FINAL REPORT

Characterization of putatively connected cells to different retinal ganglion cell terminals in the zebrafish brain

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Abstract

As described in this report, I studied the neuronal pathway that underlies a certain visuomotor transformation, the optomotor response, by anatomically characterizing the cells involved. My approach uses the larval zebrafish, an attractive model system for identifying the components of neural circuits underlying visual behavior. Because of its small size and transparency, its well studied development, a repertoire of several innate behaviors that are robust and easy to study, and the genetic tools that can be applied, it is an ideal organism for studying the function of neural circuits.

Visually induced behaviors emerge already at day three post fertilization, almost immediately after the axons of retinal ganglion cells (RGCs), the output neurons of the retina, reach their postsynaptic targets. At the other end of the circuit, there are distinct subsets of spinal projection neurons that are responsible for directing motor output (swims and turns) that constitute an essential visual response to whole-field motion. The intermediate circuit, i.e. cells in the tectum or pretectum, downstream of the RGCs and upstream of the spinal projection neurons that participate in the optomotor response, is still unknown. To this end my final report will show putative candidate cells that might be the missing link in this complete circuit.

Starting at the level of RGCs that send their axons into different regions of the brain, the so-called arborization fields, I will describe a method that allows us to identify *in vivo* the neurons downstream of the RGCs that are putatively connected to these arborization fields. Using a fast genetic recombineering system and testing different enhancer fragments for their expression patterns, I was able to identify one fragment that exclusively labels RGCs. Employing photoactivation of the panneuronaly expressed photoactivateable (PA) fluorescent protein

PA-GFP in specific regions innervated by RGC axons or spinal projection neurons, I was able to describe cells “in-between” in the pretectum and tectum. These candidate cells are putatively connected to different RGC terminals and might be involved in forwarding information downstream to spinal projection neurons.

This study is a first approach to demonstrate how many and which cells are connected to different arborization fields of genetically labeled RGCs, and which of them relay the processed information downstream to spinal projection neurons. To get a complete picture of the functionality of the connections underlying the visuomotor circuit, it will be necessary to study the output of these genetically labeled RGCs and the responses of the candidate cells identified within this thesis, by calcium imaging.

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Introduction

The larval zebrafish as a model system to study the visuomotor circuit

The zebrafish (*Danio rerio*) is a freshwater teleost native to the rivers of India and Bangladesh [1]. The larvae develop externally and are almost completely translucent at embryonic and early larval stages. The larval brain at five days post fertilization (dpf) is less than 500 mm thick and 1.5 mm long making it the ideal model organism to be studied by two-photon microscopy *in vivo* because virtually all neurons are accessible. Furthermore the zebrafish has been established as a model system in systems neuroscience because of techniques such as light gated ion-channels, functional calcium imaging, large mutation screens, and Gal4 enhancer lines to study *in vivo* the neural circuits underlying behavior in a translucent animal [2]. There is a large amount of resources that have been systematically accumulated (e.g., www.zfin.org) and that are publicly available [3]. In this thesis I will describe a method making use of high resolution two photon microscopy to characterize the anatomy of the zebrafish underlying the circuit that is involved in the sensorimotor loop, from visual input to motor output. Starting at the level of retinal ganglion cells (RGCs) that send their axons in different regions (arborization fields) in the brain [4], my results will show putatively connected pretectal cells and their downstream partners in the midbrain.

At only 1 day post fertilization (dpf), zebrafish larvae show behavioral responses to touch as well as spontaneous motor activity [5]. Visual responses emerge by day three, almost immediately after the axons of ganglion cells leaving the eye reach their targets [6]. At 5 dpf visual induced behaviors such as the optomotor response start [7]. The most

remarkable feature in the larval behavioral repertoire is hunting for paramecia just after 5 days [8]. In both behaviors, optomotor response and prey hunting, distinct population of spinal cord projection neurons have been identified that are particularly involved [7, 8]. Therefore they offer ideal starting points to look for connection partners by photoactivation.

The optomotor response (OMR)

When confronted with whole-field visual motion, fish will turn their body and swim in the direction of perceived motion – the optomotor response (OMR). This behavior can be found in the majority of animals, including insects and humans [9]. Several components of the circuit underlying the zebrafish optomotor response have been revealed [7].There are distinct subsets of spinal projection neurons that are responsible for directing the swims and turns that constitute an important visual response to whole-field motion. These specifically active neurons are possible participants in the circuit controlling the related behavior. A small subset of spinal projection neurons in the midbrain (Nucleus of the medial longitudinal fasciculus - NucMLF) and the hindbrain (Vestibular-cells - V-cells) are involved in the OMR that link sensory processing in the brain to motor output in the spinal cord. Forward-preferring neurons can be found in the hindbrain and the NucMLF. V-cells are responsible for right or left OMR turning. To elucidate the complete sensorimotor transformations, it is still necessary to identify the neurons in the pretectum and tectum, the upstream circuit elements, mediating this behavior and to see which RGCs are involved. The experimental strategy for circuit identification is achieved by photoactivation of the active neurons dendrites to see which are their putative connection partners.

The NucMLF has also been shown to be involved in prey capture. The rostral and caudal medial lateral cells (MeLc and MeLr) of the NucMLFs extend dendrites into the ipsilateral tectum and project axons into the spinal cord. Ablation studies of both neurons have shown that afterwards prey capture is impaired. Therefore MeLc and MeLr functions in series with the tectum and the NucMLF as well as the tectum are involved in coordinating prey capture movements. By identifying the arborization fields of RGCs that send inputs to the NucMlf, one population of ganglion cells might be isolated that is only involved in prey capture.

To identify neurons throughout the brain that respond to global motion patterns that elicit specific orienting behaviors, other members of the lab use a transgenic fish (Huc:GCaMP2) with panneural expressing of a genetically encoded calcium indicator [unpublished data]. The idea was to get an overview of the set of neurons potentially involved in generating a particular response. However, since the indicator is expressed pan-neuronally, it is not very informative about the anatomy and connectivity of these neurons, and my part of the project was being able to specifically manipulate activity in these neurons to probe circuit function.

To this end I was screening larvae, injected with different enhancer fragments driving a red fluorescent protein (lyn-mCherry), to find enhancers that drive expression in subsets of neurons that overlap with our groups of interest. My project consisted of three parts:
1) Using BAC recombineering or Gateway cloning to make constructs to drive expression of GCaMP, GAL4 or other markers in cell populations of interest

2) Inject these constructs into zebrafish, using the Tol2 transposase system, assess the transient expression pattern, and raise promising fish to make stable lines.
3) Characterize the anatomy of a sensorimotor circuit by finding putative connection partners within the circuit by photoactivation of photoactivateable GFP

Retinal Ganglion Cells (RGCs)

The visual system of all vertebrates consists of the retina where light transduction and signal preprocessing takes place. In the retina, the detection of light by the photoreceptors leads via bipolar cells and amacrine cells to the activation of ganglion cells (RGCs) that serve as the output layer of the retina and project into different arborization fields in the brain. The optic nerve consisting of RGC axon bundles conveys the information into several areas in the brain where neuronal signals are relayed and furthermore processed.

The retina's output is conveyed to the brain by many different ganglion cell types. There about 15 morphological different types in mammalian retinas alone that have been identified. The population from each type covers the visual field and consequently conveys a complete but processed visual image. Ganglion cells signal brightness and darkness, contrast, color, motion and other features of the visual input. Each type could therefore need a dedicated neural circuit to extract the visual feature of interest [10].

Ganglion cell types can also be sorted according to the receptive fields of ganglion cells and consequently to the inputs they receive [11]. Using this type of classification three basic types of ganglion cells are found in the catfish. The first type is a cell with a small receptive field (200-

300µm) that gives sustained on- and off-center responses to spots of light. Its receptive field is concentrically organized with a distinct center and surround receptive field. The second type is a large-field ganglion cell that gives more transient on- and off-center responses to spot illumination. Characteristic of many of these cells is an orientation preference to bars or slits of light moved through the receptive field (orientation selectivity). The third type is a large field cell giving on-off responses to illumination presented anywhere in its receptive field. The forth type of retinal ganglion cell found in the rabbit retina is an on-off ganglion cell that shows motion- and direction-sensitive responses. A variety of other ganglion cell receptive fields have been described, for example the edge-detectors, another kind of variation, seen in on-off retinal ganglion cells [10]. Most of the above mentioned cells are described by their functionality regarding the input they receive from upstream cells (amacrine and bipolar cells). Others, as mentioned before, have been described because of their different cellular morphology: differences in size and form of the cell perikarya and the dendritic tree.

It is hard to find a genetic pattern that distinguishes one ganglion cell type from another. But as each ganglion cell type has different features and develops differently, it is not far away, that they must distinguish by different intrinsic properties, e.g. expression of a protein that is not expressed by another type of ganglion cell. One example of such an approach to identifying a molecular marker for a RGC subset was described recently [12]. Several immunoglobulin adhesion molecules that are known to be selectively expressed in RGCs, were screened. One of these molecules is the junctional adhesion molecule B (JAM-B) that was demonstrated to mark OFF RGCs that are responsible for detecting

upward motion. By using marker that were identified to express in RGCs, subsets of RGCs can be identified and further studied.

I will illustrate how testing of different expression patterns from molecular markers for RGCs in zebrafish identified at least two functional enhancer fragments that show specific labeling of RGCs.

RGC enhancer fragments and their expression patterns

To make use of this approach I searched in the literature and in the zfin database [3] for markers of RGCs. Nine such different markers that I tested for their expression pattern are depicted in Fig.1

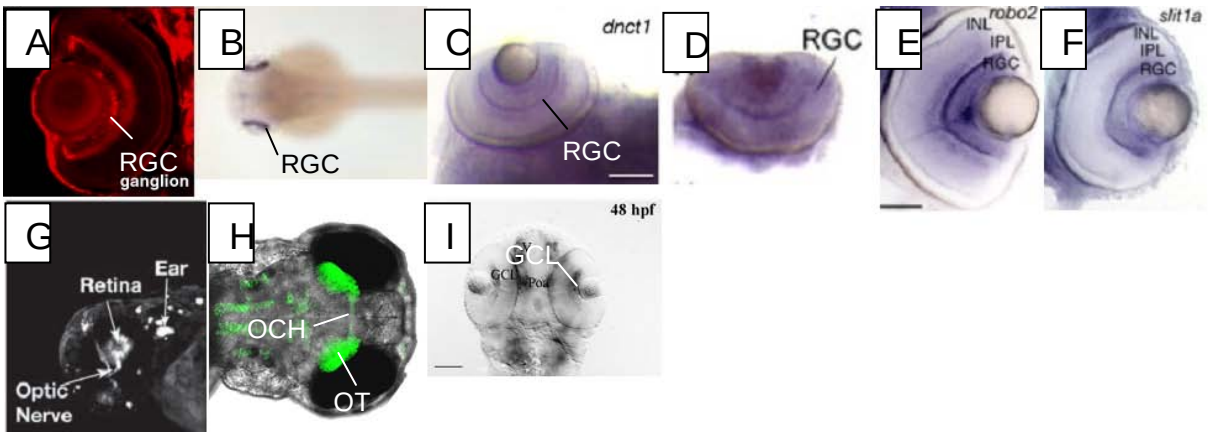


Fig.1 genetic markers of RGCs

All of the depicted figures show that the proteins are expressed to some extent in RGCs. A description in detail of those markers can be found under Results.

A: alcam-a (activated leukocyte cell adhesion molecule), immunostaining shows labeling of RGCs, lateral view of the eye of 5 day old larvae

B: itga6 (integrin alpha 6), in situ hybridization shows labeling of RGCs, dorsal view

C: dnct1 (cytolinker protein) in situ hybridization, dorsal, lateral view of the eye, RGC layer is stained dimly

D: hs6st1b (heparan sulfate sulfotransferase), in situ hybridisation dorsal, later view of the eye, RGCs are labeled (3 day old larvae)

E, F: robo2(roundabout homolog 2) and slit1a (calcium ion binding protein), lateral view of the eye, in situ hybridization, labeling weakly of the inner nuclear layer (INL), and of RGCs. inner plexiform layer (IPL) in between is not labeled

G: brn3c (transcription factor), Brn3c:GFP, dorsal view of a 6 day old larva, retina including RGCs and the optic nerve (axon bundles of the RGCs), as well as the ear are labeled by Brn3c:GFP

H: ath5 (transcription factor), Ath5:GFP, dorsal view of 5 day old larva, the optic tectum(OT) is labeled and the optic chiasm(OCH), crossing of the RGC axon bundles can be seen

I: pcp4a (Purkinje cell protein), dorsal view, in situ hybridization shows labeling of the ganglion cell layer (GCL)

I mentioned that there are different subsets of RGCs with distinct functions. It can be assumed that each RGC subset should also project their axons in a specific region of the brain.

Arborization fields (anatomy)

In the zebrafish larvae the main projection site of RGC axons is the contralateral optic tectum (Fig. 2), the visual midbrain – the mammalian homologue is the *superior colliculus.* There are nine more distinct regions, termed arborization fields in which the optic axons of RGCs arborize. Those fields have been identified by intraocular injection of DiI and tracing the RGC axons into their respective target areas [4].

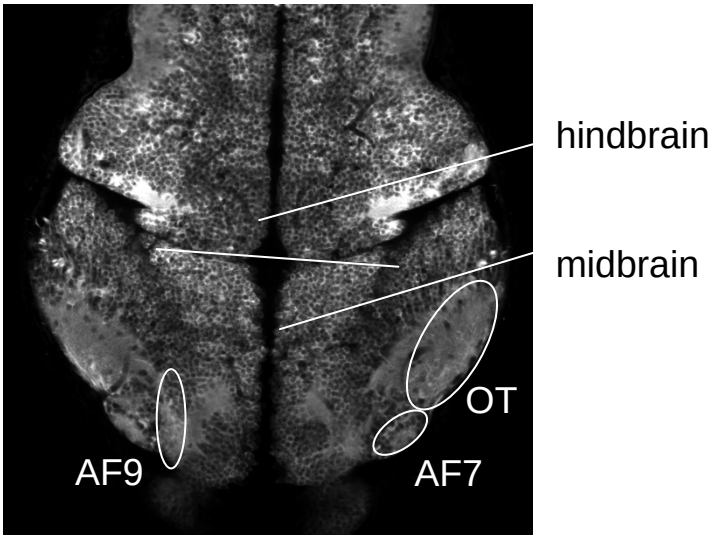


Fig.2 Visualization of the different regions of the zebrafish brain – 2 photon image

Dorsal view of the zebrafish brain (fish faces rostral). The midbrain-hindbrain border is clearly visible and marked by a white line. Three different arborization fields are depicted, the optic tectum (AF10), AF7 and AF9. OT-optic tectum, AF7 – arborization field 7, AF9 - arborization field 9

Others have started to map visual behaviors to different target areas of RGCs. Ablation of the optic tectum had only mild effects on optomotor responses but abolished orienting movements during prey capture [13]. My results will show which putative tectal and pretectal connection partners are projecting to arborization field 7 (AF7) and which putative pretectal cells are connected to arborization field 9 (AF9). I will also show by high resolution two-photon microscopy that there is one population of cells that seems to project to the NucMLF and apparently receives input from a particular arborization field.

Two photon imaging

Two-photon imaging provides access to every neuron of the zebrafish brain (Fig. 3). A number of calcium indicators of neural activity have been used with zebrafish and when combined with two-photon microscopy, it is possible to record responses to behaviorally-relevant stimuli in every potential component of the controlling circuit [7]. In the subsequent sections I will illustrate how two photon microscopy allows to get a detailed picture of the anatomy and connections in the zebrafish brain. In all the following figures the fish will always face rostral.

