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# Let there be light: zebrafish neurobiology and the optogenetic revolution

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## Abstract

Optogenetics has revolutionized the toolbox arsenal that neuroscientists now possess to investigate neuronal circuit function in intact and living animals. With a combination of light emitting 'sensors' and light activated 'actuators', we can monitor and control neuronal activity with minimal perturbation and unprecedented spatiotemporal resolution. Zebrafish neuronal circuits represent an ideal system to apply an optogenetic based analysis owing to its transparency, relatively small size and amenability to genetic manipulation. In this review, we describe some of the most recent advances in the development and applications of optogenetic sensors (i.e., genetically encoded calcium indicators and voltage sensors) and actuators (i.e., light activated ion channels and ion pumps). We focus mostly on the tools that have already been successfully applied in zebrafish and on those that show the greatest potential for the future. We also describe crucial technical aspects to implement optogenetics in zebrafish including strategies to drive a high level of transgene expression in defined neuronal populations, and recent optical advances that allow the precise spatiotemporal control of sample illumination.

**Keywords:** neurobiology; neuronal circuits; optogenetics; zebrafish.

## Introduction

The advent of optogenetics has been arguably the greatest revolution in neuroscience in the past decade (Deisseroth et al., 2006; Luo et al., 2008; Miesenbock, 2009; Zhang et al., 2010). For the first time, scientists have the ability to directly test the effect of silencing and activating neuronal circuits, while observing the activity of neuronal populations or behavior in live animals. This unprecedented approach relies on genetically encoded tools that can monitor or control neuronal activity. We will refer to these proteins as optogenetic sensors or actuators, respectively. From the initial studies, it is already evident that an ideal system in which to apply optogenetics would be transparent to allow stimulating actuators with light and detecting light signals from sensor proteins. In addition, it must be amenable to genetic manipulation so that genetically encoded optogenetic tools can be expressed from stable or transient transgenic reporter genes. Given these requirements, it is no surprise that the zebrafish has been one of the first model organisms in which this emerging set of techniques has been applied with great success (McLean and Fetcho, 2008; Baier and Scott, 2009; Friedrich et al., 2010). Zebrafish larvae have a unique combination of properties that make them ideal experimental models to exploit the power of optogenetics maximally. First and foremost, zebrafish are transparent at embryonic and early larval stages. Secondly, zebrafish larvae have a relatively small and simple nervous system, making virtually any neuron accessible to high resolution *in vivo* microscopy. Larval zebrafish develop robust and complex behaviors that can be used as the final readout of neural circuit manipulation (Saint-Amant and Drapeau, 1998; Baier, 2000; Drapeau et al., 2002; Neuhauss, 2003; Orger et al., 2004; Fleisch and Neuhauss, 2006; Sison et al., 2006). Furthermore, for over 30 years, the zebrafish has been used as a genetic system and a plethora of techniques exist to target specific neuronal populations. In particular, in recent years, the creation of efficient transgenic methods based on the Tol2 transposon system (Kawakami, 2004) and the adaptation of the upstream activating sequence (UAS)/Gal4 system widely used in *Drosophila* have enormously expanded the power of zebrafish as a model system for neurosciences (Asakawa and Kawakami, 2008; Halpern et al., 2008; Scott, 2009). This review offers an updated view of the optogenetic toolbox that has already been successfully used in zebrafish and highlights some of the new tools that hold the greatest promise for the future. We also briefly describe some technical advances that allow the precise targeting of specific neurons using both genetic and optical methods.

## Optogenetic sensors for zebrafish neuroscience

In neurons, action potential spikes trigger large influxes of free divalent calcium ions ($Ca^{2+}$) (Jaffe et al., 1992). Voltage-gated $Ca^{2+}$ channels located throughout the plasma membrane mediate these influxes. Furthermore, synaptic input triggers local $Ca^{2+}$ entry through neurotransmitter receptors at post-synaptic terminals (Muller and Connor, 1991). Therefore, the pattern of action potentials and synaptic activity both lead

