Rita Maria Santos Esteves

Licenciatura em Biologia

Development of Optogenetic Tools for Manipulating Neuronal Activity and Behaviour in Zebrafish

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Michael Orger, Principal Investigator,
Fundação Champalimaud, Vision to Action Lab
Co-orientador: Ana Catarina Certal, PhD, Head of Fish Platform,
Fundação Champalimaud

Júri:
Presidente: Dr. José Paulo Nunes de Sousa Sampaio
Arguente: Dra. Ana Rita Silva Martins Nunes
Vogal: Michael Brian Orger

Novembro de 2016

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Acknowledgments

It has been an incredible year, an amazing journey. I must say this project pushed me out of my comfort zone, both professionally and personally, but that is the beautiful thing about science, always leading us to more complex and unknown scenarios. For the safe and sound arrival to the finish line, I must acknowledge some people that were essential to my learning process and personal growth.

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Someone without whom I would certainly not be writing these words is Sabine Renninger. Thank you for the incredible mentorship. For taking the endless time and patience to teach me and make me understand how and why things are done. For never letting me go into despair when PCRs or behaviour set ups randomly don't work. For being the most amazing teacher, either by words or example. For carrying me to the finish line.

I have to thank the Orger Lab, specially Raquel Tomás, my molecular biology guru, that gained a tall shadow in my first weeks at the CCU, and kept helping me throughout the entire year, with scientific knowledge, career advice and great relaxing times! To Alexandre Laborde, that helped me with lots of technical problems and always cheered up the mood in the open lab. To João Marques, that in its own special way made me realize that I can pursue this path in scientific research, and must not be afraid of pushing my boundaries. Even not realizing so, his words and actions always came in perfect timing to keep me motivated. To Simone Lackner, for the friendship, motivation and availability to help me whenever needed. To Marta Oliveira for the company and chit chat during endless injecting mornings. Thanks to Rita Felix, Jens Bierfeld, Antonia Groneberg, Ruth Diez del Corral and Claudia Fierestein for always being available to help and for making a great lab.

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Finally, thanks to my family, specially Mãe, Pai and Rui Miguel, for being my rock. For unconditional love and support. For always believing and pushing me. For making me who I am.

Resumo

A integração de sinais sensoriais e a sua tradução em comportamento é um processo dinâmico que envolve diversas e distintas populações celulares distribuídas pelo cérebro.
De modo a estudar este tema, é crucial monitorizar e manipular em tempo real a actividade neuronal de populações celulares específicas. Isto é possível em larvas de peixe-zebra, dado o seu pequeno tamanho, o cérebro transparente e o facto de estarem disponíveis ferramentas genéticas para este modelo-animal: ferramentas de optogenética, que permitem a activação e inibição da actividade neuronal de forma reversível e dependente da luz, e indicadores de cálcio geneticamente codificados, que permitem monitorizar actividade neuronal de forma não invasiva.
Ferramentas optogenéticas mais modernas, de cinética mais rápida e maior sensibilidade, tornam possíveis a manipulação específica de actividade neuronal com precisão temporal durante o comportamento. Estas têm sido desenvolvidas de modo a serem compatíveis com os indicadores de cálcio mais recentes para, em simultâneo, manipular e monitorizar actividade neuronal.
Neste projecto, ferramentas optogenéticas - activadores ChrimsonR, C1V1(t/t) e Chronos, o inibidor Jaws, sensor de cálcio (vermelho) jRCaMP1b e os marcadores nucleares H2B-RFP e H2B-mCherry - foram optimizados para serem expressos em peixe-zebra. Estabelecemos ensaios comportamentais para caracterizar a activação e inibição das ferramentas optogenéticas ChrimsonR e Jaws. Em animais transgénicos que expressam ChrimsonR de forma estável nos neurónios trigeminais, induzimos de forma robusta uma resposta de escape de latência curta. A combinação desta ferramenta rápida, sensível e na gama do vermelho, com imagiologia de GCaMP abre a possibilidade de simultaneamente manipular e monitorizar, com elevada precisão espacial e temporal, a actividade de grandes populações neuronais de modo a estudar as suas interações durante o comportamento do peixe-zebra.

Palavras-chave: optogenética; indicadores de cálcio geneticamente codificados; peixe-zebra; neurobiologia; circuitos neuronais.

Abstract

Integration of sensory input and computation of behavioural output is a dynamic process involving diverse populations of cells often distributed throughout the brain.

To study this topic, monitoring neuronal activity from a large population of cells and manipulating targeted neuronal activity in a behaving animal is crucial. This is possible in zebrafish, due to its small and transparent larval brain and its genetic malleability, by making use of optogenetic tools that allow reversible light-dependent activation and inhibition of neuronal activity, and genetically encoded calcium indicators (GECI) that enable non-invasive activity recording.

State-of-the-art optogenetic tools with faster kinetics and higher sensitivity facilitate reliable manipulation of activity with high temporal precision during behaviour. Such tools have been developed to be compatible with better calcium indicators to successfully manipulate and optically record neuronal activity simultaneously.

In this project, the latest developed optogenetic tools - activators ChrimsonR, C1V1(t/t) and Chronos, inhibitor Jaws, red calcium sensor jRCaMP1b and nuclear markers H2B-RFP and H2B-mCherry - were optimized to be expressed in zebrafish. Behavioural assays to characterize the activating and inhibitory optogenetic tools ChrimsonR and Jaws were established. An escape response of short latency could reliably be evoked in transgenic animals with stable expression of ChrimsonR in trigeminal neurons. Combination of this fast, sensitive and red-shifted tool with GCaMP calcium imaging opens the possibility to simultaneously manipulate and record activity with high spatial and temporal precision from a large population of neurons to study their dynamic interactions during behaviour.

Keywords: optogenetics; GECI; zebrafish; neurobiology; neural circuits.

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I. Introduction

1. Neuronal Circuits: From Sensory Input to Behaviour Output

A key question in neuroscience is how the brain integrates sensory inputs and computes a behavioural output. Addressing this topic requires a multidisciplinary approach, involving, among other scientific fields, genetics, molecular biology, optics and mathematical modelling. To simplify this challenging task, studying an animal model with a relatively simple nervous system but with robust behaviour, along with the possibility of genetic manipulation, is ideal. Such a model, as zebrafish, enables labelling of neuronal populations and monitoring their activity through calcium imaging, using genetically encoded calcium indicators. Manipulation of neuronal activity can be achieved by optogenetic technology, optically activating or inhibiting neuronal activity, thus enlightening circuit connectivity and function. A great advantage of using zebrafish in neuroscience research lies in the ability to image whole-brain neuronal activity in a behaving animal, due to its small and transparent brain, which is not possible in mice, where only a subset of neurons can be recorded or manipulated in a given time. This feature enables the development of hypotheses on how large populations of neurons in different brain areas are connected and generate a behavioural response (Friedrich, et al., 2013; Sumbre & de Polavieja, 2014; Portugues & Engert, 2009).

2. Zebrafish as a Model Organism

2.1. *Danio rerio*

An organism that can be genetically manipulated and imaged live non-invasively is of great advantage in neuroscience research, as it allows monitoring neuronal activity in behaving animals.

Zebrafish, *Danio rerio,* is a small fish native to the streams of Himalayas, member of the teleostei infraclass. Its major organs are recognizable at 24 hours post fertilization and embryos develop to a free swimming and hunting larvae in 5 days, with characteristic behavioural patterns that prove the existence of well-developed neuronal circuits (Portugues & Engert, 2009; Del Bene & Wyart, 2012; Howe, et al., 2013).

Teleosts underwent an additional whole genome duplication, resulting in an advantageous partial redundancy in gene function, since mutations induced in less redundant species may result in lethal phenotypes.

Zebrafish was initially used in developmental and genetic studies since the 1980s, due to its fast generation time of 3-4 months (Fig. I.1), large clutches of over 100 embryos for mating pair, small size, larvae transparency, and external development, facilitating developmental studies, genetic and chemical screens, and husbandry in a cost-efficient manner.

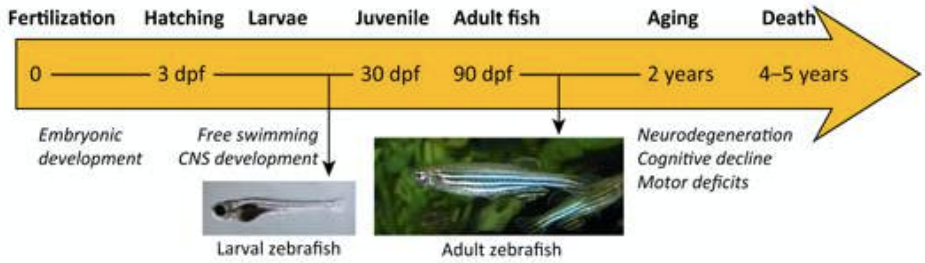

Figure I.1 – Life cycle of zebrafish. Adapted from Stewart et. al., 2014a.

Although phylogenetically distant from humans, zebrafish has a nervous system organized like all vertebrates, and has 10,660 genes in common with the human, mouse and chicken genomes and 70% homology to humans (Fig. I.2) (Howe, et al., 2013; Stewart, et al., 2014; Kalueff, et al., 2014). Genetic manipulation is accessible, through chemical mutagenesis supported by gene or enhancer-trap screens, morpholino delivery, or gene editing such as TALENs or CRISPR, and transgenesis mainly using the Tol2 system (Rinkwitz, et al., 2011; Del Bene & Wyart, 2012). The use of all these systems has generated several transgenic and mutant zebrafish lines. Several mutant lines have been identified as vertebrate models of human neural diseases, such as Alzheimer, epilepsy or autism (Stewart, et al., 2014a; Newman, et al., 2014; Stewart, et al., 2012; Stewart, et al., 2014b; Kalueff, et al., 2014).

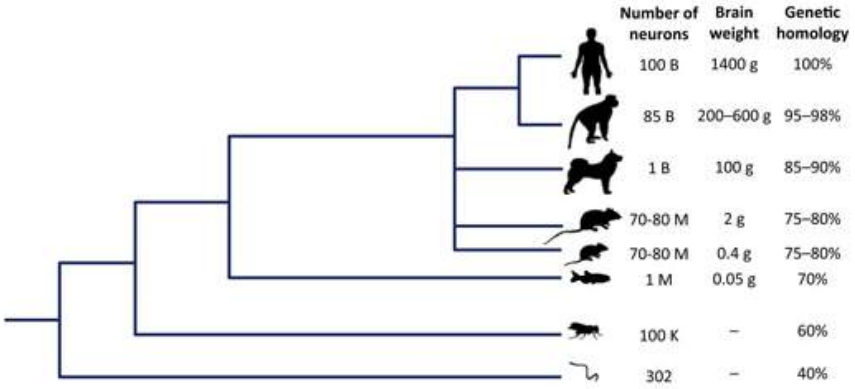

Figure I.2 - Phylogenetic tree of major model species. Adapted from Stewart et. al., 2014b.

Neuroscientists became further interested in zebrafish with the development of sophisticated imaging techniques that allow imaging of whole-brain neuronal activity at single cell resolution, and optical systems that enable stimulation of optogenetic tools in single cells or populations. The larvae's small size, transparency and cutaneous breathing make it possible to restrain it in low-melting agarose gel without paralysers and image brain-wide activity either in completely restrained or tail-free animals for relating neuronal activity to behavioural responses (Sumbre & de Polavieja, 2014).

2.2. Visual evoked behaviour

The advantages of zebrafish in genetic manipulation and live imaging made it a promising model for studying neuronal circuits underlying behaviour. Zebrafish larvae exhibit specific behavioural patterns, such as the startle response, optomotor and optokinetic responses, and prey capture, that can be elicited with visual stimuli in an artificial environment. These behaviours require acquisition of retinal image, connection between retina and the brain, and function of muscles involved in visuomotor behaviour. Correlation of stimulus input with neuronal activity and behaviour output can be used to analyse the brain circuitry and computations (Easter & Nicola, 1996; Feierstein, et al., 2015; Portugues & Engert, 2009).

The escape response can occur due to an acoustic, touch or visual startle. The visual startle response, described at 3 dpf, consists in abrupt movement within 2 seconds of the off-light stimulus (Easter & Nicola, 1996; Portugues & Engert, 2009).
The touch response is present from 1 dpf, and may be elicited with a piezoelectric tapper touch on the larvae head; the fish responds with a rapid large bend away from the stimulus followed by a return bend. Calcium imaging used by (O'Malley, et al., 1996) showed that this stimulus leads to activation of the reticulospinal neuron Mauthner cell, whose activation is correlated with an escape response. Douglass *et. al.* elicited this behaviour by stimulating Rohon-Beard and trigeminal sensory neurons in an optogenetic assay with ChannelRhodopsin2 (ChR2). Escape responses were obtained with single spike induction in trigeminal neurons (Fig. I.4). Considering that Mauthner cells, the downstream target of trigeminal neurons in the hindbrain, can drive escape responses with single spikes, Douglass, *et. al.* speculate that single action potentials (AP) in trigeminal neurons are translated into single APs in Mauthner cells.

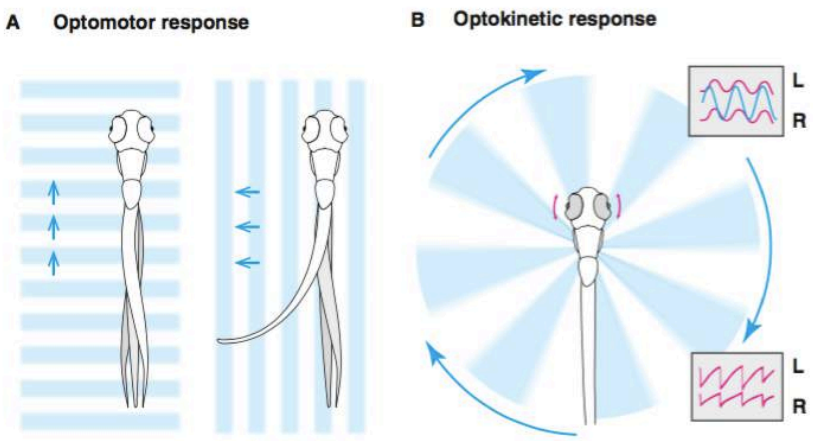

Figure I.3 – Optomotor (A) and optokinetic (B) responses in zebrafish. (A) OMR comprises orienting turns, to bring motion into a tail to head direction (right), and forward swims when the fish position is according to flow (left). (B) During a continuously rotating stimulus, eye movement alternates between slow tracking and fast saccades (pink traces, bottom box); sinusoidal rotating stimulus (pink arrows) results in consistent slow tracking movement (top box). Adapted from (Orger, 2016).

The optokinetic response (OKR) is a reflexive eye movement in response to whole-field rotational motion that stabilizes the image on the retina and permits high-resolution vision. It consists of a slow tracking eye movement and fast reset saccades (Fig. I.3) (Huang & Neuhauss, 2008; Feierstein, et al., 2015).

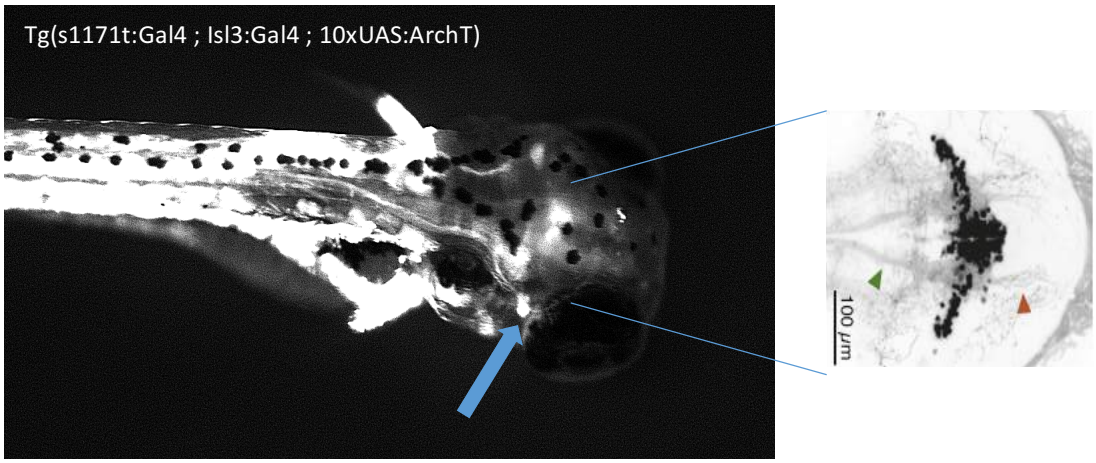

Figure I.4 – On the left, fluorescense image of transgenic fish expressing ArchT (inhibitory optogenetic tool) in trigeminal nerve (blue arrow pointing to trigeminal nucleus, with axon projecting torwards the spinal cord) driven by *Isl3* promoter, and in nucMLF (area highlighted between the eyes) driven by *s1171t* driver sequence. On the right, confocal projection of Tg(s1171t:Gal4; UAS:GFP), adapted from (Thiele, et al., 2014).

Another intrinsic zebrafish behaviour, set from 5 dpf, is the optomotor response (OMR), where larvae stabilize their position according to the optic flow. When presented with a visual stimulus of moving gratings, the larvae swim forward until reaching the stimulus velocity. If the stimulus is presented in any other direction than tail-to-head, the fish makes orienting turns until reaching the right position (Fig. I.3). The OMR is mediated by red and green cones, and reticulospinal neurons, including a small group of neurons located in the mid- and hindbrain that project to the spinal cord, organized in the nucleus of the medial longitudinal fasciculus (nucMLF) (Fig. I.4), which have a part in transducing visual information into motor output (Orger & Baier, 2005; Orger, et al., 2008; Thiele, et al., 2014; Severi, et al., 2014).

Prey capture consists of visually identifying and tracking the prey, through slow swims and J-turns, followed by a capture swim. Involvement of nucMLF in this response has been described (Borla, et al., 2002; Gahtan, et al., 2005; McElligot & O'Malley, 2005).

This set of behaviours can be elicited in both freely swimming and head-restrained larvae, through presentation of moving black and white gratings, small dots or other visual stimuli below or around the animal.

2.3. Transgenesis

The zebrafish advantages discussed above, including its easy-to-manipulate genetics, facilitates transgenic generation.

Transgenesis in zebrafish can be achieved by genome integration of the gene of interest through plasmid DNA microinjection into one-cell stage embryos, traditionally with the help of nucleases and more recently effectively with transposases, using transposition methods as the *Sleeping Beauty* (SB) transposon system or the *Tol2* transposon systems. The *Tol2* system has the highest germline transmission frequency from these systems (Kawakami, 2005).

2.3.1. *Tol2* transposon system

The *Tol2* element, identified in medaka fish, is an autonomously-active transposon of 4.7kb encoding a transposase protein. The transposase is capable of catalysing transposition of a non-autonomous *Tol2* construct, i.e., a construct that contains only the *Tol2* arms (200bp and 150bp sequences) flanking a region where a DNA of interest (up to 10kb) can be cloned (Fig. I.5). Stable transposition is achieved when the *Tol2* construct is co-injected with transposase mRNA in zebrafish one-cell stage embryos: the transposase mRNA is translated and the protein catalyzes the excision of the flanked DNA, which integrates into the genome (Supplement 1). When both mRNA and protein degrade, transposase activity is lost and the insertion gets stabilized. The only modification observed with *Tol2* integration is an 8 bp duplication at the target site.

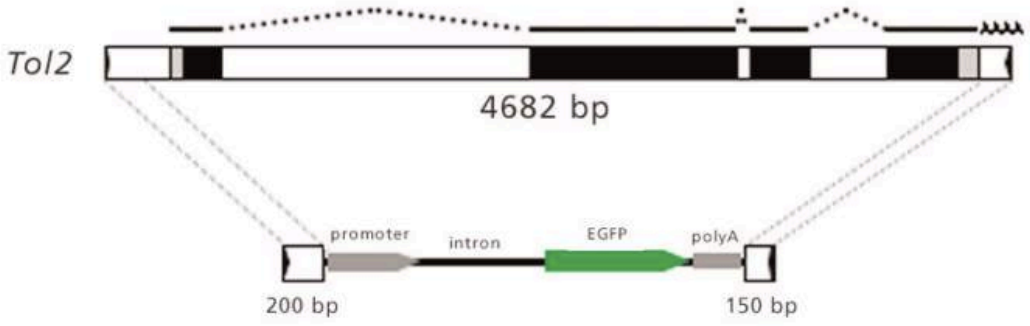

Figure I.5 – *Tol2* element (top) and minimal *Tol2* vector (bottom). Black boxes are coding sequences and white boxes introns. Terminal black arrows indicate 12 bp inverted terminal repeats. Minimal *Tol2* vector including EGFP gene in the site where any DNA fragment (up to 10 kb) can be cloned. Adapted from Kawakami et. al., 2007.

Germline transmission of the construct can be selected in F0 generation (founders), by outcrossing the injected fish (Supplement 1). GFP transgenic expression has persisted until F5, suggesting that there are less silencing effects than in other transgenesis systems (Kawakami, 2005; Kawakami, 2007).

2.3.2. Gal4/UAS Transactivation System

The Gal4/UAS transactivation system is frequently used in zebrafish to easily obtain transgene expression in a cellular population of choice, since it allows for flexible combination of driver lines with reporter lines.

Gal4 is a yeast transcription factor for genes required for galactose utilization. The Gal4 protein binds as a dimer to short DNA sequences upstream of target genes, Upstream Activating Sequence (UAS), and recruits transcriptional machinery to adjacent promoters. Gal4 can also bind cooperatively to UAS tandem repeats, enhancing gene expression (Halpern, et al., 2008; Asakawa & Kawakami, 2008).

The transcription factor was modified into several products as mini-Gal4, a truncated protein for driving expression in yeast and *Drosophila* (Carey, et al., 1989), Gal4-VP16, a fusion of the DNA binding domain of Gal4 to the transcriptional activation domain of herpes simplex virus VP16 protein that increases transcriptional activity (Sadowski, et al., 1988), or Gal4FF (GFF), containing the DNA-binding domain of Gal4 and two short transcriptional activation motifs from VP16, with weaker activity (Seipel, et al., 1992; Baron , et al., 1997) (Fig. I.6). This last variant may help diluting the described squelching phenomenon, where expression of a strong transcription factor can cause toxicity due to titration of endogenous transcription machinery (Gill & Ptashne, 1988; Halpern, et al., 2008; Asakawa & Kawakami, 2008).

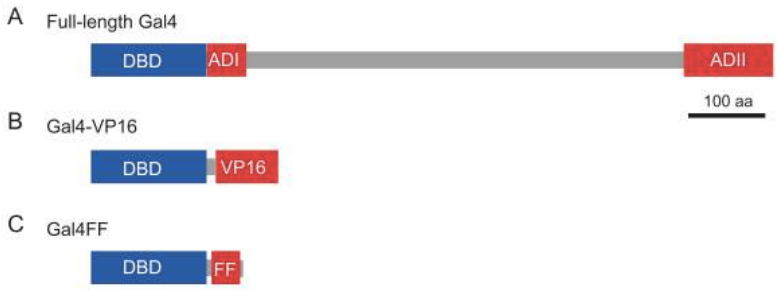

Figure I.6 – Gal4 protein and its derivatives. DBD, DNA-binding domain of Gal4, AD I and AD II, transcriptional activation domains of Gal4, VP16, transcriptinal activation domain of VP16, FF, two transcriptional activation motifs of VP16. Adapted from Asakawa & Kawakami, 2008.

