging experiments of cell ablation the animal was glued to a coverslip using the surgical glue, 2-octyl-cyanoacrylate (Dermabond) and mounted in a Petri dish which had an adjusted concentration of Metronidazole in filtered artificial seawater. The animals remained alive and active throughout the process and the immobilized body part was imagined with the Leica M205 FA epifluorescence stereoscope equipped with DsRed, GFP and CFP filter sets.

Results and Discussion

1. Injections of 1-2 cell stage and 4-8 cell stage embryos

In order to establish the Nitroreductase/Metronidazole cell ablation system in *Parhyale*, I constructed two plasmids that carry the nitroreductase (*ntr*) gene fused with the Cyan Fluorescent Protein (CFP) gene downstream of two different cell type-specific cis-regulatory elements, the DC5 and *Ph*MS (Materials and Methods, Fig 6, Fig 7). As mentioned in the introduction, the *Drosophila melanogaster* DC5 element induces the expression of the reporter gene in neural cells of *Parhyale* which protrude into the limbs. *Ph*MS is a cis-regulatory element that allows us to express the transgene (*cfp-ntr*) in muscle cells of *Parhyale.*

Each construct, pMi(3xP3::DsRed.SV40; DC5::CFP-NTR.SV40) and pMi(3xP3::DsRed.SV40; PhMS::CFP-NTR.SV40), was injected together with the Minos transposase mRNA (Materials and Methods, page 22) in 1/2-cell stage embryos, which can result in mosaic animals with wide NTR expression. Additionally, in order to narrow down the expression of nitroreductase to smaller cell populations, I injected 4-8 cell stage embryos.

The injections are summarized in the following tables:

Injections of pMi(3xP3.DsRed.SV40;DC5.CFP-NTR.SV40)

	No of injected embryos	***No of CFP$^+$ embryos***
1-or 2-cell stage	110	6
4-or 8-cell stage	691	15

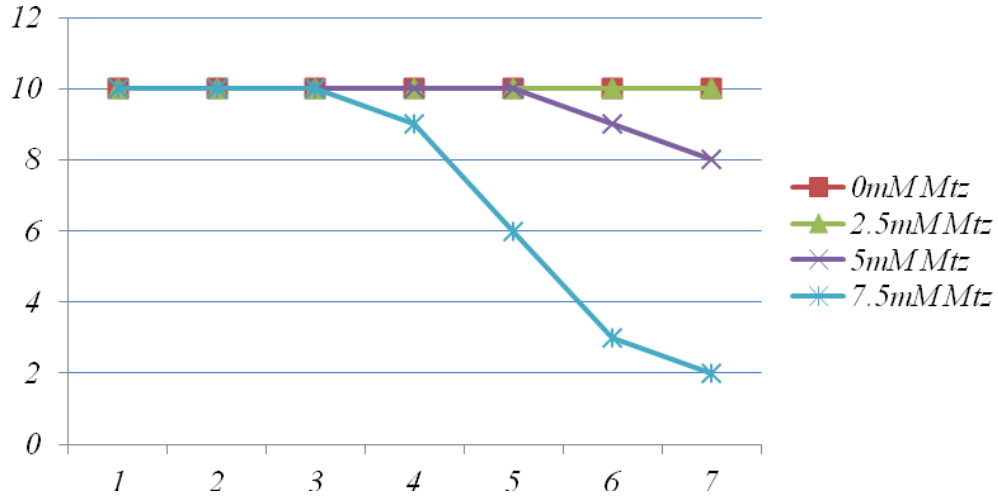
Injections of pMi(3xP3.DsRed.SV40;PhMS.CFP-NTR.SV40)

	No of injected embryos	***No of CFP+ embryos***
1-or 2-cell stage	285	30
4-or 8-cell stage	154	0

By the end of the embryogenesis the injected embryos were screened for the transformation marker (the 3xP3-DsRed transgene). The mosaic embryos were selected and raised to adulthood (about two months) before they were subjected to Metronidazole treatments. The expression of the *cfp-ntr* gene could be monitored during embryonic, larval and adult stages.