to detectable changes in $Ca^{2+}$ concentration ($[Ca^{2+}]$) (Denk et al., 1996; Yasuda et al., 2004). Intracellular imaging of $[Ca^{2+}]$ has been efficiently performed using fluorescent dyes derived from the highly selective $Ca^{2+}$ chelator BAPTA (Tsien, 1980). Currently, researchers have a large number of such dyes that vary in crucial properties such as excitation and emission spectra, $Ca^{2+}$ affinity, kinetics of association and dissociation, fluorescent intensity or wavelength ratiometric readout, and cell permeability (Paredes et al., 2008). Although these dyes have been shown to be capable of detecting $[Ca^{2+}]$ fluctuations linked to neuronal activity in over three decades of research, they present some technical limitations. Dyes can be difficult to deliver inside cells in the intact nervous system, often requiring invasive injection procedures. Labeling in the cytoplasm can decrease over time owing to active secretion or subcellular compartment sequestration, making chronic or long-term $[Ca^{2+}]$ detection in the same cells impossible. Furthermore, chemical calcium dyes cannot be targeted to specific, genetically defined cell types or targeted to particular subcellular regions. Over a decade ago (Miyawaki et al., 1997), these limitations were overcome through the generation of genetically encoded calcium indicators (GECIs). GECIs consist of modified fluorescent proteins that are intrinsically capable of varying their emission properties according to the $[Ca^{2+}]$ in their environment (Barth, 2007; Garaschuk et al., 2007; Kotlikoff, 2007; Hires et al., 2008; Mank and Griesbeck, 2008; Wilms and Hausser, 2009). GECIs can be divided into two classes, the first based on single fluorescent proteins and the second based on fluorescent protein pairs that mediate fluorescent resonance energy transfer (FRET). GECIs of the first class typically consist of circularly permutated fluorescent proteins whose spectral or fluorescent efficiency properties are altered in response to conformational changes driven by $Ca^{2+}$ binding to $Ca^{2+}$ recognition elements present in the GECI structure itself (Baird et al., 1999; Nagai et al., 2001; Nakai et al., 2001). Similar conformational changes driven by $Ca^{2+}$ binding modulate FRET efficiency in the second class of GECIs, altering emission intensity of both FRET donor and acceptor fluorophores (Miyawaki et al., 1997). GECIs can be further subdivided by the different $Ca^{2+}$ recognition elements that they use. In most cases, the $Ca^{2+}$ binding domains of calmodulin (CaM) (Miyawaki et al., 1997; Nakai et al., 2001) or troponin C (Heim and Griesbeck, 2004) have been utilized, often in engineered forms to modulate $Ca^{2+}$ binding affinity and response properties. Despite several recent improvements and intrinsic advantages, GECI performance remains inferior to synthetic calcium indicators in signal-to-noise ratio, kinetics and photostability (Knopfel et al., 2006; Mank and Griesbeck, 2008). In practice, detection of single action potentials using GECIs is difficult to reach without averaging (Mank and Griesbeck, 2008). For this reason, the use of synthetic calcium dyes in zebrafish has been predominant. Calcium dyes have been successfully applied to monitor neuronal responses in the larval visual and olfactory systems, the hindbrain, and the spinal cord (Cox and Fetcho, 1996; O'Malley et al., 1996; Friedrich and Korsching, 1997; Ritter et al., 2001; Brustein et al., 2003; Tabor et al., 2004, 2008; Bhatt et al., 2007; Mack-Bucher et al., 2007; McLean et al., 2007; Yaksi et al., 2007, 2009; Orger et al., 2008; Ramdya and Engert, 2008; Sumbre et al., 2008; Bollmann and Engert, 2009; Niessing and Friedrich, 2010).

Higashijima and colleagues published the first study in which a GECI was applied in zebrafish to detect neuronal activity (Higashijima et al., 2003). The FRET based calcium sensor yellow cameleon 2.1 (YC2.1) (Miyawaki et al., 1999) was used to image and measure activity in spinal cord neurons in larvae by confocal microscopy. In this pioneering research, the YC2.1 protein was expressed in the cytoplasm using a general neuronal promoter in transient and stable transgenic experiments. Action potentials produced either by electrical stimulation or escape responses to touch were detected in Rohon Beard (RB) sensory neurons, primary motoneurons, and CiD interneurons in the spinal cord (Higashijima et al., 2003). Owing to the ratiometric response of the YC2.1 indicator and because motion artifacts do not constitute a major problem, the authors could image neuronal activity in the escape response in partially restrained animals. In RB neurons, electrical stimulation that elicited a single spike could be detected by YC2.1. This result represents an ideal situation and is unlikely to be a general feature of other neurons. Because of the very long decay kinetics of the FRET signal (sometimes >10 s) and the rapid saturation of the response, series of action potentials could not be resolved nor the total number of spikes determined. Because a pan neuronal promoter was used, neurons could only be identified by morphology and position within the spinal cord, and in many regions of the central nervous system cell labeling was too dense to allow single cell imaging. However, stable transgenic animals were normal in their development and behavior, confirming that the expression of GECIs at high levels in the majority of neurons has little to no effect on the physiology of the animals. A statistical method has also been proposed to increase the signal-to-noise ratio of YC2.1 expressed in zebrafish neurons, but has so far only been applied to dissociated transgenic spinal cord neurons in culture (Fan et al., 2007).