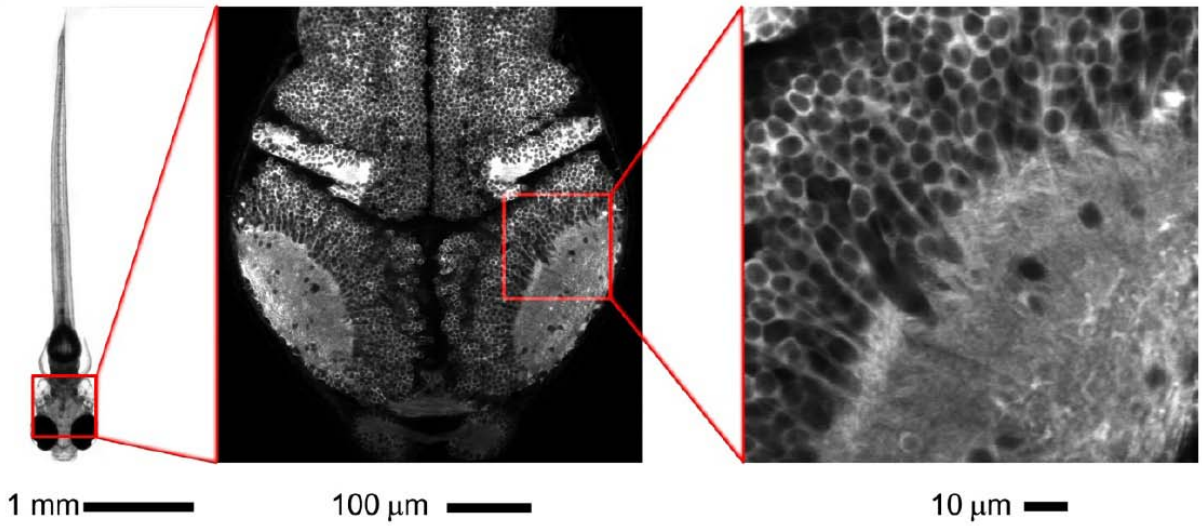


Fig.3 Two photon image of the zebrafish brain (with permission of Adam Kampff)

The transgenic fish Huc:YC2.1 labels most of the neurons in the brain. A single dorsal section through the optic-tectum, cerebellum, and hindbrain (middle) was acquired at high spatial resolution, allowing every individual neuron to be resolved (zoom-in into one optic tectum, right).

Reporter

Photoactivateable(PA) GFP

Photoactivateable GFP has been used in drosophila preparations to trace individual neurons [14]. Two-photon microscope-mediated activation of PA-GFP provides adequate spatial resolution and photoconversion-energy to expose the neuronal processes of defined neuronal populations and individual neurons in the fly brain. Two photon microscopy allows targeted illumination of PA-GFP with submicrometer three-dimensional precision and therefore permits non-random, optically guided labeling of individual neurons. Photoactivation of the neuropil resulted in labeling of the dendritic arbors of the population of neurons of interest. Diffusion of PA-GFP from the illuminated dendritic arbors allowed to reveal the cell bodies and axonal projections of multiple of those neurons.

Within my thesis I will show that PA-GFP can be photoactivated in neurons in the living brain of zebrafish to study a defined neuronal population in the pretectum and tectum. Further studies will show that photoactivation of PA-mCherry [15] can be used to label cells and to do calcium imaging at once from those labeled cells. I make use of a panneuronal expressing PA-GFP transgenic zebrafish line [unpublished data] that allows for photoactivating of nearly every single neuron in the brain and compare the connections to transgenic labeled populations of RGCs with mCherry and to with Texas red dextran dye labeled reticulospinal neurons.

Calcium Indicators

GCaMP3

GCaMP is a genetically encoded calcium indicator that consists of a GFP that has been circularly permuted. The N terminus of EGFP was connected to the M13 fragment of myosin light that calmodulin (CaM) binds to in the presence of calcium. The C terminus is fused to calmodulin. The name comes from GFP with a CaM inserted into it (G-CaM-P). GCaMP is very dim but upon binding calcium, it increases its fluorescence because of a conformational change in EGFP. The new version of GCaMP, GCaMP3, has between two to five times better signal to noise ratio than GCaMP2, its kinetics are faster and it is stated that it is more photostable than fluorescence resonance energy transfer (FRET) indicators [16]. But GCaMP3 is not perfect because it can only resolve individual action potentials in vivo up to 6 Hz. GCaMP3 can be used to study a whole population of cells simultaneously in the zebrafish brain, to see which cells are active during a set of different behaviors.

Synaptophysin GCaMP

GCaMP2 is targeted to the cytoplasmatic side of synaptophysin at the outer surface of synaptic vesicles [17]. This localization permits the fluorescence signal to be restrained to the presynaptic terminal containing a high density of voltage-sensitive calcium channels and therefore calcium fluxes in response to action potentials are high. Targeting to synaptophysin improves the response magnitude of GCaMP2 and allows optical recording of synaptic inputs by single action potentials. Combining both the targeting strategy with synaptophysin together with GCaMP3 that has a higher signal to noise ratio and faster

kinetics, in a reporter that I subcloned, should allow looking at signaling at the presynaptic site of RGC axon terminals. Therefore the destination vector that I created (see materials and methods) makes it possible to test this reporter under a variety of enhancer fragments that label RGCs.

GAL4-UAS system

The yeast transcriptional activator GAL4 can be used to drive transgenes linked to the target UAS of the GAL4 protein. Once a stable Gal4 line driven under a certain enhancer fragment is established, it can be used to drive expression of any UAS linked reporter. By crossing to stable lines driving UAS linked reporters or injection of reporters linked to the UAS target sequence, the same “Gal4 enhancer” can be used to test different reporters. For the enhancer trapping a GAL4 construct is used that is linked to a 5’basal promoter which only drives expression when the GAL4 construct inserts near an endogenous enhancer [18.] This intends to drive tissue-specific expression in the next generation in case the construct integrates in the genome in front of an enhancer. For my purposes, I am using a variant of Gal4, Gal4FF, which was shown to be less toxic in zebrafish [19] and to test for a higher level of expression of my constructs.

Cmcl2 heart GFP.

A cmcl2 enhancer fragment of 200 bp driving GFP expression reliably labels only the heart [20]. I used this enhancer fragment in a plasmid to be coexpressed with my reporter (GCaMP or Gal4) under different enhancer fragments labeling RGCs. Coexpression then should allow fast screening for transgenes by looking for a bright heart fluorescence. This

coexpression-system is especially useful to create Gal4 lines, as there is no other way to screen for, than coinjection with an UAS vector.

Results

To identify different populations of RGCs needs a sophisticated approach. One of these approaches is the GAL4 enhancer trap [18]. But to screen thousands of fish, and not being able telling immediately after screening if the next generation of fish, will still express in the same population of cells, and that in subsequent generations the expression can become mosaic, is a huge backlash of this system.

A different approach is to use well known promoters that are involved in driving the expression of proteins in RGCs. Enhancer bashing (testing of the ability of cis-acting DNA elements upstream of the start codon to drive expression) and trying different enhancer fragments varying in size, is a powerful tool, using well known proteins involved in RGC development and function.

Gateway cloning and gap repair – methods to test different enhancer fragments

For this purpose I used a recombineering system called Gateway cloning [21], see materials and methods. This recombineering system is very useful for fast testing of many different enhancer fragments, and to drive immediately after a simple recombineering step different reporters. Moreover once one reporter is within a destination vector, it can be used to test different enhancer fragments. The destination vector contains the reporter and the Tol2 arms, which by injection of the plasmid together with Tol2 transposase facilitates germline integration.

Insertion of a sequence with negative or no regulatory activity in the entry vector will not lead to expression of the reporter. The system only

allows identifying positive regulatory elements that can drive transcription on their own. Modification of the system, using the minimal promoter cfos, as described in [21], make it possible to look at enhancer fragments that only have regulatory activity without being able to drive transcription on their own. Different enhancer fragments were examined for their expression patterns without the minimal promoter, since expression to some extent was shown in most of the cases.

Enhancer fragments tested for the labeling of RGCs

*Atonal homolog 7 (atoh7, **ath5**, lakritz)*

The basic helix-loop-helix transcription factor ath5 has been shown to be involved in RGC differentiation. So far in the literature Ath5 enhancer fragments labeled not only RGCs but tectal cells too [22]. The one enhancer fragment described by Masai et al contained untranslated regions of 7 kb of 5' and 3'genomic fragments. I used this enhancer fragment contained in the Ath5:GFP plasmid [22] as template to make a pcr reaction creating a shorter 2kb long version that was also shown in *medaka* to faithfully recapitulate ath5 expression.

The 2kb fragment that I am using in comparison to the 7kb fragment drives the expression of mCherry exclusively in RGCs and not in cells in the tectum. I tested the expression patterns of both enhancers (Fig. 5) by crossing Ath5:GFP fish to Ath5 2kb:mCherry. The results show that not the complete arborization field is covered. As Ath5:GFP also labels the dendrites of tectal cells within one arborization field, it is still unclear whether Ath5 2kb:mCherry covers the whole population of RGCs because it cannot be distinguished between how much volume the dendrites of the tectal cells take in comparison to the RGC axons in one arborization field (see Fig. 4).

I also tested a 4kb and 5 kb long enhancer fragment of Ath5. Both did not show any expression, apparently they must have contained a sequence with negative regulatory activity. Eight different fish expressing ath5 2kb mCherry showed transient expression of interest and were grown up. Two of them showed to be founders and all following experiments were conducted with these two fish lines. One line Ath5 2kb mch line 1 labeled broader AF9 but the general expression pattern of the other AFs was weaker. The other line Ath5 2kb mch line 2 was brighter with weaker expression in AF9. One of the special feature of my lines is labeling of the pineal gland, which is very useful because it allows fast screening for expression, already at day 2 (Fig. 4).

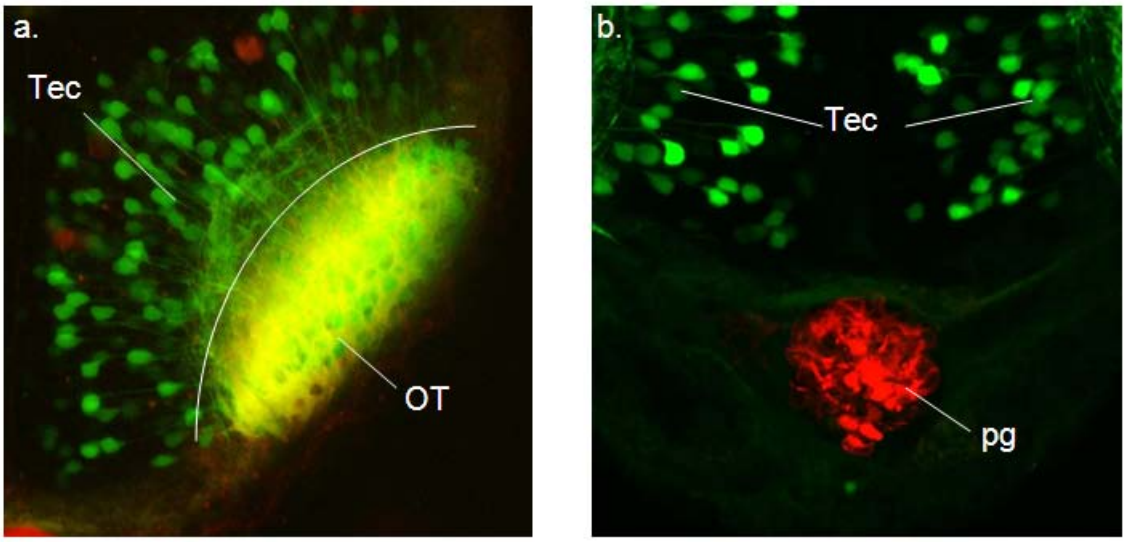


Fig. 4 expression pattern of Ath5 2kb:mCherry in comparison to Ath5:GFP

a. Shows overlapping expression in the tectum without labeling any of the tectal cells that send their dendrites into the arborization field.

b. Tectal cells are labeled by Ath5:GFP. Ath5 2kb:mCherry labels ganglion cells (not seen here) and the pineal gland.

Tec – tectum, OT - optic tectum, pg – pineal gland

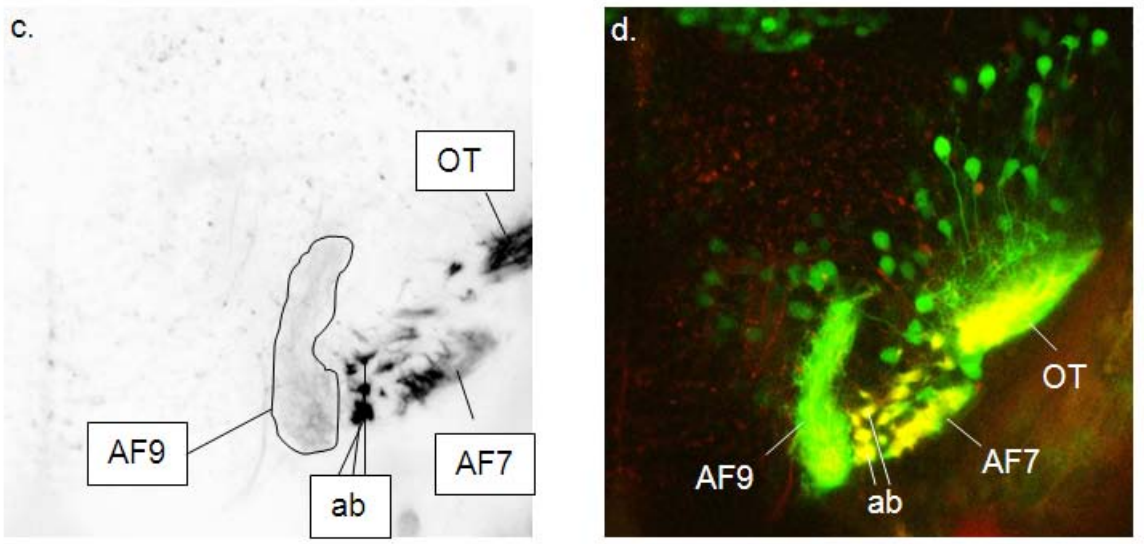


Image is depicted in inverted luminance for visual clarity

c. Ath5 2kb labels arborization field 9, more dimly than in comparison to the Ath5:GFP line (see overlap in d.)

d. bright labeling of all of the RGC axon bundles, AF10 and AF7, and dimmer expression of Ath5 2kb in AF9. It is still unclear whether AF9 receives more dendrites than the other AFs and is therefore labeled to a lesser extent, or because the 2kb do not cover a population of RGCs labeled in the 7kb enhancer fragment.

OT – optic tectum, AF – arborization field, ab – axon bundles

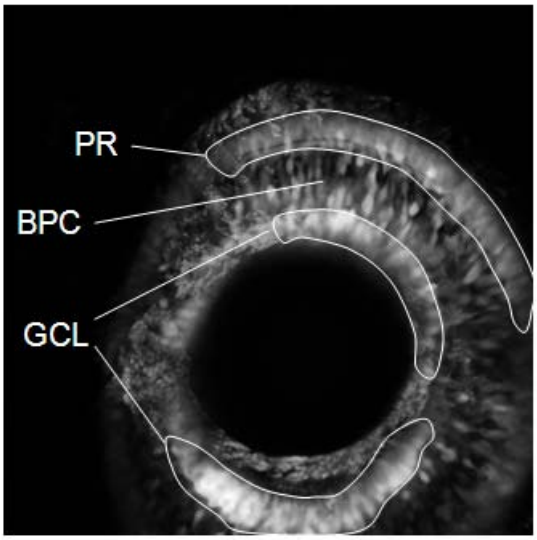


Fig. 5 RGC cell bodies labeled by Ath5:GFP

A z-stack of 50µm with a lateral view of the eye shows labeling of different retinal cells of the Ath5:GFP line. The inner circle shows retinal ganglion cells, the outer circle shows photoreceptors and in between labeling of bipolar cells can be seen.

GCL – ganglion cell layer, PR – photoreceptors, BPC – Bipolar cells

Imaging with the retina facing the objective shows that most of the RGCs, if not all, in Ath5:GFP are labeled, but also bipolar cells and photoreceptors (Fig.5). The reason why I was not able to compare the expression pattern of Ath5 2kb with Ath5:GFP was that for imaging of mCherry through the retina the power of the laser was not high enough for excitation (also mentioned later in detail).

*Activated leukocyte cell adhesion molecule a (Neurolin-a, **alcam-a**)*

Alcam-a is a protein of the immunoglobulin superfamily with functions in axon growth and guidance [23]. Onset and progression of alcam-a expression parallels the pattern of RGC differentiation. In mature neurons alcam-a is only expressed at RGC cell contact sites and synapses, at earlier developmental stages it is expressed all along RGC

axons. Alcam-a is also essential for RGC survival and for the differentiation of all other retinal neurons.

A 10 kb fragment spanning the 5' untranslated region in front of the ATG start codon showed to label to some extent RGCs, two putative arborization fields, the ocular muscle, a population of cells in the midbrain of unknown identity and reticulospinal neurons (Fig. 6).