2. *Metronidazole (Mtz) toxicity curve*

In order to estimate the optimum concentration of Metronidazole (Mtz) and to avoid side-effects of Mtz (toxicity), I subjected juvenile wild type (wt) animals to different Mtz concentrations for a time period of seven days. We used three different concentrations of Mtz: 2.5mM, 5mM and 7.5mM. The animals were kept in darkness during the treatment because Mtz is photosensitive and each animal was kept separately at 1ml volume. Additionally, during the treatment they were not fed. The following diagram displays the number of survived animals (y axis) at different Mtz concentrations, during the seven days of treatment (x axis):



After three days of exposure at different concentrations of Mtz the *Parhyale* wt animals were alive. From the 5th till the 7th day of exposure, 2 out of 10 animals that were exposed to 5mM Mtz, died and between 3rd and the 7th day exposed at 7.5mM Mtz, 8 out of 10 died. All wt animals treated at 2.5mM Mtz survived.

Because of the high lethality for animals exposed to 7.5mM Mtz (8 out of 10 equal to 80%), I excluded 7.5mM concentration for the following experiments. Moreover, I decided to use the 2.5 and 5mM Mtz for the mosaic animals.

3. *Mtz exposure for mosaic transgenic animals:*

i. *Muscle-specific CFP-NTR^{+} animals and time-lapse video imaging*

To ablate muscle cells with the use of NTR/Mtz system five *Parhyale hawaiensis* Muscle-Specific (PhMS) mediated CFP-NTR^{+} mosaics were subjected to 2.5mM Mtz and five were subjected to 5mM Mtz, for 24hrs without light at RT.

From the five mosaic animals that were exposed to 2.5mM Mtz, one died and the rest four animals are shown in Fig 10 before (a, c, e, g) and after (b, d, f, h) the treatment.

The images were taken for each animal with the same settings before and after the treatment.

