

# Optogenetics in a transparent animal: circuit function in the larval zebrafish

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Optogenetic tools can be used to manipulate neuronal activity in a reversible and specific manner. In recent years, such methods have been applied to uncover causal relationships between activity in specified neuronal circuits and behavior in the larval zebrafish. In this small, transparent, genetic model organism, noninvasive manipulation and monitoring of neuronal activity with light is possible throughout the nervous system. Here we review recent work in which these new tools have been applied to zebrafish, and discuss some of the existing challenges of these approaches.

## Addresses

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Current Opinion in Neurobiology 2013, 23:119–126

This review comes from a themed issue on Neurogenetics

Edited by Ralph Greenspan and Christine Petit

For a complete overview see the [Issue](#) and the [Editorial](#)

Available online 12th December 2012

0959-4388/\$ – see front matter, Published by Elsevier Ltd.

<http://dx.doi.org/10.1016/j.conb.2012.11.001>

## Introduction

Understanding how neuronal circuits generate behavior is a critical yet complex endeavor. The circuits involved typically span multiple brain regions, which may be spatially distributed and difficult to access. The recent field of optogenetics relies on a set of tools that are well suited for simultaneously monitoring neural activity in large populations of neurons and causally testing their role by modifying their electrical properties and perturbing their spiking patterns. The use of light both for imaging and manipulation facilitates non-invasive access following the expression of transgene-derived proteins that either emit photons (such as fluorescent or bioluminescent reporters of activity) or are sensitive to light (such as light-gated channels or pumps).

The larval zebrafish is gaining prominence as a model organism within systems neuroscience. It is relatively small (about 4 mm long), translucent and amenable to

genetic modifications. Zebrafish have an archetypal vertebrate brain plan [1] and exhibit a wide range of simple but reliable behaviors. These include a diversity of spontaneous locomotor maneuvers, such as slow forward swims and routine turns [2] and reflex-like responses, such as touch mediated escape responses [2–4]. Zebrafish larvae also exhibit a variety of visually driven behavior [5] such as the optokinetic response (OKR) where objects moving across the visual field evoke stereotyped tracking eye movements [6], the optomotor response (OMR) where larvae turn and swim in the direction of perceived whole-field visual motion [7], prey tracking and capture [8–11], as well as associative learning [12], and motor adaptation, where the larvae adapt their locomotor output to compensate for changes in the amount of visual feedback they receive while performing a forward swim [13,14<sup>••</sup>].

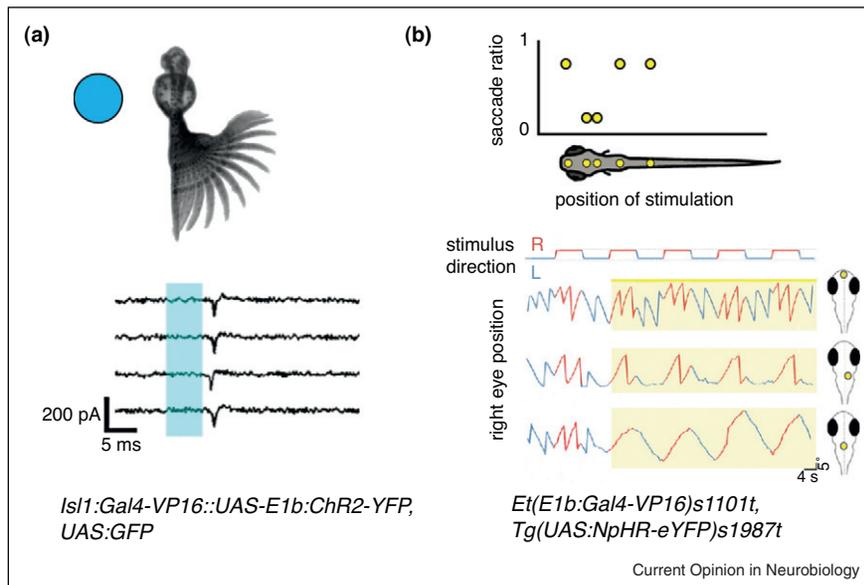
In this review we will discuss recent work in which optogenetic approaches have been applied in larval zebrafish to explore the neural basis of behavior. Optogenetics not only allows us to monitor neural activity but also to perform loss-of-function and gain-of-function experiments to test the necessity and sufficiency, respectively, of neural activity to elicit specific behavior.