Because of the small signals that cameleon proteins show *in vivo* compared to their *in vitro* performance, recent research in zebrafish has focused on other GECIs such as inverse pericam (IP) (Nagai et al., 2001), G-CaMP1.6 (Ohkura et al., 2005), and G-CaMP2 (Tallini et al., 2006), which are single-wavelength indicators that lack the benefits of ratiometric approaches. IP transgenic fish were used to detect $[Ca^{2+}]$ variations in olfactory bulb neurons in intact larvae starting at 2.5 days post-fertilization, and in the dissected adult olfactory bulb in response to odor stimulation (Li et al., 2005a). In contrast to IP, YC2.1 did not show any signal variation in similar preparations. Given that both GECIs were expressed under the same pan neuronal promoter and their expression patterns were indistinguishable, the authors hypothesize that IP possesses a dynamic range better matching the $[Ca^{2+}]$ variations normally occurring in olfactory bulb neurons (Li et al., 2005a).

Two other studies have demonstrated the potential of the optogenetic sensor G-CaMP1.6 (Ohkura et al., 2005) when combined with zebrafish genetic tools. Distinct stages of

cardiac conduction in the developing heart were identified and analyzed using a transgenic line expressing G-CaMP1.6 under a cardiac specific promoter (Chi et al., 2008). This tool was also used for a forward genetic screen to isolate novel, conduction-specific mutations, in the first application of a GECI for such an approach.

Neuronal activity in the zebrafish larva visual system has also been monitored using G-CaMP1.6 (Sumbre et al., 2008). In this study, the authors used a calcium indicator dye to observe rhythmic activity among specific tectal neuronal ensembles following repetitive visual conditioning, but the additional use of a GECI allowed them to address a fundamental issue. Targeting the expression of G-CaMP to retinal ganglion cells only ruled out that the observed rhythmic activity originated in the retina and, instead, supported an intratectal mechanism.

A modification to the G-CaMP protein allows specific targeting to presynaptic terminals to detect synaptic activity *in vivo* (Dreosti et al., 2009). Tallini and colleagues fused the G-CaMP2 (Tallini et al., 2006) coding sequence to the presynaptic protein synaptophysin. The resulting reporter localized to presynaptic terminals, enabling their visualization *in vivo* and had a linear response over a wide range of spiking frequencies. This latter property is due to the specific localization of the G-CaMP protein that senses only the brief calcium transient in the presynaptic compartment rather than changes in the bulk calcium concentration in the cytoplasm. This construct was expressed in both tectal neurons and in retina bipolar cells. The presynaptic localized G-CaMP protein performed equally well in both locations, recording activity from the spiking neurons of the optic tectum and the activity patterns across dozens of ribbon synapses in the retina bipolar cells that display a graded voltage signal. In a follow-up study, an interesting, semi-automated method was devised to identify synapses automatically, extract dynamic signals, and assess the temporal and spatial relationships between active units, and was validated on retinal bipolar cells in transgenic larvae (Dorostkar et al., 2010). This is another interesting modification of G-CaMP2 fusion with the membrane-tethering domain of Lck kinase (Shigetomi et al., 2010). The resulting protein, Lck-GCaMP2, had a 10-fold increase in the level near the plasma membrane where calcium transients are maximal. This resulted in an improved signal-to-noise ratio allowing detection of calcium transients in astrocytes that were missed by conventional cytoplasmic G-CaMP2. Additionally, because of its membrane localization, Lck-GCaMP2 offers superior labeling and visualization of fine processes such as dendrites and axonal terminals.

New and better GECI sensors are quickly being developed. A promising new tool application to zebrafish is G-CaMP3, the latest member of the G-CaMP family of calcium sensors (Tian et al., 2009). It has been reported to show increased baseline fluorescence, 3-fold greater dynamic range, and higher affinity for calcium. G-CaMP3 performed better than any other GECI tested in pyramidal cell dendrites and was capable of detecting a single action potential. It has been tested in model organisms including *Caenorhabditis elegans*, *Drosophila* and mice, with zebrafish waiting to be added to the list. Recently, a novel sensor named GCaMP-HS has also been successfully applied in zebrafish spinal cord neurons, showing a greatly improved performance *in vivo* (K. Kawakami, personal communication).