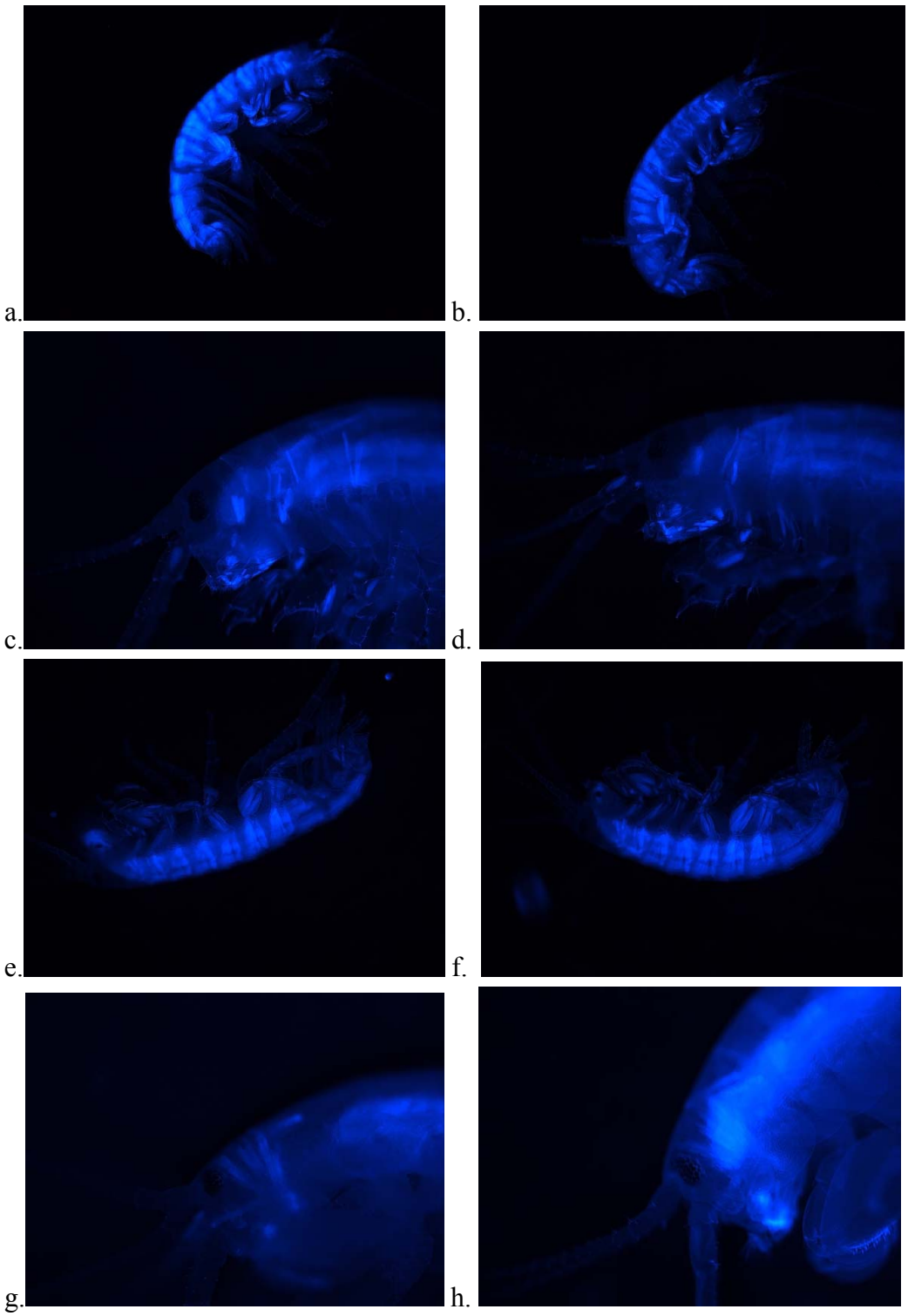


Fig 10: PhMS-NTR.CFP mosaics exposed to 2.5 mM Mtz for 24 hours. (a, b) The ‘n-3’ animal with widely CFP signal at all muscle of right side of its body before (a) and after (b) 2.5mM Mtz exposure for 24hrs, (b, d) The ‘n-20’ animal with CFP fluorescence at some muscle fibers near the head before (c) and after (d) 2.5mM Mtz exposure for 24hrs, (e, f) The ‘n-10’ animal with CFP signal at all muscle of the right side of its body before (e) and after (f) 2.5mM Mtz exposure for 24hrs, and (g, h) The ‘n-

25’ animal with CFP fluorescence at some muscle at head before (g) and after (h) 2.5mM Mtz exposure for 24hrs.

Images were processed by using ImageJ software and integrated density value of each image was calculated.

Integrated Density			
BEFORE		***AFTER***	
Fig 10a	1612.696	***Fig 10b***	1279.242
Fig 10c	3163.927	***Fig 10d***	2327.851
Fig 10e	1541.192	***Fig 10f***	1619.95
Fig 10g	2742.331	***Fig 10h***	4754.221

The integrated density values for Fig 10a – Fig 10f were grouped as ‘BEFORE’ and ‘AFTER’ and I performed t-test. The integrated density values for Figures 10g and 10h were not used because these images were taken in different medium (water and clove oil). The data were found to be statistically significant reduction of fluorescence with p-value of 0.58.

From CFP+ animals that were exposed to 5mM Mtz for 24hrs at RT in darkness, 4 out of 5 animals died. According to the toxicity curve the concentration of 5mM Mtz is not harmful to wt animals for 72hrs of Mtz exposure. So, I thought that the high percentage of death is not due to Mtz toxicity. Another explanation of CFP+ animals’ death could be the extensive NTR expression. As I mentioned in introduction, the *Ph*MS regulatory element enables the expression of our transgene in all muscle of animal. Furthermore, the stage of the injected embryo and the timing of the transgene integration are resulting in different patterns of mosaic animals. The CFP-NTR+ animals which were used for the 5mM concentration had extended transgene expression (all muscle or muscle at half of the animal) and I assume that the extended ablation of muscle resulted in its death.

Although, the CFP-NTR^{+} mosaic animal which survived the 5mM treatment, presented a strong decrease in CFP fluorescence, as shown in Fig 11. The images Fig11a and Fig11b were processed by using ImageJ software and the integrated density values were calculated 2136.803 and 1262.752, respectively. As it is obvious there is a reduction of CFP signal after Mtz exposure, and some muscle fibers appeared to be completely eliminated (see arrowhead on thoracic leg 7).