## Probing neural circuits with light-induced neuronal excitation

Channelrhodopsin-2 (ChR2) [15] is a light-gated channel that, upon absorption of blue light, allows the non-specific flow of cations into cells. When expressed in the nervous system, it can be used to depolarize neurons (Na<sup>+</sup> ions are conducted twice as efficiently as K<sup>+</sup> ions) and control their firing with high temporal precision [16,17<sup>••</sup>,18]. In zebrafish, ChR2 was first used in the somatosensory system of 1 day post fertilization (dpf) embryos [19<sup>•</sup>]. Transient mosaic expression of ChR2 was driven by the *islet-1* promoter from plasmids injected at the one-cell stage resulting in expression in trigeminal and Rohon-Beard neurons. It was shown that photo-activating these genetically defined populations of neurons could trigger an escape response. Furthermore, a single action potential in one sensory neuron was sufficient to evoke escape behavior (Figure 1a).

Targeting optogenetic tools to neuronal subpopulations was subsequently facilitated by the isolation of transgenic driver lines from Gal4 enhancer trap screens [20<sup>•</sup>,21<sup>•</sup>]. A variety of these transgenic lines have been used to localize expression of the light-gated ionotropic glutamate

Figure 1



Using optogenetic tools to test the sufficiency and necessity of neurons in generating different types of motor output. **(a)** An embryo expressing ChR2 in somatosensory neurons under the *is/1* promoter responds to neuronal stimulation by a blue light flash (adapted with permission from [19]). *Bottom*: A 5 ms blue light pulse typically elicits a single spike. In some of the cases, a single spike in a single Rohon-Beard cell was sufficient to generate an escape response. **(b)** Mapping brain regions necessary for saccade generation using yellow-light activation of NpHR (adapted with permission from [30]). The ratio between saccade number in expressing versus non-expressing larvae decreases when light hits a certain part of the hindbrain, suggesting that it is necessary for saccade generation. *Bottom*: Eye position during left and right motion during suppression of neuronal activity in different areas. Behavior could be bilaterally or unilaterally affected depending on where NpHR was activated.

receptor LiGluR [22,23] — combined with an exogenous co-factor, MAG, to depolarize neurons — within the spinal cord of 5 dpf larvae [24<sup>••</sup>]. This intersectional gene expression approach helped identify a particular type of neuron, the Kolmer-Agduhr (KA) cells, whose photo-stimulation was sufficient to induce a slow forward swim [24<sup>••</sup>]. Upon massive stimulation, the KA neurons can modulate ventral spinal circuits comprising the central pattern generators for locomotion in the larval zebrafish.

### Optically silencing neural activity

Both ChR2 and halorhodopsin (NpHR) [25–28] have been used to investigate the initiation of locomotion in larvae [29]. NpHR is a light-driven chloride pump that allows reversible silencing of neurons and functionally has the opposite effect of ChR2. The authors used enhancer trap lines to generate transgenic larvae expressing NpHR in most of the neurons and observed that swimming was inhibited by widespread NpHR stimulation and was triggered when this inhibition was released. NpHR stimulation was focalized to regions of  $\sim 30 \mu\text{m}$  using an optic fiber. This allowed the identification of the neuroanatomical region responsible for the induction of the rebound locomotion: the *commissura infima Halleri*, which is located at the boundary between the hindbrain and the spinal cord. By co-expressing ChR2 and NpHR in the same neurons, it was shown that activating them led to

swim initiation and silencing them led to larvae terminating swimming.