The Gal4/UAS system allows for combination of a promoter sequence with a gene of interest (GOI), by crossing a promoter:Gal4 driver line with a UAS:GOI reporter line (Fig. I.7).

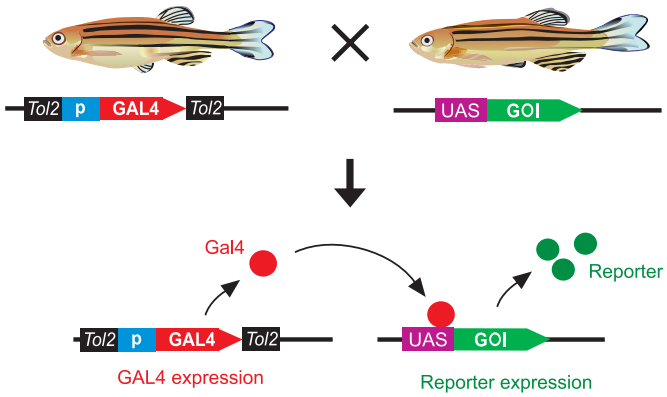

Figure I.7 – Gal4/UAS transactivation system in zebrafish. A Gal4 driver line crossed with a UAS:reporter line results in double transgenic embryos expressing the reporter protein in Gal4 expressing cells. Adapted from Asakawa & Kawakami, 2008.

Therefore, gene expression in a desired cell population is simply achieved by one breeding event, given that a set of transgenic driver lines with the desired promoter sequences are available.

3. Optogenetics as a Tool for Neuronal Circuitry Analysis

Optogenetics is a technology which combines optical tools and genetic targeting techniques for reversible activation or inhibition of identified neurons or populations of neurons by light. Such manipulations are key to studying how neuronal circuit dynamics shape an animal's behaviour (Del Bene & Wyart, 2012; Knafo & Wyart, 2015; Deisseroth, 2015).
This technology relies on: i) optogenetic actuators that elicit electrical current across a membrane upon light delivery; ii) strong and specific genetic targeting of actuator expression in a cellular population of interest; iii) light delivery for optical stimulation with high spatial and temporal resolution in a behaving animal (Deisseroth, 2015).

Zebrafish larvae exhibit complex visuomotor behaviours, as exemplified above, that involve computation in many brain areas and different cell types. The larvae's transparency and small size was used to monitor and manipulate neuronal activity throughout the entire brain while animals were behaving (Ahrens, et al., 2013; Panier, et al., 2013; Dunn, et al., 2016). These experiments helped to identify circuit components and generate hypothesis about their functions (Friedrich, et al., 2013).

3.1. Optogenetic Actuators

There are two major classes of optogenetic actuators: microbial opsins and engineered neuronal receptors/channels tethered to a chemical photoswitch.

Microbial opsin genes, from algae and archaebacteria, encode for rhodopsin-like proteins, ion channels and pumps that elicit electrical current across membranes, in response to light, mediating phototaxis and photophobic behaviour. In contrast, vertebrate rhodopsin proteins mediate

phototransduction in the eye, through signalling cascades that indirectly influence ion channels. Microbial rhodopsin-like proteins directly transduce photons into electrical current (Harz & Hegemann, 1991; Deisseroth, 2015).

There are three types of microbial opsins used in optogenetics: bacteriorhodopsins, halorhodopsins and channelrhodopsins (Fig. I.8). When expressed in neurons, bacteriorhodopsins pump protons in a light-dependent manner out of the cell and halorhodopsins pump chloride ions into the cell, both mechanisms resulting in hyperpolarization and thus inhibition of action potential firing. In contrast, channelrhodopsins are light-gated cation channels used for depolarization and thus activation of neurons due to the cation flow down the electrochemical gradient (Yizhar, et al., 2011) (Fig. I.8).

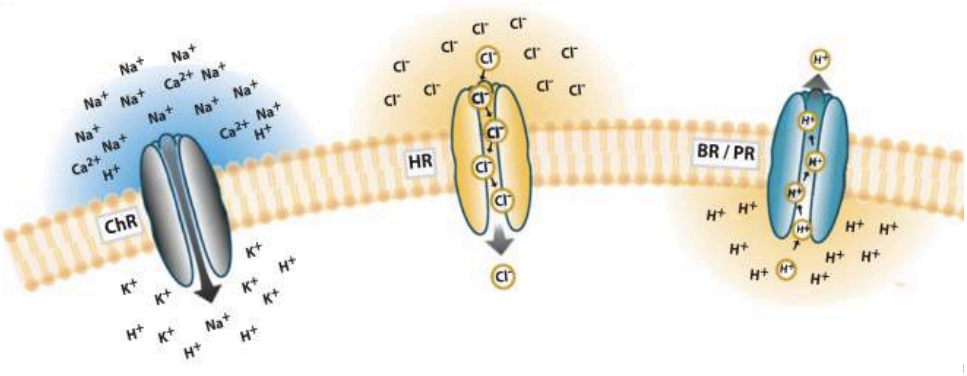

Figure I.8 - Microbial opsins families used as optogenetic actuators. ChR, channelrhodopsins, HR, halorhodopsins, BR/PR, bacteriorhodopsins. Adapted from Yizhar et. al., 2011.

The most widely used opsins so far in zebrafish are channelrhodopsin ChR2 from green algae *Chlamydomonas reinhardti*, that responds to blue light (460 nm) at low intensity (1 mW/mm^2) (Nagel, et al., 2003) and the chloride pump halorhodopsin NpHR from *Natronomonas pharaonis*, that responds to yellow light (Schobert & K., 1982). ChR2 was used in zebrafish to trigger escape responses (Douglass, et al., 2008) and study behaviours like the optokinetic response (Schoonheim, et al., 2010). NpHR-driven silencing helped to identify neurons responsible for initiating locomotion (Arrenberg, et al., 2009).

Since the discovery of these opsins, several variants have been found in nature and engineered in the lab to develop proteins with faster kinetics, higher light sensitivity and response to different light wavelengths, such as activators ReaChR (Lin, et al., 2013), ChrimsonR (Klapoetke, et al., 2014), Chronos (Klapoetke, et al., 2014) and C1V1 (t/t) (Yizhar, et al., 2014), and inhibitors ArchT (Han, et al., 2011) and Jaws (Chuong, et al., 2014).

Other types of optogenetic actuators are natural channels or receptors that are genetically engineered to bind *in vivo* to an exogenous chemical photoswitch. This photoswitch reversibly isomerizes when illuminated with UV and green light. A ligand functions either as an agonist, antagonist or blocker of

the protein, and the photoisomerization by UV/green light moves the ligand from and to its binding site, controlling protein function (Fortin, et al., 2008; Wyart & Del Benne, 2011). An example of these actuators is the light-gated glutamate receptor LiGluR where UV light opens the channel and green light closes it by changing glutamate ligation to the binding site, when the photoswitch MAG is present (Volgraf, et al., 2006). LiGluR was used in zebrafish for activation of spinal cord neurons (Wyart, et al., 2009; Wyart & Del Benne, 2011).
The advantages of using this approach are the higher expression level of these channels in the neuronal plasma membrane, and the higher conductance compared with microbial opsins. However, efficient delivery of MAG is a challenge, that in zebrafish larvae can be effectively overcome by injection into the spinal cord or bath application, due to the larvae's high permeability to small molecules that can diffuse through the blood-brain barrier.

Optogenetic actuators have to be efficiently targeted to neuronal populations of interest, which can be achieved in zebrafish taking advantage of the Gal4/UAS transactivation and *Tol2* transposon systems, which together enable integration of the optogenetic tool in the fish genome by the Tol2 transposase, and its expression in a population where the activator Gal4 is present, as discussed previously. The broad library of Gal4 zebrafish driver lines available with specific promoters identified by enhancer trap screens or through conventional cloning gives access to diverse neuronal cell populations to express the latest optogenetic actuators and study their role in visuomotor behaviour (Scott, et al., 2007).

To understand the computational principles in complex neuronal networks, it is very useful to manipulate cell activity while monitoring network activity, which can be achieved with optogenetic activation/inhibition and calcium imaging with genetically encoded calcium indicators. Zebrafish is an ideal candidate for this approach as whole brain imaging is achievable in the small transparent larvae, as discussed before.

3.2. Optogenetic Reporters: Genetically-Encoded Calcium Indicators

Genetically-Encoded Calcium Indicators (GECIs) are tools that enable *in vivo* optical activity recording from genetically-targeted neuronal populations. Neuronal activity can be measured by recording changes in intracellular calcium concentration, since calcium ions (Ca^{2+}) presence is correlated with action potential firing and synaptic input (Akerboom, et al., 2013).
GECIs use fluorescence or chemiluminescence to monitor intracellular Ca^{2+} concentration, using fluorescent proteins (as GFP) or Ca^{2+}-binding photoproteins (aequorin), respectively.
Some proteins have an intracellular calcium-binding domain, EF-hand, a helix-loop-helix motif that coordinates one calcium or magnesium ion. Aequorin contains EF-hand Ca^{2+}-binding sites, that when bound to Ca^{2+} promote a conformational change in the protein that induces oxidation of coelenterazine, resulting in emission of blue light (λ=470nm) (Kendall & Badminton, 1998) (Fig. I.9).
On the other hand, fluorescent proteins in their native form are not sensitive to changes in Ca^{2+}, therefore Ca^{2+}-binding properties of other proteins have to be recruited. In single fluorophore GECIs, a

fluorescent protein is fused with the calmodulin (CaM) EF-hand Ca^{2+}-binding motif at its C-terminal, and at the N-terminus the M13 peptide from myosine light chain kinase. When Ca^{2+} is present, M13 binds to CaM, which results in a conformational change of GFP and increase of its fluorescence emission. (Fig. I.9)

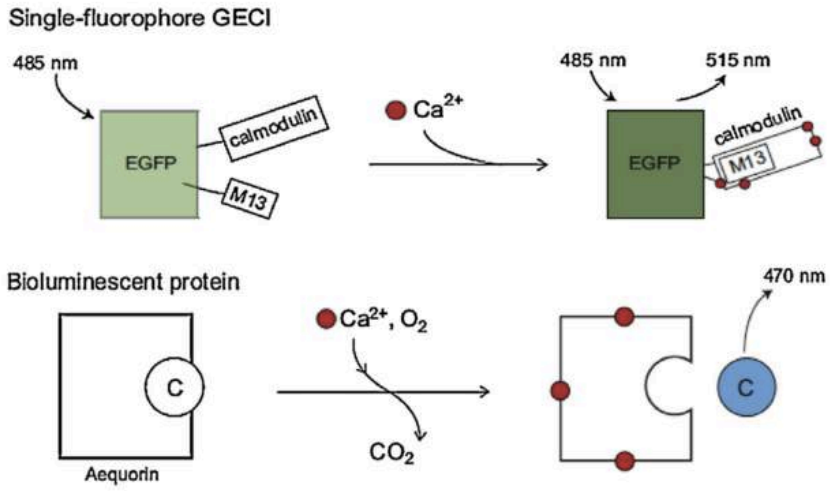

Figure I.9 – Genetically-encoded calcium indicators: in single-fluorophore GECIs calcium binding induces conformational change and increases emitted fluorescence; in bioluminescent GECIs calcium binding leads to oxidation of coelenterazine (C) and energy transfer between aequorin and GFP leads to emission of a green photon. Adapted from Knafo et. al., 2015.

These Ca^{2+} indicators can be targeted to subtypes of neurons through cell-type specific promoters, subcellular targeting sequences (e.g. membrane trafficking signals) and transgenic methods, resulting in expression of a calcium sensor *in vivo*, with cellular specificity, that allows non-invasive, chronic imaging of neuronal activity.

A GECI should ideally have a low baseline fluorescence and be highly sensitive to low-level Ca^{2+} changes due to firing of one action potential.

Aequorin lacks background bioluminescence and allows imaging freely moving animals, but has the limitation of being coelenterazine-dependent and having no spatial resolution: image is captured with a large-area photo-detector positioned above the behaviour arena, that receives light emitted from any point of the arena (Naumann, et al., 2010).

Fluorescent proteins have been engineered so that mutations in its sequence, changes in the calmodulin or M13 linker, and addition of certain tags improve the biosensor performance (Ca^{2+} affinity, kinetics, protein stability, expression/degradation, signal level), thus improving the initially developed GCaMP GECI (λ_{exc}=490nm), designed from EGFP (Nakai, et al., 2001). The GECIs with the best performance so far are those from GCaMP6 family - GCaMP6s and GCaMP6f - which have higher sensitivity and faster kinetics than the previously engineered ones (Chen, et al., 2013). These GCaMP are excited by blue light, which makes them incompatible with ChR2 that is sensitive to this light wavelength.

Another key feature that can be modified by genetic engineering is the sensor colour. GFP was replaced through several mutations, mainly in its chromophore, to create fluorescent proteins with

different excitation/emission properties (BFP, CFP, YFP, RFP – blue, cyan, yellow, red) that can be fused to biosensors to obtain a GECI. This allows combination of GECIs for imaging different neuronal populations simultaneously or combining activity recording with optical stimulation of optogenetic actuators. Usage of red-shifted indicators is preferable as imaging at longer wavelengths reduces problems like tissue scattering, phototoxicity and background fluorescence (Mank & Griesbeck, 2008; Akerboom, et al., 2013). Red-shifted GECIs developed from mApple and mRuby fluorescent proteins (RCaMP1, R-GECO, RCaMP2) (Akerboom, et al., 2013; Zhao, et al., 2011) were improved to originate brighter sensors, non-susceptible to photoswitching by blue light, namely jRCaMP1a, jRCaMP1b and jRGECO1a (Dana, et al., 2016).

The spectrum of GECIs available enables monitoring of neuronal activity, either driven by specific stimuli or in response to activation or inhibition of certain populations by optogenetic actuators, using advanced imaging acquisition techniques.

4. Aims

We aimed to generate transgenic zebrafish expressing new optogenetic tools, which are more sensitive, faster and red-shifted, to use in combination with GECIs, and develop protocols for optogenetic modulation of zebrafish behaviour. The specific aims of this project are:

- To clone new enhanced optogenetic tools into zebrafish expression vectors, specifically: the activators ChrimsonR (red-shifted), Chronos (faster kinetics and higher sensitivity) and C1V1(t/t) (better off-kinetics and sensitivity); the inhibitor Jaws (red-shifted); GECI jRCaMP1b (red); and the nuclear markers H2B-RFP and H2B-mCherry;
- To inject fluorescence-tagged constructs in zebrafish one-cell stage embryos to test the expression driven by specific neuronal promoters, and generate stable transgenic lines using the Tol2 transposon system;
- To establish a functional assay to test the efficacy of the optogenetic tools in zebrafish larvae, and optimize the protocol for optogenetic stimulation. Activating tools will be expressed in trigeminal sensory neurons and screened for an escape response, and silencing tools expressed in motor neurons and screened for changes in swimming.

II. Material and Methods

1. Cloning

1.1. Gene isolation

Plasmids encoding different optogenetic tools were obtained from Addgene (TableII.1) and the gene of interest (GOI) was isolated to be cloned into a Tol2 Gateway destination vector (Supplement 2).

Table II.1 - Original plasmids containing the optogenetic tools; antibiotic resistance and bacteria growth temperature.

Gene of Interest	Original Plasmid	Resist.	Growth Temp.
ChrimsonR-tdTomato	pCAG-ChrimsonR-tdTomato[1]	amp	37°C
Chronos-tdTomato	pAAV-Syn-Chronos-tdTomato[2]	amp	37°C
C1V1(t/t)-TS-mCherry	pAAV-CaMKIIa-C1V1(t/t)-TS-mCherry[3]	amp	37°C
Jaws-KGC-GFP-ER2	pAAV-CaMKII-Jaws-KGC-GFP-ER2[4]	amp	30°C
NES-jRCaMP1b	pGP-CMV-NES-jRCaMP1b[5]	kan	37°C
H2B-mCherry	mCherry-H2B-6[6]	kan	37°C
H2B-RFP	H2B-RFP in pENTR1A[7]	kan	37°C

Bacterial cultures were grown overnight in LB medium with the appropriate antibiotic at specific temperature. DNA extraction was carried out with QIAprep Spin Miniprep Kit (QIAGEN).

1.1.1. Polymerase Chain Reaction (PCR)

The GOI was isolated by PCR reaction, using specifically designed primers containing restriction sites for cloning into Tol2 Gateway destination vector. Kozak sequence was added in the forward primer.

fw primer: 5' SpeI-KozakSequence-ATG…[GOI]… 3'
rv primer: 5' SacII-StopCodon…[GOI]… 3'

[1] pCAG-ChrimsonR-tdT was a gift from Edward Boyden (Addgene plasmid # 59169)
[2] pAAV-Syn-Chronos-tdTomato was a gift from Edward Boyden (Addgene plasmid # 62726)
[3] pAAV-CaMKIIa-C1V1 (t/t)-TS-mCherry was a gift from Karl Deisseroth (Addgene plasmid # 35500)
[4] pAAV-CaMKII-Jaws-KGC-GFP-ER2 was a gift from Edward Boyden (Addgene plasmid # 65015)
[5] pGP-CMV-NES-jRCaMP1b was a gift from Douglas Kim (Addgene plasmid # 63136)
[6] mCherry-H2B-6 was a gift from Michael Davidson (Addgene plasmid # 55056)
[7] H2B-RFP in pENTR1A (w507-1) was a gift from Eric Campeau (Addgene plasmid # 22525)

To clone Chronos-tdTomato into Tol2 Gateway destination vector, a different pair of restriction enzymes was used:

Chronos fw primer: 5' SpeI-KozakSequence-Chronos… 3'
Chronos rv primer: 5' PacI-Chronos… 3'
Tol2 fw primer: 5' PacI-SacII-Tol2… 3'
Tol2 rv primer: 5' PacI-SpeI-Tol2-… 3'

To clone Jaws-mCherry, by replacing GFP with mCherry in the original Jaws-GFP construct, primers were designed to obtain two overlapping fragments that were fused by Gibson Assembly:

Jaws-GFP fw primer: 5' SpeI-KozakSequence-Jaws… 3'
Jaws-GFP rv primer: 5' /mCherry-Jaws… 3'[8]
UAS-Lyn-mCherry fw primer: 5' /Jaws-mCherry… 3'
UAS-Lyn-mCherry rv primer: 5' SacII-Stop-mCherry… 3'

Primers sequence, polymerase and annealing temperature used for each PCR are described in Supplement 3. Further PCR conditions were according to the recommended protocol for each polymerase: Phusion HF DNA Polymerase (NEB), Platinum Taq DNA Polymerase (Invitrogen), AccuPrime Pfx Supermix (Invitrogen).

PCR products were analysed and isolated in 1% agarose gel (SeaKem LE agarose, Lonza) electrophoresis, ran at 80-130V. Fragment size was estimated using a 1kb DNA ladder (2-log DNA Ladder (0.1-10.0kb), NEB). Samples were loaded with Gel Loading Dye Purple (NEB) 1:5, and gel stained with GreenSafe Premium (nzytech).

1.1.2. Gibson Assembly

Gibson Assembly is a method to assemble multiple DNA fragments. The Gibson assembly master mix includes an exonuclease that creates 3' overhangs, a polymerase that fills in the gaps after overlapping region annealing, and a DNA ligase that seals nicks in the assembled DNA (Gibson Assembly Master Mix – Instruction Manual, NEB).
Jaws and mCherry PCR products were fused into Jaws-mCherry by Gibson Assembly (protocol details in manufacturer manual of Gibson Assembly Master Mix (NEB)). The assembly product was used for PCR amplification to increase fragment yield for cloning.

[8] [/] stands for a portion of the gene sequence, the begining in mCherry, and the end in Jaws.

1.2. Tol2-GOI cloning

Both GOI PCR products and Tol2 vector were digested with either SpeI/SacII or SpeI/PacI restriction enzymes (NEB), purified (QIAquick PCR Purification Kit, QIAGEN) and then ligated with T4 DNA ligase (NEB). Tol2-GOI constructs were transformed into ccdb survival cells (One Shot ccdb survival, Invitrogen) and selected for ampicillin resistance. Tol2-GOI successful ligation was confirmed by control digestion and sequencing (STAB VIDA), after DNA extraction.

1.3. Tol2-Regulatory Sequence-GOI cloning

To clone the desired regulatory sequences (4xUAS, 10xUAS and *elavl3* panneuronal promoter) upstream the gene of interest, the gateway cloning technology, a fast and flexible cloning system, was used.

1.3.1 Gateway Cloning Technology

The Gateway Cloning Technology is a recombination system based on the site-specific recombination system of phage λ to integrate its DNA in *E. coli* and switch between lytic and lysogenic pathways. (Gateway Technology User Guide, Invitrogen) (Fig. II.1)

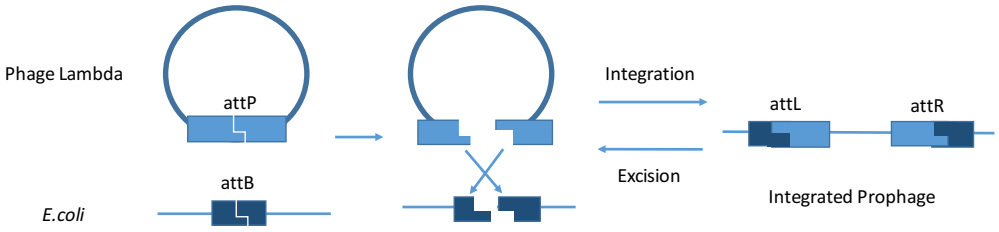

Figure II.1 – Site-specific recombination system of phage λ. attP sites of phage λ recombine with attB sites in *E. coli* and DNA integration is achieved, originating attL and attR sites. The process is reversible.

The Gateway cloning includes the excision and integration of DNA fragments *in vitro*, where the adapted att1 and att2 sites recombine in a directional and site-specific manner: attB1 reacts with attP1, attB2 reacts with attP2 (BP reaction), and the same occurs in attL and attR sites (LR reaction) (Fig. II.1).