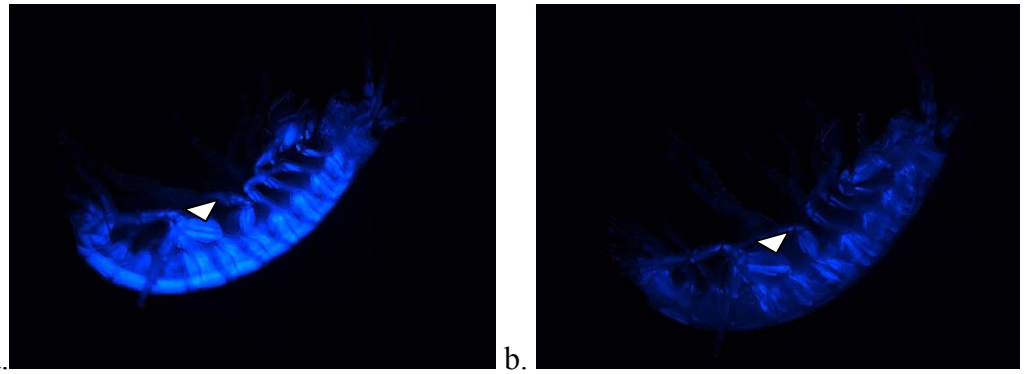


Fig 11: The presence of CFP fluorescence in 'n-2' animal before (a) and after (b) its exposure at 5mM Mtz for 24hrs.

Time-lapse video imaging was also performed on a mosaic animal exposed at 5mM Mtz at RT without light. Images were taken every 10min by using the Leica M205 FA epifluorescence stereoscope with the DsRed, GFP and CFP filter sets. The animal expressed CFP-NTR only in one muscle in Ant1 (Fig 12A). After 14hrs and 30min of Mtz exposure I could observe a decrease of the CFP signal (comparing Fig 12A and Fig 12B) and after two more hours, the CFP fluorescence was completely abolished (Fig 12C). The time period of two hours is in agreement with the duration of apoptosis, which had been confirmed as the mechanism of NTR/ Mtz-mediated cell ablation in zebrafish (Curado et al, 2007; Curado et al, 2008).

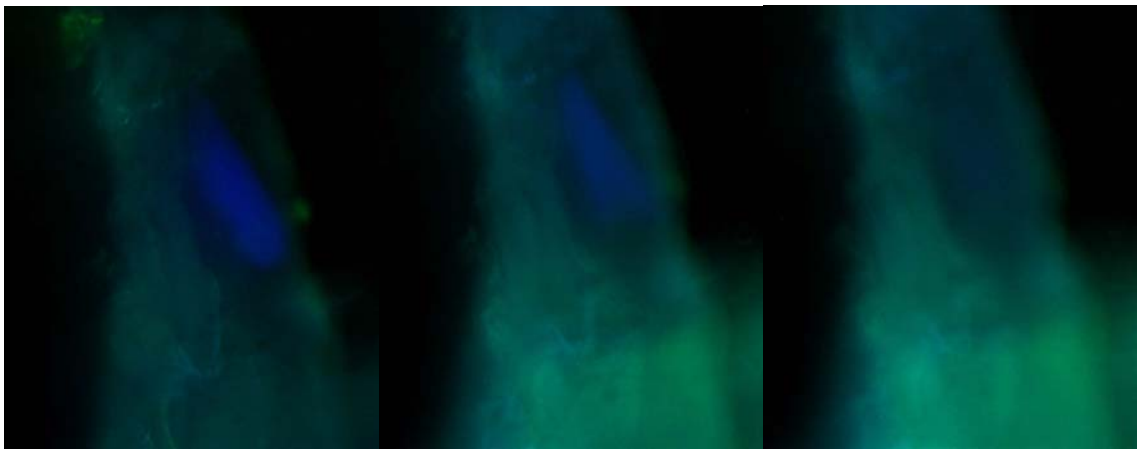


Figure 12: The ablation of CFP+ antennal muscle of n-36 animal. The images were taken before (A) and after 14hrs and 30min (B) and 16hrs 40min (C) of Mtz exposure (5mM) at RT.