The same strategy was used to identify brain regions associated with an oculomotor behavior, the fast resetting saccades of the OKR [30]. Silencing activity by localized stimulation of NpHR in a subregion of rhombomere 5 was enough to abolish saccades (Figure 1b). In addition, the authors were able to perform a gain of function experiment by expressing ChR2 in the relevant neurons in the double indemnity (*didy*) mutant. This mutant was originally identified by its inability to perform saccades during the OKR, but saccades could be evoked by blue light stimulation of ChR2. Silencing neuronal activity in these same larvae in a slightly more caudal region around rhombomeres 7/8 was enough to decrease gaze stability, suggesting that the neural integrator for eye position resides in this area [31<sup>•</sup>].

### Restricting activation to subpopulations of neurons with genetics and light patterning

Several of the experiments mentioned above took advantage of the diversity of Gal4 enhancer trap lines generated by large screens from the Baier and Kawakami laboratories [21<sup>•</sup>,32] and the high expression levels obtained with the Gal4/UAS transcriptional regulatory system. A different approach, based on the Tet system

[33,34], was successfully implemented [35] to generate transgenic lines with strong, stable expression in a sparse subset of the neurons targeted by the promoter driving the Tet activator (*elav13* (HuC) and *Dlx4/6* were used in this study). This system has the additional feature that expression can be regulated pharmacologically by administering doxycycline. Larvae expressing ChR2 under Tet control were stimulated with blue light [35], which evoked distinct swimming behavior.

Studies in the olfactory bulb have been carried out using adult zebrafish brain explants. In an elegant study where light patterns were controlled spatially and temporally using digital mirror devices [36\*\*], a transgenic line expressing ChR2 in sensory afferents was used to stimulate inputs to the bulb. The authors studied coding of olfactory responses between mitral cells and the dorsal telencephalon, and thereby demonstrated a role for spike timing in the coding of stimuli throughout the olfactory pathway.

A different transgenic line expressing ChR2 at high levels in interneurons of the glomerular layer was used in [37] to show that these cells release both GABA and dopamine. While short trains of blue light evoked only GABA-mediated inhibitory currents in mitral cells, more prolonged trains also activated a slow hyperpolarizing dopamine-mediated current. The results suggest that GABA is involved in dynamic odor processing, whereas dopamine is implicated in the slow adaptation of circuit function, such as filtering out slow variations in background odors while retaining sensitivity to novel ones.

In the above studies, photostimulation of the light-gated channel or pump was carried out in different ways using combinations of light patterning [38,39\*\*], and genetic targeting of the light-gated channels [19\*,24\*\*,36\*\*]. In one case a blue spot of 50  $\mu\text{m}$  diameter was generated using an iris in the epifluorescence path [19\*]. In other studies, a small optic fiber further reduced the diameter of the illumination spot to 30  $\mu\text{m}$  [29,30]. Friedrich and colleagues achieved complex non-synchronous stimulation of mitral cells using digital mirror devices [36\*\*]. This approach allows millisecond switching of two-dimensional illumination patterns. However, none of these methods of illumination allow restriction in the  $z$ -direction [29].

Whether the intent is to excite or inhibit populations of neurons, challenges remain in targeting light quickly, precisely, and in three dimensions. Single photon illumination tends to activate groups of neurons unless the targeted neurons are sparse or spread in a 2D plane. In order to specifically activate single cells and reduce scattering of stimulation light, multi-photon techniques would be ideal, with the added benefit of interfering less with the visual system. Two-photon excitation of ChR2

was first achieved in cultured neurons in [40\*\*] and successfully implemented in an adult zebrafish brain explant [35]. If successful, *in vivo* multi-photon excitation could provide relatively fast (tens of milliseconds) activation of neurons distributed across a large field of view within a restricted resolution in  $z$ , temporally limited only by the spiral scan time over a soma. There are no reports of two-photon activation of light-gated pumps silencing neurons.