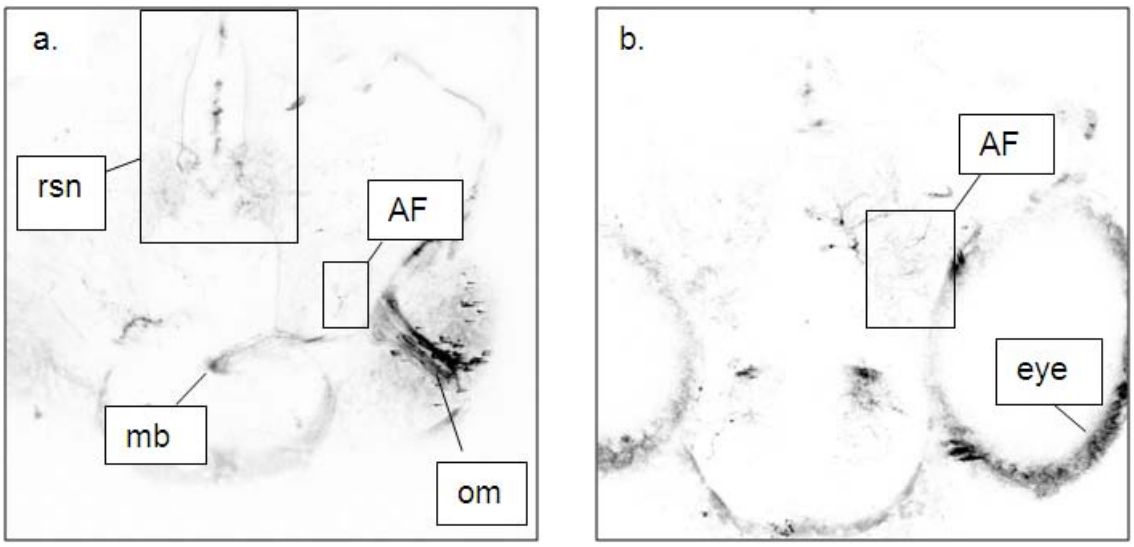


Fig. 6 expression pattern of transient alcam-a 10kb:mCherry

Images are depicted in inverted luminance for visual clarity.

a. The occular muscles can be seen in the left eye. A population of neurons in the midbrain and reticulospinal neurons and a putative arborization field of RGCs are depicted

b. A more ventral view shows another putative arborization field and a population of cells on the midbrain-hindbrain border, just next to the putative arborization field.

om – ocular muscle, rsn, reticulospinal neurons, mb – midbrain, AF – arborization field

*POU domain, class 4, transcription factor 1 (pou4f1, **brn3a**)*

Members of the class IV POU domain transcription factors were all shown to be involved in retinal ganglion cell development. Brn-3b (Pou4f2, Brn3.2) and Brn-3c (Pou4f3, Brn3.1) are essential for the normal differentiation and maturation of RGCs and Brn3c is also involved

in the expression of hair cells of the auditory system [24]. The expression pattern of a 5kb as well as the 3kb enhancer fragment of Brn3a was quite broad, including most of the tectum [data not shown], and no specific RGC labeling was seen in this enhancer fragment. The PCR reaction was done using as a template Tg(brn3a-hsp70:GFP) [24], described in materials and methods.

*POU domain, class 4, transcription factor 2 (pou4f2, **brn3b**)*

Brn-3b is highly expressed in the developing retinal ganglion cell layer and in the optic tectum [25]. None of the Bacs PAC clones BUSMP706A1597Q2 and BUSMP706N19174Q2 described, containing the enhancer fragments of Brn3b are available anymore. Therefore I tried to make a pcr reaction from genomic DNA to get the Brn3b sequence (see materials and methods).

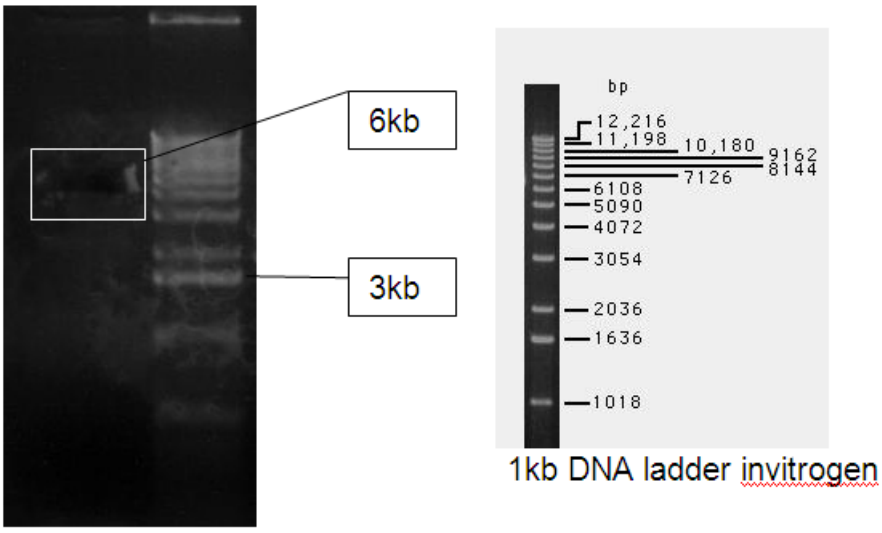


Fig. 7 Gel showing a 6kb PCR fragment of Brn3b(cut out)

Although the PCR fragment had the right size (Fig.7), I was unable to create any entry vector, most likely because of the low quantity yield of

the PCR. Multiplying the purified PCR fragment by PCR again, resulted in higher quantity of the PCR fragment but apparently not enough to allow for a recombineering reaction to get the PCR fragment into the entry vector.

*POU domain, class 4, transcription factor 3 (pou4f3, **brn3c**)*

Brn3c was shown to label one subset of RGCs that projects into one of the four retinorecipient layers of the tectum and into a small subset of the extratectal arborization fields [26]. The 6 kb enhancer fragment I used had the same plasmid Brn3c:GFP as template as described before. The labeling showed mostly hair cells and parts of the tectum, neither any ganglion cells were labeled nor AF-6, AF-7, AF-8 or the optic tectum, the arborization fields that should be labeled [data not shown]. One explanation is that it depends where the construct is integrated into the genome to get a specific expression pattern. I used the same enhancer fragment starting from the BspEI restriction enzyme site at the 5 prime end and the translation start at the 3 prime end as described [26] but each PCR reaction can make mutations into the enhancer fragment.

heparan sulfate 6-O-sulfotransferase 1b (hs6st1b)

Expression of the hs6st1b promoter at 48 hpf can be seen in retinal ganglion cells [27]. mRNA in situ hybridization with antisense riboprobes specific to hs6st1b, showed labeling of the RGC layer (Fig. 1). I tried a 5kb long enhancer fragment for its expression pattern. The expression was either not strong enough, or not in a population visible under the fluorescent scope (20 fold magnification), or most plausible did not contain the right enhancer.

slit homolog 1a (slit1a) and roundabout homolog 2 (robo2)

Slit-Robo signaling is known for retinal axon guidance but also plays a later role in mediating retinal ganglion cell arborization and synaptogenesis [28]. robo2 is expressed in RGCs as they navigate toward their main target, the optic tectum. Slit 1 is weekly expressed in RGCs and strongly expressed in the tectum.

I tried both gap repair to get 10kb and enhancer fragments of 5kb length. For Slit1a it was very hard to find a BAC containing the enhancer fragment because of the different annotations in the USCS university of california genome browser and the ensembl genome browser. I did not succeed in getting any PCR products, possible because of a wrong annotation and therefore I was not able to test their expression pattern, so far. Different smaller enhancers of Robo2 did not show any expression patterns and gap repair did not work because of recombineering of the template with its own ends.

integrin, alpha 6 (Itga6)

Searching the zfin database [3] for proteins that show expression patterns, mostly in RGCs, I also discovered an integrin which is expressed in RGCs. Enhancer bashing and injection however did not lead to any results.

dynactin 1a (mok, dctn1a)

Retinas of *mok* mutants have an expanded ganglion cells layer [29] and dynactin 1a was shown to be expressed to some extent in the ganglion

cell layer. 5kb and 6kb long enhancer fragments did not show any expression pattern of interest [data not shown].

*Purkinje cell protein 4a (p*cp4a)

Pcp4a is a calmodulin binding protein and it is expressed in the central portion of the ganglion cell layer in the retina [30]. I tried 2 kb, 4kb and 5kb long enhancer fragments, all without success. Also the gap repair did not work. The bacteria contained a plasmid mediating resistance but apparently it recombineered with itself because the size of the plasmid was wrong. Both primers (as well as all the other primers that I used for gap repair) have shown to have a strong similarity given that 14 bp (ttgtacaaagttgg) of the primer contain the same sequence because of the AttL recombineering site (see materials and methods).

I therefore tried different primers using parts of the Ath5 enhancer fragment as spacer instead of bp contained in the AttL recombineering site for primer design to get reduced recombineering with itself. All of these homology arms were not able to fetch the 10kb enhancer fragment, I still got recombineering with itself.

Junctional adhesion molecule B (JAM-B)

Since enhancer fragments can be conserved between species, and there is little information and incomplete sequences about JAM-B in the database and no BACs containing a putative enhancer available, I tried to inject the promoter described by Kim *et al*.[12]. This enhancer fragment drives the expression of CRE-ER and CRE-ER is integrated randomly into the BAC, so there is not much information about the putative enhancer sequence. Therefore I got the construct from In-Jung Kim. The BAC is floxed, therefore I had to get rid of the flox sites in order

to inject the BAC. BAC transgenesis efficiency is quite low for getting a possible germline integration. But in my case I was just interested in transient expression to see if this enhancer fragment in principle could drive the expression of a population of neurons at all. To test this I used a transgenic zebrafish line Tg(eab2:[EGFP- T-mCherry] expressing loxP mCherry which switches to expression of GFP if CRE Recombinase is induced by Tamoxifen mediated by expression of the BAC in a certain population of cells (see materials and methods). I could not see any changes in fluorescence, therefore the enhancer is most likely not driving expression in zebrafish.

Destination vectors mediating expression patterns in RGC populations

Lyn-mCherry

To test all the mentioned enhancer fragments above. I made use of a lyn-mCherry reporter that labels the cell membrane. The idea was to first assess the expression pattern in a reporter and to grow up the fish which show expression in cells of interest. Once they have stable germline integration, they can be crossed to panneuronaly expressing PA-GFP fish to photoactivate the processes of neurons of interest and see their putative interaction partners.

To evaluate calcium signaling in this population of cells, a possible approach would have been to cross the fish expressing mCherry in RGCs directly to a existing panneuronal expressing GCaMP2 line under the Huc promoter to immediately study activity. But since the spatial resolution of the two photon microscope is possibly not high enough to distinguish signals in axons from the nearby dendrites, I started creating

fish expressing GCaMP3 and synaptophysin GCaMP2 under the Ath5 2kb enhancer and the 10kb alcam-a fragment. Furthermore I created a synaptophysin GCaMP3 destination vector version by exchanging GCaMP2 with GCaMP3.

A pitfall of mCherry is that its excitation wavelength with the 2 photon has its maximum at 1040nm. The laser in contrast cannot go beyond 1040nm and its power is strongly reduced at its maximum wavelength. After testing the imaging quality of simultaneous imaging of GFP and mCherry, 980 nm turned out to be the best wavelength to get most efficient emissions from both mCherry and GFP (which has its excitation maximum with the two photon at 920nm). For this reason I worked also on an alternative red fluorescent protein dTomato, which has been shown to have a lower excitation wavelength than mCherry [31]. dTomato namely has its excitation maximum at 554 and mCherry at 587nm (for one photon excitation).

Synaptophysin GCaMP3

To access different signals from axon terminals by calcium imaging, I cloned synaptopyhsin GCaMP2 into a destination vector. Driving this destination vector with the Ath5 2kb enhancer fragment and injections of the construct did not show any transient expression. Synaptopyhsin GCaMP2 should label only axonal terminals and the expression therefore might not be high enough to see it under the fluorescent scope with the low numerical aperture that I used for screening. Therefore I used an expression system with a self-cleaving 2A-Peptide [32] in which dTomato is coexpressed with a brighter version of GCaMP, synaptophysin-GCaMP3. I have chosen dTomato as mentioned before,

because it is a putative better alternative for two photon imaging to mCherry because of its lower excitation wavelength.

To test if a self cleaving 2A-Peptide is working in zebrafish I created a destination vector dTomato-2A-syGCaMP3 (see materials and methods) under the 2kb enhancer fragment. In order to access if the 2A-peptide works in general and to test it at a broader expression level I used the panneuronal promoter Huc to express dTomato-2A-syGCaMP3. dTomato was expressed in most of the neurons, as is the case for the Huc promoter, but there was no GCaMP expression [data not shown].

GCaMP3

GCaMP3 is an improved GCaMP calcium indicator which was not tested yet in zebrafish [16]. Injections of the GCaMP3 destination vector driven under the Ath5 2kb fragment did not show any transient expression. To test if higher expression levels of the construct could show transient expression, I used the Gal4 UAS system. By means of the gateway system I recombineered a GAL4FF destination vector under the Ath5 2kb enhancer fragment and created another destination vector containing UAS mCherry to test for the expression pattern before using UAS GCaMP3. Coinjection of both constructs (Ath5 2kb Gal4FF together with UAS mCherry) have shown to broaden the expression pattern and exposed non-specific labeling [data not shown]. The other approach that allows testing for higher expression levels after integration of GCaMP3 into the germline is described below. As transient expression of the Ath5 2kb GCaMP3 is invisible, it does not allow selecting for successful injections. This was achieved by means of identifying transient expressing larvae using a cmcl2 heart GFP marker that is coexpressed after successful injections and germline integration.

Cmcl2 heart GFP, a marker used for screening of transgenes

The idea of using a marker that labels the heart with GFP very brightly was to coexpress the marker and designing a system that allows easy identification of successful injections and later on fast screens in the G1 for successful germline integration. For the following destination vectors Gal4FF and GCaMP3, I created plasmids attached with a sequence encoding cmcl2 heart GFP in antisense direction (Fig. 8). All the cloning steps are described in Materials and Methods.

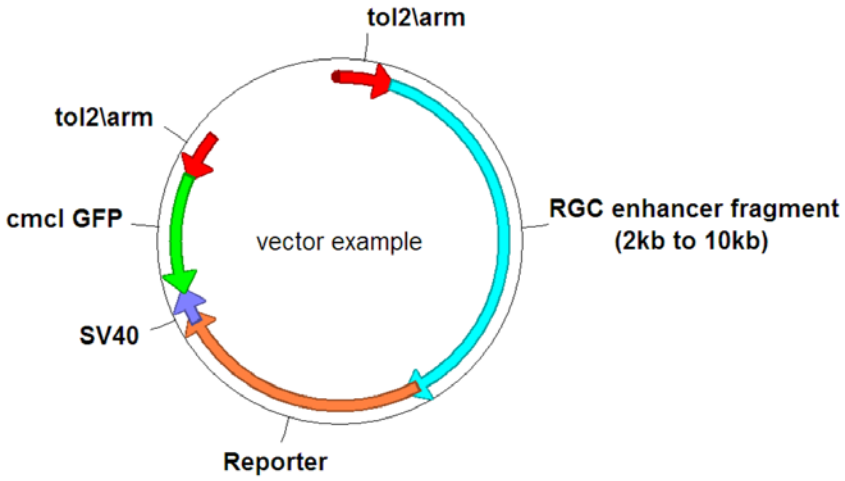


Fig. 8 Tol2 destination vector example containing a cmcl2 GFP marker (arrow shows direction of transcription)

Plasmid destination vector, containing the RGC enhancer fragment driving the expression of either reporter (Gal4FF or GCaMP3) with a SV40 polyadenylation signal. Cmcl GFP is transcribed independently of the other reporter in the antisense direction. The tol2 arms are necessary for successful integration into the genome

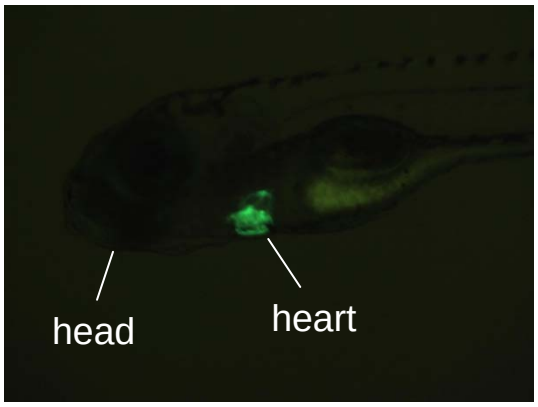


Fig. 9 F1 with stable germline integration expressing GFP in the heart exclusively

Lateral view of the transgenic zebrafish larva Ath5 2kb GCaMP3 cmcl2 heart GFP. GFP is expressed brightly in the heart, but no expression of GCaMP in RGCs.