In conclusion, the NTR/Mtz system is effective in ablating muscle cells at 5mM Mtz for a maximum of 24hrs in *Parhyale hawaiensis*.

ii. Neural-specific CFP-NTR^{+} animals and imaging

To ablate the neural CFP-NTR$^+$ cells, first I subjected five DC5-mediated CFP-NTR$^+$ animals to 5mM Mtz for 24hrs at RT without light and I did not observe any change in CFP fluorescence. Secondly, I exposed these mosaic animals to 10mM Mtz for 14hrs. From the five animals which were exposed to Mtz, three died and the other two are shown in the Fig 13 (a, b).

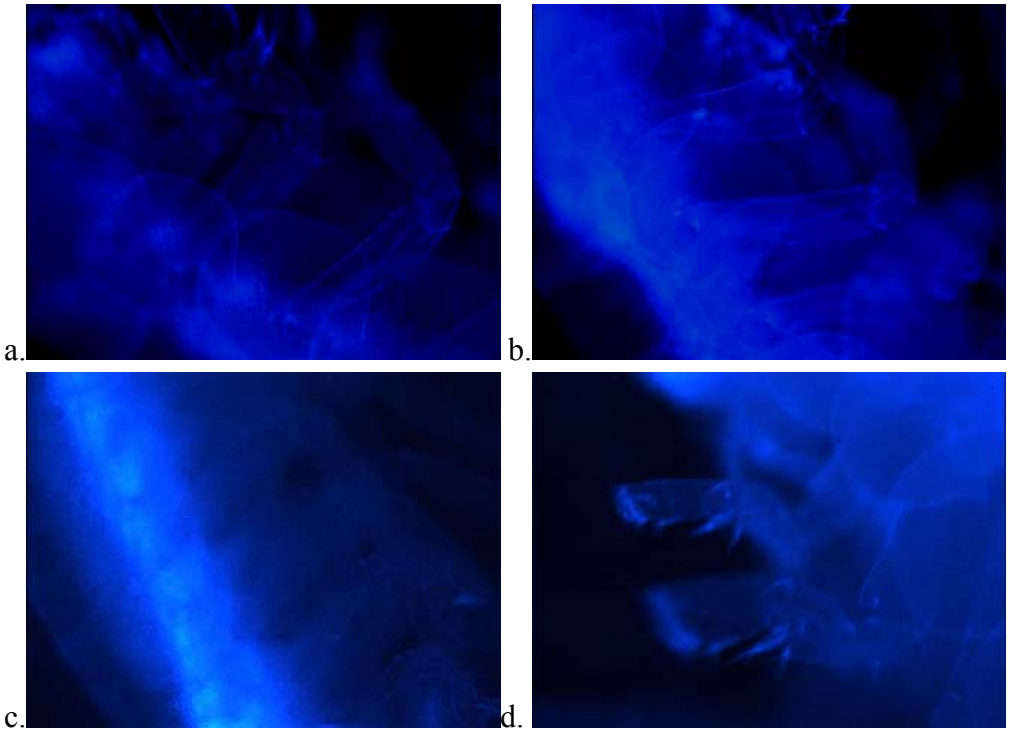


Fig 13: (a, b) The neural specific-CFP$^+$ animal 'n-35' is shown before (a) and after (b) 10mM Mtz treatment for 14hrs, (c, d) The animal 'n-21' with CFP expression at protrusions ending at the limbs is also shown before (c) and after (d) 10mM Mtz treatment for 14hrs. Before Mtz treatment, its limbs were amputated.

As Fig 13a and Fig 13b show, I did not observe decrease of CFP fluorescence at the NTR$^+$ protrusions in *Parhyale* limbs and the CFP signal was not abolished. Moreover, although amputation of limbs was performed for 'n-21' animal (Fig 13c, d) before Mtz treatment, the limb CFP+ cells were not abolished. A possible reason of unsuccessful neural ablation could be the penetration of Mtz to neural tissues.