The combination of temporal focusing and digital holography [38,39\*\*,41] permits multi-photon activation of larger groups of selected neurons in the single millisecond time range. Temporal focusing greatly improves axial resolution in the excitation pattern, and can be used in combination with other techniques. Digital holography utilizes iterative algorithms and a liquid crystal spatial light modulator (LC-SLM) to modulate the input beam's wavefront, eventually producing the desired, often complicated, patterns of light (*both topics are reviewed in* [42]). These new techniques improve temporal resolution and allow the simultaneous imaging of neuronal activity and 3D optical stimulation in different planes [43]. Improvements of devices for 3D spatiotemporal delivery of light will be very useful for the optogenetically mediated activation of defined groups of neurons in zebrafish.

### Calibration

A current challenge when using optogenetic tools involves calibrating the system to understand what effect a given light intensity has on the membrane potential of a neuron. This is important to ensure reproducibility of the stimulus across both neurons and larvae. Theoretically, this would involve integrating the local power density of the light and knowing the expression levels of the optogenetic effector at the soma. In practice it may be possible to calibrate these tools using electrophysiology, although doing so across a population of neurons is not feasible. Another possibility is to co-express genetically encoded voltage sensors [44\*,45] and use them to estimate the light-induced effects at the population level.

### Imaging neuronal activity with genetically encoded calcium indicators

Fetcho and colleagues were the first to demonstrate the possibility of monitoring neuronal activity in zebrafish with the genetically encoded calcium indicator (GECI), cameleon, by imaging motor neurons, spinal interneurons and Rohon-Beard cells in intact zebrafish [46]. These cells were shown to respond to touch and electric stimulation. Since this first *in vivo* demonstration, the use of GECIs has been extended to circuits involved in several behavioral paradigms [14\*\*,36\*\*,37,47\*\*,48,49].

The optic tectum (OT, homologue of the mammalian superior colliculus) is a layered structure, which in larval zebrafish is involved in the tracking of small, visual

stimuli and contributes to prey capture [8], a complex feeding behavior. How does the OT select small prey-like stimuli over larger ones? This question was addressed using two versions of the GCaMP family of indicators (GCaMP1.6 and GCaMP3) and transgenic lines identified in [21<sup>•</sup>], to determine the receptive fields of the superficial and deep layers of the OT to large and small visual stimuli [47<sup>••</sup>]. Deep tectal layers constitute the output of the tectum and project to pre-motor areas. Excitatory neurons in deep layers receive active retinal inputs when both small and large visual stimuli are presented. On the other hand, superficial inhibitory neurons are preferentially activated by large visual stimuli and this inhibition is fed-forward to the deeper layers. This circuit may silence the output of the OT when large visual stimuli are presented and thereby allow it to preferentially select small moving stimuli over large ones. Consistent with this hypothesis, larvae in which superficial inhibitory neurons were silenced using the tetanus toxin light chain were less effective at catching small prey.

In the described experiments, the sensitivity of detection relies on the affinity and kinetics of the calcium indicator used. Guided by the protein structure of the original GCaMP indicator, targeted mutagenesis and high-throughput screening have led to great improvements in recent years [50]. The latest variant is GCaMP5 [51], a new indicator with increased dynamic range. GCaMP5 shows a 2-fold to 4-fold improvement (Figure 2) compared to GCaMP2 [52] or GCaMP3 [53] in a variety of experiments involving both transient and stable

expressions in zebrafish larvae. The high sensitivity and signal to noise ratio allow identification of a comparatively larger number of active cells in experiments involving single trial analysis. The continuous improvement of both green and red GECIs [51,54<sup>•</sup>] raises the hope that calculations of neuronal spiking may be possible across a wide range of firing rates in the future.