After injection of Ath5 2kb GCaMP3 cmcl2 heart GFP growing up of larvae showing transient labeling of the heart, screening of 4 founders (400 eggs) demonstrated that most of the fish expressed GFP very brightly in the heart (brighter than the transient expressing parents), but there was no expression of GCaMP3 in any of the larvae. The problem and why this system is not applicable might be that cmcl heart GFP is expressed, and apparently inhibiting the transcription of the reporter under the enhancer fragment. Even after waiting for a generation, there was not any correspondence between the expression of cmcl2 heart GFP and the integration of GCaMP3 labeling RGCs in the germline (Fig. 9). Since I was not able to detect GCaMP3 expression before under the Ath5 2kb promoter, it is still unclear if the system is not working and expression is suppressed by the cmcl2 promoter or if GCaMP3 expression is too low to detect under the dissection scope.

Photoactivateable m-Cherry

For simultaneous calcium imaging in green and anatomical studies of the cells by photoactivation in red, I cloned photoactivateable (PA)-mCherry into a destination vector. To create a transgenic fish that labels most of the neurons I put the destination vector under an alpha tubulin 1 enhancer fragment. Alpha tubulin 1 is known to label most of the neurons

[32]. This enhancer described cannot be used within the Gateway system, since it includes the first exon and intron of alpha tubulin1. So the strategy was to make a pcr reaction directly from the plasmid containing the alpha tubulin enhancer fragment (see materials and methods). PA-mCherry will be extremely useful for simultaneous calcium imaging of driven GCaMP (in green) and to unravel the anatomical connection of a cell that is activated during a certain behavior.

Photoactivation of Photoactivatable GFP in different arborization fields, the hindbrain and the midbrain

Arborization Field 7 and its putative pretectal connection partners

The 2kb Ath5 mCherry line labels RGC axons in different arborization fields. One of these arborization fields is nicely labeled in the 2kb Ath5:mCherry line and was described as arborization field 7 (AF7) [4]. It is still unclear in which behaviors AF7 is involved. Ablation studies have shown that AF7 is putatively not involved in the optomotor response [13]. Crossing of the 2kb Ath5:mCherry line to a panneuronal expressing photoactivateable GFP line under the alpha tubulin promoter allows to specifically photoactivate the dendrites sent into one arborization field. Aiming for the RGC axons and photoactivation of nearby dendrites show that there are two populations of cells that are putatively connected with the RGC axons in AF7 (Fig.10a,b and d). One of this populations are tectal cells that sit right on top of the arborization field. The other populations are pretectal cells near the midline that send their long processes into the arborization field (Fig. 10d). It is still unclear whether the tectal cells receive information or modulate the signal because photoactivation does not tell if the photoactivated processes are dendrites or axons. Nevertheless the results are interesting because they

show that there are just a few cells that are involved in receiving or sending signals into the arborization field which can be tested. Therefore future experiments that involve electroporation of the labeled cells either with retrograde transported virus to see in which direction the information flow goes, and together with red calcium indicators will show their involvement in testing a series of different behaviors.

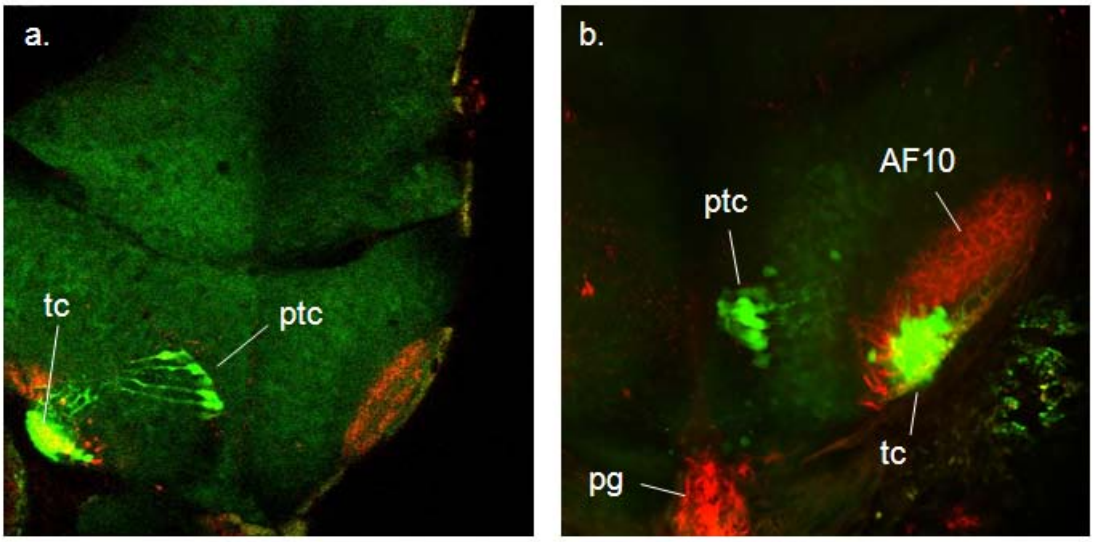
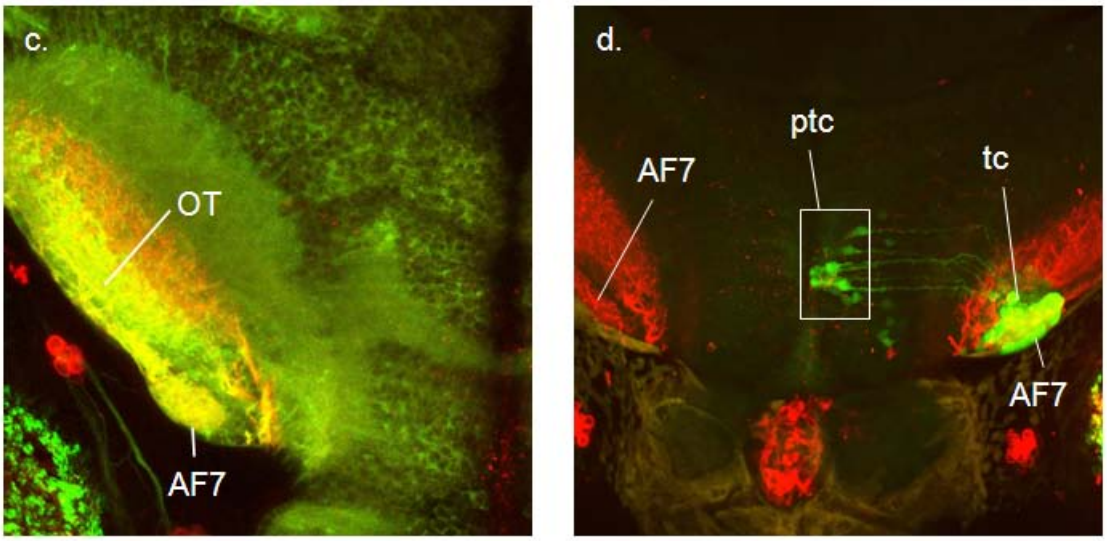


Fig. 10 Ath5 2kb: mCherry, alphatubulin:PA-GFP: Photoactivation of AF7 (a,b, and d)

a. Larvae(5 day old fish) faces rostral and is tilted to the right side. Photoactivation in the arborization field 7 shows constant and reliable labeling of the same two populations of cells – tectal cells and pretectal cells,

b. 4 day old straight fish, same two populations of cells are lightening up after photoactivation, Ath5 2kb:mCherry also labels the pineal gland and AF10.



c. Ath5 2kb mCherry in comparison to Huc:YC2.1,

overlap of AF7 and the optic tectum. Huc:YC2.1 labels the dendrites and axons (dense) in the optic tectum and all arborization fields, as well as tectal and pretectal cells.

d. Another overview of the rostral part of the fishbrain. Pretectal cells are framed. Tectal cells in green, more ventral AF7 (in yellow) next to the left eye, both photoactivated, in comparison to non-photoactivated AF7 next to the right eye.

ptc – pretectal cells, tc – tectal cells, AF – arborization field, pg – pineal gland, OT – optic tectum

Arborization Field 9 and its putative pretectal connection partners

Arborization field 9 is much harder to photoactivate, first since it is more dimly labeled and second because of its anatomy (eggplant shape – see Fig. 4c,d). I tried multiple photoactivations starting from the rostral end. Fig.11 demonstrates the first phototactivation of the rostral end of AF9 labeling two pretectal cells next to the midline.

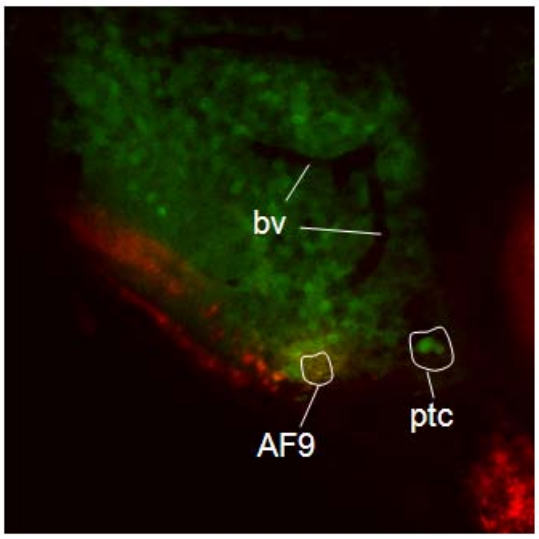


Fig. 12 After Photoactivation of AF9 (high contrast)

After photoactivation of a small lower part (in yellow) of arborization field 9 (see Fig 4c and d for structural detail), two pretectal cells light up near the midline. The two black lines are blood vessels.

AF- arborization field, ptc - pretectal cells, bv- blood vessels

In principle it is possible to photoactivate one candidate cell in the pretectum again, to see both its processes, axon and dendrites, and see the other projection field than the RGC arborization field. This method for assessing candidate cells can also be done by photoactivation with PA-mCherry, to see directly if the pretectal cells labeled in a panneuronal expressing calcium indicator line is also active during a specific behavioral setup that will be tested. Another approach to see if this pretectal cells is connected to a specific reticulospinal neuron downstream that transfer information from the brain to the spinal cord, is photoactivation of these reticulospinal neurons to see with which pretectal cells they might be interconnected.

From the spinal cord to the arborization field

Several reticulospinal neurons are involved in the opto motor response: the. NucMLF in the midbrain is active during forward swimming, the V-cells in the hindbrain are active specifically during turning, but also during forward swimming [7]. To label these populations, I injected Texas red dextran (invitrogen) into the spinal cord. Texas red dextran will label

most of the neurons that project into the spinal cord (therefore most of the reticulospinal neurons) [7].

Injection of Texas red dextran into a Huc:YC2.1 background gives a great anatomical map of the position of the reticulospinal neurons in the hindbrain and midbrain compared to the arborization fields and pretectal and tectal neurons.(Fig. 12 a. and b.)

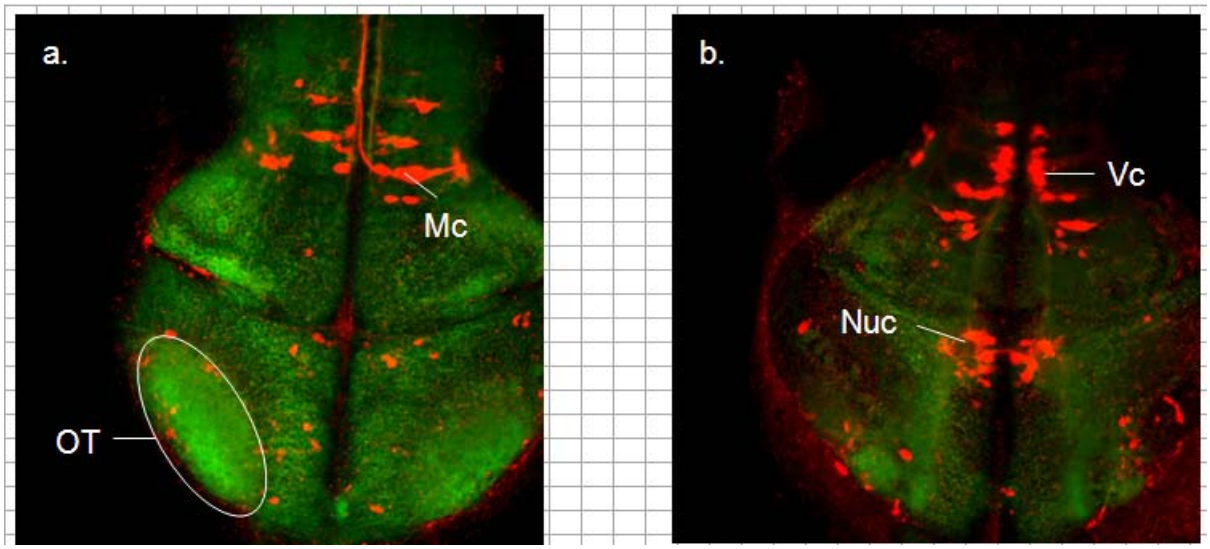


Fig. 12 Huc:YC2.1 injected with Texas red dextran into the spinal cord

a. A more dorsal view of the brain shows the optic tectum in green (framed oval), and the Mautner cell and its homologs in red in the hindbrain.

b. a more ventral view: in red: V-cells in the hindbrain(caudal) and NucMLF in the midbrain (rostral).

OT – optic tectum, Mc – Mautner cell, Vc – V-cells, Nuc - NucMLF

To test putative interaction partners I injected Texas Red Dextran into larvae expressing photoactivateable GFP under the alpha tubulin promoter. Since I was the first to try photoactivation of single cells in zebrafish, I needed to establish a protocol that tells how much laser power, for how long, in which wavelength, and in which volume of the cells allows for single cell activation (see materials and methods). For

this purpose I started with the biggest cell available, the Mautner cell. Fig. 13a shows photoactivation of the right Mautner cell, in comparison to photoactivation of the left V-cell. Both cells are nicely labeled showing the axons projecting to the spinal cord.

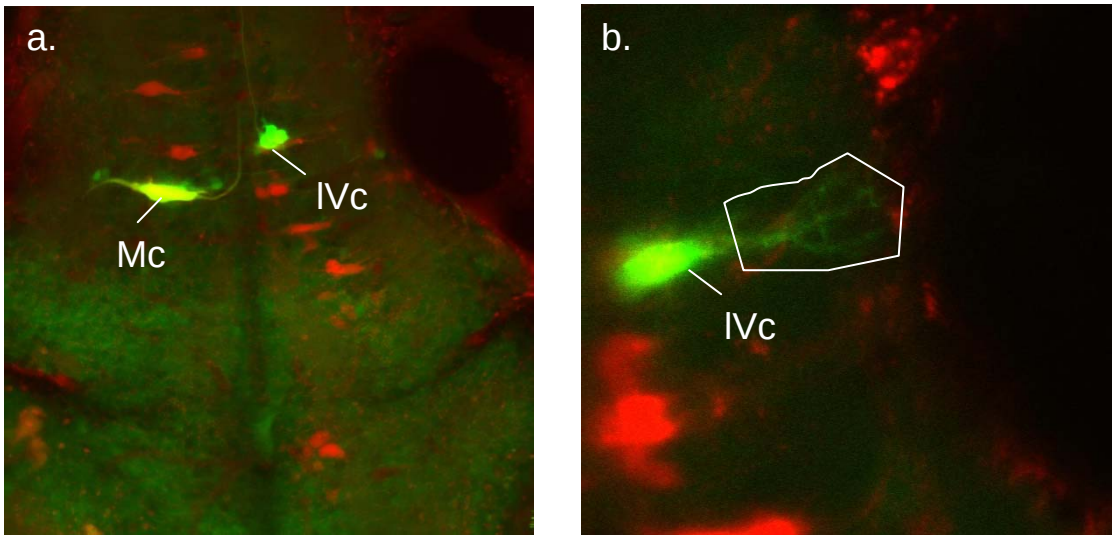


Fig. 13 Single cell photoactivation after injection of Texas red dextran to label spinal projection neurons

a. Photoactivation of the Mautner cell (big cell on the left): axon is going caudal to the spinal cord, the dentrites bundle is going to the lateral side) These results shows that the protocol that I developed allows for single cell labelling and its dentrites as well its axons by diffusion of the GFP. Photoactivation of the V-cell (on the right)

b. photoactivation of the left V-cell (in another larvae) The dendrites are nicely labelled (axon is also labelled but because the image is taken in another z-level, it cannot be seen). The framed region depicting dentrites is seen in Fig.14 in higher magnification.