Axon insulation and action potential propagation are important aspects of a functional and fast responding nervous system. As vertebrates, invertebrates have evolved by utilizing the benefits of nerve ensheathment resulting in insulation of nerve system from the surrounding environment. Blood-brain barrier (BBB) and blood-nerve barrier (BNB) for central (CNS) and peripheral nervous system (PNS), respectively, ensures this insulation through nerve axonal myelination. Studies in *Drosophila melanogaster* (Banerjee et al, 2006), annelids and malacostraca crustaceans like palaemonid shrimp (Heuser and Doggenweiler, 1996), panaeid

shrimp (Xu and Terakawa, 1999) and alpheid shrimp (Govind and Pearce, 1988) showed the presence of myelinated nerves of the peripheral nervous system. Moreover, three families of copepods belonging to crustacean phylum have myelinated axons (Davis et al, 1999; Lenz et al, 2000; Weatherby et al, 2000).

Interestingly, Otopalik et al (2012) showed recently that the nervous system of the crustacean *Cherax destructor* has a charge-selective intracerebral blood-brain interface. By using two differential polar magnetic resonance imaging (MRI) agents, they showed a differential uptake of these two probes and specifically, selectively uptake of the positive charge manganese ions (Mn(II)) confirming the presence of ion-selectiveness for blood-brain interface (Otopalik et al, 2012). According to Isles et al (2001) by using another NTR substrate, CB1954, they ablated successfully NTR-expressing neurons of transgenic mice.

Because of the small number of mosaic DC5-mediated CFP-NTR^{+} animals I could not perform NTR/Mtz-mediated ablation in DC5 neural-specific cells. The experiment has to be repeated at different concentrations and with and without limb amputation in order to overcome the Mtz permeability problem. Morover, to overcome an ion-selective blood-brain interface, the CB1954 drug could be an alternative substrate for neural-specific ablation.

4. Testing the FRT/Flp recombination assay

Because of the extensive NTR expression under MS or DC5 regulatory element, we were interested in inducing CFP-NTR expression in a more controllable way. Use of a Heat Shock promoter upstream of a site-specific recombinase (SSR) like Cre (from bacteriophage P1) or Flp (from *Saccharomyces cerevisiae*) and induction of clonal CFP-NTR expression by heat-shock could allow me to express this transgene in a time controllable way and narrow the NTR^{+} cell population.

Flp and Cre SSRs recombine specific DNA sequences, FRT and lox sites, respectively, in dividing and post-mitotic cells. According to the recombination mechanism, the orientation of these sequences to each other results in gene deletion, insertion, inversion or exchange (Branda and Dymecki, 2004). If FRT or lox sites have the same orientation in cis, SSR deletes the DNA sequence between them (Branda and Dymecki, 2004). Moreover, an inducible or tissue specific cis-regulatory element upstream of SSR gene drives the expression of SSR in a spatially and/or time-controllable way.

Additionally, the time of recombination event can be regulated by expressing the DNA recombinase under the control of synthetic hormones (Logie and Stewart, 1995; Feil et al, 1996; Zhang et al, 1996). FLPER(T) and CreER(T) recombinases whose open reading frame was fused to a mutated form of human estrogen receptor ligand binding domain (LBD), are inducible by synthetic anti-estrogen 4-hydroxytamoxifen (which does

not respond to the natural ligand estrogen) in mice (Feil et al, 1996), human cultured cells (Angrand et al, 1998) and in *Xenopus laevis* (Werdien et al, 2001).

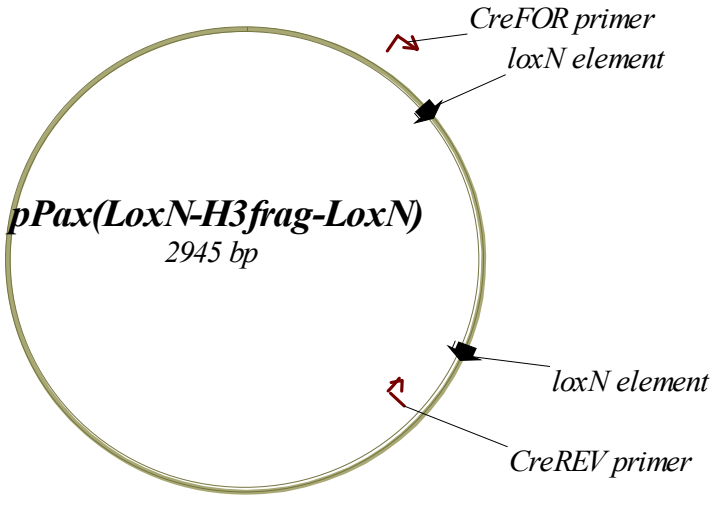
To assess the efficiency of these systems in *Parhyale*, I performed an excision assay. I injected 1- or 2-cell stage embryos with a plasmid which carries pairs of target sequences of the recombinase (loxN or FRT) in the same orientation, together with the corresponding recombinase mRNA (Cre or Flp). If the recombination event is happening in *Parhyale*, the sequence between the two target sites will recombine and the intervening sequence will be excised.