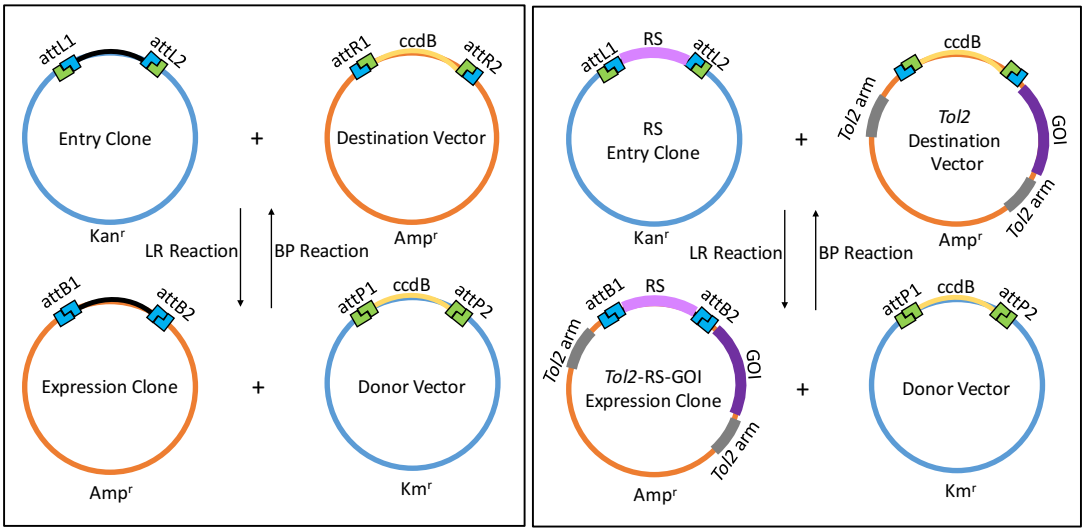

Figure II.2 - Gateway cloning system (left) can be used with a *Tol2* destination vector expressing a gene of interest (GOI) recombined with an entry clone expressing a regulatory sequence (RS), resulting in an expression clone with a *Tol2* backbone, expressing a GOI driven by a RS (right).

Selection against ccdB toxicity and for antibiotic resistance permits identification of successful recombination.

The genes of interest were cloned into a *Tol2* destination vector that contains the *Tol2* arms and attR1 and attR2 sequences. Regulatory sequences (RS) available in entry clones (Supplements 4-6) were inserted upstream the GOI through the Gateway LR reaction (Gateway LR Clonase II, Invitrogen), generating the expression clone Tol2-RS-GOI (Fig. II.2).

Tol2-RS-GOI constructs were transformed into TOP10 competent cells (One Shot TOP10, Invitrogen) and selected for ampicillin resistance. Successful LR reaction was confirmed by control digestion and sequencing (STAB VIDA), after DNA extraction.

1.4. Transformation

2μL of vector was incubated in a 50μL aliquot of chemically competent cells for 30min on ice. Heat-shock was applied by emerging the sample in a 42°C bath for 30sec, followed by 2min on ice. 500μL of LB medium was added and bacteria recovered for 45min, at 400rpm, 37°C. 100μL of the transformation mixture was plated on LB-agar plates with ampicillin and incubated at 37°C overnight.

2. Transgenesis

2.1. Fish Husbandry

The fish were maintained at a facility kept at 25°C, 50%-60% humidity, 14h:10h light:dark cycle with 200-300lux ambient light intensity. Fish water was at 28°C, and pH, salinity and dissolved gases were kept in physiological conditions (Martins, et al., 2016). Embryos were kept in E3+Methylene Blue embryo medium (see Methods topic 5: Solutions) at a maximum density of 50 embryos/dish,

incubated at 28°C, until 6dpf when they entered the water recirculation system. Raised embryos were bleached at 1dpf.

The larvae used for behaviour experiments were kept at 20 fish/dish, protected from direct light, fed with rotifers from 4dpf on, and the E3 medium (1x) (see Methods topic 5: Solutions) was refreshed daily.

All animal experiments were done according to the CF Animal Welfare Body guidelines and under a CF Ethics Committee-approved project.

2.2. Microinjection in one-cell stage zebrafish embryos

Injection of the final construct Tol2-RegulatorySequence-GeneOfInterest in one-cell stage embryos was performed according to (Kikuta & Kawakami, 2009). The injection mixture comprised of 15-20ng/μL of Tol2-RS-GOI and 0.1μg/μL of Tol2 transposase mRNA in E3+PhenolRed. Constructs with 4xUAS and 10xUAS regulatory sequences were injected in Tg(Isl3:Gal4$^{(+/-)}$) embryos (all Gal4 driver lines encode for a Gal4FF protein), and *elavl3* regulated constructs in *nacre*$^{(+/-)}$ embryos. Injected embryos were incubated at 28°C.

2.3. Transient expression

Injected embryos were selected for transgenic transient expression between 3-5 dpf (days post fertilization), by fluorescence imaging using a PentaFluor-equipped V8 stereoscope (Zeiss). Embryos were screened for panneuronal (*evalv3* driver) or trigeminal nerve (*Isl3* driver) fluorescence (green for GFP tag and red for mCherry, RFP and tdTomato tagged constructs) and raised into adulthood.

2.4. Screening for stable expression

When the positive injected fish reached sexual maturity (2 to 3 months), an outcross with Tg(Isl3:Gal4$^{(+/-)}$) or *nac*$^{(+/-)}$ fish was performed, for UAS:GOI and evalv3:GOI transgenic fish, respectively. Animals with positive progeny were kept. From the positive progeny (F0, founders), stable transgenic lines are established.

3. Behaviour assay

3.1. Transgenic fish tested

Activating optogenetic tools were tested by optically stimulating the trigeminal nucleus, in Tg(Isl3:Gal4;10xUAS:GOI) larvae obtained from *Isl3:Gal4* fish, and inhibitory tools were tested with stimulation of nucMLF in Tg(*s1171t:Gal4;10xUAS:GOI)* larvae from Tg(*s1171t:Gal4*) fish.

3.2. Setup

Larvae with 6 or 7dpf were embedded in 1.6% low-melting agarose (UltraPure LMP Agarose, Invitrogen) in E3 medium on sylgard (Dow Corning) dishes, and the tail was freed.

For optogenetic stimulation a 590nm LED was coupled either to a 200μm diameter fiber (multimode fiber, ceramic ending, Doric), a 50μm or 25μm diameter fiber (multimode fibers, 0.22NA and 0.1NA

respectively, 0.7m, FC/PC in one end, open plain cut in other end, Thorlabs). The 635nm laser (squared laser, MLL-III, Photontec, 500mW) was fed into the 50μm or 25μm diameter fiber, focused and aligned with lens and XY stage (Fig. II.3).

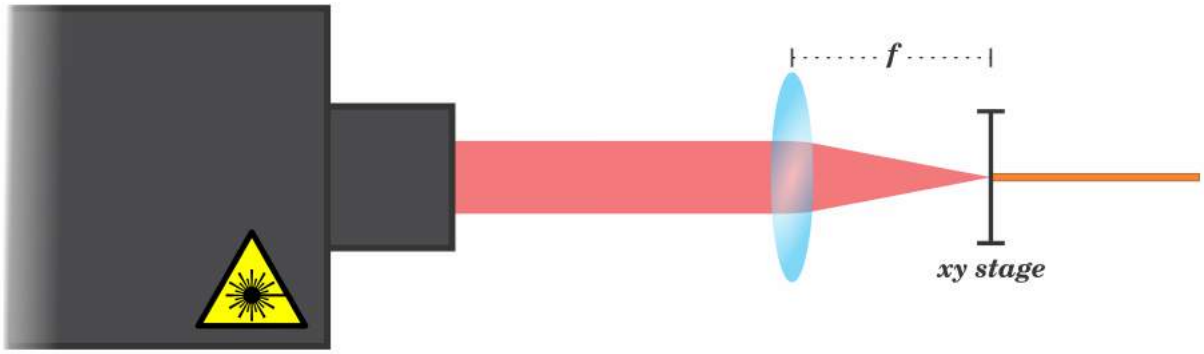

Figure II.3 – Scheme of 635nm laser feeding into fiber. (f) focal distance. Courtesy of Alexandre Laborde.

Visual stimuli were projected on a screen 0.5cm below the fish using a LED projector (optoma ML 550). Sinusoidal stationary or moving gratings with spatial frequency of 1cm were presented (Fig. II.4).

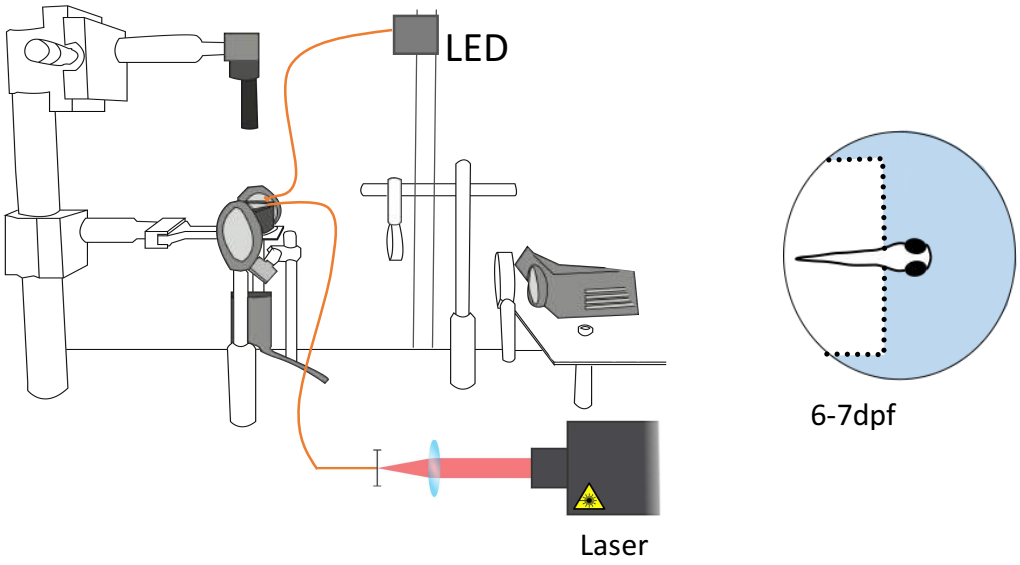

Figure II.4 – Behaviour assays set up: 6-7dpf larvae embeded in low-melting agarose with tail free is placed on a stage with visual stimulus coming from a projector presented below the larvae; light delivered by fiber coupled to LED or laser; camera placed on top of the larvae to record behaviour.

Visual stimulation, optogenetic manipulation and behavioural recording were controlled by software custom written in Visual C# (Microsoft). Homogeneous infra-red illumination (M780L, Thorlabs) was provided for tracking of tail movements. Images were recorded at 700 frames per second using a Mikrotron EoSens (MC1362) camera fitted with Xenoplan 2.8/50-0902 lens. Swim bladder, tip of the tail and eye position were manually defined and tail angle values from 16 points evenly spaced between swim bladder and tip of the tail were saved for behavioral analysis (Fig. II.4).

4. Data analysis

Behavioural data was analysed in Matlab. Individual swim bouts and half beats were detected similar to (Marques, J. 2016). Briefly, tail angle values were smoothed and frame-to-frame changes in curvature for each of the 16 points along the tail were determined and the cumulative sum of the absolute value along the tail was calculated. The 80^{th} percentile of each fish's tail motion measure was used to identify the start and end of a bout (Marques, J. 2016). For each fish, bout duration and latency after stimulus onset was determined and number of half beats were evaluated. For half beat detection tail curvature values were smoothened using a boxcar filter and a dynamic threshold was determined for each bout to identify beginning and end of half beats by calculating the most extreme values of the tail for each tail angle.

5. Solutions

- LB medium: distilled water with 1.0% bactotritone, 0.5% yeast extract, 0.5% NaCl, pH=7.0 (adjusted with NaOH 5N). Sterilized.
- LB-agar: 1.5% bacto-agar in LB medium. Sterilized.
- E3+ Methylene Blue:

50x:	1x:
- 29.38g NaCl	- 400mL 50x E3
- 1.26g KCl	- 60mL 0.01% Methylene Blue Solution (0.05g Methylene Blue
- 4.86g $CaCl_2.2H_2O$	powder in 500mL MQ water)
- 8.14g $MgSO_4.7H_2O$	- system water to 20L

- E3 (60x): 5mM NaCl, 0.17mM KCl, 0.33mM $CaCl_2$, 0.33mM $MgS0_4$, pH=7.2. Sterilized.
- E3-PhenolRed: 0.0025% PhenolRed in E3. Filtered.

III. Results

1. Optimization of state-of-the-art optogenetic tools for zebrafish expression

Several genes of interest obtained from addgene (Table II.1) were cloned into a zebrafish expression system: the *Tol2* vector with attR sites that enable integration of a regulatory sequence into the vector through the gateway technology. The genes of interest include optogenetic actuators, reporter, and nuclear markers, in detail explained below.

1.1. Genes of interest

The microbial opsins used as optogenetic actuators should have on-, off- and recovery-kinetics fast enough to precisely control neuronal spike timing and consistency. To evoke action potential firing, channelrhodopsins need photocurrents sufficient to depolarize the neuron cell membrane above its spike threshold (Klapoetke, et al., 2014).
In order to optogenetically manipulate and simultaneously monitor neuronal activity, it is essential to use optogenetic actuators and reporters with non-overlapping excitation spectra. Therefore, recently developed red-shifted actuators are of great interest for combination with state-of-the-art GCaMP calcium imaging methods, and red GECIs are useful for combination with the classical blue/green opsins.

ChrimsonR-tdTomato
Chrimson is a channelrhodopsin derived from *Chlamydomonas noctigama* algae. The opsin is red-shifted, with spectral peak at 590nm, and highly sensitive (photocurrents of 674pA with 660nm light induction). The original Chrimson opsin has slow off-kinetics. The K176R mutant, ChrimsonR, described by (Klapoetke, et al., 2014) has faster off-kinetics of 15.8ms (comparing to Chrimson) without compromising its red-shifted action spectrum. The faster kinetics allow spike frequency of 20Hz without a depolarization block observed with Chrimson. The red-shifted and narrow action spectrum of ChrimsonR makes it favourable for combination with GCaMP6 calcium imaging (λ_{exc}=470nm) (Klapoetke, et al., 2014).
As ChrimsonR itself is a non-fluorescent protein, a red tdTomato reporter gene was fused C-terminally for visualization of transgenic expression.

Chronos-tdTomato
Chronos is a channelrhodopsin from *Stigeoclonium helveticum* algae with spectral peak at 550nm. It has very fast on-kinetics (2.3ms) and off-kinetics (3.6ms). Induced with 530nm green light, Chronos mediated spiking is similar to electrically mediated spiking, between 5 and 60Hz (other opsins tested in a previous study can only reliably evoke spikes up to 20Hz). Its kinetics make Chronos suitable to probe neuronal circuit functions during behaviour. Chronos may be combined with either other

actuators for simultaneous activation/inhibition or manipulation of different populations, or with red excitable calcium indicators for neuronal activity readout (Klapoetke, et al., 2014).
The construct contains a red tdTomato reporter gene.

C1V1(t/t)-TS-mCherry

C1V1(t/t) is a chimeric channelrhodopsin engineered from ChR1 and VChR1 with spectral peak at 535nm. It contains E122T/E162T mutations that reduce off-kinetics (34ms), as compared with the firstly developed C1V1, and membrane trafficking sequence (TS) Kir2.1 that increases photocurrent (1104pA). The red-shifted action spectrum makes the channelrhodopsin compatible with simultaneous GCAMP6 imaging (Yizhar, et al., 2014; Yizhar, et al., 2011).
The construct contains a red mCherry reporter gene.

Jaws-KGC-GFP-ER2

Jaws is a cruxhalorhodopsin derived from *H. salinarum*. This red-shifted hyperpolarizing chloride pump has its spectral peak at 600nm. Point mutations K200R and W214F to the original Halo57 opsin significantly increased photocurrents (~270pA), and KGC and ER2 membrane trafficking sequences were added (Chuong, et al., 2014).
The construct contains a green GFP reporter gene.

NES-jRCaMP1b

jRCaMP1b is a red genetically-encoded calcium indicator based on mRuby, fused to CaM and M13 peptide. Mutagenesis on the RFP/CaM and CaM/M13 interfaces, and CaM itself, gave rise to jRCaMP1b, a sensitive red GECI with a broad dynamic range that does not exhibit photoswitching. NES nuclear export sequence was added to sense calcium in the cytoplasm (Dana, et al., 2016). This red GECI is compatible with green/blue optogenetic actuators.

H2B-RFP

H2B-RFP is a nuclear marker, with human histone H2B fused to HcRed fluorescent protein from *Hecteratis crispa* (λ_{exc}=598nm, λ_{em}=645nm) (Anson, 2007).

H2B-mCherry

H2B-mCherry is a nuclear marker, with human histone H2B fused to mCherry red fluorescent protein (λ_{exc}=587nm, λ_{em}=610nm), derived from DsRed, cloned from *Discosoma coral* (Anson, 2007).

1.2. Expression Vector Cloning

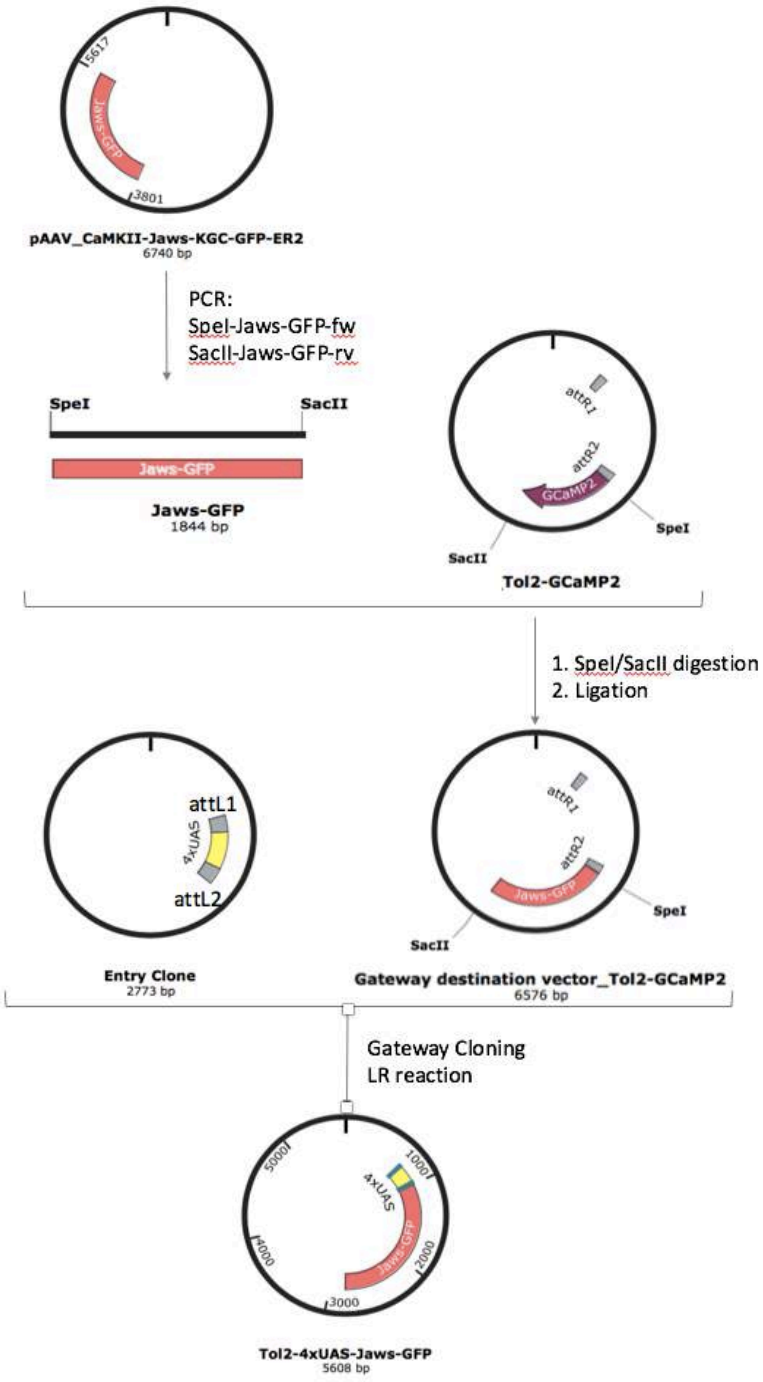

Figure III.5 - General cloning strategy of final expression vector Tol2-Regulatory Sequence-Gene of Interest.

General Cloning Strategy

DNA sequences corresponding to each optogenetic tool were isolated from the original plasmid by PCR with primers designed to have SpeI and SacII restriction sites flanking the PCR product and a functional Kozak sequence 5' to the ATG-side of the GOI. PCR fragments were cloned into a *Tol2* gateway destination vector containing the same restriction sites, by restriction digestion and ligation. Recombination between Tol2-GOI and the entry clone through Gateway LR reaction, resulted in the final expression clone Tol2-Regulatory Sequence-Gene of Interest. (Fig. III.1, Table III.1).

Entry clones with regulatory sequences *elavl3*, a panneuronal promoter sequence, and 4xUAS and 10xUAS, driving expression in Gal4 dependent manner, were used.

Chronos-tdTomato cloning

To clone Chronos-tdTomato into the *Tol2* vector, a different pair of restriction enzymes (SpeI and PacI) was used, since Chronos sequence included a SacII restriction site. For this, both the GOI and the *Tol2* destination vector were amplified by PCR using primers designed to generate PacI-Chronos-tdTomato-SpeI and SpeI-Tol2-PacI PCR products (Fig. III.2).

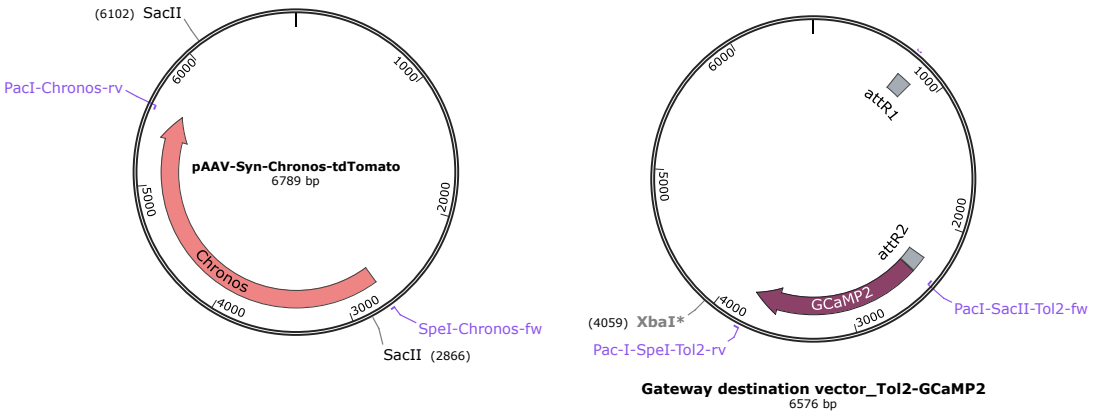

Figure III.2 – Chronos-tdTomato and Tol2 primer design. Isolation of Chronos-tdTomato fragment and modification of Tol2 vector with compatible endings (PacI and SpeI) to ligate them without resourcing to SacII restriction enzyme.

Both products were digested and ligated to generate Tol2-Chronos-tdTomato vector, and LR reaction was performed to obtain final expression vectors.