Mc – Mautner cell, lVc – left V-cell

The next step is to find putative upstream neurons in the midbrain, or pretectum by photoactivating dendrites. Photoactivation with the two photon makes it possible to photoactivate certain parts of the dendrites and to see if they are connected to any axons. One example of

photoactivation in V-cells can be seen below. In this case photoactivation in this part of the dendritic tree did not lead of the labelling of any nearby axons.

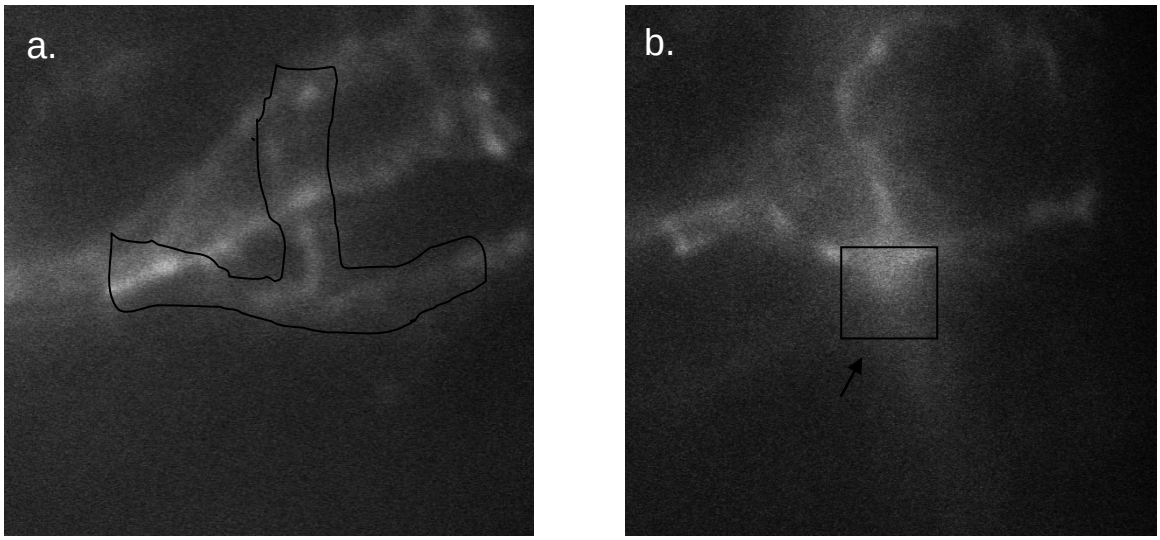


Fig. 14 dendrites of the V-cell after photoactivation of the cell depicted in Fig. 13b

a. Zoom in into the dendrites depicted in Fig.13b (all images were taken with low resolution and with as less power as possible to avoid possible photoactivation

b. 2nd photoactivation (marked with the arrow) in the middle of the depicted "T-branch" framed in **a.**

Another example of photoactivation where I could identify putative connection partner by photoactivation, is the MeLc in the NucMLF, involved in forward swimming of larvae (Fig. 15).

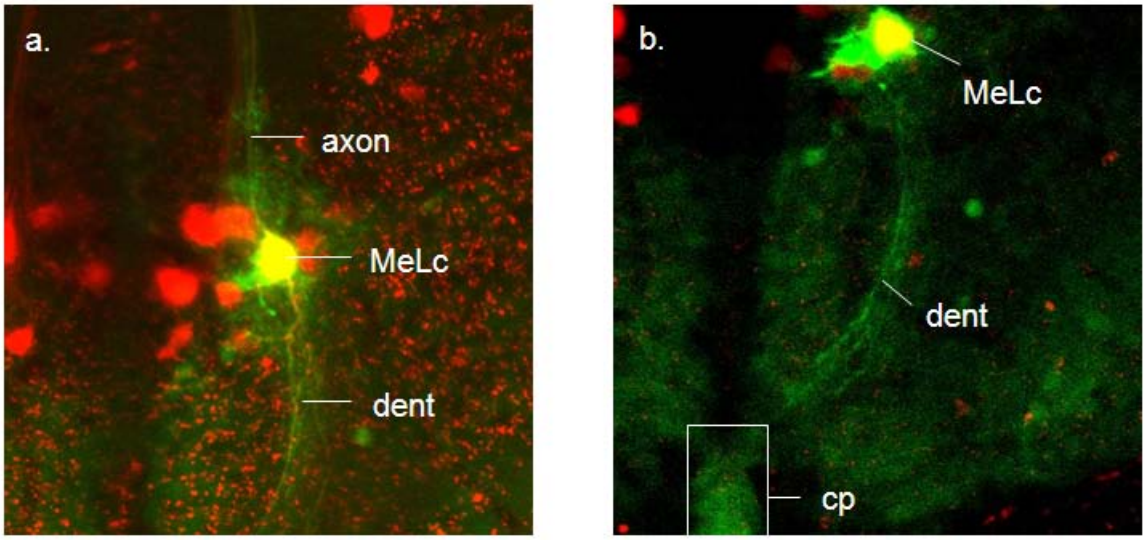
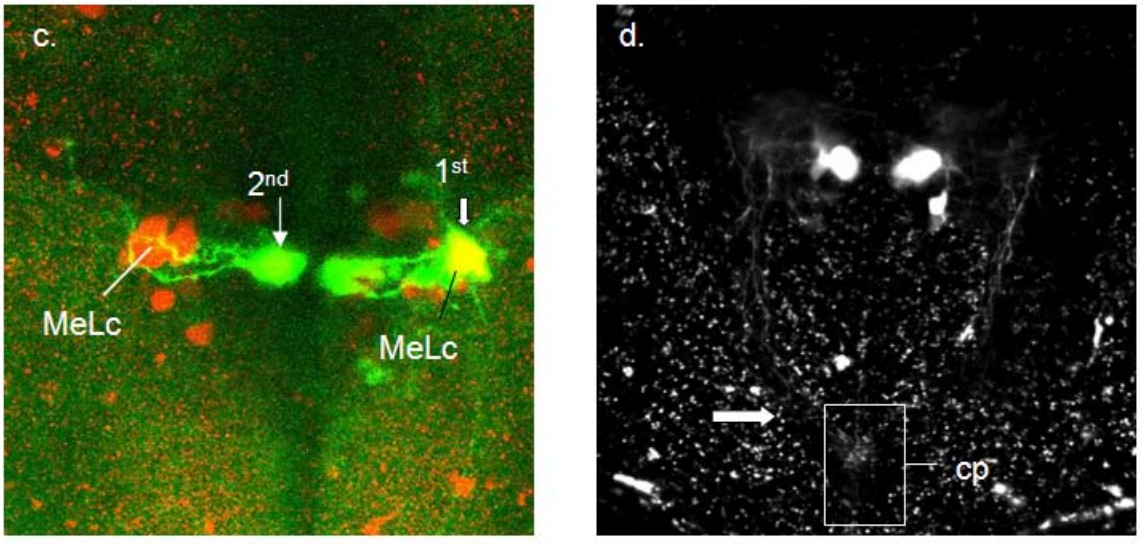


Fig. 15 Photoactivation of NucMLF and putative connection partners in the pretectum

a. photoactivation of MeLc showing its axon and dendrites,

b. NucMLF and its putative dendrite projecting into an anatomical structure that seems to act as a center piece connecting both sides of the brain.

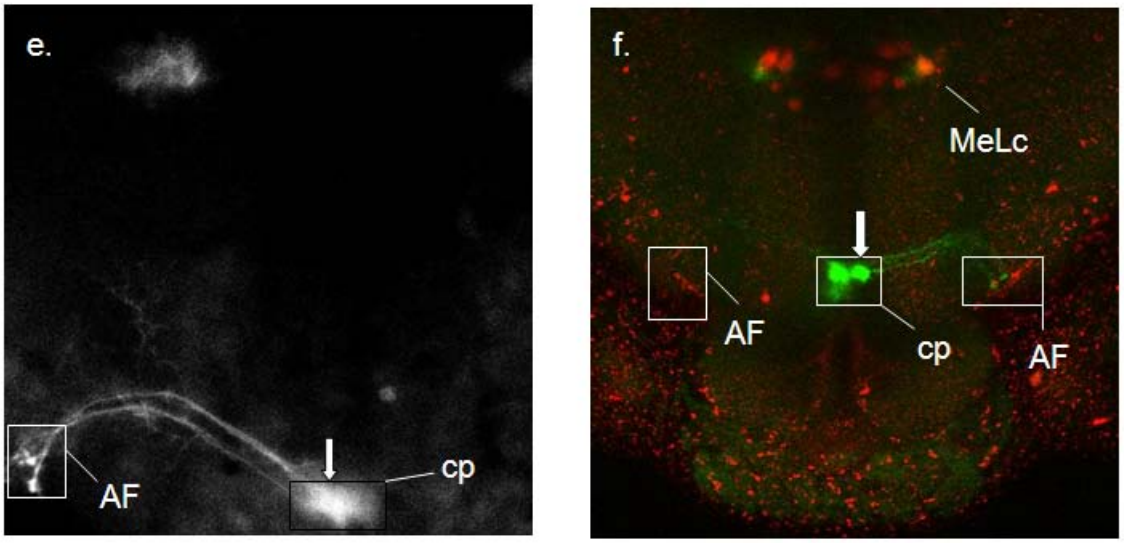


c. Zoomed in image after 2nd photoactivation (same cell as in **b.** but z-stack)

1st photoactivation of MeLc in the NucMlf has labeled a candidate cell (lateral yellow cell).

2nd photoactivation of the same candidate cell on the contralateral side shows that it might send its processes to the cell body of MeLc. Also suggested is that MeLc sends dendrites to the pretectum.

d. Texas red dextran staining labels the dentrites of the NucMLF that are projecting into the center piece (marked by the arrow)



e. Photoactivation (marked by arrow) of the dendrites in the center piece labels two candidate cells that seem to project into one arborization field.

f. After photoactivation of the controlateral MeLc (marked in 15 **c**) at the dendritic ends of both MeLs (dentrites are depicted in **d**., one dendrite going rostral can be seen in **a.** and **b.**), photoactivation (marked by arrow) shows another two candidate cells on the contralateral side that seem to project into the same arborization field on the other side.

dent – dentrites, MeLc - Medial lateral caudal cell, cp – center piece, AF – arborization field

By photoactivation of the NucMLF dendrites I identified cells in an anatomical structure that seem to act as a center piece connecting both sides of the brain (Fig. 15b). These cells within the center piece (also on the controlateral side) seem each to project into a RGC arborization field (Fig 15f).

I also photoactivated larvae of the Ath5 2kb mCherry genotype crossed to alpha tubulin PA-GFP. In this double transgenic fish I injected again Texas red dextran. I compared photoactivation of the NucMLF and its possible connection partners in the center piece to RGC axon

terminals, to see if this group of cell is going into a specific arborization field.

Since Texas red dextran is very bright, the PMT (Photon multiplyer tube) has to be run with lower power (lower gain). This though does not allow to make high resolution stacks with mCherry, because Texas red dextran has a lot of unspecific background (also seen in Fig 16f.). Therefore I could not depict any results that I got, showing that these cells indeed seem to project into one of the arborization fields. Injection of dyes (in different color) or not as bright as Texas red dextran, or with a high molecule weight version of Texas red (that apparently does not lead to background staining) will allow for high resolution images.

Discussion

The first goal of my diploma thesis was to characterize enhancer fragments that label RGCs, and in the best case subpopulations of ganglion cells. Therefore I tested several enhancer fragments for their expression pattern and showed that a small enhancer fragment of Ath5 can drive populations of retinal ganglion cells (Fig. 4c and d). It is still difficult to assess which fragment is the right one, how long it has to be to drive expression and what is the necessary length that it is still specific and labels the population of interest as described for the whole promoter. As there are regulatory elements in a certain range of the entire promoter, it is necessary to try different enhancer fragments of size. In cases in which the tested enhancer fragments did not show any labeling, as was shown for the Ath5 4kb and Ath5 5kb element, the fragments might have contained a negative regulatory element inhibiting expression.

Also different sizes of the pcp4a enhancer fragment that I tested, did not show any expression, in that case it might be that the enhancer alone is too weak to drive expression and an alternative approach by driving the enhancer element under a minimal promoter like cfos could show some expression level. Larger enhancer fragments were tested for expression and for specificity in labeling RGCs. The Ath5 7kb enhancer fragment is able to express in a broad and less specific population of cells, namely in tectal and ganglion cells, compared to a 2kb enhancer fragment of the same promoter that labels only RGCs (Fig. 4 c and d). It was the case for several tested enhancer fragments of 10kb size (pcp4a, robo2) that recombineering using gap repair to get longer enhancer fragments showed self-recombineering and no entry vectors could be

generated. The reason for self recombineering is still unclear, because gap repair with similar primers worked for alcam-a to get a functional 10kb long enhancer fragment which resulted in labeling of RGCs (Fig. 6). Unspecific labeling was shown for Brn3a and Brn3c enhancer fragments and originates most likely from missing of negative regulatory elements within the enhancer or integration in the wrong site of the genome.

At this point enhancer bashing together with Gal4 enhancer traps are the best genetic methods available to find and label populations of interest in zebrafish. There is still no homolog recombineering or site-specific integration [39] available to the zebrafish toolbox. As could be seen within my thesis, 2 fragments out of at least 30 (also considering size) different fragments tested, showed labeling of RGCs. Because expression depends on regulatory elements within a certain enhancer fragment but also on the position of the genome where the enhancer element integrates, sometimes plenty of injections are necessary to get the integration of the construct in the right position to see the expression of interest. To work with site-specific integration would provide a remedy, getting the same integration site (one that is known for strong expression) for every injected construct. Such techniques are available in other organism [39], but have not yet been developed for zebrafish.

In order to assess different expression patterns of putative RGC enhancer elements I worked with the red fluorescent protein mCherry as a reporter. My results demonstrate that mCherry can be used for simultaneous imaging with green fluorescent proteins under the two photon microscope (e.g. Fig. 4b). But it also showed its limitations, e. g. imaging through the eye of the zebrafish. Trying to detect how many RGCs and their cell bodies are labeled within the Ath5 2kb mCherry line, did not work because of restrictions of the laser power. For this reason

the generation of a dTomato destination vector was a reasonable alternative as for having a red fluorescent protein that shows a lower excitation wavelength and therefore less laser power is needed for excitation. Even simultaneous imaging with green fluorescent proteins can be done closer to the excitation maximum of GFP.

Another reporter system that I tested involved a calcium indicator only expressed at synapses synaptophysin GCaMP3. None of the tested enhancer fragments showed any expression. The reason was that the vector I worked with coded for a rat-synaptophysin instead of the zebrafish synaptophysin. Apparently synaptophysin is not conserved enough between species and therefore it is not expressed at axon terminals. Exchanging the synaptophysin in the destination vector will allow testing of the calcium indicator.

The other calcium indicator tested was GCaMP3. Expression of GCaMP3 under the Ath5 2kb enhancer did not work. The first possibility why GCaMP3 was not expressed, might be that it is to dim to see it in a sparse population of cells that I drove with my enhancer fragment. The second possibility is that the expression level was not high enough. On this account I used the UAS-Gal4 system and a labeling method that involves cmcl2 heart GFP. The UAS-Gal4 system was a setback as it broadened the expression pattern. Also after selection for germline integration with the cmcl2 heart marker, GCaMP3 expression was not detectable (Fig. 9). GCaMP3 is a calcium indicator that was developed for worms, flies and mice [16]. Therefore there is the risk that this calcium indicator does not work in zebrafish. Given that there are already other versions of GCaMP better than GCaMP2, and different to GCaMP3, these calcium indicators will be the next candidates to test for their functionality and expression level in zebrafish.

The marker I used to test for coexpression, cmcl2 heart GFP, corresponded to successful injections and germline integration but there was not any coexpression with GCaMP. It might still be extremely advantageous in scanning through thousands of eggs, because the bright GFP expression in the heart should correspond to a successful integration of the construct of interest in the germline, especially for constructs containing only a very dim expression of any kind of GCaMP. Moreover in many cases different enhancer fragments might only label a subset of neurons, which might not have been detected easily. Testing the cmcl2 heart-GFP together with another enhancer fragment or under another reporter that shows broader and easier detectable expression should show if the cmcl2-heart-GFP marker system works in principle. On the other hand there is no need for this system if expression patterns can be easily detected also without it. So far my results show that this system at least in certain cases is not applicable for fast screening of coexpression.