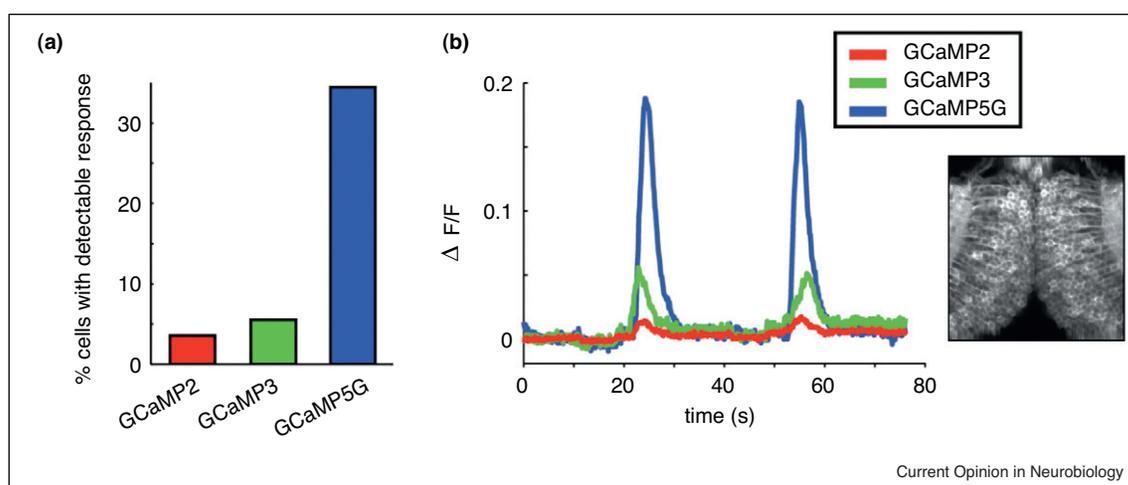
### Recording activity with bioluminescence

Recording of neuronal activity in freely moving animals has a rich history in rats [55] and other animals, and has the obvious advantage of capturing neuronal activity while the test subject is in its natural state. A recent technique permits completely non-invasive neural recordings in freely swimming zebrafish by expressing the protein GFP-Aequorin [56<sup>•</sup>] in targeted populations of neurons (Figure 3a). The GFP-Aequorin protein is bioluminescent, and (after larvae are bathed in coelenterazine) emits green photons when it binds to calcium, which, due to the transparency of the larva, can be detected with photosensitive devices. Aequorin was targeted to hypocretin-expressing neurons of the hypothalamus and neuronal discharge was elevated during periods of increased locomotion [57<sup>••</sup>]. Since current techniques only allow for the monitoring of gross population activity, it will be useful to combine this neuroluminescence technique with targeting of specific subsets of neurons using novel genetic methods [32,35,58<sup>••</sup>,59].

### Imaging activity during virtual behavior

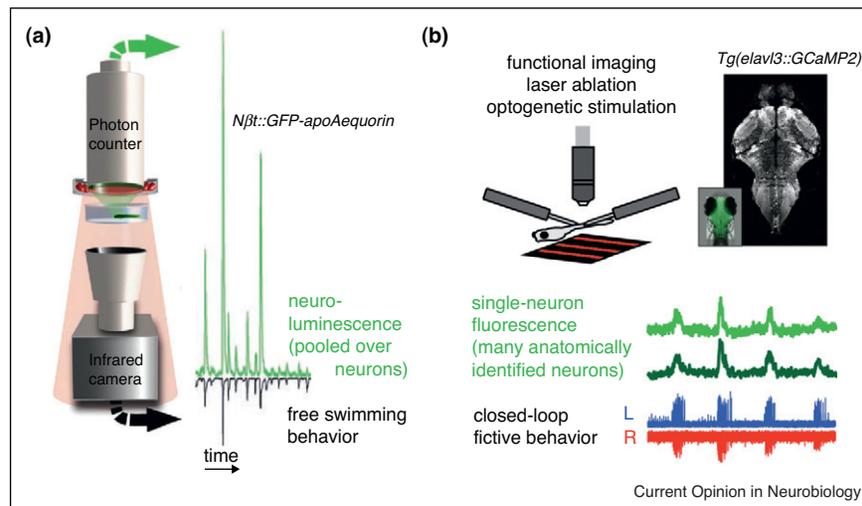
A new approach for monitoring neuronal activity using GECIs has recently been developed in zebrafish larvae