Despite the improvements that have been made and can be expected with GECIs, variation in $[Ca^{2+}]$ remains an indirect way to assess neuronal function. Even with the best recording devices, $Ca^{2+}$ kinetics are an approximation of the real action potential activity and membrane potential changes of excitable cells. To overcome this limitation, various genetically encoded sensors have been developed to detect membrane voltage changes based on fluorescent proteins (Baker et al., 2008; Siegel and Isacoff, 2010). Their application *in vivo* has been limited owing to their relatively low signal-to-noise ratios and slow kinetics. Recently, new improved FRET-based voltage sensors were described (Lundby et al., 2008; Tsutsui et al., 2008) based on the voltage sensing domain of the *Ciona intestinalis* voltage-containing phosphatase (Murata et al., 2005). These sensors display up to 30% change in emission ratio per 100 mV in cultured neuronal cells. Their fast on-off kinetics allow the recording of spikes comparable to action potentials with appreciable changes (2–7%) in emission ratio. The first application of one of these voltage sensors, named Mermaid, (or of any genetically encoded voltage sensor) in an intact organism *in vivo* was to monitor voltage dynamics in the developing zebrafish heart (Tsutsui et al., 2010). These sensors have also successfully applied to record voltage changes in intact mouse brain (Akemann et al., 2010) and they are highly promising for the future applications to record activity from neurons in the intact larval brain.

We conclude this overview of optical sensors for recording neuronal activity by mentioning a recently developed technique in zebrafish that uses bioluminescence in freely behaving animals (Naumann et al., 2010). Because this approach does not require incident light to emit, it is not formally an optogenetic tool, but nevertheless highlights a technical innovation. In this study, neuronal activity was detected from zebrafish in which genetically defined neuronal populations expressed the bioluminescent protein (GFP)-Aequorin (Baubet et al., 2000). This protein in the presence of an appropriate substrate (coelenterazine) catalyzes an oxidizing reaction that emits a photon as a byproduct. Because the reaction is $Ca^{2+}$ dependent and Aequorin has no basal activity at resting $[Ca^{2+}]$ levels, photon emission is a measure of neuronal excitation. The recording setup involves a large area photomultiplier tube in a light-proof enclosure, which does not provide any spatial information about the source of the emitted photons within the animal and therefore relies for its specificity solely on the promoter used to drive *aequorin* gene expression. This approach allows, for the first time, long-term monitoring of activity of a genetically specified neuronal population in freely behaving larvae.

## Optogenetic actuators for zebrafish neuroscience

Understanding the relevance of dynamic firing of neuronal networks to behavior requires genetic targeting of actuators

that enable remote control of neuronal activity with spatial and temporal accuracy. This novel technology has been applied to awake, behaving animals and has enabled the role of neuronal firing in determining behavior (Douglass et al., 2008; Arrenberg et al., 2009; Wyart et al., 2009; Schoonheim et al., 2010) and in establishing short and long range synaptic connectivity (Petreanu et al., 2007, 2009; Cruikshank et al., 2010) to be tested for the first time *in vivo*.

There are two major classes of light-gated actuators: (i) the microbial opsins (Ebnet et al., 1999; Nagel et al., 2003; Boyden et al., 2005; Zhang et al., 2007a, 2008; Chow et al., 2010), where the retinal binds to the channel or pump to control the photo-isomerization and gating of the protein; and (ii) the engineered neuronal receptors/channels (Banghart et al., 2004; Trauner and Kramer, 2004; Chambers et al., 2006; Volgraf et al., 2006; Gorostiza et al., 2007; Szobota et al., 2007; Fortin et al., 2008) tethered to a chemical photoswitch which controls their gating under light pulses.

### Microbial opsins used for activating neurons

The microbial opsins are channels or pumps normally found in nature in light-sensitive cells of marine algae that exhibit phototaxis and photophobic behavior (Beckmann and Hegemann, 1991; Deininger et al., 1995; Holland et al., 1997; Braun and Hegemann, 1999; Ebnet et al., 1999; Ehlenbeck et al., 2002; Nagel et al., 2003, 2005a). They can operate at the millisecond timescale and do not need the addition of an exogenous chemical cofactor because their chromophore, all-*trans* retinal, is present at sufficiently high concentration in the extracellular space of vertebrates. Because their conductance is relatively small, they require a high level of expression at the membrane for reliable control of spiking.