The second part of my diploma thesis consisted of analyzing putative connection partners to the RGCs that are labeled by the Ath5 2kb:mCherry line. Ath5 2kb:mch labels at least AF9, AF7, and the optic tectum (Fig 2c), the biggest arborization fields that can be easily detected. Within the second series of experiments I showed that photoactivation of dendrites and photoactivation at the single cell level is a good approach to identify candidate cells interconnected and possibly underlying a common neuronal circuit. I demonstrated that arborization field 7 is possibly connected with two different regions in the brain, one is the nearby tectum and another is the pretectum innervated by long processes sent from AF7. Furthermore I identified two cells that putatively project into arborization field 9. All these identified pretectal

cells are the most likely candidates to project to the reticulospinal neurons downstream.

To complete the circuit from the other end I showed possible interaction partners in the pretectum that are connected with the processes of distinct subsets of reticulospinal neurons. By means of spinal cord injections with Texas red dextran I labeled reticulospinal neurons that have been demonstrated to be involved in the OMR. Photoactivation of one of the V-cells, and subsequently a part of its dendrites, did not label any other cell processes. Activation of different parts of the dendrites therefore need to be tested, and there also two more V-cells [7] that can be photoactivated to look for their connection partners.

After photoactivation of the NucMLF, I mapped out in detail putative connection partners that are interconnected with the MeLc and send their projections into a possible RGC arborization field. I demonstrated that photoactivation with a two-photon microscope can be done in a very small volume of the cell, or even in the branching of a dendrite and therefore shows reliably the same labeling, depending on the position of photoactivation *in vivo*. This method allows to photoactivate a single cell of interest and by diffusion its axon and dendrites. All these results show that photoactivation of a photoactivateable fluorescent protein is a good approach to get a picture of the cells involved in a certain circuit. Once the candidate cells are identified, they can be tested in functional imaging and their role in the animal's stimulus-response function can be uncovered.

The photoactivation approach is very useful to demonstrate where and how the signal from the RGCs might be transferred to the tectum and

pretectum, and to classify candidate tectal and pretectal cells that send their projections into the arborization fields. Subsequent experiments will give predictions of the different cells receiving and sending information from and to the arborization field by analyzing results from anatomical studies in combination with the correlation of functional data obtained from calcium signals in RGC axon terminals. For this purpose I will make use of the vector that I cloned with the photoactivateable(PA)-mCherry under the alpha tubulin promoter to generate transgenic fish. The alphatubulin1:mCherry line will label most of the neurons and allows for simultaneous calcium imaging of identified candidate cells. Photoactivation of the neurons in the pretectum that show activation (measured via GCaMP), and following the axons and dendrites, will suggest if they are connected to specific arborization fields. Functional connectivity between such arborization fields and the photoactivated pretectal neurons can then be tested by correlating the calcium signals in the ganglion cell-axons (measured with syGCaMP2), with GCaMP2 calcium signals in pretectal somata. To this end I will make use of the Ath5 2kb enhancer element to drive expression of synaptophysin GCaMP in RGC axon terminals.

It is still unclear if single axonal terminals of RGCs already contain direction selective activity, or if direction selectivity arises later in the tectum and pretectum. So far there is no proof that there are direction-selective ganglion cells in the zebrafish retina, and direction selectivity could arise at a later stage, for instance in the tectum. The RGC enhancer element Ath5 2kb that I uncovered will allow for the first measurement of RGC direction selectivity in zebrafish.

In future I will use the established enhancer fragments driving calcium indicators in different types of RGCs to test a set of different behaviors.

The first step before testing different behavioral setups will be to make control experiments with the different calcium indicators for their functionality and compare them to synaptophysin GCaMP2 [17]. It is crucial to see which calcium indicator gives the highest signal to noise rate and saturates more gradually as a function of number of spikes *in vivo*.

The behavioral setup that I am most interested to study in future essays is the optomotor response (OMR). Circuit level descriptions of this behavior do not exist, but the robustness of the behavior, combined with the array of available genetic tools, make it possible to understand this behavior at the level of circuits that span the entire brain. I plan to apply synaptophysin GCaMP exclusively expressed in RGCs and together with photoactivation of PA-mCherry to reveal both the anatomical structure and connectivity of the circuit, and the function of this circuit in the whole brain. With this assay and the tools that I have developed I should be able to look at subsets of retinal ganglion cells in the arborization fields that are active during each response of the movement conducting the OMR. This will be achieved by doing functional calcium imaging in the axon terminals of these cells.

The optomotor response should either be mediated by a single arborization field of retinal ganglion cells, or by multiple arborization fields. The Ath5 2kb enhancer driving synaptophysin GCaMP will make it possible to compare different response properties within and between arborization fields and see which arborization fields are involved in the OMR. Collecting data from the presynaptic terminals of retinal ganglion cell axons, will permit demonstrating which arborization fields are activated during the OMR, and more specifically, to distinguishing between signals from different axon terminals within one arborization

field that is active during forward motion, left or right turns. This should reveal which subtype of ganglion cell responds to the stimuli, and which is their intrinsic selectivity in processing and projecting information downstream to induce forward swimming, left or right turns.

The enhancer fragment driving RGCs that I discovered and the new calcium indicator synaptophysin GCaMP and its variants will help to dissect the complete sensory-motor loop that underlies the OMR of the zebrafish larva.

Photoactivation of cells, in combination with calcium imaging, is a good approach for finding candidate neurons and synaptic connections in the network involved in the OMR. However, to achieve completely robust conclusions, it should be proved that there are monosynaptic connections between pairs of cells. Moreover, in certain cases it might be unclear if the identified candidate cell in the tectum or pretectum sends its axon into the arborization field, or receives input via dendrites (Fig. 10d). A possible approach is to use monosynaptic retrograde labeling with Rabiesvirus [40]. Using this virus it will be possible to identify whether neurons are synaptically connected to RGCs, by electroporating Rabies-mCherry into a candidate neuron, previously labeled by photoactivation of PA-GFP in the arborization field. If a synaptic connection exists, the green and red labeling in certain RGCs will overlap. The combined anatomical and functional imaging approaches would be a powerful tool for identifying both structure and function of the neural circuit.

Ten different arborization fields have been identified using dye injections [4], so far I have shown three of them labeled in the Ath5 2kb:mch line (Fig. 4c). Members of the lab are focused to decipher

different behaviors like prey capture and phototaxis. Since there is strong agreement that different behaviors are mediated by different groups of cells, it will be interesting to place my techniques at the disposal of other members in the lab who have expertise in different behavioral setups. For example, it is known that the NucMlf is also involved in prey capture, so by identifying the arborization fields that send inputs to the NucMlf, one population of ganglion cells might be isolated that is only involved in prey capture [8].

It is also well known that different behaviors can be identified by the movement of the tail, e.g. that prey capture is conducted by J-turns [41], and phototaxis elicits O-bends [42]. Therefore a genetic system driving Channelrhodopsin (ChR) under the Ath5 enhancer element should allow activating one, or multiple, arborization fields at a time to see if one arborization field is specifically involved in the behavioral output of interest. A new variant of ChR (CHETA) [43], containing a signal peptide at its N-terminus [unpublished] allows higher expression and therefore activation in axons. ChR activation of single or combinations of arborization fields will show their sufficiency for given behaviors. Second, in combination with the signals obtained from a panneuronaly expressed calcium indicator, it will tell us which neurons are activated downstream of the retinal ganglion cells [44]. Additionally, by driving Halorhodopsin (NpHR) [45] under the Ath5 promoter to inhibit different arborization fields specifically, or by making use of laser ablations [7], future results can prove the necessity of different arborization fields for an array of behaviors.

In the first part of my diploma thesis I showed that a 2 kb long enhancer fragment is able to label most or all RGCs and this makes it

possible to drive a reporter that only label RGCs and to work with a marker that is expressed only in the axon terminal (synaptophysin GCaMP). This enhancer-reporter system allows addressing the question which population of RGCs is active during the OMR.

In the second part I showed that by photoactivation of dendrites next to labeled ganglion cell axons within different arborization fields I can unravel the putative connections of the tectal and pretectal cells that send their dendrites to the arborization fields. Anatomical connections can then be accompanied by strong correlations between calcium signals; thus this technique provides dual, independent measures for connectivity. This will indicate which population of cells is downstream receiving the input from RGC-axon terminals by sending their dendrites to the activated arborization field.

Analysis of the activation pattern of an entire ensemble of neurons in the context of the OMR or other behaviors in the future will finally show how information gets processed from visual input through the ganglion cells, from different arborization fields to the tectum and pretectum and from there to the midbrain and hindbrain. These results should show the necessary components of the neural circuit responsible for controlling an innate response to a sensory stimulus which is the ultimate goal of use for the techniques and fish lines that I have been developing during my diploma thesis.

Materials and methods

Gateway cloning and gap repair – methods to test different enhancer fragments

The Gateway system consists of three steps. In the first step the enhancer fragment is enclosed by two recombineering sites (attB1 and attB2) by a PCR reaction. As templates for the different enhancer fragments I either used plasmids or the Bacterial Artificial Chromosome (BAC) containing the enhancer fragment. In certain cases where no BACS were available PCR was done from genomic DNA directly. The different enhancer fragments that I tested for expression in RGCs are described in results. The primers are all listed below. In the second step: the enhancer PCR fragment is recombineered in the tube in the entry vector. The entry vector is a plasmid that contains, after recombineering, the enhancer fragment and the recombineering sites (attL1 and attL2) that are necessary for the integration of the enhancer fragment in front of the reporter in the destination vector. In the third step the entry vector is used in another recombineering step together with the destination vector to create a plasmid containing the enhancer fragment driving the reporter.

In certain cases enhancer fragment up to 5kb size are not able to drive expression, and it is useful to test bigger fragments. Therefore I made use of a simple method called “gap repair” which make use of a recombineering step within bacteria. First by using an entry vector as a template, a PCR is done with primers that have 50bp homology arms to each of the 5’ and 3’ end of the new 10kb long enhancer fragment. The PCR product with 50bp at the ends complementary to the enhancer fragment is electroporated into bacteria containing the BAC coding for

the enhancer fragment. Within the bacteria the open plasmid (PCR product) is filled with the 10kb in a recombineering step. The bacteria are finally selected for the resistance which is mediated by the entry vector only after a successful recombineering step. After plasmid purification the new entry vector can be used in the Gateway system to drive different reporters.

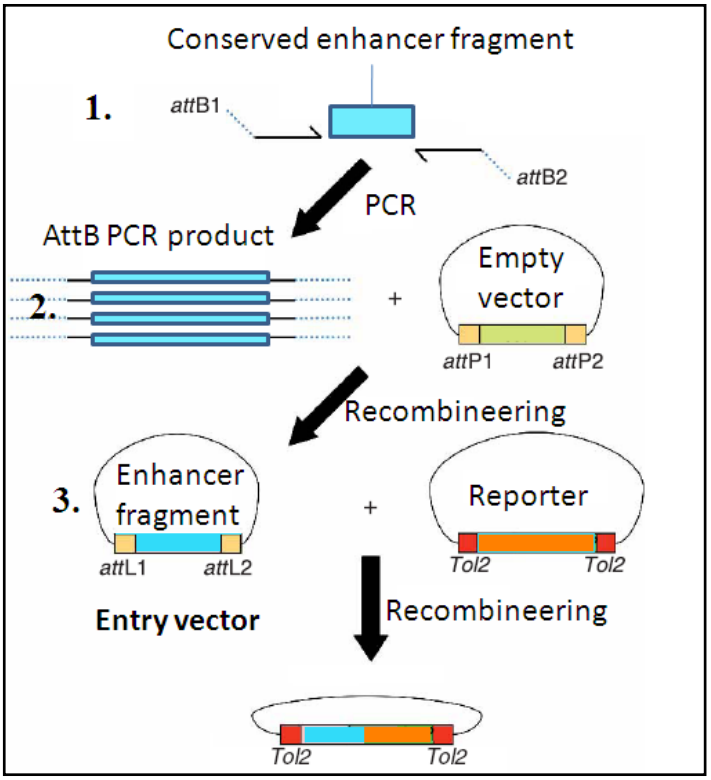


Fig.16 Gateway Cloning

1. The first step is to find conserved enhancer fragments between medaka, goldfish and zebrafish and to design primers to do the PCR from a template containing the enhancer fragment.

2. In the second step the PCR product is used in a simple recombineering reaction in the tube to get it into a plasmid backbone surrounded by recombineering sites to generate the entry vector containing the enhancer fragment.

3. In the third step the entry vector and a plasmid coding for the reporter, the destination vector are put in tube for recombineering. The final plasmid then contains an enhancer fragment driving a reporter enclosed by Tol2 transposon arms and is ready to inject into zebrafish eggs.

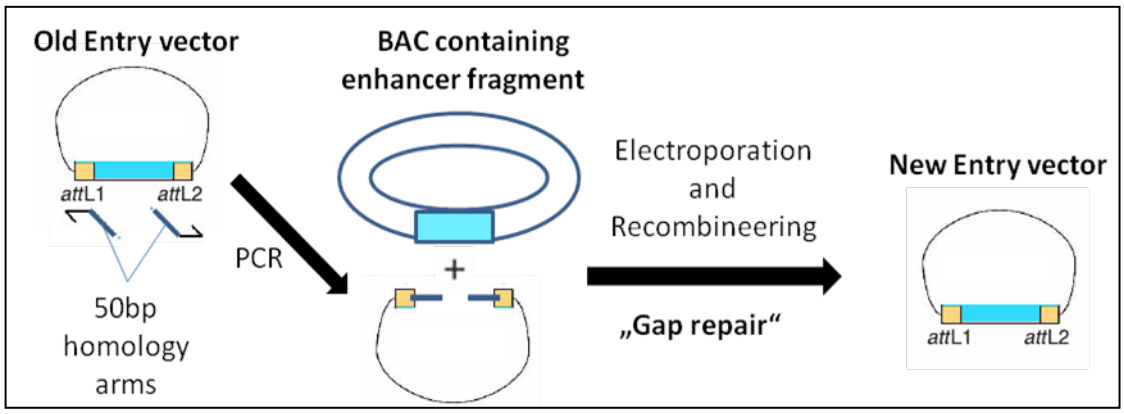


Fig.3 Gap repair.

To get enhancer fragments containing up to 10kb, a PCR product is created containing 50bp homology arms. This PCR product can be electroporated into bacteria containing the Bacterial Artificial Chromosome (BAC) coding for the enhancer fragment. In a recombineering step "Gap repair", the plasmid is "filled" with the sequence between the two 50bp homology arms and a new entry vector containing a 10kb enhancer fragment is created.

Primer design, and genetics

Each primer was designed comparing genomic sequence of the USCS university of california genome browser (assembly: Dec. 2008) and the ensembl genome browser (assembly: Dec 2008).

Depending on commercial available sequenced Bacterial Artificial Chromosomes (BACs), sequence and primer design was chosen correspondently.

<u>BACs</u>

alcam: CH211-285G11

hs6st1b: CH211-135P4

slit1a: dkey-175n21

int alpha 6: DKEY-251J19

robo2: CH211-253D13

dctn1a DKEY-31G16(HUKGB735G0131Q)

Plasmids used as template to make pcr reactions from enhancer fragments

Ath5:GFP [22]

Brn3a plasmid: Tg(brn3a-hsp70:GFP) [24]

Brn3c:mGFP [26]

a1TIpEGFP (Alphatubulin1:GFP) [32]

Brn3b:

Genomic DNA was isolated after a method for isolation of PCR-ready genomic DNA from zebrafish tissues as described [33].

Each primer pair was designed carrying the adjacent sequence to the ATG start codon and the reverse complementary primer was distanced either 2kb, 3kb, 5kb up to 10kb.

Primer pairs were designed using Primer 3.0 [34] and tested for melting temperature (Max Tm Difference: 1°C, Primer GC% 40-60%). Hairpin and self primer-dimer formation was tested with IDT SciToolsOligoAnalyzer3.1 [35].

Primer design to make pcr reactions of enhancer fragments using the above described plasmids, BAC, and genomic DNA:

4 guanine (G) nucleotides were added to the 5'end of the forward primer, followed by 25bp AttB1 site, followed by 18-25bp of template-specific

sequence (5’ GGGGACAAGTTTGTACAAAAAAGCAGGCT-template-specific sequence-3’)

For the reverse primer, 4 guanine (G) nucleotides were followed by 25bp attB2 site, followed by 18-25bp of template-specific sequence (5’-GGGGACCACTTTGTACAAGAAAGCTGGGT- template-specific sequence-3’) as described [21].