Figure 2



Comparison of sensitivity of GCaMP indicators in zebrafish larvae. The same experimental protocol was applied to zebrafish larvae expressing one of three GCaMP indicators — GCaMP2, 3 or 5 (specifically GCaMP5G) — under the pan-neuronal promoter *elav/3* (HuC) ( $n = 11, 9, 9$ ). Larvae were paralyzed, and activity in tectal cells was monitored with a two-photon microscope, while a bright spot on a dark background was moved from left to right then right to left. (a) The percentage of tectal cells with detectable responses differs by a factor of approximately 10 between GCaMP2 and GCaMP5, with GCaMP5 significantly more sensitive ( $t$ -test,  $p < 0.01$ ). (b) The average response of the top 50% of responding cells shows that GCaMP5 has a much improved signal to noise ratio. See [51] for further details. Right: Two-photon image of the optic tectum where the data were acquired (field of view =  $150 \mu\text{m} \times 150 \mu\text{m}$ ).

Figure 3



Novel strategies for recording neural activity in populations of neurons in awake larvae during active or fictive locomotion. **(a)** The neuroluminescence system (adapted with permission from [57\*\*]). A zebrafish larva expressing GFP-Aequorin swims freely in a Petri dish. A photomultiplier tube above the animal detects green photons that are emitted during raised activity levels in the target neurons, while a camera underneath the Petri dish films the larva as it swims. In this way, behavior and neural activity can be monitored simultaneously in freely swimming larvae (*right*). **(b)** Fictive virtual-reality system for recording whole-brain activity during behavior (adapted with permission from [14\*\*]). A zebrafish larva was paralyzed and fictive motor output registered via two electrodes attached to each side of the tail, recording from the motor nerve roots. The intended motor output was used to simulate locomotion through a virtual environment, projected underneath the larva. In this way, recordings from large populations can be recorded at once during closed-loop behavior.

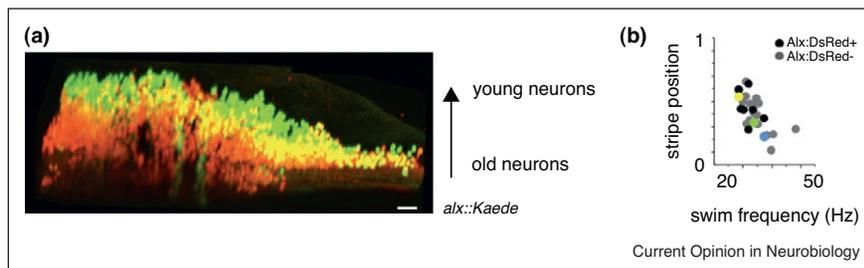
behaving in a virtual reality environment [14\*\*] (Figure 3b). Inspired by virtual reality systems for mice [60] and fruit flies [61,62], this paradigm enables recording of neuronal activity from all areas of the brain with cellular resolution. A conventional two-photon microscope images neuronal GECI fluorescence in a larva immersed in a virtual environment. The larva is paralyzed with  $\alpha$ -bungarotoxin, thereby yielding the brain completely stationary. Large electrodes record neuronal activity (through the skin) from the bundle of motor neuron axons in the nerve root along the tail [63,64]. By analyzing these nerve root signals, it is possible to estimate the corresponding motor output. The intended locomotion is used in turn to drive movement in the virtual environment, and has been used to study whole-brain activity during a simple form of motor learning [13,14\*\*]. When changing the strength of visual feedback following an intended swimming event, the animals respond by compensatory adjustments of their locomotor drive. GCaMP2 was expressed throughout the brain to investigate the neuronal activity patterns underlying this response [14\*\*] (using the pan-neuronal *elavl3* (HuC) promoter [46,65]). Calcium imaging in sequential planes through the entire brain volume revealed that activity was particularly concentrated in the olivo-cerebellar system. Lesions of the inferior olive reduced the ability of the larva to perform simple forms of motor learning. This study raises many questions about the mechanisms by which sensorimotor integration leads to changes in behavior and paves

the way toward recording whole-brain neuronal activity during diverse behavioral responses.