One day after injection, I performed plasmid DNA extraction from the injected embryos and by the use of primers which were upstream and downstream of FRT sites (Fig14a, Fig14b) I checked with PCR if recombination events had happened.

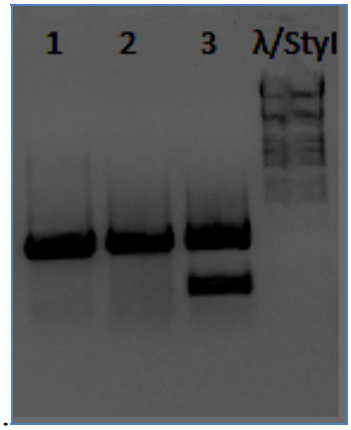
For this experiment I used two plasmids that Nikos Konstantinides had constructed: the pPax(loxN-H3flag-loxN) and pflipout constructs (Fig 14a, c). We produced the Cre and Flp recombinase mRNAs (Materials and Methods: In vitro synthesis of mRNA) using the pCS(SP6::*Cre*) and pCS(SP6::*Flp*) constructs linearized with MluI and NotI digestions, respectively. I performed the injections at 1- or 2-cell stage embryos with the following mixtures:

Mix 1 (N=47)	***Mix2 (N=49)***	***Mix3 (N=40)***	***Mix4 (N=90)***
3μg plox	3μg plox	3μg pflipout	3μg pflipout
-	1.5μg *Cre mRNA*	-	1.5μg *Flp mRNA*
1μl phenol Red	1μl phenol Red	1μl phenol Red	1μl phenol Red
H_2O to 10μl	*H_2O to 10μl*	*H_2O to 10μl*	*H_2O to 10μl*

After one day I performed plasmid DNA extraction for the injected embryos and the PCR reactions (Materials and Methods) were run on 1% agarose gel.



a.



b.