List of primers for different RGC enhancer fragments

Ath5 5kb 5 prime GGGGACAAGTTTGTACAAAAAAGCAGGCTTTCACACAGGAAACAGCTATGAC

Ath5 5kb 3 prime GGGGACCACTTTGTACAAGAAAGCTGGGTgatggttcttaatcgcttctgttc

Ath5 4kb 5prGGGGACAAGTTTGTACAAAAAAGCAGGCTTCACAATACCGTCCGTGATACC

Ath5 2kb 5prGGGGACAAGTTTGTACAAAAAAGCAGGCTCTGTGACTGTCTGAATCTGCTTTG

Ath5 promoter 3pr GGGGACCACTTTGTACAAGAAAGCTGGGTTGTCACCGATGTTCTTGGGATG

Brn3a 5 prime 5kb GGGGACAAGTTTGTACAAAAAAGCAGGCTAGTTATTACTAGCGCTACCGG

Brn3a 3 prime GGGGACCACTTTGTACAAGAAAGCTGGGTGGTCCGGCTCAGATAAAGTG

Brn3a 5 prime 3kb GGGGACAAGTTTGTACAAAAAAGCAGGCTTGCATTCAACGGAAGAAATTCA

Brn3b 5kb 5 prime
GGGGACAAGTTTGTACAAAAAAGCAGGCTTTGTGCCAGATGGGCTGGTCTGAG

Brn3b 5kb 3 prime GGGGACCACTTTGTACAAGAAAGCTGGGTATTTGCGACCGAGCTTCGGCGAG

Brn3c 6kb 5 prime GGGGACAAGTTTGTACAAAAAAGCAGGCTGGAATTAAATGGCTCATTAGCAG

Brn3c 3 prime GGGGACCACTTTGTACAAGAAAGCTGGGTCTTCGTTGCGCACCTTGCAG

alcam 10kb 5prime
CAAACCGATGGCTCAAAGATGTTCCGACAGCCAGAATGAGTGCGTTTTGGagcctgctttttgtacaaagttgg

alcam 10kb 3 prime
TTGAGACTGTCGCCGGACTGTATAAAGGAGAACCGGGGTTTTCTTTAAGGacccagctttcttgtacaaagttgg

alcam 5kb 5 prime GGGGACAAGTTTGTACAAAAAAGCAGGCTATCTGACCGTCGAACCATGTGTC

alcam 5kb 3 prime
GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTAAAGAAAACCCCGGTTCTCCT

robo2 10kb 5 prime

GTGGTTCACTAGTTGATCTGCACAACAAACCCTGCCTTTAGGGAAGATGTagcctgctttttgtacaaagttgg

robo2 10kb 3 prime

AAACGTGTTCTGGGGTTGAGAACTGAGGTGTGGATGTGGACTATGACAGGacccagctttcttgtacaaagttgg

robo2 4kb 5 prime GGGGACAAGTTTGTACAAAAAAGCAGGCTGCAGAGACAACATGAAGGAATTG

robo2 5kb 5 prime GGGGACAAGTTTGTACAAAAAAGCAGGCTGTGGTCCTGGTGTTCGGGTATC

robo2 5kb 3 prime GGGGACCACTTTGTACAAGAAAGCTGGGTCCTGTCATAGTCCACATCCACAC

hs6st1b 10kb 5 prime

CACCCGGTGTGGTCTTCTGCTTCTGCTACTGTAGCCCATCTGCCTCAAGagcctgctttttgtacaaagttgg

hs6st1b 10kb 3 prime

GCAAGGCACCGCAGAAGCACCGCGGACTGTTGTCTGAGAAATGATAACAAacccagctttcttgtacaaagttgg

hs6st1b 5kb 5 prime

GGGGACAAGTTTGTACAAAAAAGCAGGCTCCTTGCTCATTATAGGAACTTGAAC

hs6st1b 3 prime

GGGGACCACTTTGTACAAGAAAGCTGGGTTGTTATCATTTCTCAGACAACAGTC

dctn1a 10kb 5 prime

GTGTAAAACATATACTGGATGAGTTGCCGATTGTTGCGCAGCAGGTAGTGagcctgctttttgtacaaagttgg

dctn1a 10kb 3 prime

TATTTGTGTGTGTGTTTGTGTCAGCTGTGGAGTGGCACGCTCGGTAAGacccagctttcttgtacaaagttgg

dctn1a 5kb 5prime

GGGGACAAGTTTGTACAAAAAAGCAGGCTCACTTTTCGCGGCCTTGCAGTTTCA

dctn1a 5kb 3 prime

GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACCGAGCGTGCCACTCCACAGCT

dctn1a 6kb 5prime

GGGGACAAGTTTGTACAAAAAAGCAGGCTGTTGTCCGATCAGGTCCATGTGTG

dctn1a 6kb 3prime

GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACCGAGCGTGCCACTCCACAG

itga6 10kb 5 prime

TGAAATTTGAAGAAACGAACAAATTGTGTGTCTACTTACTTGAACCCCCCagcctgctttttttgtacaaagttgg

itga6 10kb 3 prime

TCAAAAAAACAAGGGCTATATTTTTCATACAACAGCATTCATTTCAGGCAacccagctttcttgtacaaagttgg

itga6 5kb 5 prime

GGGGACAAGTTTGTACAAAAAAGCAGGCTAAAGCTCTCGCCTGATTTTTACCTC

itga6 4kb 5 prime GGGGACAAGTTTGTACAAAAAAGCAGGCTCACTTGAACTCCTCCATCATCCAG

itga6 5kb 3 prime

GGGGACCACTTTGTACAAGAAAGCTGGGTGCCTGAAATGAATGCTGTTGTATG

slit1a 10kb 5 prime

ACTATCGGCTGGGTTTAGGGAAGGTGGTGGGTCTATGCATCAGTCGGTTGagcctgctttttttgtacaaagttgg

slit1a 10kb 3 prime

TGGTCCATGCTGGTTCCAGTAGGTCTTCTGCAGTATTGGTGATGATTGGGacccagctttcttgtacaaagttgg

slit1a 5kb 5 prime

GGGGACAAGTTTGTACAAAAAAGCAGGCTACTCCACACAGAAATGCCAACTGAC

slit1a 5kb 3 prime

GGGGACCACTTTGTACAAGAAAGCTGGGTCCCAATCATCACCAATACTGCAGAAG

slit1a 6kb 5 prime GGGGACAAGTTTGTACAAAAAAGCAGGCTGCTTACAACCAAACCAATGCAAC

slit1a 6kb 3 prime GGGGACCACTTTGTACAAGAAAGCTGGGTCCCAATCATCACCAATACTGCAG

pcp4a 5 prime 4kb GGGGACAAGTTTGTACAAAAAAGCAGGCTTTGCAGCCCTACAACTGAAATC

pcp4a 3 prime GGGGACCACTTTGTACAAGAAAGCTGGGTCATTCACAAAGCATCTCCGTATG

pcp4a 10kb 5 prime caaccttgctccgtgcatatcctgaatgtttacaaactggtatttcatctagcctgctttttttgtacaaagttgg

pcp4a 10kb 3 prime cttctgcccaatccagccttcttccttcatcttcatactcttcatccagcacccagctttcttgtacaaagttgg

pcp4a 10kb 5 prime 3bp Ath5

caaccttgctccgtgcatatcctgaatgtttacaaactggtatttcatctCAGagcctgctttttttgtacaa

pcp4a 10kb 3 prime 3bp Ath5

cttctgcccaatccagccttcttccttcatcttcatactcttcatccagcACAacccagctttcttgtacaa

pcp4a 10kb 5 prime 5bp Ath5

caaccttgctccgtgcatatcctgaatgtttacaaactggtatttcatctCACAGagcctgctttttttgtac

pcp4a 10kb 3 prime 5bp Ath5

cttctgcccaatccagccttcttccttcatcttcatactcttcatccagcTGACAacccagctttcttgtac

Primers used in different reporter systems

SyGCaMP2

SpeI SyGcamp 5 prime CCactagtcATGGACGTGGTGAATC

SacII SyGcamp 3 prime CCTTCCGCggATTATGATCTAGAGTC

Alpha tubulin 1

BAMHI alpha 1 tub forward

cgGGATCCagatcgctcccggactca

PCR syGC f

cttttgccttttttcttcacagGATGTTGCCAACCAGTTGGTC

syGC SV40 BssHII ttggcgcgcGTATCGATAAGCTTGATTTAAG

PCR PA-mCh f

cttttgccttttttcttcacaggtgagcaagggcgaggagg

NotI-Pa-mCh r

ATAAGAATgcggccgcttacttgtacagctc

cmcl2 heart GFP

CMLC2 Bglii forward gggAGATCTcgcAAAGCTTAAATCAGTTGTG

CMLC2 Bglii reverse gggAGATCtggatcCTTGTTTATTGCAGCT

CMLC2 Mlu1 forward gggACGCGTcgcAAAGCTTAAATCAGTTGTG

CMLC2 Mlu1 reverse gggACGCGtggatcCTTGTTTATTGCAGCT

cmcl2 SV40 right wrong way 5 prime tggtatggctgattatgatcctct

cmcl2 right way 3 prime GCTCTCCAAATCAGCAGACTTAAC

cmcl2 SV40 wrong way 3 prime

GGTCACTGGCTTACTAATGGAGTC

PCR was done using following protocols depending on the difficulty of the PCR

Takara Polymerase was used for simple PCR reactions, Phusions for the reactions were Takara Polymerase failed.:

Phusion™ High-Fidelity in GC Buffer

1µl of each Primer [10mM]

25µl PCR 2x Master Mix containing 0.2 mM dNTPs and 1.5 mM $MgCl_2$

98,5°C 2min30

30 cycles: 98,5°C 15s; 72°C 2min30

72°C 10min

Total volume: 50µl

Takara LA Mix

95°C 5min

35 cycles: 95°C 1min, 58°C 1min, 72°C 5min

72°C 3min

10kb PCR

Template: 0,5 µl of Ath5 2kb entry vector

LB medium liquid

5g NaCl, 5g Bactotryptone, 2,5g Yeast extract

LB plates

7,5g Agar, 5g NaCl, 5g Tryptone, 2,5g Yeast extract

Cmcl2 heart GFP

pDest GC3 and pDestGal4FF were cut with AscI, pDest cfos was cut with BglII,
cmcl2 pcr from clone ZFOS-207B3 with either MluI or BglII
All ligation was done with the Takara ligation mix (for one hour).

Integration in the right direction (antisense for the cmcl2 GFP fragment) was tested with primer cmcl2 SV40 right wrong way 5 prime and cmcl2 right way 3 prime

Gap repair

1.step

Bacs were isolated using Quiagen Kit Buffers P1, P2 and P3

300µl P1, 300µl P2 1min, 300µl P3 on ice 5'

spined 10' @ 11,5k @ cold room

800µl isopropanol (pre-cooled) were added

mixed 10-15' on ice

spinned 14k 10' cold room

washed with 500µl 70% EtOH

let column dry for 20'

added 100µl H2O

conc between 2000 and 5000 µg/µl

5 to 10µl were used and electroporated into elctrocompetent SW102

recovery, selection on Chlor plates [12,5µg/µl], single colonie was picked inoculated in 5ml LB 12,5µg/µl Chlorampicilin on and glycerol stock, storred at –80°C

SW102 containing the BAC were made electrocompetent again

http://recombineering.ncifcrf.gov/protocol/Protocol2_CKO_vectors.pdf

2.step

For 10kb primer the eV Ath5 2kb was used as template

PCR fragments of 2600kb with homology arms were electroporated into electrocompetent SW105 containing the corresponding BAC

after homologous recombination and gap repair,

tested for Kan resistance and restriction enzyme digest to test for the correct enhancer fragment.

Protocol to make SW102 containing the BAC electrocompetent:

add 100µl of *on* culture to 500ml LB chlor 30°C
grow up to OD 600,
take 100ml, heat shock 20’ 42°C
spin 10’ @ 4000 rpm
decant supernatant
resuspend in 5ml 10% glycerol
wash twice
resuspend in 200µl 10% glycerol
50µl in each tube into liquid nitrogen, stored at –80°C

modified protocol.
washed in dH2O
and used directly for eletroporation

electrocompetent SW102 cells were prepared after the following protocol:
http://recombineering.ncifcrf.gov/protocol/Protocol3_SW102_galK_v2.pdf
and stored in 50µl aliquots at -80°C.

DH5alpha competent cells were prepared after the following protocol:

TB (transformation buffer)
3.0g PIPES (final 10mM)
2.2g $CaCl_2.2H_2O$ (final 15mM)
18.6g KCl (final 250mM)
950ml H_2O
Adjust pH to 6.7 by KOH
Then add 10.9g of $MnCl_2.4H_2O$ (or 9.3g of $MnSO_4.4H_2O$) (final 55mM)
Add H_2O to total 1L
Filtration (0.22µm)
Keep at 4˚C

Experimental procedure
1. Saturate *E.coli* in LB
2.Add 1:10000 of cells to fresh LB medium (e.g. 20µl to 200ml LB)
3. 18˚C for ~24hr (~150-200 rpm/min)
4. Wait until it reaches OD=0.4-0.9. It is about 48hr for slow-growing

DH5alpha. Efficiency does not change dramatically in this OD range.

5. Sit on ice for 10min
6. 3000rpm, 10min, 4˚C
7. Remove sup and resuspend the cells gently with 1/3 vol (67ml) of ice-cold TB
8. Sit on ice for 10min
9. 3000rpm, 10min, 4˚C
10. Remove sup and resuspend the cells gently with 16ml of ice-cold TB
11. Add 1.2 ml of DMSO (final 7%)
12. Sit on ice for 10 min
13. 200µl x 85tubes
14. Freeze cells by liquid nitrogen
15. Store at -80˚C
16. Enough high efficiency for at least several months

heat shock protocol

20 min on ice competent DH5alpha or competent ccdB cells (invitrogen)

45 sec 42°C

Sit 2 min on ice

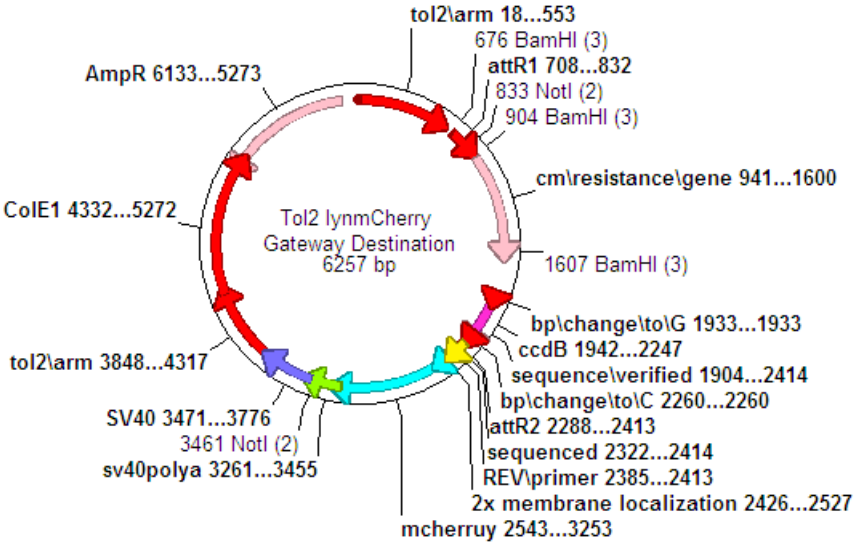
add 10x volume SOC medium

1h recovery @ 37°C

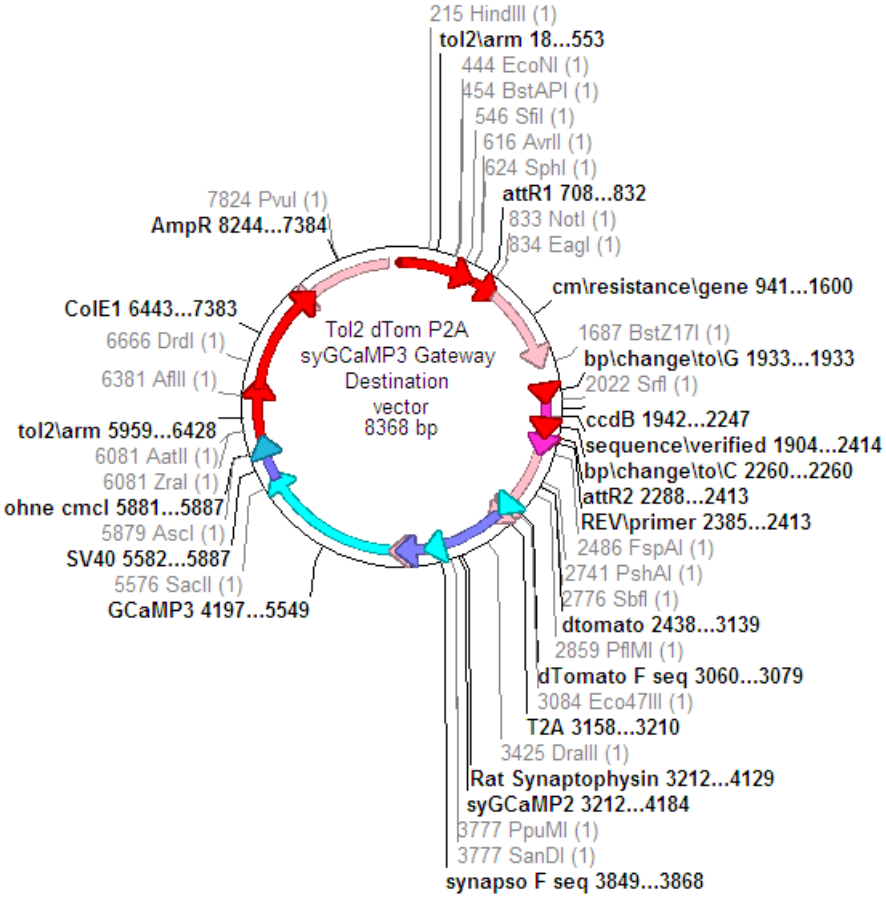
sequencing was done by Dana-Farber/Harvard Cancer Center DNA Resource Core