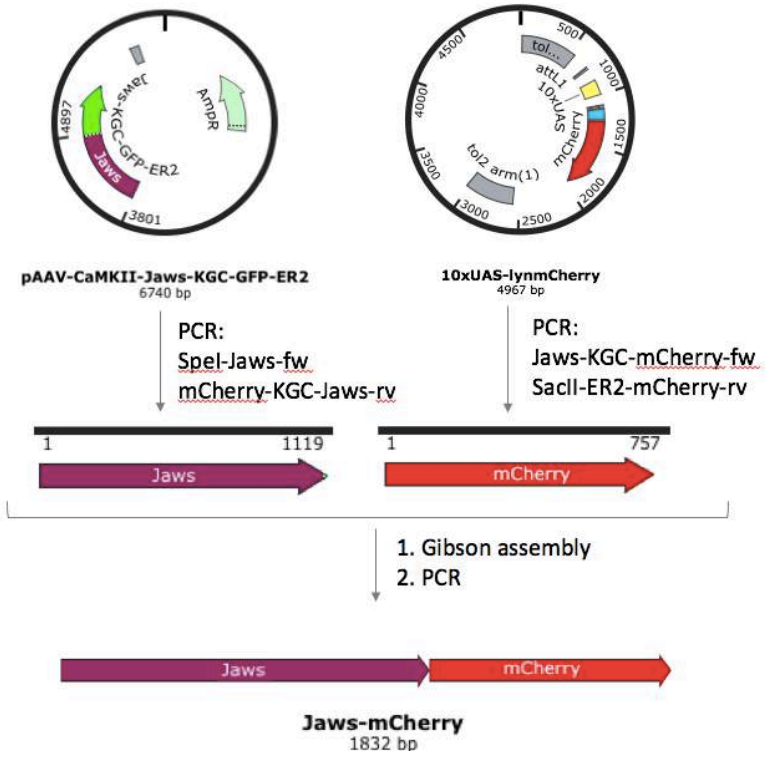

Figure III.3 – Jaws-mCherry cloning strategy.

Jaws-KGC-mCherry-ER2 cloning

The original Jaws construct obtained from addgene was tagged with GFP as a reporter gene (Jaws-KGC-GFP-ER2). To facilitate visualization of Jaws expression in transgenic conditions in which a green fluorescent calcium sensor such as GCaMP is co-expressed, the GFP-tag was replaced by mCherry.

Primers were designed to obtain Jaws-KGC and mCherry-ER2 fragments with overlapping endings and restriction sites to clone into *Tol2* destination vector. Both fragments were fused through Gibson Assembly and the fusion product was amplified by PCR to increase fragment yield for subsequent cloning steps (Fig. III.3). Jaws-KGC-mCherry-ER2 was cloned into Tol2 vector through digestion and ligation, and final expression vectors were obtained after LR recombination with *elavl3* or UAS entry vectors.

H2B-RFP cloning

To identify the correct H2B-RFP start site for cloning of H2B-RFP into the *Tol2* expression vector, the addgene vector "H2B-RFP in pENTR1A" was aligned to the H2B-mCherry sequence. A second ATG start site adding extra 63bp sequence upstream of the H2B gene from "H2B-RFP in pENTR1A" original plasmid was found (Supplement 7). Therefore, two H2B-RFP constructs were cloned:

- H2B-RFP1: starting at the ATG common to both H2B sequences
- H2B-RFP2: starting at the upstream ATG, with the extra 63bp

Both versions of final expression vector were injected to assess differences in expression.

Table III.1 – Expression vectors cloned.

Backbone	Driver	Optogenetic tool
Tol2 Gateway destination vector	4xUAS 10xUAS *elavl3*	ChrimsonR-tdTomato
		Chronos-tdTomato
		C1V1(t/t)-TS-mCherry
		Jaws-KGC-GFP-ER2
		Jaws-KGC-mCherry-ER2
		NES-jRCaMP1b
		H2B-mCherry
		H2B-RFP1
		H2B-RFP2

2. Generation of transgenic zebrafish lines

Final expression vectors with optogenetic tools or nuclear markers were injected in one-cell stage Tg(Isl3:Gal4$^{(+/-)}$) embryos in case of UAS constructs or *nacre*$^{(+/-)}$ embryos in case of *elavl3* constructs (see Table III.2) in order to test expression efficiency of the tools in zebrafish and generate transgenic lines for experiments.

Table III.2 – Optogenetic tools expression vectors, injected into one-cell stage zebrafish embryos.

Zebrafish Embryos	Expression Vector
Isl3:Gal4$^{(+/-)}$	T2-10xUAS-ChrimsonR-tdTomato
	T2-10xUAS-Chronos-tdTomato
	T2-10xUAS-C1V1(t/t)-TS-mCherry
	T2-10xUAS-Jaws-KGC-GFP-ER2
	T2-10xUAS-Jaws-KGC-mCherry-ER2
	T2-10xUAS-NES-jRCaMP1b
	T2-10xUAS-H2B-mCherry
nacre $^{(+/-)}$	T2-HuC[9]-Jaws-KGC-GFP-ER2
	T2-HuC-H2B-mCherry
	T2-HuC-H2B-RFP1

Larvae were screened for fluorescence from 3dpf onwards and embryos with good transient expression of the respective fluorescent reporter gene (Fig. III.4) were raised. For the construct T2-10xUAS-Chronos-tdTomato more than 400 animals were screened without any expression.

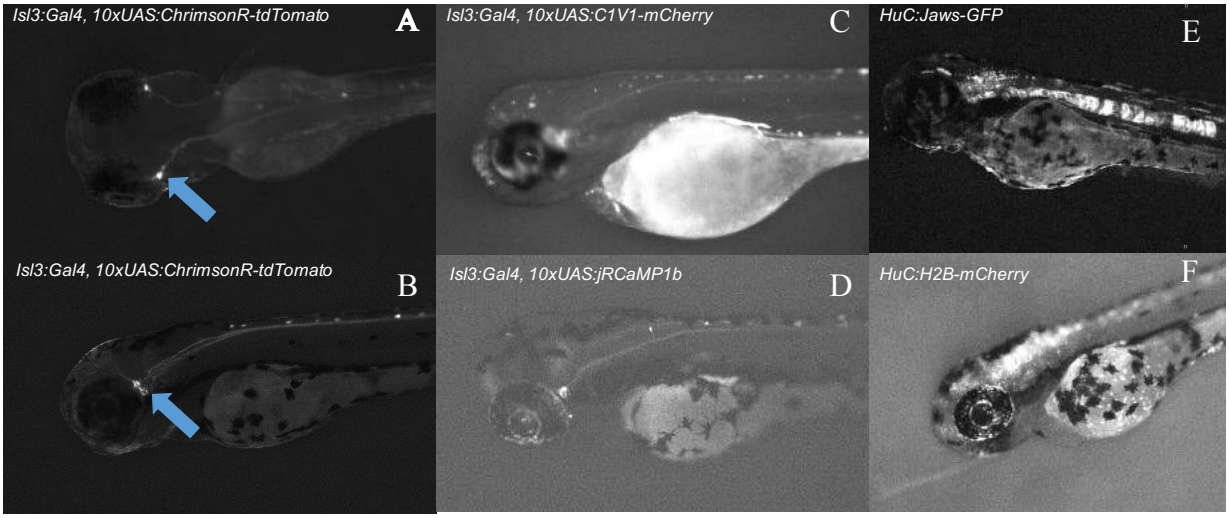

Figure III.4 – Transient expression of constructs injected in Tg(Isl3:Gal4$^{(+/-)}$) or *nacre*$^{(+/-)}$ zebrafish one cell stage embryos. Animals selected at 3-5 dpf. A-D, expression in trigeminal nerve, with one trigeminal nucleus pointed by blue arrow, that projects to the spinal cord; E-F, panneuronal expression pattern.

When reaching sexual maturity (approximately 60 days old), fish that had been previously selected for their transient expression were outcrossed: Tg(Isl3:Gal4$^{(+/-)}$,10xUAS:GOI) crossed with Tg(Isl3:Gal4$^{(+/+)}$) and Tg(HuC:GOI*)* with *nacre*$^{(+/-)}$. Stable integration of the construct in the fish germ

[9] Different nomination for panneuronal promoter *elavl3.*

cells leads to transmission of the transgene to its progeny. Positive progeny was selected to be raised (F0, founders) (Fig. III.5) to establish stable transgenic lines.

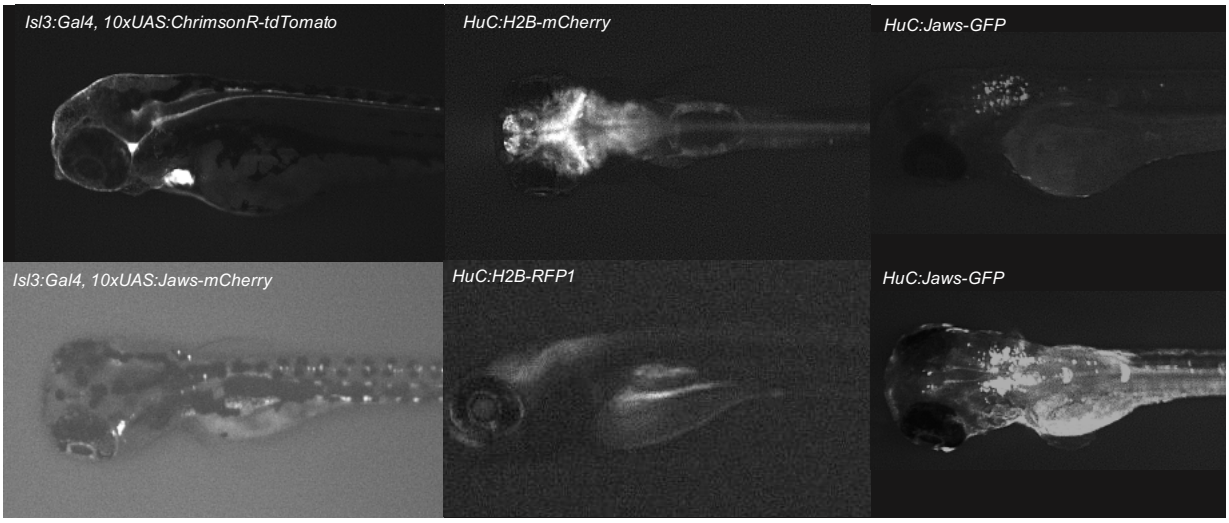

Figure III.5 - Stable expression of constructs in founder larvae (3-5 dpf), obtained from outcrossess of injected fish with Isl3:Gal4$^{(+/+)}$ or *nacre*$^{(+/-)}$.

Table III.3 – Percentage of stable transgene insertion (germline transmission) for each construct (# positive F0 larvae / # injected fish screened x 100).

Optogenetic Tool	% of stable insertion
10xUAS:ChrimsonR-tdTomato	30%
10xUAS:Jaws-GFP	10%
HuC:Jaws-GFP	60%
10xUAS:Jaws-mCherry	20%
10xUAS:C1V1-mCherry	10%
HuC:H2B-mCherry	40%
10xUAS:H2B-mCherry	20%
HuC:H2B-RFP1	30%

Animals with stable transgenic expression of actuator ChrimsonR and the inhibitor Jaws were used in behaviour assays to characterize the tools' efficacy and develop protocols for reliable modulation of neuronal activity during zebrafish behaviour.

3. Functional characterization of optogenetic tools in behaviour assay

To test the efficiency of the optogenetic tools expressed in zebrafish, behaviour assays were established, making use of the knowledge about neuronal activity correlated with a certain behavioural response. Stimulation of the trigeminal nucleus results in an escape response, and manipulation of nucMLF neurons modulates forward swimming, so both these populations were used to test the activating and inhibitory tools. Precisely, excitatory ChrimsonR was expressed in trigeminal nerve

(driven by Isl3:Gal4) and light was delivered to the trigeminal nucleus; inhibitory Jaws was expressed in nucMLF and light was targeted to that area (Fig. I.4).

3.1. Inactivating tools

3.1.1. Establishing optogenetic inhibition of behaviour using ArchT

In order to establish behavioural assays for testing optogenetic inhibition protocols, we made use of a transgenic line Tg(Isl3:Gal4;10xUAS:ArchT) previously generated in the lab. These transgenic fish express a proton pump named ArchT (Han, et al., 2011), which allows for neuronal silencing with light in the spectrum of 480-590nm. Fish were crossed to a driver line Tg(s1171t:Gal4) for expression of the optogene in the nucMLF, a population of neurons implicated in larval swimming (Severi, et al., 2014). Progeny was screened at 3-4dpf and only double transgenic fish expressing ArchT in the nucMLF (driven by the *s1171t* driver sequence) were used in the behaviour assay. Siblings, used as control fish, did not have any fluorescence, and could be double negative or only s1171t:Gal4 positive or 10xUAS:ArchT positive, with no ArchT expression. As the Tg(Isl3:Gal4) driver line used has a heart marker associated with the construct, these siblings were excluded.

Larvae were first tested with a 200μm diameter fiber coupled to a 590nm LED (wavelength correspondent to ArchT action spectra maximum peak). The stimulus protocol (Fig. III.6) consisted of 30s moving gratings to evoke an optomotor swimming response (larvae swimming in the direction of the moving grating) and a 10s interstimulus time; optogenetic stimulation was delivered during every other moving gratings step, in order to compare differences in bout number and nature in no-light vs. light trials. The light power used was 650μW, the maximum power obtained at the incidence point with the 200μm diameter fiber coupled to the 590nm LED. Light was modulated in ON/OFF pulses of 100ms/100ms.

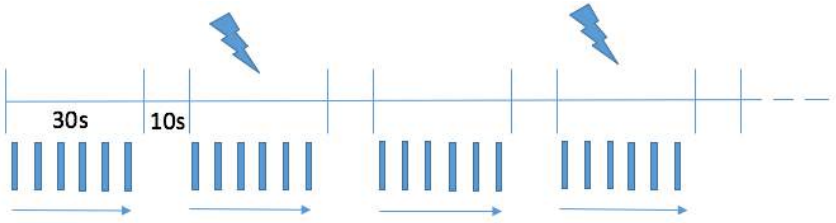

Figure III.6 – Stimulus protocol for ArchT: 30s moving gratings/10s stationary gratings/30s moving gratings with light delivery (100ms/100ms ON/OFF light pulses)/10s stationary gratings; 10 repetitions of the 4 step stimulus; light power at incidence point of 650μW.

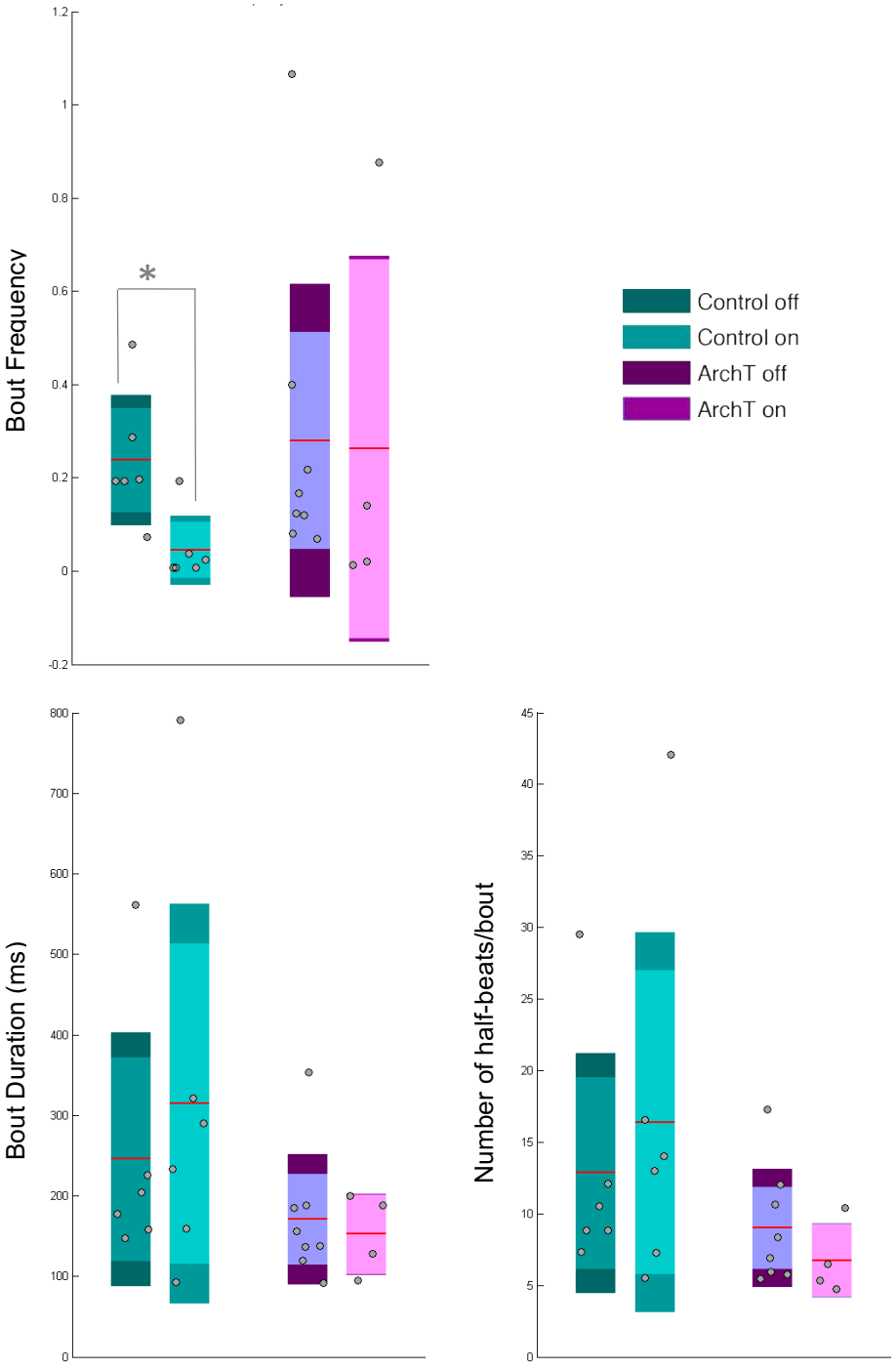

Figure III.7 - Bout frequency, duration and number of half-beats per bout per Tg(s1171t:Gal4;10xUAS:ArchT) fish, tested with 650μW light power, delivered by a 200μm diameter fiber coupled to a 590nm LED, in 100ms/100ms ON/OFF light pulses, during 30s. N=6 for control fish and N=8 for ArchT fish. Only the 4 ArchT larvae that performed bouts are plotted in the light-on trials.

Zebrafish larvae swim by discrete bouts (11 types identified) (Marques, J. 2016), which comprise oscillations of the tail, and interbout periods with no tail movement (Budick & O'Malley, 2000). The parameters used for behaviour analysis were the bout frequency, bout duration, and number of half-beats per bout (gives the frequency of the tail oscilations for one bout), calculated for each fish tested.

Using light stimulation by 590nm LED and 200μm diameter fiber (Fig. III.7), the bout frequency in control fish, during light-on trials is significantly decreased comparing to light-off trials (p=0.031, Wilcoxon signed ranked test). This raises the hypothesis that some inhibition by the light flash is happening, which could bias the interpretation of results of inhibition by the optogenetic tool.

To characterize this non-specific effect in more detail, wild-type (Tuebingen, TU) larvae were tested to assess if the inhibitory effect was a result of the light flash (Fig. III.9). The stimulus was adjusted to have a 10s interval of light delivery instead of the initial 30s (Fig. III.8), and the power levels used were 120μW and 650μW, with light pulse duration of 100ms/100ms ON/OFF.

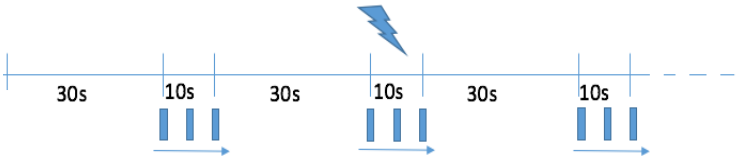

Figure III.8 – Stimulus protocol for TU: 30s stationary gratings / 10s moving gratings / 30s stationary gratings / 10s moving gratings with light delivery (100ms/100ms ON/OFF light pulses); 10 repetitions of the 4 step stimulus; light power at incidence point of 650μW and 120μW.

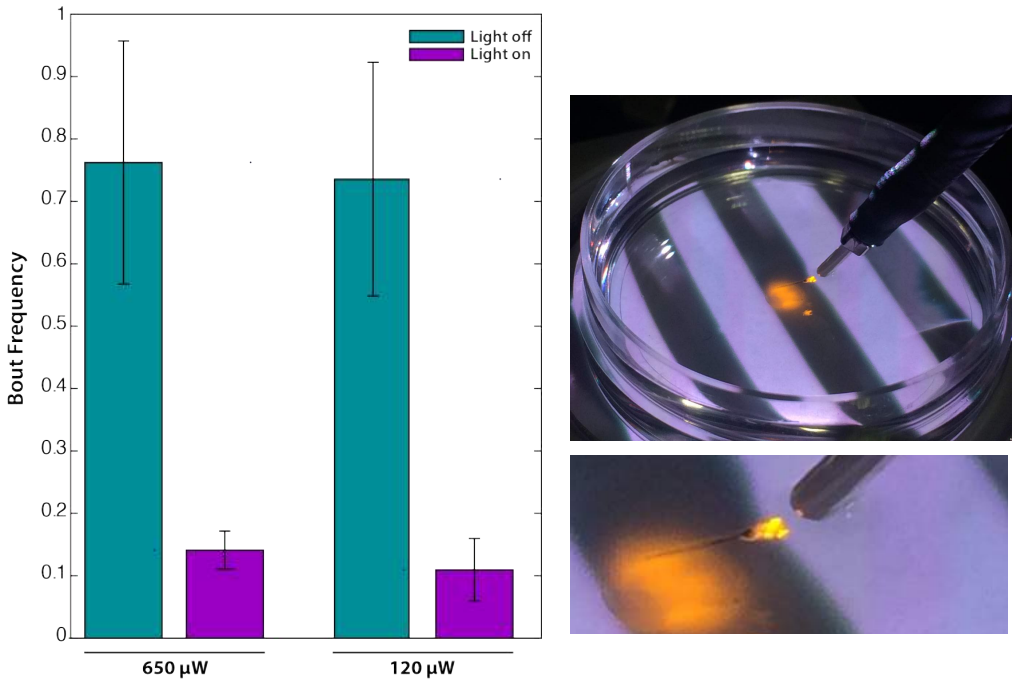

Figure III.9 – Left: bout frequency of wildt-type fish tested with two light powers (650μW and 120μW), delivered by a 200μm diameter fiber coupled to a 590nm LED, in 100ms/100ms ON/OFF light pulses, during 10s. N=8 for 650μW and N=6 for 120μW. Right: Light cone delivered with 200μm diameter fiber.

Bout frequency in TU fish stimulated with either 120μW or 650μW, from LED combined with 200μm diameter fiber, was significantly decreased compared to trial where no light was presented (Fig. III.9), as observed in the ArchT assay (Fig. III.7).

To reduce the diameter of the incident light cone (which covered the whole head of the larvae (Fig. III.9) we switched to a smaller fiber of 50μm diameter cut blunt at the ending. The maximum light power provided by this fiber coupled to the LED was 13μW, light level not sufficient for optogenetic stimulation. The fiber was thus coupled to a laser (635nm, 500mW) providing light powers of 1mW to 80mW for stimulation.