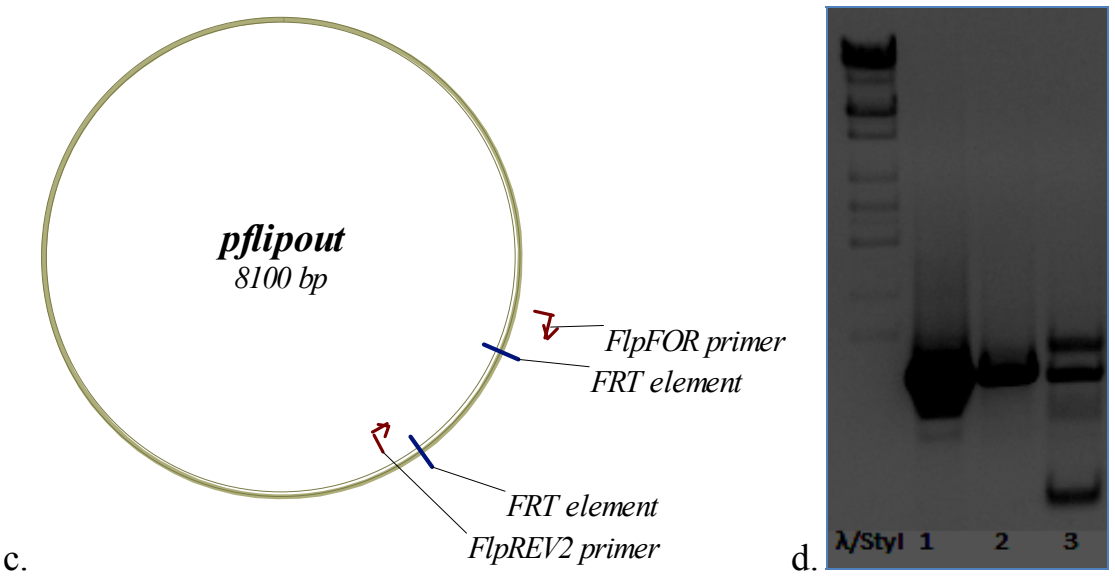


Fig 14: a. pPax (LoxN-H3flag-LoxN) plasmid which carries two loxN sequences and the positions of CreFOR and CreREV primers which were used for the PCR, b. The PCR products with plasmid DNA extracted from injected embryos with mix1 (2) and mix2 (3). As negative control (1), we performed PCR with the plox plasmid, c. pflipout plasmid which carries two FRT sequences and the positions of FlpFOR and FlpREV2 primers which were used for the PCR, d. The PCR products with plasmid DNA extracted from injected embryos with mix3 (2) and mix4 (3). As negative control (1), we performed PCR with the pflipout plasmid.

The sizes of the PCR products for the plox plasmid were 320bp and 839bp with and without recombination, respectively, and for pflipout plasmid 462bp and 1162bp with and without recombination respectively. The presence of 320bp band for plox (Fig 14b, line 3) and 462bp band for pflipout (Fig14d, line 3) demonstrate the activity of both Cre and Flp recombinase systems in *Parhyale hawaiensis*. Moreover, by comparing the intensity ratios for 320bp/839bp (Fig14b, line 3) and 462bp/1162bp (Fig14d, line 3) I concluded that FRT/Flp system is more effective than Cre/loxN in *Parhyale hawaiensis*.

In order to induce CFP-NTR+ clones with the use of Heat-Shock inducible Flp or Flp-ER recombinase, I will generate stable transgenic lines that express DsRed under the tissue-specific promoter, which is flanked by FRT sites on either side followed by *cfp-ntr* coding sequence, which upon Flp-mediated recombination drives CFP-NTR expression (Fig 15).

In order to make the stable transgenic lines, I constructed the pBME(/DsRed.SV40/FRT/CFP-NTR.SV40) and the pMi(3xP3::DsRed.SV40; PhMS/FRT/DsRed.SV40/FRT/CFP-NTR.SV40) constructs. By using the last construct we will make a transgenic line expressing DsRed in all muscle. Crossing it with a stable line carrying the *flp* gene under the heat shock promoter, the DsRed+ heterozygous offspring could be subject to heat shock resulting in CFP-NTR+ muscle clones (Fig15).

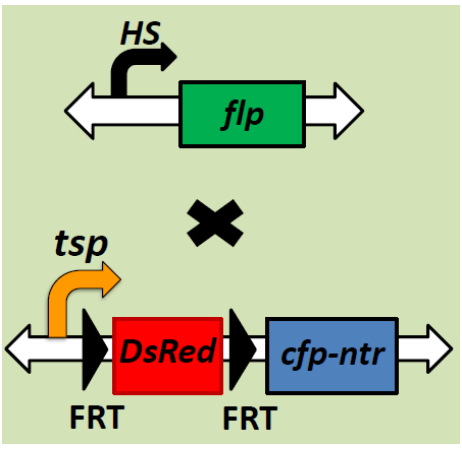


Fig 15: The two transgenic lines which will carry the HS::flp and tissue-specific promoter (tsp)/FRT/DsRed/cfp-ntr transgenes will be crossed and their progeny will used to be subjected to clonal analysis and cell ablation.

5. Conclusions

The results of this master thesis can be summed up in five points:

- I was able to express the *cfp-ntr* fused gene under tissue-specific promoter in *Parhyale*.
- I estimated a range of usable Mtz concentrations for *Parhyale*, which do not affect its viability in the absence of an NTR transgene.
- I was able to ablate the muscle CFP-NTR+ cells with 24hrs exposure to 5mM Mtz at RT in darkness, however neurons were resistant to ablation under these conditions.
- I showed that both Cre/loxN and FRT/Flp recombinase systems are working in *Parhyale hawaiensis*.

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