Destination vectors

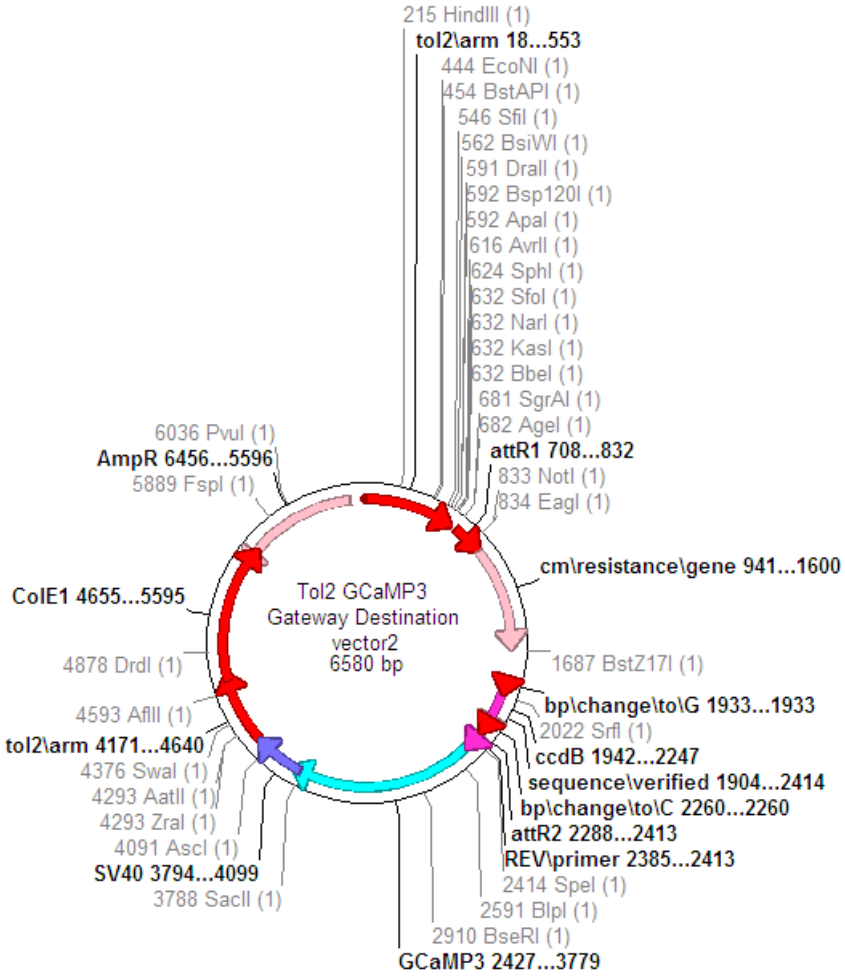
Tol2 Lyn-mCherry destination vector



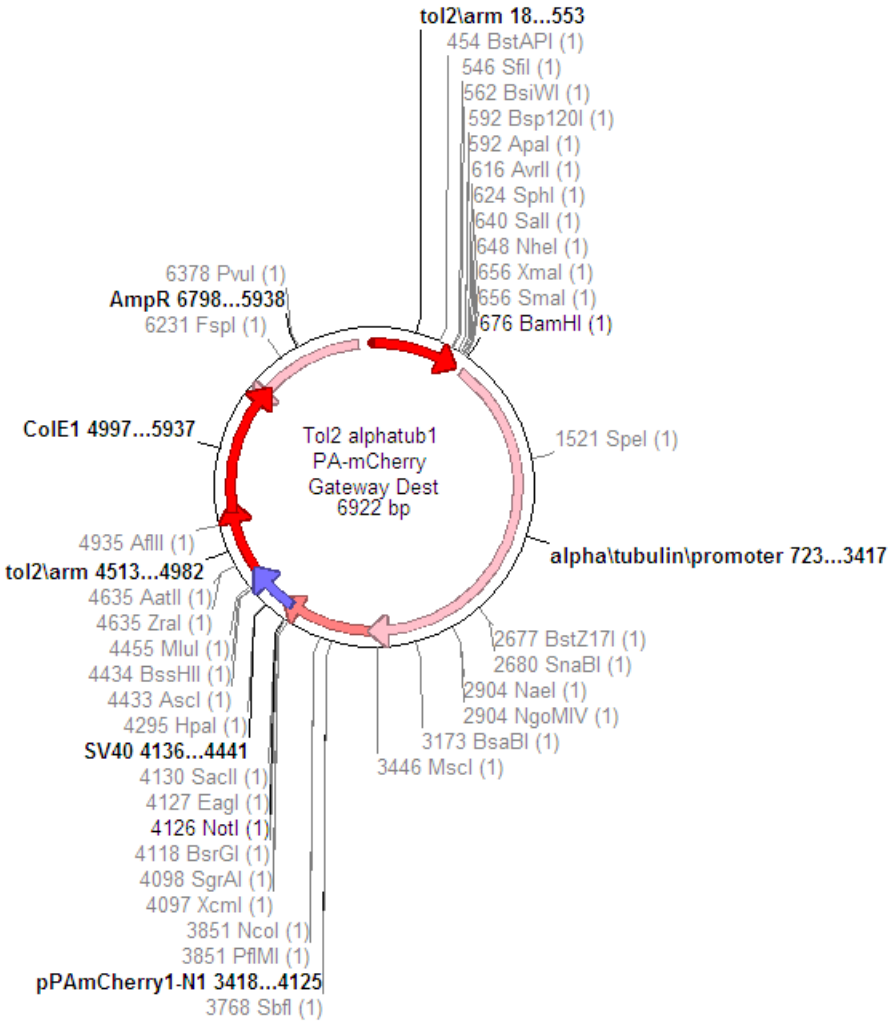
Tol2 dTomato P2A Zebrafish-Synyptophysin GCaMP3 Gateway Destination vector



Tol2 GCaMP3 Gateway Destination vector



Tol2 alpha tubulin 1 PA-mcherry Gateway Destination vector



New generated destination vectors were selected on carb chlor plates in ccdB competent cells. Once the destination vector was recombineered with an entry vector, it lost its ccdB and chlor resistance if the enhancer successfully integrated in front of the reporter.

Synaptophysin GcAMP3

cDNA + 3bp-BamHI-Sy-cDNA-r and 7bp-NdeI-SpeI-Sy-cDNA-f

cDNA was used and primer as described in Lagnado et al.

PCR purification with quiagen kit

direct digestion with NdeI and BamHI

cDNA coding for synaptophysin was put into a GcAMP3 destination vector, cut with SpeI and BlpI

Alpha tubulin PA-mCherry

BAMHI alpha 1 tub forward

cgGGATCCagatcgctcccggactca

PCR PA-mCh r alpha cctcctcgcccttgctcacctgtgaagaaaaaggcaaaag

PCR PA-mCh f

cttttgccttttttcttcacaggtgagcaagggcgaggagg

NotI-Pa-mCh r

ATAAGAATgcggccgcttacttgtacagctc

By creating a template of photoactivateable (PA)-mCherry with 25bp of homology arms and together with the first PCR product as a second template, a PCR fragment was created that was sequently cloned into the Ath5 2kb lyn-mcherry destination vector replacing Ath5 2kb lyn-mcherry with alpha tubulin PA-mCherry.

JAM-B

In-Jung created a BAC containing the JAM-B promoter with integrated with CRE-recombinase together with a floxed neomycin. So the first step was to pop-out the floxed Neomycin after the following protocol:

http://recombineering.ncifcrf.gov/protocol/Protocol2_CKO_vectors.pdf

Isolation of BAC was isolated with the Quiagen Kit (Maxiprep) and injected into FlEx-Based-transgenic reporter lines [36].

Cre-ER was induced with 50mM Tamoxifen as described [37]. by Hans et al.

Fish

Zebrafish embryos were collected and raised according to established procedures [38] and kept on a 12 hr 'on-off' light cycle, with light-on synchronized to embryo collection.

All zebrafish were obtained from the Harvard MCB zebrafish colony.

The Ath5:GFP line was a gift from the Masai lab [22].

Tg(eab2:[EGFP- T-mCherry] was a gift from the Chen lab [36]

All other transgenic lines were made by the Engert lab on a mitfa+/- (nacre) /AB background.

Injections

Needles: borosilicate glass capillaries GC150F-10 (1.5mm O.D) and 0.86mm I.D

Needles were pulled in two steps (75 °C and 65°C)

injection into the one cell stage using plasmid DNA [30ng/µl]

for coinjection each plasmid DNA [15ng[µl],

together with Tol2 RNA [180ng/µl]. Tol2 RNA was made as described [21]

filled in aliquots of 2 µl and stored at -80°C.

screen for successful injections was done at day3 to day5 because only then the RGC enhancer fragments are expressed for sure. MS-222 was used [0,1µg/µl] for anaesthetizing the larvae, since they start swimming in high speed, once they are hatched.

Transient fish that showed expression pattern of interest were grown up and crossed after three months to screen for germline integration. From each possible founder at least 100 eggs were screened.

Screening was done on a Olympus BX51 microscope.

Texas red dextran (Invitrogen) was injected into 4 day old larvae. The larvae were anaesthetised by bathing in high concentration of MS-222 [1µg/µl] for one minute. Afterwards they were put onto a small petri dish filled with agarose surrounded by a small film of water, so they would not move while injecting. Injections were done into the most caudal part of the spinal cord and the fish was positioned lying sideways.

Photoconversion

PA-GFP based neuronal tracing was performed with Ath5 2kb mch line 1 and Ath5 2kb mch line 2 each crossed to fish expressing PA-GFP panneuronally under the alpha-tubulin promotor. All fish had mitfa -/- nacre background, meaning no pigmentation in the skin, and most were PTU treated so that they would also lack pigmentation in the eye.
Embedded into 1,8% Agarose, on silgard dishes, treated with 1% PTU, they were either anesthetized in 1% MS-222 (Sigma Aldrich), and freed of the agaraose if it was of interest to grow them up (in case they were founders). Otherwise they bathed for 30 min in 1mg/ml high dosis Bungarotoxin (Invitrogen) (reused)

Image was focused at the focal plane, seeing the axons, (excitation wavelength: 980nm). Imaging at this wavelength because of its low power was not enough to excite PA-GFP.
zoomed in into one aborization field (zoom 0,03 x 0,03), which corresponds to half of the arborization field.
Single cell photoactivation was done under (zoom 0,01 x 0,01) which is around 0.5 µm, Photoconversion for 1 to 2min of the neuropil; Laser power of 100mW with 790nm

(in the same way dentrites of the NucMlf were photoactivated.)

Z-stack @ 980nm for both channels was taken right away.
There was no need to wait for diffusion of PA-GFP as described [14], most likely because imaging was done in vivo.

All imaging was performed on a custom made two-photon-laser scanning microscope [7], using a pulsed Mai-Tai laser and an Olympus 20x water immersion objective.

Huc:YC2.1 together with Ath5 2kb mCherry was imaged at 1040nm.

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Abbreviations

RGCs - Retinal ganglion cells

PA-GFP - Photoactivateable green fluorescent protein

Dpf - day post fertilization

NucMLF - Nucleus of the medial longitudinal fasciculus

MeLc - Medial lateral caudal cell

MeLr - Medial lateral rostral cell

V-cells - Vestibular-cells

OMR - The optomotor response

BAC – Bacterial Artificial Chromosom

AF – Arborization Fields

kb – kilobases

mch - mCherry

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Appendix

Zusammenfassung

Diese Arbeit beschäftigt sich mit der anatomischen Untersuchung der Neuronen die im neuronalen Netzwerks des Opto-Motor-Reflexes eine Rolle spielen, welches verantwortlich ist für die Umsetzung von visuellem Input in Bewegungsmuster. Zu diesem Zweck werden Zebrafischlarven verwendet, die ein sehr gutes Modellsystem für das Entschlüsseln des visuellen Verhaltens und den zugrunde liegenden Nervenzellen bieten. Der Zebrafisch ist im Larvenstadium klein und transparent, und besitzt ein Repertoire an verschiedenen angeborenen Verhaltensmustern, die leicht auszulösen sind und ständig wiederkehren.

Eine Reaktion auf visuelle Stimuli kann bereits drei Tage nach der Fertilization beobachtet werden auf Grund dessen, dass Axone von retinalen Ganglienzellen ihre postsynaptischen Ziele erreichen. Ab diesem Augenblick ist das neuronale System verantwortlich für die Verarbeitung von visuellen Reizen. Am anderen Ende des neuronalen Netzwerks sitzen Zellen, die ihre Axone in die Wirbelsäule senden. Diese Nervenzellen sind verantwortlich für die Bewegungsmuster (Schwimm – und Dreh-Bewegungen) welche dem Opto-Motor Reflex zu Grunde liegen. Die Nervenzellen, die sich im Tectum und Pretectum zwischen der Retina und den Zellen befinden, die in die Wirbelsäule projizieren, sind bis jetzt unbekannt. Meine Diplomarbeit zeigt auf, dass mehrere Kandidaten dieser tectalen und pretectalen Nervenzellen möglicherweise in dem bis jetzt noch nicht entschlüsselten Opto-Motor Reflex involviert sind.

Dieser Nachweis erfolgt durch die Identifikation von Nervenzellen *in vivo,* welche Signale von Ganglienzellen erhalten. Ganglienzellen senden ihre Axone in verschiedene Regionen im Gehirn, welche nach

ihren Verzweigungsmustern identifiziert werden können. Dabei zeige ich wie mit einem Rekombinationsystem Ganglienzellen genetisch markiert werden können. Dieses ermöglicht Elemente von verschiedenen Promotoren auf ihr Expressionsmuster zu untersuchen. Dadurch habe ich ein Promoterfragment identifziert, das ausschließlich Ganglienzellen markiert. Durch Photoaktivierung von photoaktivierbarem grün-fluoreszierendem Protein in den Bereichen, die diese von mir markierten Ganglienzellen oder anders markierte Wirbelsäul-projezierende Zellen innervieren, beschreibe ich die Anatomie einer Population von Neuronen, die sich im Tectum und Pretectum befindet. Dieses Experiment zeigt also durch die Fluoreszenz, dass eben diese Nervenzellen mit den Ganglienzellen verbunden sind. Darüber hinaus besteht daher die Möglichkeit, dass sie auch dafür verantwortlich sind, die Information von der Retina zu den Wirbelsäule-projizierenden Zellen weiterzuleiten.

Diese Arbeit versucht zu klären, wie viele und welche Zellen mit den axonalen Enden der genetisch markierten Ganglienzellen verbunden sind. Um eine vollständige Aufschlüsselung der Funktionaltiät dieses Netzwerks zu erhalten, sind weitere Versuche nötig, die diese von mir mittels genetischer Markierung von Ganglienzellen und deren Photoaktivierung identifzierten pretectalen und tectalen Kandidaten, auf ihre Aktivität innerhalb des Opto-Motor-Reflexes testen.

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