This test in TU larvae using the 50μm diameter fiber coupled to a 635nm laser was performed using four different light powers - 2mW, 13mW, 20mW and 25mW – and 100ms/100ms ON/OFF light pulse duration, with the same visual stimulus protocol as in TU tested with 200μm diameter fiber with 590nm LED (Fig. III.8).

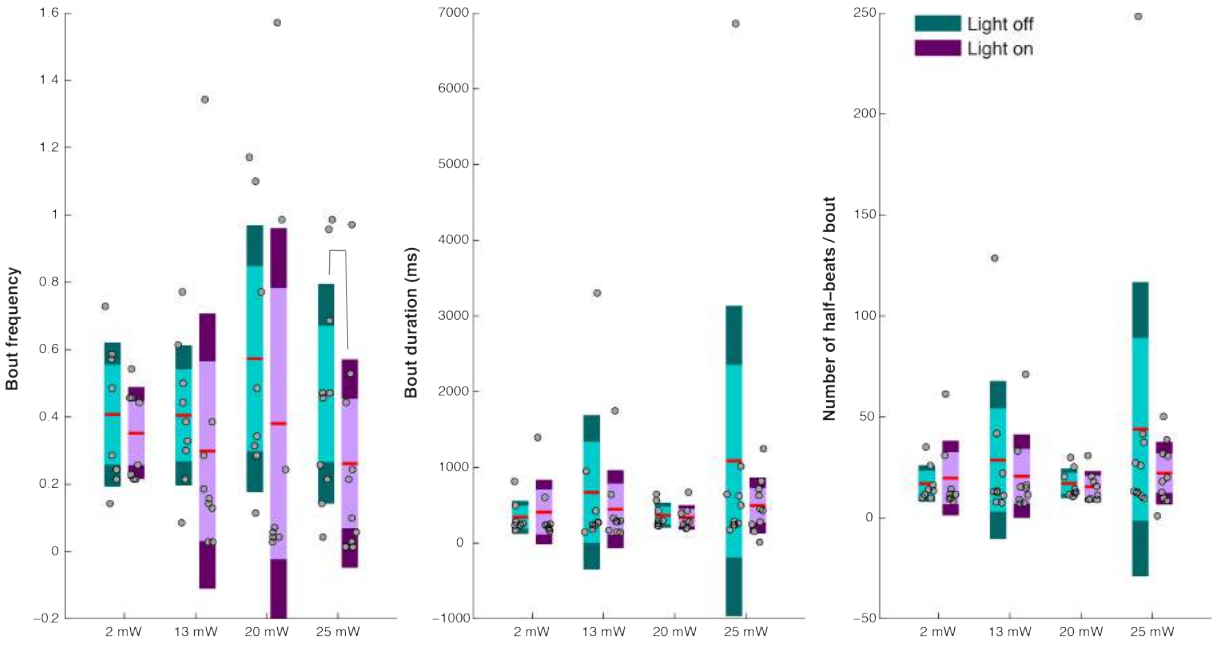

Figure III.10 - Bout frequency, duration and number of half-beats per bout per TU fish, tested with 2, 13, 20 and 25mW light power, delivered by a 50μm diameter fiber coupled to a 635nm laser, in 100ms/100ms ON/OFF light pulses, during 10s. N=9.

The difference between bout frequency in light-off trials compared to the light-on trials was not significant except for the highest power level of 25mW (p=0.006, Wilcoxon signed rank test) (Fig. III.10). Although the bout frequency is decreased in light-on trials, at higher power levels, the bout duration and number of half-beats remains unchanged (Fig. III.10), which indicates that the bout nature itself is not modulated by the light flash.

As the laser provided enough power, we aimed for a higher spatial resolution of the stimulating light applied and used a 25μm diameter fiber coupled to the 635nm laser. With this approach, the light cone was reduced even more and allowed to target the region of interest, the nucMLF, more specifically.

3.1.2. Characterizing Jaws-dependent inhibition of zebrafish behavior

Jaws inhibitory tool was tested in founders, selected from the progeny of the injected fish positive for transient expression in Tg(Isl3:Gal4;10xUAS:Jaws-mCherry) x Tg(s1171t:Gal4). Only fish expressing Jaws in the nucMLF were used in the behaviour assay. Control fish used did not have any fluorescence, which could be double negative or only s1171t:Gal4 positive, with no Jaws expression.
These larvae were tested with the 25μm diameter fiber coupled to the 635nm laser. The visual stimulus protocol used was the same as in TU tested with the 50μm and 25μm diameter fiber laser coupled (Fig. III.8) with a power level of 1mW, 5mW or 15mW.

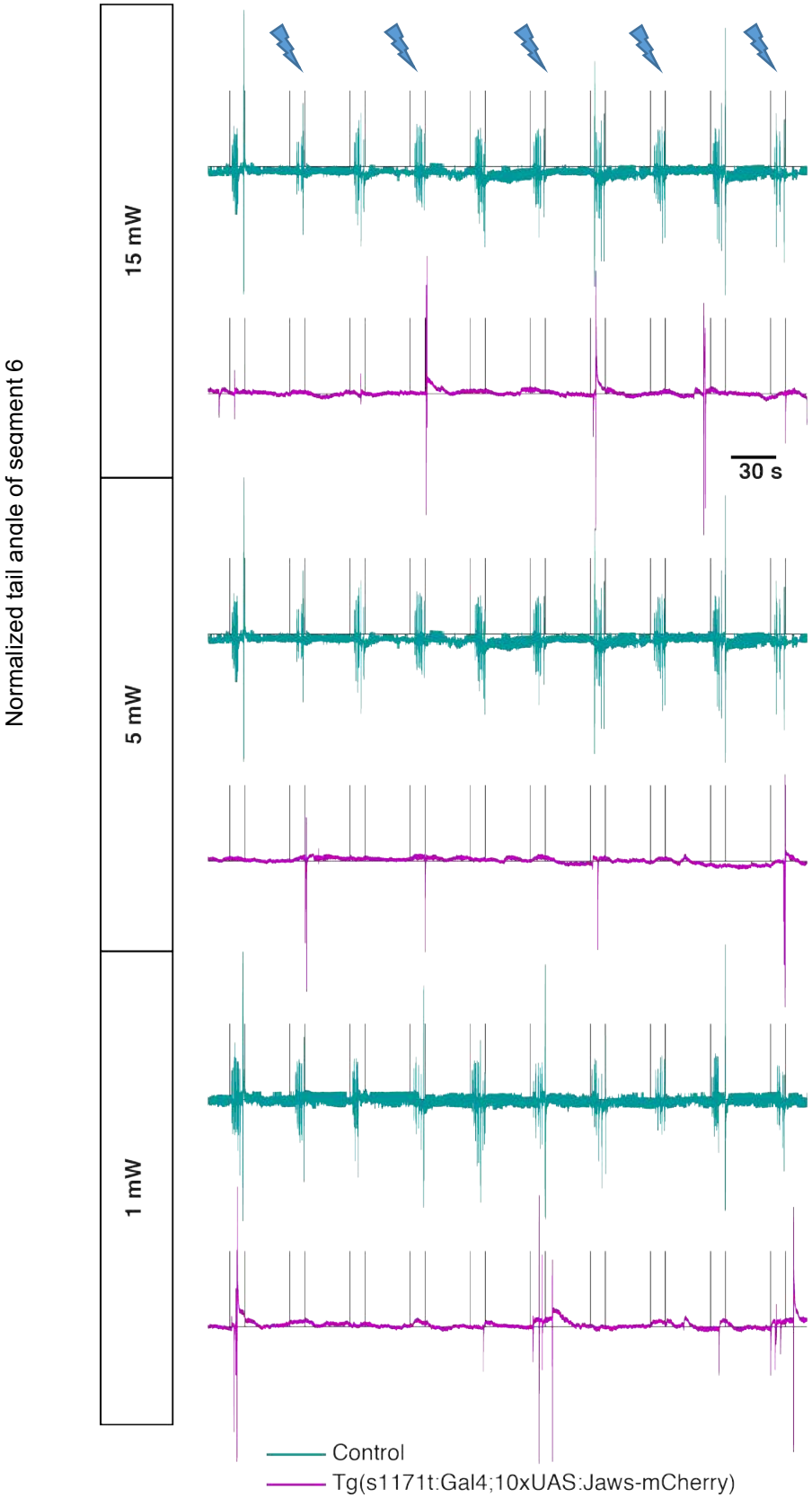

Figure III.11 - Bout traces (normalized tail angle of segment 6) for one Tg(s1171t:Gal4;10xUAS:Jaws-mCherry) fish tested with 1, 5 and 15mW light power, delivered by a 25µm diameter fiber coupled to a 635nm laser, during

10s every other moving grating stimulus. ON/OFF light pulses of 200ms/200ms for Jaws fish and 100ms/100ms for control sibling.

From the Jaws assay, the bout traces of the control sibling show consistent swimming movement in the 10s of moving gratings presentation, opposite to the 30s stationary gratings when almost no activity occurred (Fig. III.11). This indicates that the higher spatial control gained with the smaller fiber eliminated the inhibition effect of light flash described before in control fish, but more larvae would have to be tested to confirm. The Jaws expressing larva showed no reliable swim movement neither in control trials nor in trials with optogenetic stimulation (Fig. III.11).

3.2. Activating tools

3.2.1. Establishing optogenetic activation of zebrafish behaviour using ReaChR

To establish behavioural assays for testing optogenetic activation protocols, we made use of a transgenic line Tg(Isl3:Gal4;10xUAS:ReaChR), previously generated in the lab. These transgenic fish express a channelrhodopsin named ReaChR (Lin, et al., 2013) that allows induction of neuronal activity with light between 590-630nm. Fish were crossed to a driver line Tg(Isl3:Gal4) for optogene expression in the trigeminal nerve, where optogenetic activation is expected to elicit a short escape response (Easter & Nicola, 1996; O'Malley, et al., 1996). Progeny was screened at 2-4dpf and only double-transgenic fish expressing ReaChR in trigeminal nerve (driven by *Isl3* promoter sequence) were used in the behaviour assay. The siblings used as control did not have any fluorescence, meaning that ReaChR was not expressed.
Larvae were first tested using a 50μm diameter fiber coupled to a 635nm laser. Light was delivered every 10s (Fig. III.12), in a single pulse of 50/100/200ms duration, with a power level of 1mW, 5mW or 15mW. Below the fish, stationary gratings were presented.

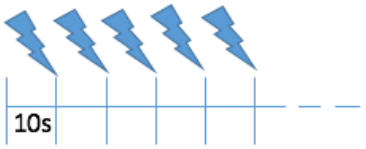

Figure III.12 – Stimulus protocol for activating tools: one light pulse of 50ms/100ms/200ms is delivered every 10s, with a power level of 1mW, 5mW or 15mW. Stationary gratings are presented below the embedded larvae.

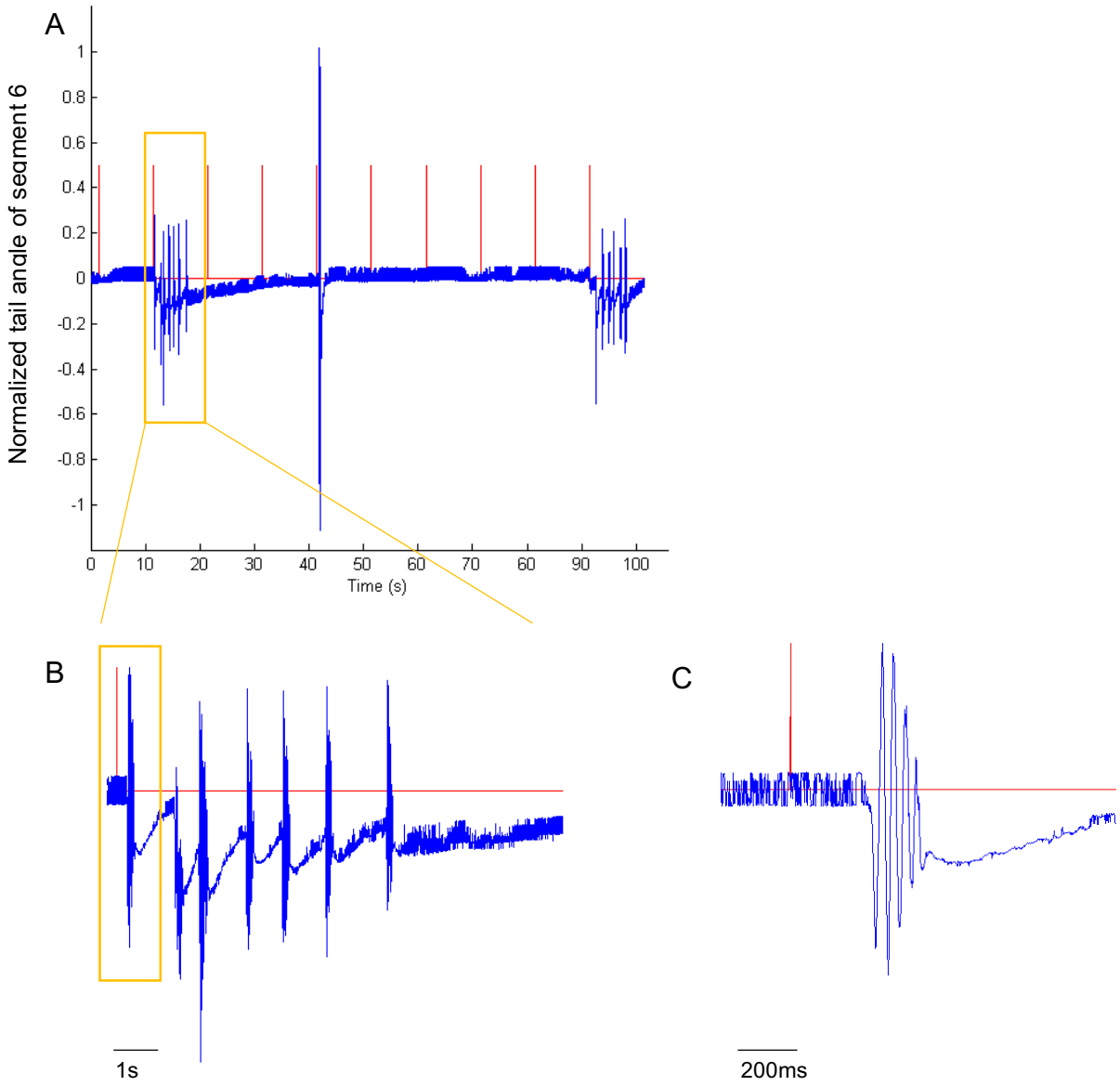

Figure III.13 – (A) Behaviour assay for ReaChR stimulated with 635nm laser and 50μm fiber. In blue, normalized tail angle of segment 6; in red, light stimulus, delivered every 10s. (B) zoom of the highlighted area in (A); (C) zoom of the highlighted area in (B).

With these settings, the response to the light onset was not reliably evoked (Fig. III.13A), and the bout was not locked to the stimulus (Fig. III.13C). Zooming into a response with multiple bouts after light pulse (Fig. III.13B), the swimming activity stops for a long period after the 7 bouts.

To improve on the stimulus efficiency and obtain higher light stimulus spatial control, ReaChR was tested with the 25μm diameter fiber coupled with the 635nm laser, to target stimulation light to trigeminal nerve either on the left or right side of the brain.

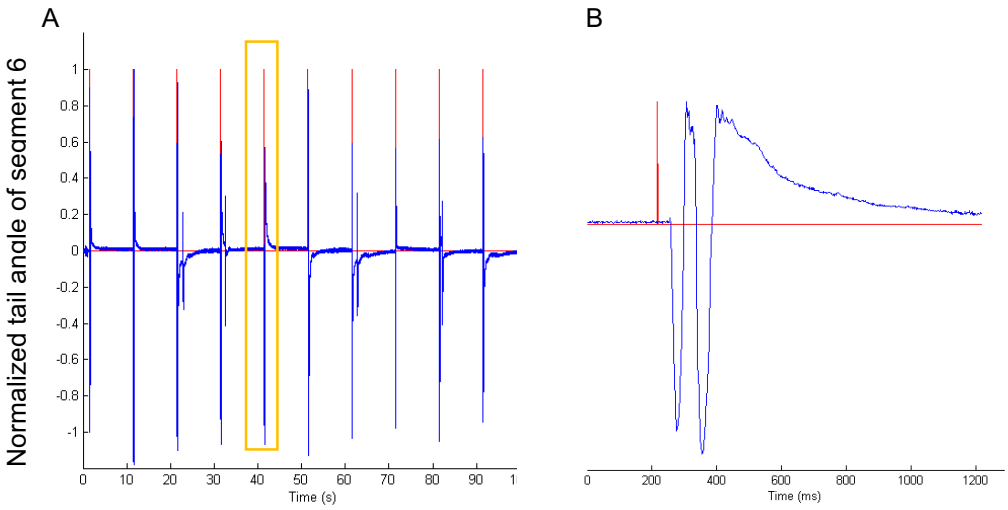

Figure III.14 - (A) Behaviour assay for ReaChR stimulated with 635nm laser and 25μm fiber. In blue, normalized tail angle of segment 6; in red, light stimulus, delivered every 10s. Only the first second after light onset was ploted. (B) zoom of the highlighted area in (A).

The assay with Tg(Isl3:Gal4;10xUAS:ReaChR) stimulated with 25μm fiber and 635nm laser (same protocol as in ReaChR larvae tested with 50μm diameter fiber (Fig. III.12)) resulted in a much more reliable response than the one seen using the 50μm diameter fiber. Each light pulse (in red) elicited a bout with short latency (Fig. III.14). Only two fish were tested, so more fish would have to be assayed to insure the consistency of the response to the light induction of this channelrhodopsin.

3.2.2. Characterizing ChrimsonR-dependent activation of zebrafish behaviour

ChrimsonR is a channelrhodopsin with action spectrum peak at 590nm, inducible with red light. It is preferable to ReaChR in the way that it has higher photocurrents and a narrower action spectrum, allowing combination with GCaMP6 GECI (λ_{exc}=470nm), which is not possible with ReaChR since it is still inducible at those wavelengths.

ChrimsonR activating tool was tested in founders, selected from the progeny of the injected fish positive for transient expression Tg(Isl3:Gal4;10xUAS:ChrimsonR-tdtTomato) x Tg(Isl3:Gal4).

These larvae were tested with the 25μm diameter fiber coupled to the 635nm laser. The stimulus protocol used was the same as the one used to test ReaChR (Fig. III.12) with a power level of 1mW, 5mW or 15mW and a light pulse duration of 50ms/50ms, 100ms/100ms and 200ms/200ms ON/OFF.

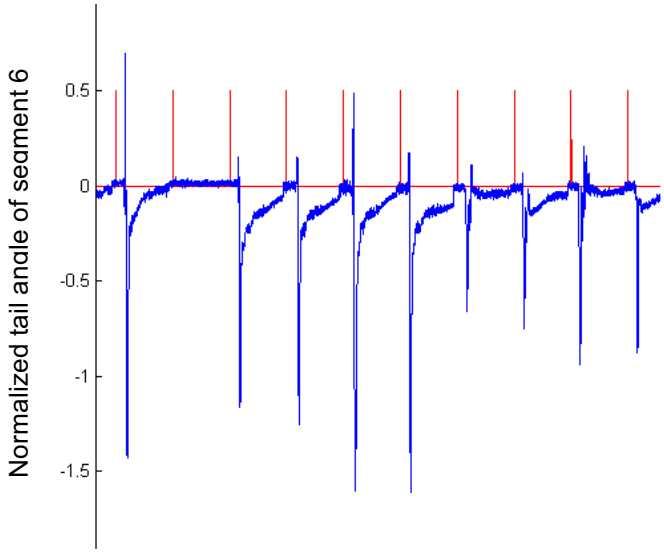

Figure III.15 - Behaviour assay for one Tg(Isl3:Gal4;10xUAS:ChrimsonR) fish stimulated with 635nm laser and 25μm fiber. In blue, normalized tail angle of segment 6; in red, light stimulus, delivered every 10s. Only the first second after light onset was ploted.

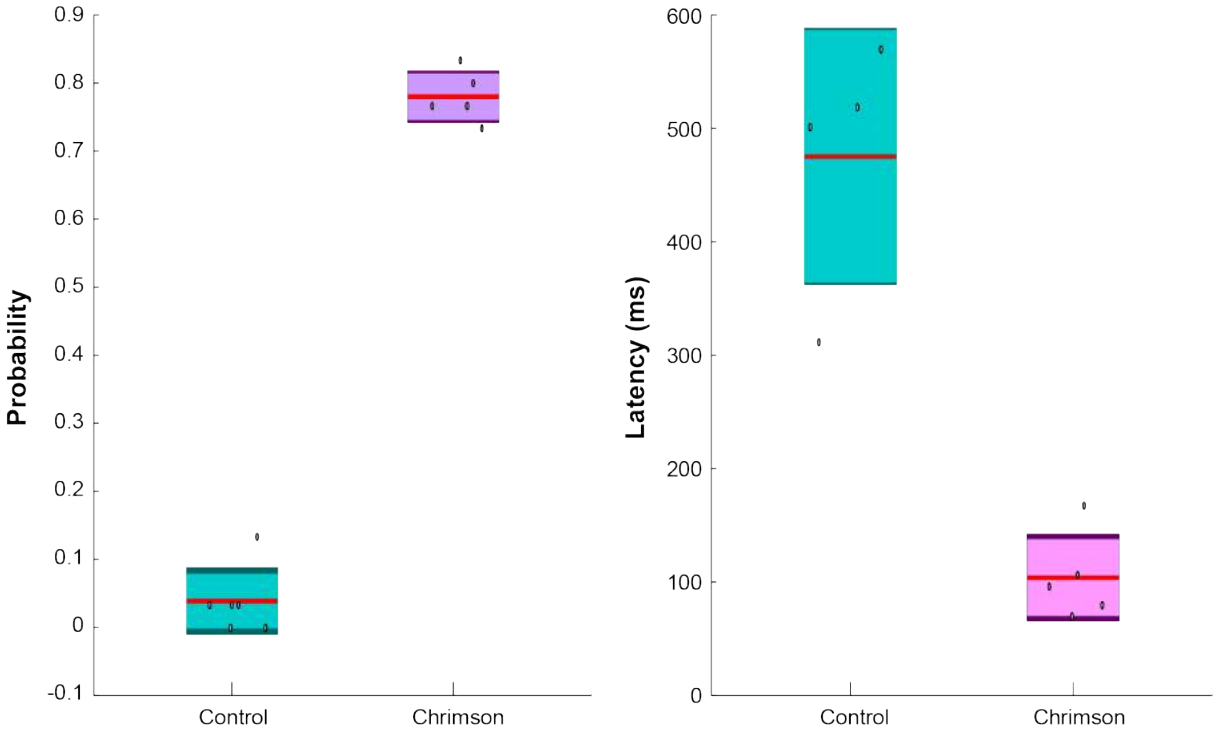

Figure III.16 - Behaviour assay for ChrimsonR stimulated with 635nm laser and 25μm diameter fiber. On the left, probablility of a bout happening within 1s after light flash (mean with 95% confidence interval). On the right, latency of first bout after light onset (mean with 95% confidence interval). Control: N=6, ChrimsonR: N=5.