### Photoconvertible fluorescent proteins and the interface between neuroscience and development

Within the first week of life, zebrafish larvae exhibit different locomotor patterns and by 5 dpf they can perform slow swims and feed. Fetcho and colleagues recently observed that a well-defined structural and functional ground-plan in the hindbrain is involved in the control of swimming at different frequencies at the larval stage. Groups of glutamatergic neurons, organized in sagittal planes, discharge in synchrony with swim patterns [66\*\*,67\*\*]. These neurons likely drive motor output. Using *chx10:Kaede* transgenic animals [68], the fluorescent protein Kaede can be photo-converted from green to red at early stages of development. Thus, early differentiating neurons contain more red (photo-converted) fluorescent protein while the more recent differentiating cells contain only green fluorescent protein. This analysis revealed that dorsal interneurons were younger than ventral ones (Figure 4). Ventral neurons were characterized as having lower input resistances and were recruited at higher swimming frequencies. These observations are consistent with the development of swimming patterns from embryonic to larval stages, and suggest that additional circuitry from late-born neurons in the larva is responsible for slow swimming locomotion. Studies combining

Figure 4



Estimation of the age of hindbrain neurons using the photoconvertible protein Kaede (adapted with permission from [66\*\*]). (a) In an *alx::Kaede* line, Kaede is photo-converted from green to red by UV illumination at two dpf and imaged at three dpf. Neurons that are born after two dpf only contain the green original Kaede while neurons born before two dpf also contained red photo-converted Kaede. By changing the time of photo-conversion, the age of hindbrain neurons could be estimated. Newly born neurons are added dorsally demonstrating a ventral-dorsal organization of the hindbrain. (b) There is a ventral-dorsal ordering in recruitment of neurons during swimming at different frequencies. Ventral, older neurons are recruited during fast swims, and dorsal, younger neurons during slow swims.

anatomy, electrophysiology and behavior illustrate how multiple experimental approaches are critical for gaining fundamental insights into the developing nervous system underlying locomotion and other behavior.

## Prospects

Optogenetic tools have developed quickly since neuronal activity was first monitored in larval zebrafish using calcium imaging [46]. The recent work we have reviewed here shows that it is now possible to monitor activity in a large number of neurons and perform loss-of-function and gain-of-function experiments *in vivo*. In some of the cases this allows us to directly link neuronal activity with behavior. Yet most of the perturbations we have described lack the specificity required to manipulate single neuron activity at will and therefore do not provide us with a fine enough scalpel to dissect circuitry with the detail we would desire. We believe that future advances, in particular on two fronts, will greatly aid this endeavor.

Firstly, our current knowledge of genetic tools that label different populations of neurons is limited yet it is important to be able to target expression of the optogenetic tool of choice to the specific cell type of interest. Gal4 enhancer trap lines and the Tet activation system have proved to be useful, but we expect that new technologies, such as the recently developed TALENs [58\*\*,69\*] will allow better control in the future. Secondly, we need to quickly activate cells located in different z-planes, as dissecting circuit function requires the manipulation of neuronal activity of both single and multiple neurons. Even though the dynamic manipulation of neuronal circuits seems difficult in freely moving animals, the possibility of mimicking behavior in a virtual reality environment should facilitate light delivery for performing optogenetic dissection of circuits underlying behavior. Although exciting technique advancement such as digital holography and temporal focusing continues to push the

technical boundaries, limitations still exist. Further developments in optics, combined with the ever-improving optogenetic toolbox [51,70], will open new paths of investigation.

## Acknowledgements

We thank Florian Engert, Marnie Halpern and Loren Looger for critical reading of this review.

CW and KS benefited from a chaire d'excellence from the Ecole des Neurosciences de Paris (ENP), the Atip-Avenir fund from the CNRS and Inserm funded by the Fondation Bettencourt Schueller, the Fondation Institut du Cerveau et de la Moelle épinière (ICM), the Emergence grant from the Mairie de Paris, the NERF region, the FYSSEN foundation and the International Reintegration Grant (IRG) from the Marie Curie Action and the European Research Council project 'Optoloco'. MA was supported by the Howard Hughes Medical Institute and by the Wellcome Trust.

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