Stimulation of one trigeminal nerve with light from a 25μm diameter fiber coupled to the 635nm laser resulted in a reliable lateralized swimming response with very short mean latency of 104ms (Fig. III.15). The probability of a bout occurring within 1s after light pulse is significantly higher in ChrimsonR expressing fish than in control ($p<0.05$, two-sampled t test), and its latency is significantly lower in ChrimsonR-expressing larvae than in control ($p<0.05$, two-sampled t test), which indicates that the response is correlated to the stimulus.

IV. Discussion

How the brain integrates sensory stimuli and translates them into behaviour is a dynamic process involving diverse populations of cells often distributed throughout the brain. To identify these cells and understand their function, single cell or multi-unit recordings along with ablation experiments already provided insights into functional units and their computational principles. Recent technical advances now allow studying the dynamic interactions with single cell resolution within large populations of cells and allow to interfere with these dynamics in a non-invasive and reversible manner. State-of-the art optogenetic tools with faster kinetics and higher sensitivity facilitate reliable activation or inhibition of neuronal activity with high temporal precision during an animal's behaviour. Most excitingly, genetically engineering of their excitation spectra made them compatible with state-of-the art calcium sensors for simultaneous optical recording and manipulation of neural activity. Such experiments are particularly attractive in zebrafish research as the animal's transparent brain makes it easy to deliver patterns of light for manipulations and activity recordings and its small size allows to record simultaneously from all neurons located within the same depth of the brain.

In this thesis I adapted the latest optogenetic tools for transgenic expression in zebrafish and generated stable transgenic lines that can be flexibly used for optogenetic manipulation of different populations of neurons during behaviour and/or neural activity recording. I developed behavioural assays to characterize these lines and establish protocols for manipulation experiments.

Optimizing state-of-the-art optogenetic tools for expression in zebrafish

Optogenetic activators ChrimsonR, Chronos and C1V1(t/t) and inhibitor Jaws were optimized to be expressed in zebrafish. ChrimsonR is a highly sensitive activator with a narrow red-shifted action spectrum (Klapoetke, et al., 2014); Chronos is a fast activator induced by green light (Klapoetke, et al., 2014); C1V1(t/t) is a fast and sensitive activator with red-shifted action spectrum (Yizhar, et al., 2011); and Jaws is a sensitive red-shifted inhibitor (Chuong, et al., 2014).

The fluorescent tag of Jaws, which allows for visualization of the transgenic expression, was optimized for simultaneous expression of green fluorescent calcium indicator GCaMP6. The GFP tag in the original Jaws vector from addgene was replaced by mCherry. For this purpose, Jaws and mCherry genes were isolated from the original vectors by PCR amplification using primers with overlapping endings, for fusion of Jaws and mCherry and flanking restriction sites (SpeI-Jaws and mCherry-SacII) to clone into the destination vector. Gibson assembly was used for fragment fusion, taking advantage of its exonuclease, polymerase and ligase activities that result in a double stranded fully sealed DNA molecule, with higher efficiency than the classical PCR approach (Fig. III.3).

All optogenes were cloned into a *Tol2* destination vector for gateway cloning. This vector enables random integration of constructs flanked by the tol2 arms in zebrafish genome, in presence of Tol2 transposase. Tol2-mediated transgenesis in zebrafish allows for the highest efficacy in germline

transmission frequency from the systems developed in the past (Kawakami, 2005). The *Tol2* vector (Supplement 2) is adapted for gateway cloning, in the way that it has attR sites that enable easy and fast recombination for the insertion of a (regulatory) sequence upstream the GOI cloned. SpeI and SacII restriction sites between the attR2 site and the second *Tol2* arm were used for classical restriction cloning to insert a GOI in the expression vector. Thus, ChrimsonR, C1V1 and Jaws were amplified with primers introducing these restriction site sequences with SpeI added 5' and SacII 3' to the sequence of the GOI (Fig. III.1). Further, the Kozak sequence was added to the forward primer directly 5' the ATG to the GOI to increase translation efficiency and thus expression of the GOI. Recognition of this sequence by the ribosome is necessary to initiate the translation process.

A different set of restriction enzymes was used to clone Chronos-tdTomato into the *Tol2* vector because Chronos' sequence included a SacII restriction site. A PacI site was introduced to the Tol2 destination vector by amplification of the entire vector sequence using a specific primer. Similarly, SpeI and PacI sites were added to Chronos-tdTomato by PCR amplification with primers designed for this purpose (Fig. III.2, Supplement 3). Both PCR products were fused by restriction digestion followed by ligation.

Cloning these state-of-the-art optogenetic tools into zebrafish Tol2 destination vectors makes them easy and flexibly usable in different expression contexts. This vector can be recombined with a library of different entry vectors containing various regulatory sequences to drive expression in specific neuronal populations or in a Gal4/UAS dependent manner.

Generating tools for cell identification

Whole brain imaging of zebrafish neuronal activity comes with the challenge to separate cells from neuropil and identify cells reliably for analysis or in order to target light for optogenetic manipulations. To facilitate cell identification, two nuclear markers were cloned into zebrafish expression vectors, H2B-RFP and H2B-mCherry. The markers have slightly different excitation and emission spectra, λ_{exc}=598nm, λ_{em}=645nm for H2B-RFP and λ_{exc}=587nm, λ_{em}=610nm for H2B-mCherry, and may thus be optimal for different experimental conditions.

Two H2B-RFP sequences were cloned into *Tol2* vector because two ATG start sites were identified when aligning "H2B-RFP in pENTR1A" addgene vector with the H2B-mCherry sequence (Supplement 7). The extra nucleotides present in the H2B-RFP original vector could be leftovers from cloning and not influence expression, or a sequence that enhanced expression (although no identity was found in sequence blasts), and thus both sequences were cloned to be injected in zebrafish embryos and access possible differences in expression.

Regulation of optogenetic tool expression in zebrafish

Transgenic expression in zebrafish is mainly achieved taking advantage of the Gal4/UAS transactivation system, in which Gal4 protein activates the Upstream Activating Sequence, i.e., Gal4 driver lines induce expression of transgenes in UAS reporter lines. This means that the GOI can be

flexibly expressed in different populations of cells as long as the Tg(promoter:Gal4) is available for the population of choice. Generation of Gal4 driver lines is somewhat straightforward, through methods such as promoter cloning, promoter or enhancer-trap screens, Tol2 or BAC transgenesis, or gene editing using with TALENS or CRISPR (Rinkwitz, et al., 2011) (Del Bene & Wyart, 2012). The use of all these systems by different labs has generated several Gal4 driver lines that can be used to target interesting neuronal populations.

The regulatory sequences chosen to drive GOI expression were 4xUAS, 10xUAS and panneuronal promoter *elavl3* (HuC).

The amount of tandem repetitions of UAS sequences correlates with increased expression levels of downstream genes (Akitake, et al., 2011). Each UAS repeat contains a 17bp long CGG-N_{11}-CCG palindromic sequence. The CpG dinucleotides are essential for Gal4 binding and are a prominent target of DNA methylation, what might result in increased silencing of highly repetitive UAS constructs. Although such silencing effects are not present in transient expression upon injection of UAS constructs into zebrafish embryos, when integrated into the genome the sequence is prone to CpG methylation, and the minimal silencing in the first generation increases significantly upon generations. 4xUAS sequence has been shown to drive high levels of expression and be less susceptible to methylation than 14xUAS (Akitake, et al., 2011). Therefore, both 10xUAS and 4xUAS sequences were cloned upstream the optogenetic tools sequence, to balance between efficient expression levels of the GOIs and minimal silencing in stable transgenic lines.

The regulatory sequences were available in gateway entry clones (Supplements 4-6) and were inserted in the *Tol2* gateway destination vector by the Gateway LR reaction (Fig. III.1).

Expression of optogenetic tools in zebrafish

A selected set of final expression vectors cloned (Table III.2) were injected in zebrafish one cell-stage embryos to test expression efficiency in zebrafish and generate transgenic lines for experiments.

For the construct T2-10xUAS-Chronos-tdTomato more than 400 embryos were screened without any transient expression. Inspection of the DNA sequence revealed a 448bp sequence with similarity to the human synaptophysine hSyn promoter between the Kozak sequence and the potential ATG site. New primers were therefore designed to generate a new expression clone directly fusing the Kozak sequence to the ATG of Chronos site. The lack of transgene expression could have been a consequence of the Kozak sequence misplaced position or even its nature, since different organism have different codon usage and variations in the Kozak sequence are silent as long as some key nucleotides are maintained. Another possibility for the inability to identify positive larvae could also be due to the fluorescence filter coupled to the stereoscope not being optimal to screen tdTomato reporter gene fluorescence, since differences in brightness level in red fluorescent reporters were observed during screening, as is the case of H2B-RFP and H2B-mCherry. RFP filter has an excitation peak at 545nm and emission at 606nm, values closer to mCherry activity than RFP (mCherry: λ_{ex}=587, λ_{em}=610; RFP: λ_{ex}=598, λ_{em}=645).

To obtain successful transgenic expression of Chronos in zebrafish, primers were designed to have the Kozak sequence immediately adjacent to 5' Chronos. Adittionally, since this optogene is a green-

light driven very fast activator highly attractive to drive neuronal firing with high temporal precision, and its excitation spectra overlaps with green GECI imaging, Chronos could be cloned in a way that its tdTomato reporter gene was replaced by a green tag (GFP), enabling co-expression of the optogenetic tool with red GECIs.

When screening the injected embryos for transient expression of reporter genes by fluorescence, different levels of brightness were detected (Fig. III.5). This could be explained either by the capability of detecting fluorescence using the filter available in the stereoscope or the amount of DNA injected – either the number of copies integrated were variable or plasmid DNA was unevenly distributed upon cell division. The concentration of expressing vector used for injection was varied, since some of them appeared to be too lethal to the fish when injected in higher concentration, or had lower integration efficiency. Specially in larvae injected with *elavl3* regulated constructs, mortality was very high, possibly due to the over excitation of panneuronally expressed activators and inhibitors during larvae development because of incubation light conditions. When this was noticed, all transgenic fish expressing activators/inhibitors where protected from direct light. Another critical factor might be the size of the HuC constructs with the promoter being comparably large (8kb) with respect to the UAS sequences (4xUAS: ~200bp; 10xUAS: ~500bp), which might lead to higher toxicity and lower integration efficiency.

Transgenic fish with transient expression were outcrossed when reaching mating age in order to establish stable transgenic lines for behavioural manipulation experiments. If integration of the injected construct occurs in the germline, the construct is transmitted to the progeny, that becomes the F0 or founder. The founders may exhibit different expression patterns due to positional effects (Akitake, et al., 2011). It is therefore crucial to individualize the founders and establish stable lines from the ones with the preferred (most complete) expression pattern. As seen in Fig. III.5, different Isl3 and HuC expression patterns were obtained in founders. The level of expression of the optogenetic tool is an essential criteria for effective behavioural manipulations, that can depend on the number or position of gene copies integrated.

Establishing optogenetic manipulation of zebrafish behaviour

To characterize the efficiency of the cloned optogenetic tools expressed in zebrafish and establish protocols for effective optic manipulation of neuronal activity and larval behaviour, we made use of existing UAS lines expressing conventional modulators, ReachR for activation and ArchT for inhibition, in genetically defined populations of neurons. Targeting of the tools was based on prior knowledge about the function of neurons. Activating tools were tested by expressing them in a Gal4/UAS dependent manner in the trigeminal nerve (*Isl3*:Gal4) which is known to reliably elicit a fast escape response upon tactile stimulation (Easter & Nicola, 1996; O'Malley, et al., 1996), and inhibiting tools were tested by expressing them in the nucMLF (*s1171t:Gal4*), a population involved in forward swimming motion (Severi, et al., 2014).

Transgenic Tg(s1171t:Gal4;10xUAS:ArchT) fish express a proton pump named ArchT (Han, et al., 2011), which allows neuronal silencing with light in the spectrum of 480-590nm. Larvae were first tested with a 200μm diameter fiber coupled to a 590nm LED (wavelength correspondent to ArchT action spectrum maximum peak). In control larvae tested in this assay, bout frequency was significantly decreased during light-on trials comparing to light-off trials ($p<0.05$) (Fig. III.7). This modulation of behaviour in control animals was unexpected and may be due to experimental conditions. The stimulation light delivered by a 200μm diameter fiber illuminated the entire head of the animal (Fig III.9), including the eyes, and thereby might interfere with the visual perception of the moving grating driving optomotor swimming or itself be a visual stimulus. Restrains were given by the ceramic ferrule ending of the fiber which did not allow to approach the fiber closer to the fish without interfering with behavioural recordings. To examine this effect in more detail, wild-type larvae were tested with the same visual and stimulation protocol with the difference of having a shorter light stimulation time of 10s, to avoid potential rebound excitation (Kravitz & Bonci, 2013) as 30s of light stimulation of Tg(s1171t:Gal4;10xUAS:ArchT) animals was sometimes followed by a strong behavioral response (not quantified). Bout frequency in wt fish stimulated with the two different intensity conditions was again significantly decreased in light-on trials, compared to trials where no light was presented (Fig. III.9). Application of a smaller fiber of 50μm diameter and open ending did not allow to provide sufficient power levels for effective optogenetic manipulations (max power 13μW). We therefore changed to a 500mW red-laser (635nm) to obtained power levels compatible with optogenetic stimulation. The bout frequency of wt fish tested with the smaller fiber was not significantly different comparing light-on with light-off trials at 2, 13 and 20mW light power delivered (Fig. III.10). On the other hand, bout duration and number of half-beats were not affected by the light flash, which indicates that the bout nature itself is not modulated by the light onset.

To further reduce these non-specific effects of light stimulation and achieve higher spatial control of stimulation, a smaller fiber of 25μm diameter coupled to the 635nm laser was used for light delivery. This approach enabled targeting the nucMLF region more specifically. The wavelength of the laser was chosen for experiments effectively driving Jaws while imaging neuronal activity with blue light, and is outside the optimal range for ArchT activation.

Preliminary results indicate that the non-specific effect of optogenetic stimulation might be circumvented by using the smaller fiber as the bout traces of a control animal showed normal swimming in both light-on and light-off trials (Fig. III.11). Yet, a higher number of animals is required to confirm that there is no statistical significant difference between both conditions.
As screening for stable transgenic expression for Jaws already identified positive 10xUAS:Jaws-mCherry fish, we were able to test manipulation of modulation of optomotor swimming in Tg(s1171t:Gal4;10xUAS:Jaws-mCherry) animals. Behaviour of the Jaws expressing larva was not reliable as moving gratings without optogenetic stimulation could not effectively evoke optomotor swimming (Fig. III.9). Whether this is a consequence of continuous Jaws expression throughout development or individual variability in behaviour will be assessed by testing more animals. General

inspection of positive larvae revealed some anatomical and behavioural impairment, which might be due to the over inhibition throughout development, since this inhibiting tool is highly light sensitive and the *s1171t* driver leads to unspecific muscle and fin expression. This might be overcome by expressing the optogene in a more restrict cell population.

To establish behavioural assays for testing optogenetic activation protocols, a transgenic line Tg(Isl3:Gal4;10xUAS:ReaChR), previously generated in the lab, was used. These transgenic fish express a channelrhodopsin named ReaChR (Lin, et al., 2013) that allows induction of neuronal activity with light between 590-630nm.
Larvae were first tested using a 50μm diameter fiber coupled to a 635nm laser, which resulted in a non-reliable response to the light onset with long periods of no activity (Fig. III.13). With these conditions, the trigeminal nucleus located caudal to the eye on both sides of the brain was activated. We therefore made use of the higher spatial control of optogenetic stimulation when presented with the 25μm diameter fiber, which allowed specifically targeting the trigeminal nerve on one side of the brain. This optogenetic stimulation reliably evoked a response of very short latency (Fig. III.14). These first results encouraged us to use the assay to characterize lines with stable transgenic expression of ChrimsonR identified by screening.

A 200ms flash of light of 15mW power was sufficient to elicit reliably a highly lateralized response of short latency in Tg(Isl3:Gal4;10xUAS:ChrimsonR-tdtTomato) animals (Fig. III.15, Fig. III.16). This lateralized response is consistent with the escape responses described when activating the trigeminal nucleus (Easter & Nicola, 1996; O'Malley, et al., 1996). This lateralized response was more obvious in ChrimsonR than ReaChR fish, probably due to the higher spatial resolution achieved with the 25μm diameter fiber that enabled stimulation of only one trigeminal nerve, instead of the 50μm diameter fiber illumination of both nuclei.

Both activating channelrhodopsin ChrimsonR and inhibitor chloride pump Jaws were tested in behaviour assays that show promising results.
ChrimsonR is a highly sensitive red-shifted depolarizing channelrhodopsin, with narrow action spectrum peaking at 590nm, which makes it favourable for combination with blue/green opsins or green GECIs calcium imaging, for simultaneous manipulation of different populations or simultaneous activity manipulation and recording.
Jaws is a red-shifted hyperpolarizing chloride pump with higher photocurrents and narrower action spectrum than ArchT, enabling efficient combination of Jaws inhibitor with other optogenetic tools.

Key conditions for successful activation of optogenetic tools

Throughout the optimization of the behaviour manipulation protocols, some important aspects came up as essential for successful optogenetic manipulation: the expression level of the optogenetic tool, and the light targeting to the appropriate population of cells.

After each experiment, fish were observed under the fluorescent scope to confirm their expression pattern, and the high expression level (accessed by brightness of the fluorescent reporter) and complete expression pattern were highly correlated with the animal's performance in the behaviour assay, i.e., its response to the light stimulus (data not shown). This observation highlights the importance of using the optogenetic tools in stable transgenic zebrafish. The transgenic lines expressing the tool should be selected to have the most proximity to the desired expression pattern, since different lines of the same transgene may have different expression patterns due to positional effects or silencing of the UAS repetitive sequence rich in CpG, a methylation target.
The light delivery method was the other essential aspect to successful activation of the optogenetic tools, since a reduced incidence point of the light-stimulus decreases side effects of the stimulation and allows targeting to the cellular population of choice. The light delivery conditions were optimized by reducing the diameter of the fiber coupled to the light source, which seemed to be sufficient to reliably manipulate behaviour in such experimental set-up. However, when we tried a combination of activity manipulation with recording using a panneuronal GCaMP6f, using the light-sheet microscope (LSM), positioning of the fiber was extremely difficult and activity manipulation wasn't possible probably due to the insufficient power level obtained (data not shown). A system developed by (Hernandez, et al., 2016) may be the answer to efficient light delivery: spatial light modulators are used to shape the light distribution focused on the TF grating, and control the axial position of the spatiotemporal focal plane in the sample volume. This results in the generation of a spatiotemporally focused light pattern with uniform distribution, with cellular resolution targeting.

Summarizing, the activator ChrimsonR and inhibitor Jaws optogenetic tools developed for expression in zebrafish show potential for manipulation of activity and behaviour with high efficiency and temporal precision, and combination with activity recording with green GECIs. To manipulate activity with precise spatial control, light delivery methods have to be optimized, to enable brain mapping experiments that take advantage of state-of-the-art optogenetic tools and advanced imaging techniques available (Packer, AM, et al, 2015).

V. References

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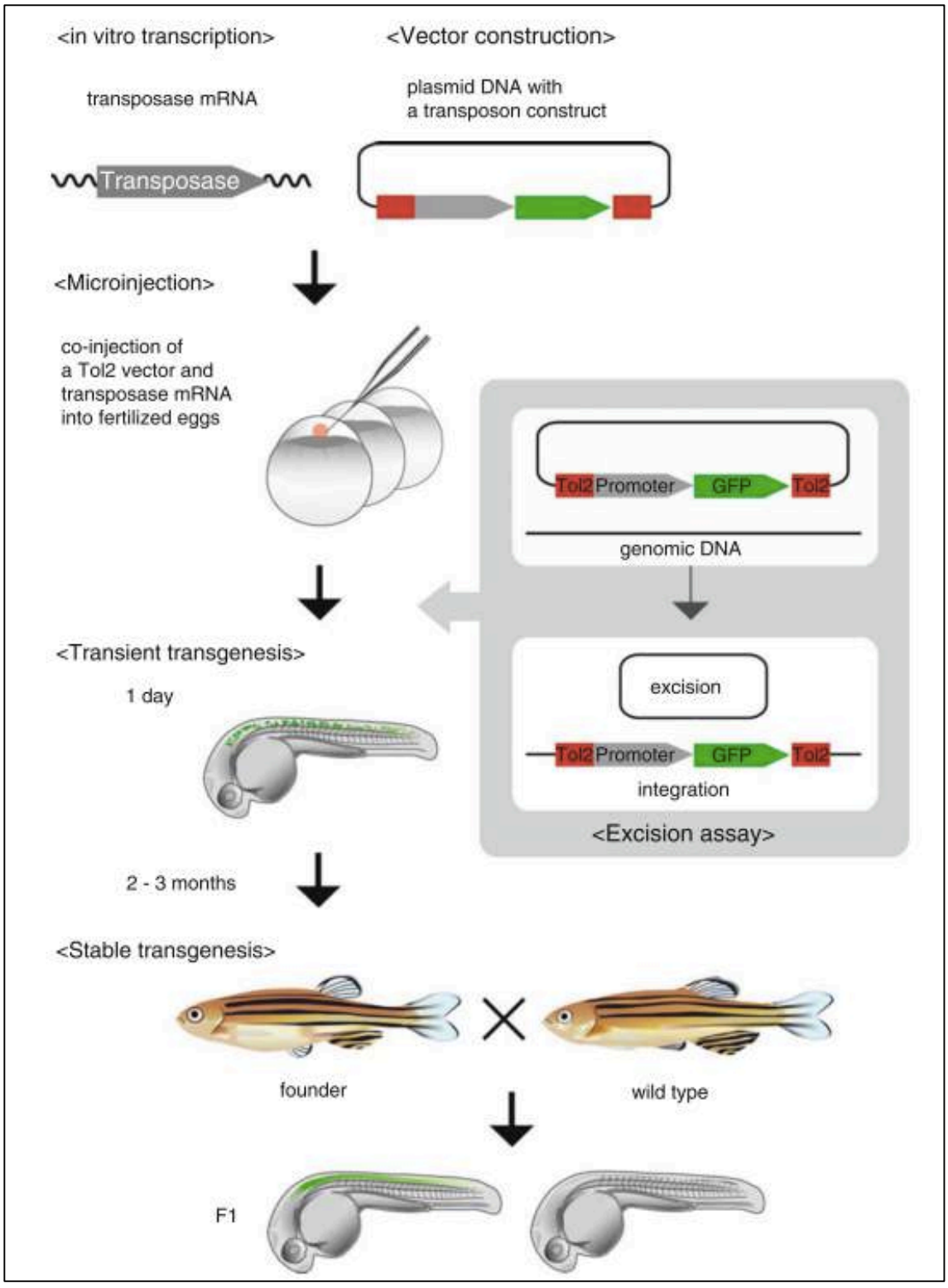
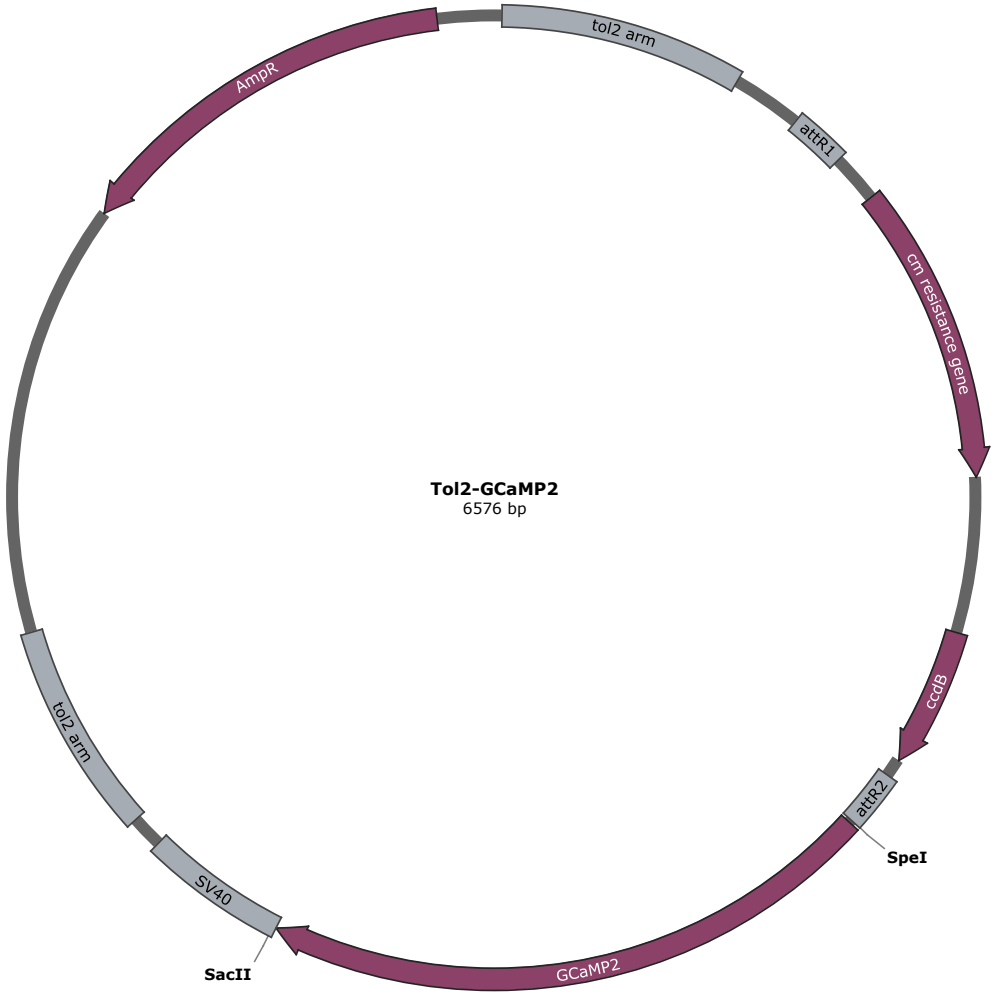
VI. Supplements

Supplement 1 – Transient and stable transgenesis in zebrafish. From Kikuta & Kawakami, 2009.



Supplement 2 - *Tol2* Gateway destination vector map.

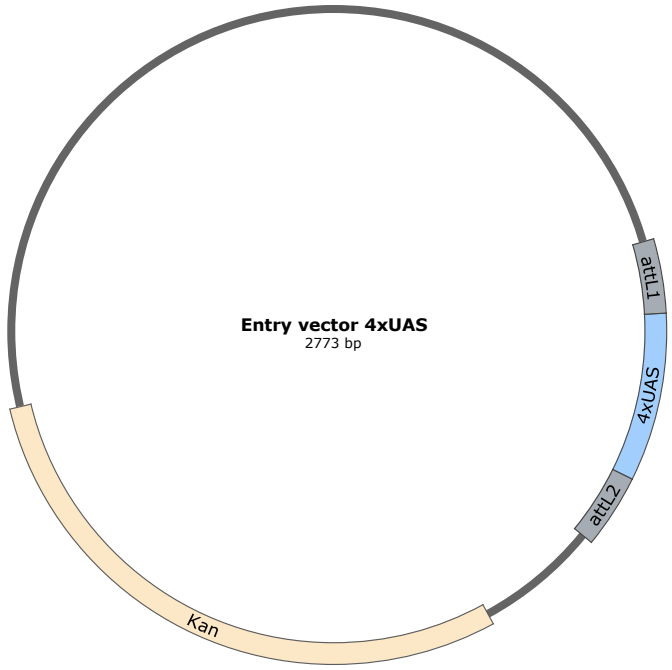


Supplement 3 - Primers and PCR conditions for each fragment isolation.

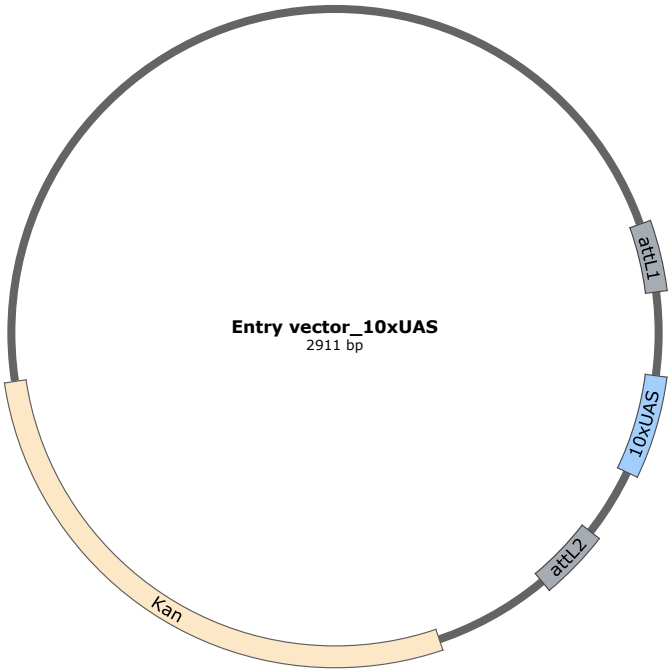
Fragment	Template	Primers		Polymerase	Ta
ChrimsonR-tdTomato	pCAG-ChrimsonR-tdTomato	fw	5’ GCATACTAGTGCGGGCACCATGGCTGAGCTGATCAG 3’	Phusion	68,5°C
		rv	5’ GCATCCGCGGTTACTTATACAGCTCATCCATGCCGTACA 3’		
Chronos-tdTomato	pAAV-Syn-Chronos-tdTomato	fw	5’ GCACTAGTTCCGCCACCATGGAAACAGC 3’	Phusion	72°C
		rv	5’ ATCCTTAATTAATTACTTATACAGCTCATCCATGCCGTACAGAAAC 3’		
C1V1(t/t)-TS-mCherry	pAAV-CaMKIIa-C1V1(t/t)-TS-mCherry	fw	5’ GCATACTAGTGCCGGCACCATGTCGCGGAGGCCA 3’	Phusion	72°C
		rv	5’ GCATCCGCGGTTACTTGTACAGCTCGTCCATGCGGC 3’		
Jaws-KGC-GFP-ER2	pAAV-CaMKII-Jaws-KGC-GFP-ER2	fw	5’ GCATACTAGTGCCGCCACCATGGGGACCTGGATGC 3’	Platinum	60°C
		rv	5’ GCATCCGCGGTTACACTTCATTCTCGTAGCAGA 3’		
Jaws-KGC	pAAV-CaMKII-Jaws-KGC-GFP-ER2	fw	5’ GGACTAGTGCCGCCACCATGGGGACCTGGATGCTGACGAAGGCTC 3’	AccuPrime	68°C
		rv	5’ TGTTATCCTCCTCGCCCTTGCTCACTGCCCCTGCAGGTGCAACA 3’		
mCherry	UAS-lyn-mCherry	fw	5’ TGTTGCACCTGCAGGGGCAGTGAGCAAGGGCGAGGAGGATAACA 3’	Phusion	72°C
		rv	5’ TCCCCGCGGTTACACTTCATTCTCGTAGCAGAACTTGTACAGCTCGTCCATGCCGC 3’		
Jaws-KGC-mCherry-ER2	Gibson assembly product	fw	5’ GGACTAGTGCCGCCACCATGGGGACCTGGATGCTGACGAAGGCTC 3’	AccuPrime	68°C
		rv	5’ TCCCCGCGGTTACACTTCATTCTCGTAGCAGAACTTGTACAGCTCGTCCATGCCGC 3’		

NES-jRCaMP1b	pGP-CMV-NES-jRCaMP1b	fw	5’ GCATACTAGTGCCGCCACCATGCTGCAGAACGAGCTT 3’	Phusion	62°C
		rv	5’ GCATCCGCGGCTACTTCGCTGTCATCATTTGT 3’		
H2B-mCherry	mCherry-H2B-6	fw	5’ GGACTAGTGCCGCCACCATGCCAGAGC 3’	Phusion	72°C
		rv	5’ TCCCCGCGGTTACTTGTACAGCTCGTCCATGCCG 3’		
H2B-RFP1	H2B-RFP in pENTR1A	fw	5’ GGACTAGTGCCGCCACCATGCCAGAGC 3’	Phusion	72°C
		rv	5’ GCATCCGCGGTCAGTTGGCCTTCTCGG 3’		
H2B-RFP2	H2B-RFP in pENTR1A	fw	5’ GCATACTAGTGCCGCCACCATGCCAACTTTGTACAAAAAAG 3’	Phusion	72°C
		rv	5’ GCATCCGCGGTCAGTTGGCCTTCTCGG 3’		
Tol2	Tol2-GCaMP2	fw	5’ GCTCTAGATGATCACCGCGGTGGAGCTCGAATTAATTCATC 3’	Phusion	72°C
		rv	5’ GCTCTAGAACTAGTGACCACTTTGTACAAGAAAGCTGAACGA 3’		

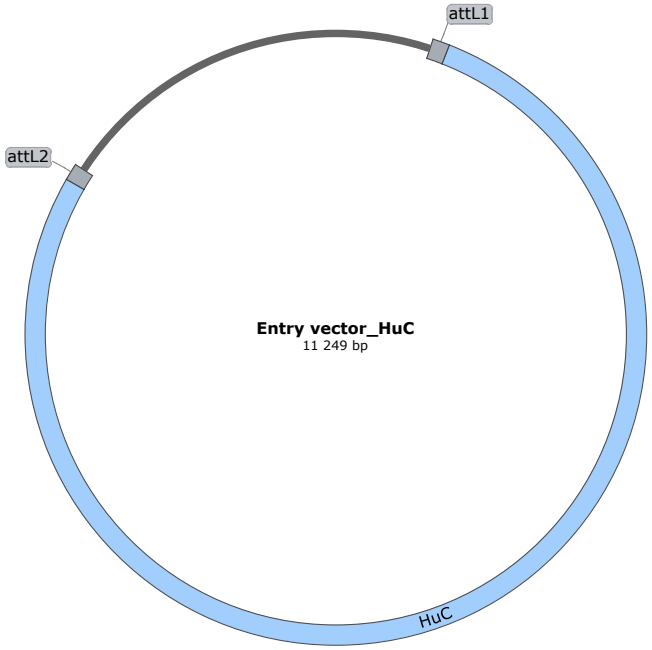
Supplement 4 - Map of Gateway entry clone with 4xUAS regulatory sequence.



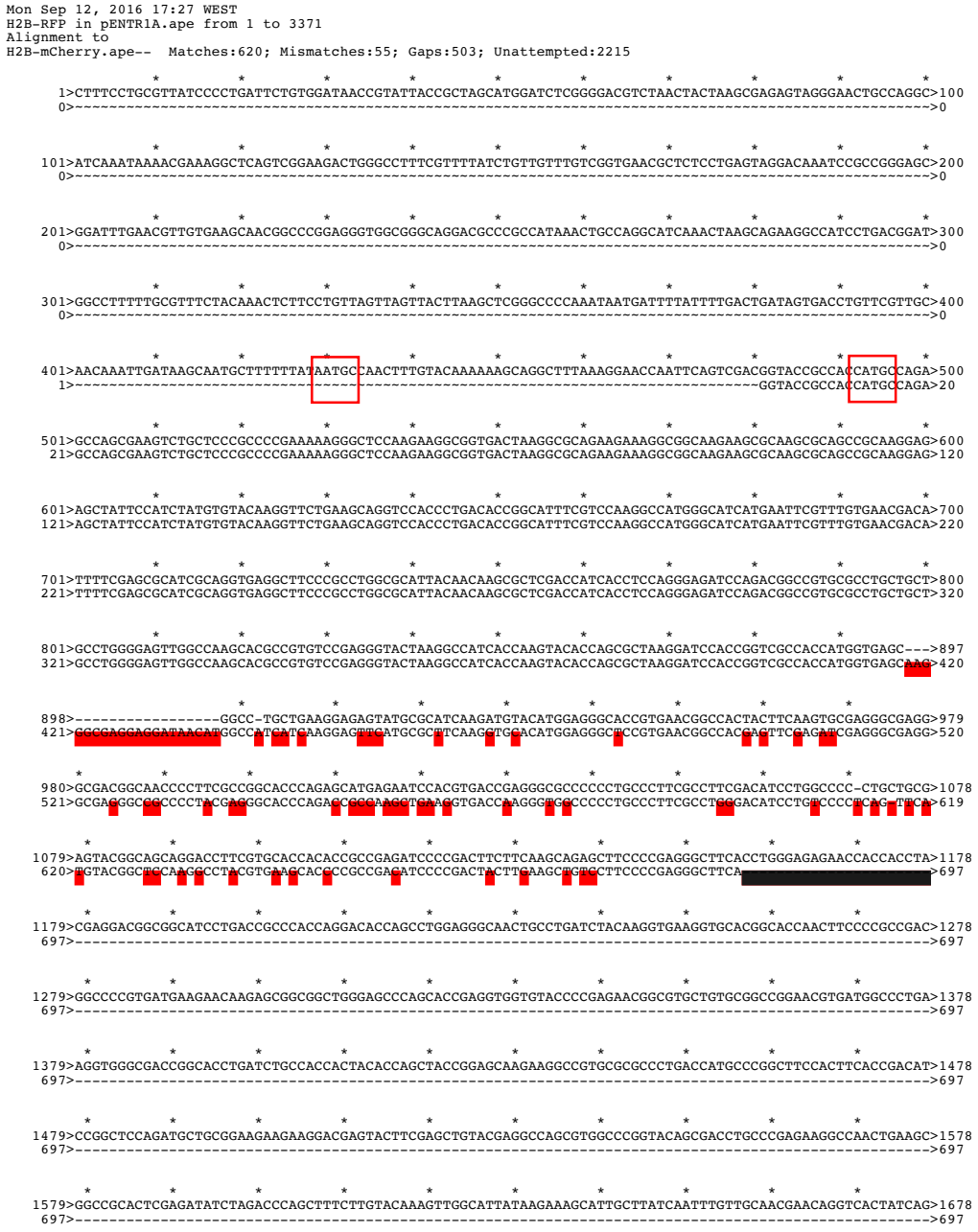
Supplement 5 - Map of Gateway entry vector with 10xUAS regulatory sequence.



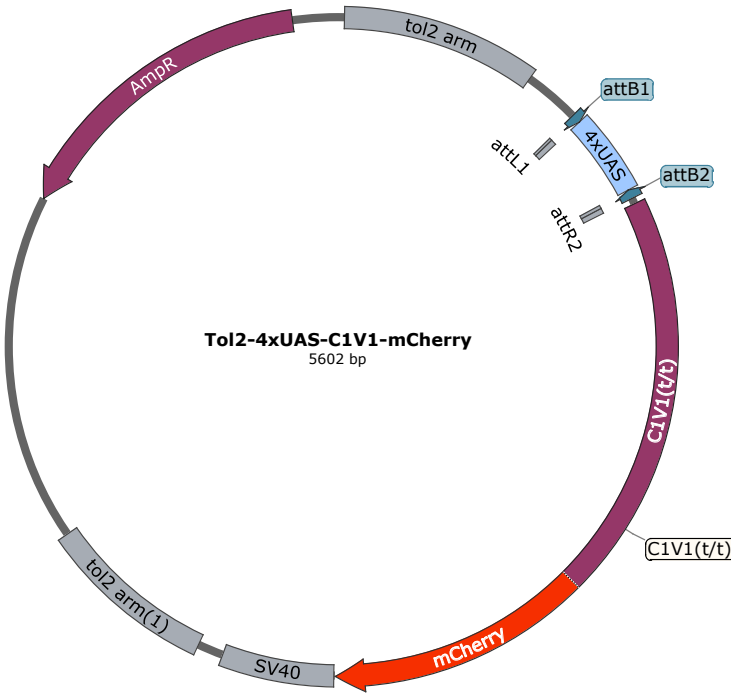
Supplement 6 - Map of Gateway entry clone with *HuC* (*elavl3)* promoter sequence.



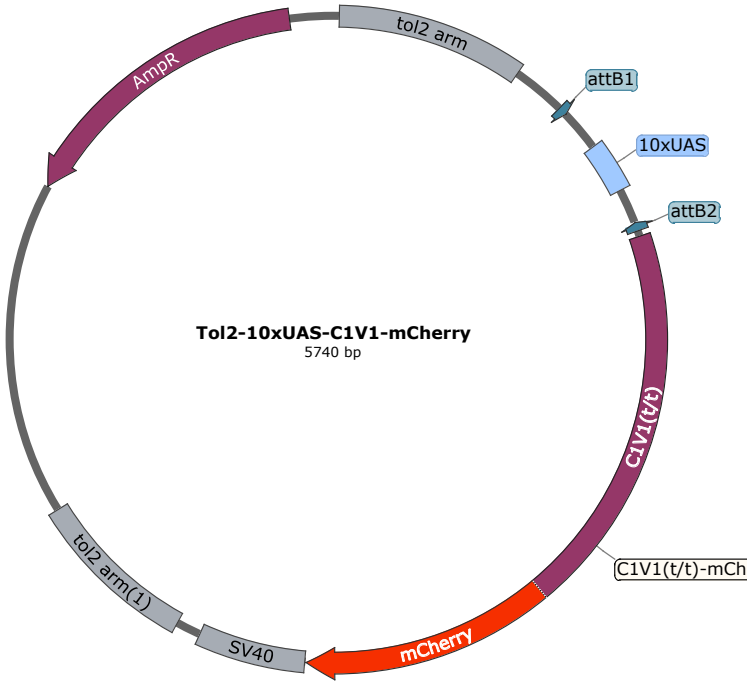
Supplement 7 – Alignment of H2B-RFP in pENTR1A to H2B-mCherry, two ATGs highlighted in red.



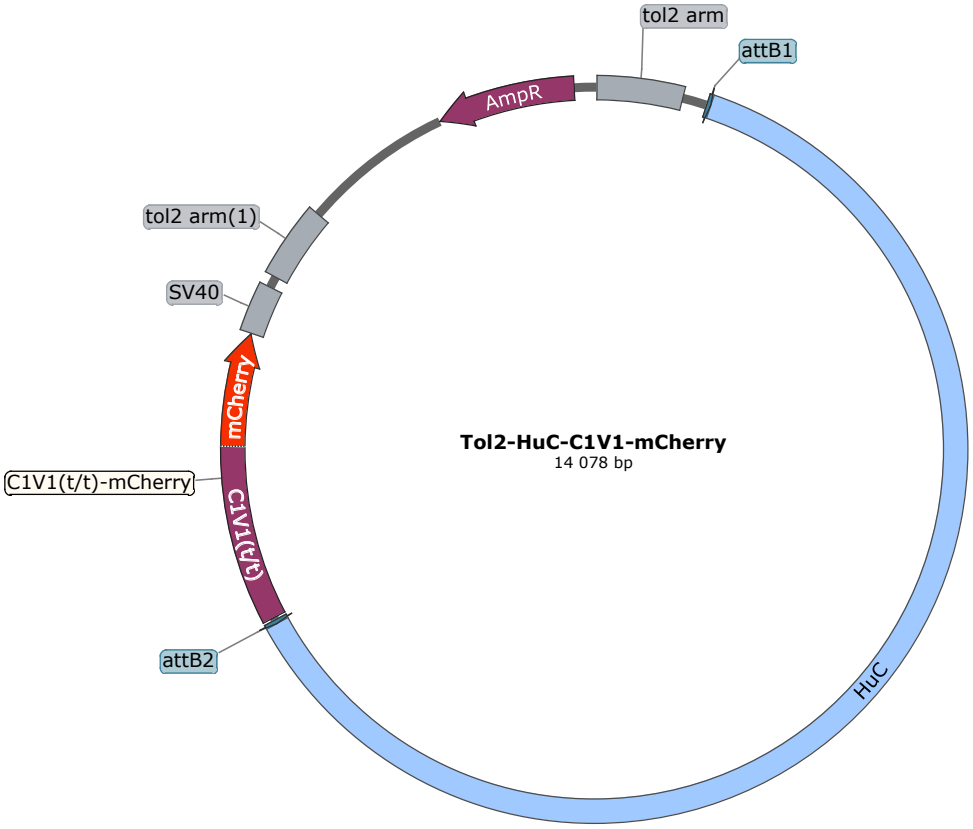
Supplement 8 – Tol2-4xUAS-C1V1(t/t)-mCherry vector map.



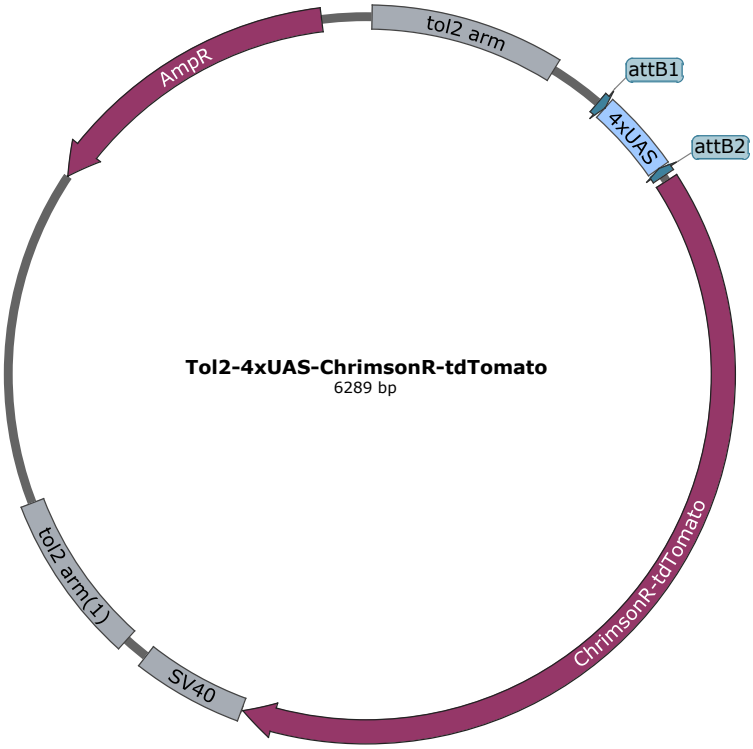
Supplement 9 – Tol2-10xUAS-C1V1(t/t)-mCherry vector map.



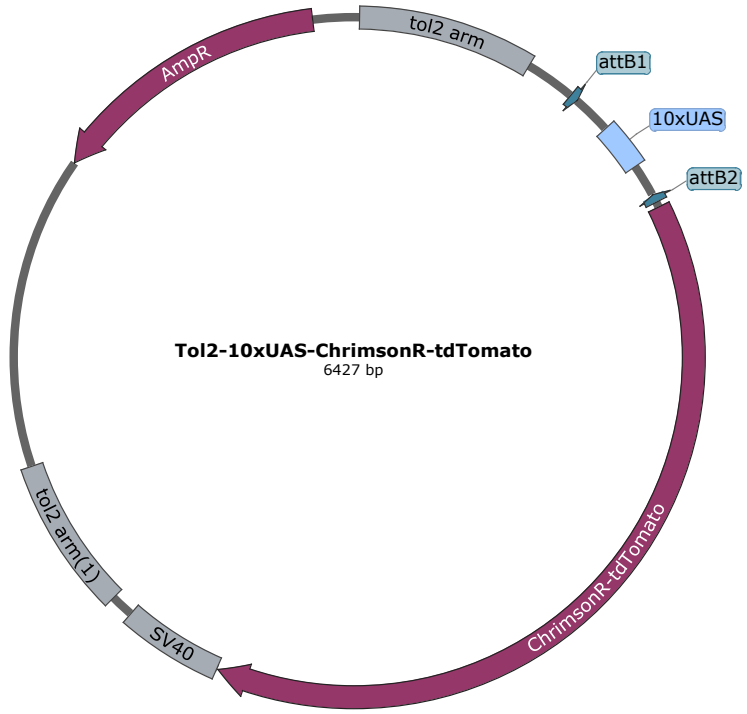
Supplement 10 – Tol2-HuC-C1V1(t/t)-mCherry vector map.



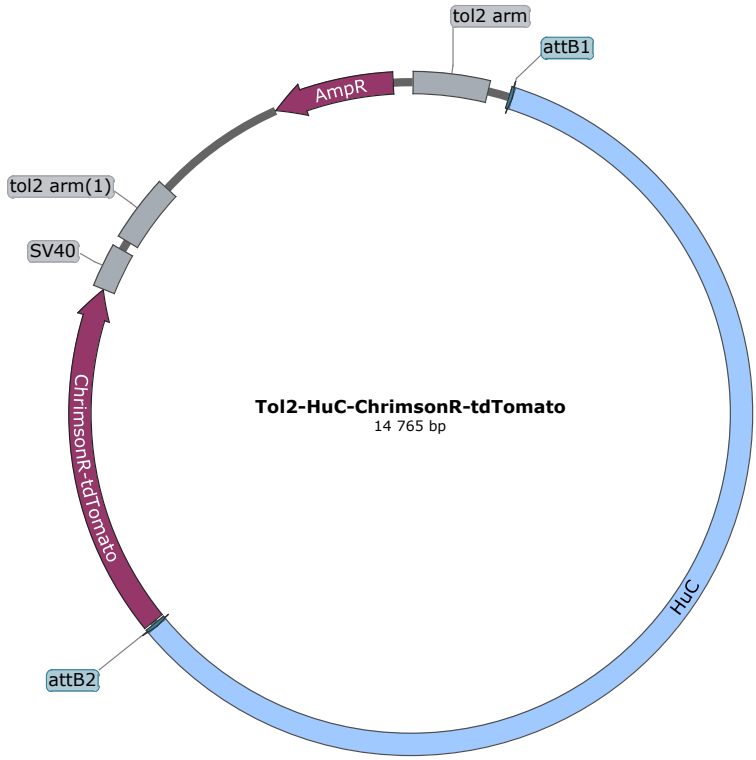
Supplement 11 – Tol2-4xUAS-ChrimsonR-tdTomato vector map.



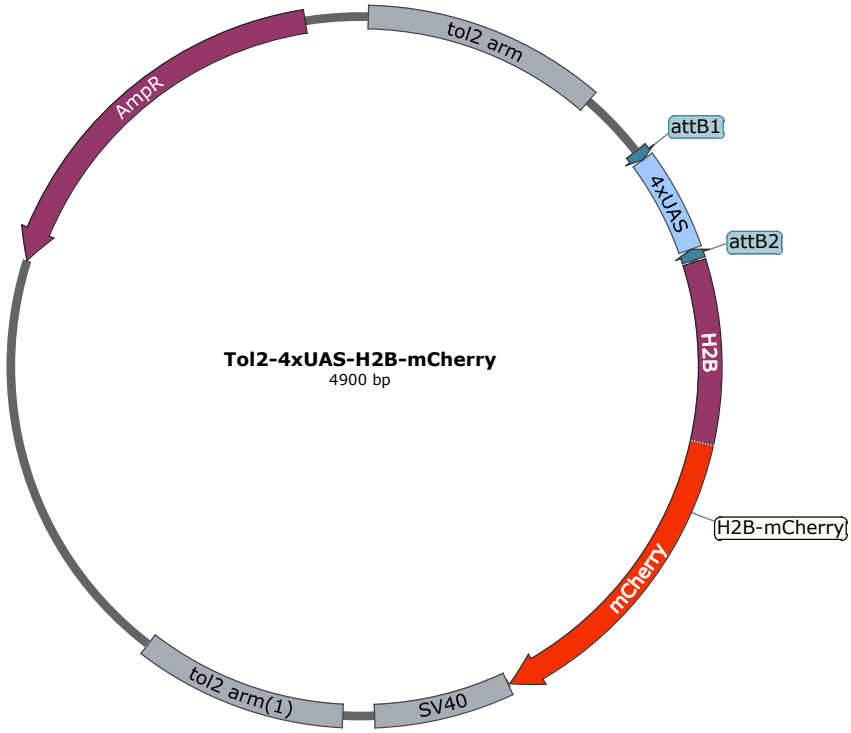
Supplement 12 – Tol2-10xUAS-ChrimsonR-tdTomato vector map.



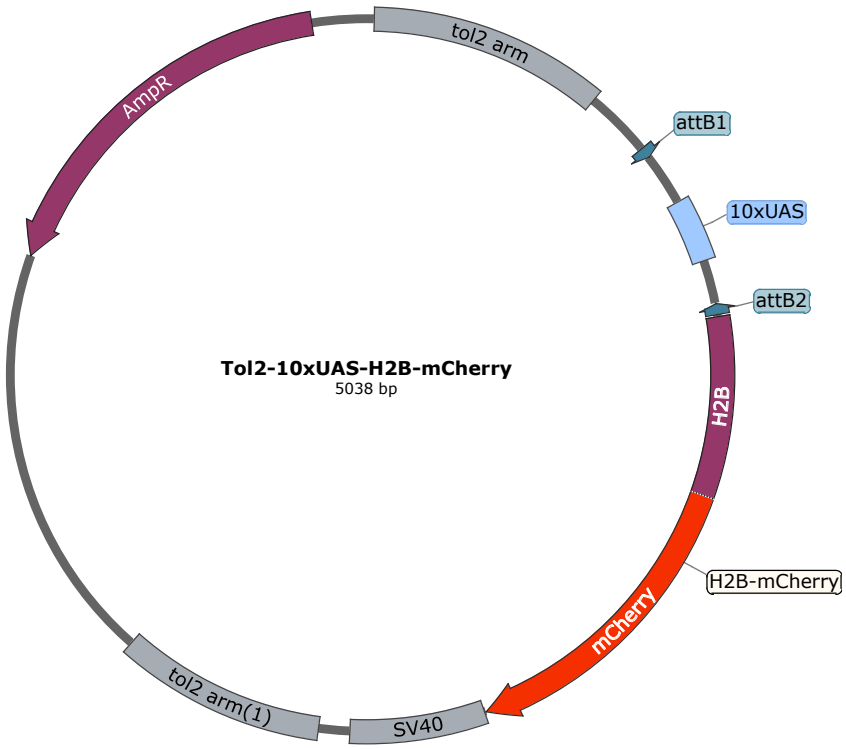
Supplement 13 – Tol2-HuC-ChrimsonR-tdtomato vector map.



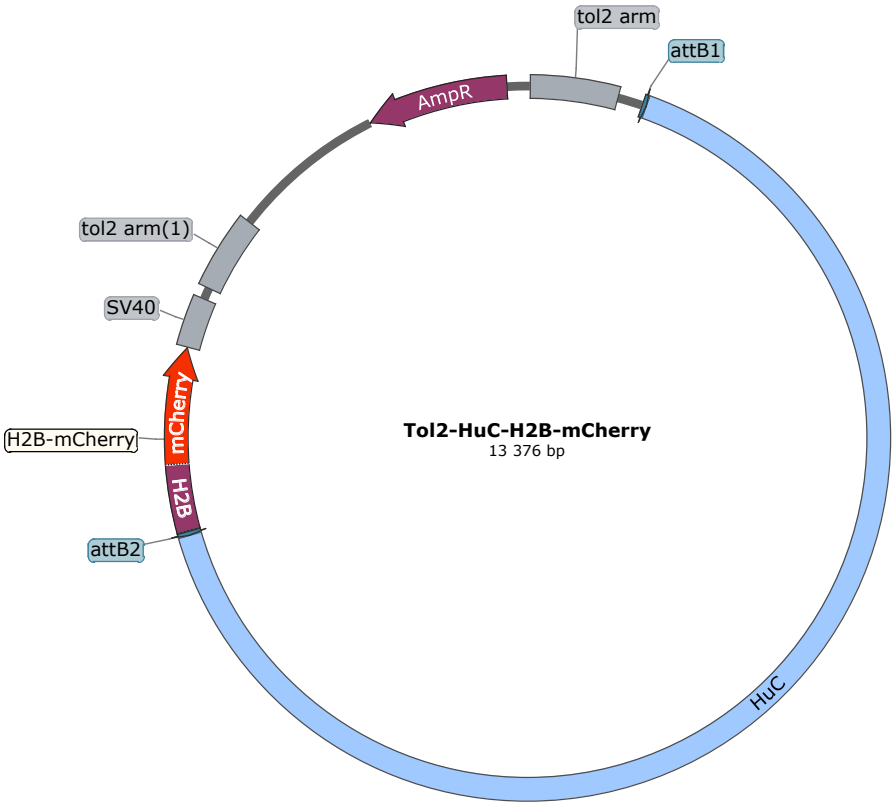
Supplement 14 – Tol2-4xUAS –H2B-mCherry vector map.



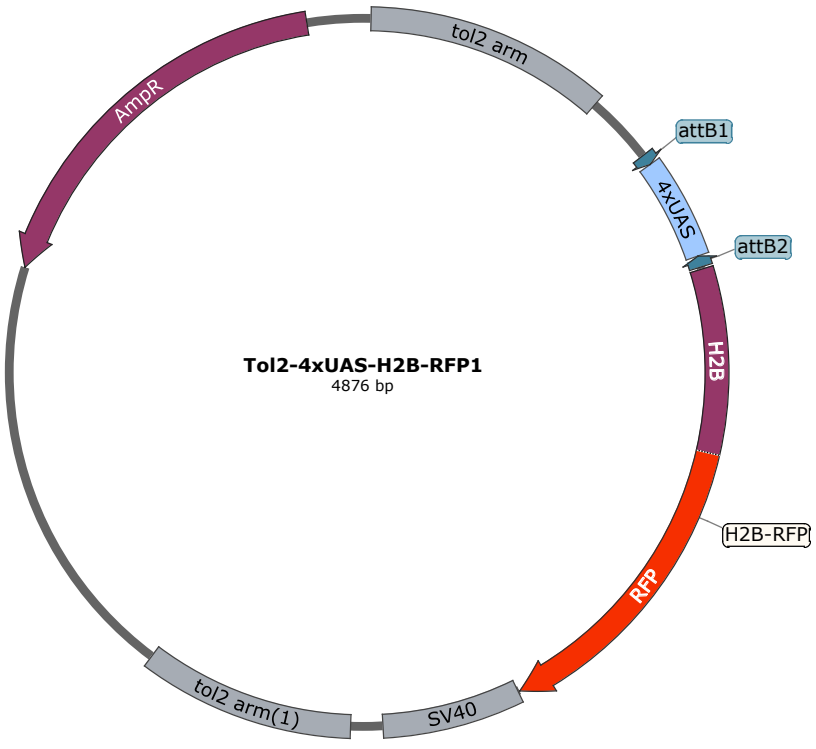
Supplement 15 – Tol2-10xUAS-H2B-mCherry vector map.



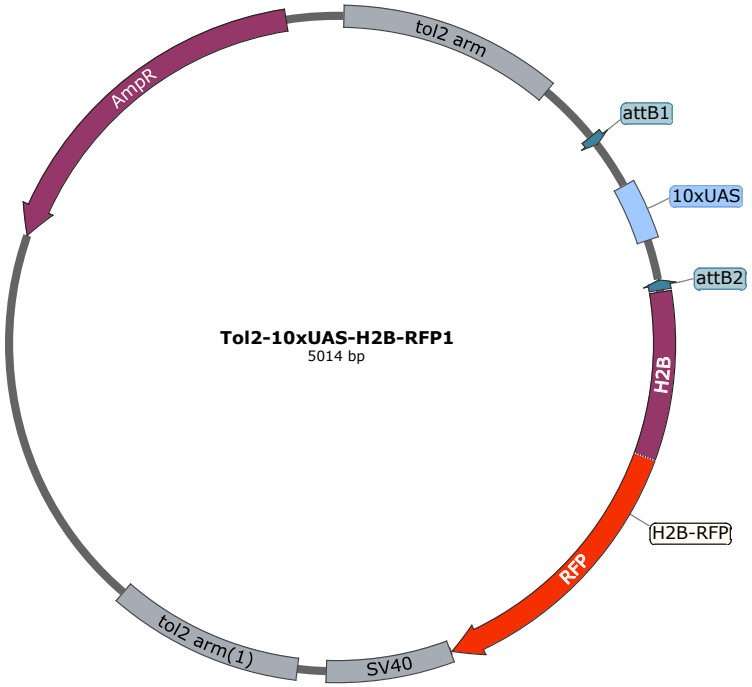
Supplement 16 – Tol2-HuC-H2B-mCherry vector map.



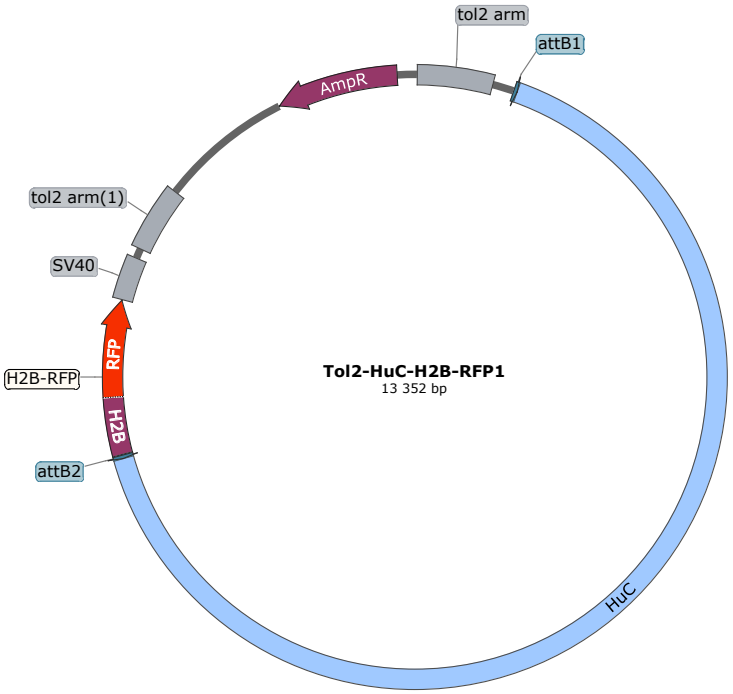
Supplement 17 – T2-4xUAS-H2B-RFP1 vector map.



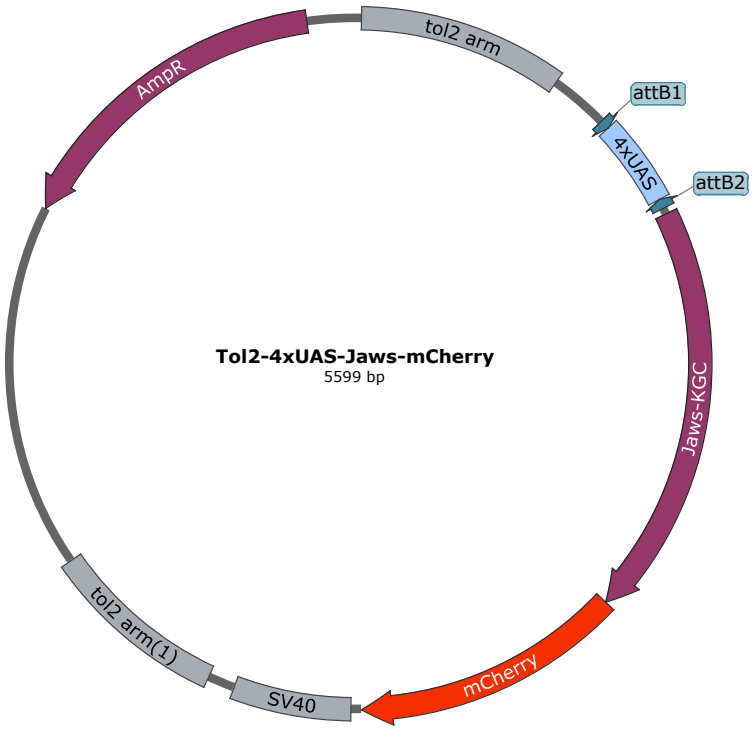
Supplement 18 – Tol2-10xUAS-H2B-RFP1 vector map.



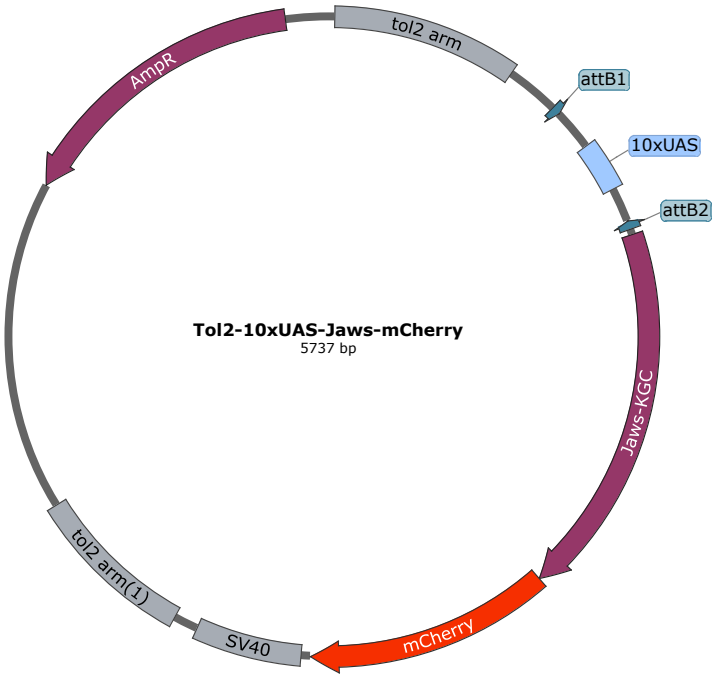
Supplement 19 – Tol2-HuC-H2B-RFP1 vector map.



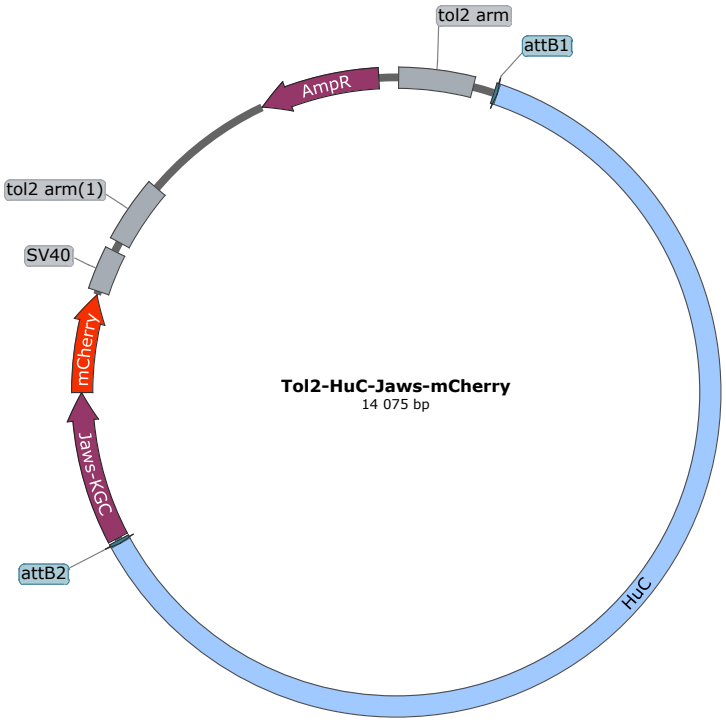
Supplement 20 – T2-4xUAS-Jaws-mCherry vector map.



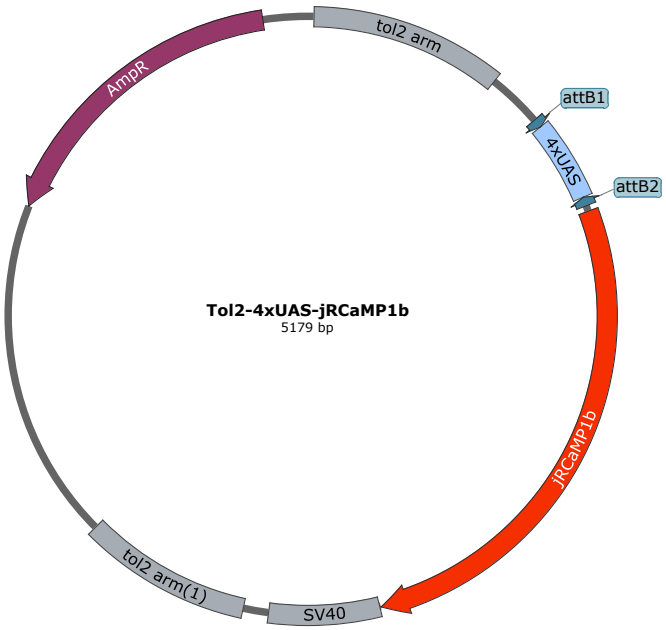
Supplement 21 – T2-10xUAS-Jaws-mCherry vector map.



Supplement 22 – Tol2-HuC-Jaws-mCherry vector map.



Supplement 23 – Tol2-4xUAS-jRCaMP1b vector map.



Supplement 24 – Tol2-10xUAS-jRCaMP1b vector map.