The major channel used for triggering spiking in neurons is channelrhodopsin (ChR2), a blue light-sensitive cationic channel isolated from the green algae, *Chlamydomonas reinhardtii* (Nagel et al., 2003). Under blue light of relatively low power (on the order of 1 mW/mm$^2$), this cationic channel opens, leading to a depolarization of the cell (Nagel et al., 2003, 2005a; Ernst et al., 2008). The fast kinetics of ChR2 enable reliable temporal precision, reaching spiking rates of approximately 40 Hz in neurons (Boyden et al., 2005; Li et al., 2005b; Deisseroth et al., 2006; Arenkiel et al., 2007; Gradinaru et al., 2007; Zhang et al., 2007a,b). In zebrafish, the original eChR2 was expressed transiently in sensory neurons mediating touch (RB neurons) using an enhancer element from the *islet 1* gene, to demonstrate that activation of a single sensory cell could trigger the escape response at early stages of development (24 h post-fertilization; Douglass et al., 2008).

A variant of eChR2 with a single mutation (H134R) showing larger stationary currents was characterized and validated in *C. elegans* (Nagel et al., 2005b). Recently, eChR2-H134R was used in zebrafish to determine that rhombomere 5 was sufficient in controlling the motor output underlying the optokinetic response (Schoonheim et al., 2010). Another microbial opsin, VChR1, with similar characteristics to ChR2 but red shifted, has been identified in *Volvox carteri* (Ebnet et al., 1999; Zhang et al., 2008).

Novel cationic opsins or variants of existing cationic opsins have been recently characterized and offer attractive experimental possibilities in zebrafish. New forms of eChR2 have been optimized for fast kinetics such as Chief and Chef that were generated by fusion of ChR2 and ChR1 (Lin et al., 2009) and an ultra-fast variant with modification of the site E123. This variant with faster off kinetics, termed 'ChETA', enables reliable firing at 200 Hz in mammalian cells (Gunaydin et al., 2010). By contrast, bistable channelrhodopsins have been engineered by a mutation in site C128 of ChR2 (Berndt et al., 2009; Schoenenberger et al., 2009; Stehfest et al., 2010) which leads to an extended lifespan of the open state while retaining the precise timing of ON and OFF using light pulses of different wavelengths (Berndt et al., 2009). Owing to their long-lasting effect after short light pulses, these variants could be of particular interest for triggering immediate early genes (Schoenenberger et al., 2009) and investigating neuromodulatory and neurosecretory mechanisms in zebrafish.

### Microbial opsins used for silencing neurons

Hyperpolarization of the membrane leading to inhibition of spiking in neurons can be achieved by an efflux of potassium, an influx of chloride or an efflux of protons. The first microbial opsin used for silencing neurons was a light-activated chloride pumping halorhodopsin from *Natronomonas pharaonis* (NpHR) (Hegemann et al., 1985; Oesterhelt et al., 1985). Under yellow light, the pump transports chloride ions into the cell, leading to hyperpolarization of the membrane (Han and Boyden, 2007; Zhang et al., 2007a). An accumulation of NpHR in the intracellular compartment has been reported to lead to aggregation and cell toxicity. Variants of the original NpHR with improved targeting to the cytoplasmic membrane have since been engineered that produce slightly higher currents and reduced toxicity (Gradinaru et al., 2008) (eNPhR). This form has been shown to reduce spiking of neurons efficiently during yellow light pulses in zebrafish larvae (Schoonheim et al., 2010). However, a rebound of high frequency spiking is observed immediately after the yellow light pulse (Arrenberg et al., 2009), making eNpHR difficult to use for strictly silencing neurons. The amplitude of this rebound appears larger with longer pulses of yellow light observed in different cell types and animal models (Han and Boyden, 2007; Zhang et al., 2007a), which is probably owing to the efflux of chloride ions following pump activation. There are three limitations of NpHR: the high power of yellow light necessary to induce efficient silencing (in the range of 50 mW/mm$^2$) can by itself lead to spiking in some neurons in wild-type zebrafish, toxicity owing to accumulation in the endoplasmic reticulum, and a long-lasting inactive state of the pump in response to light.

Improvements over NpHR have been made recently with the discovery of novel proton pumps, Arch from *Halobacterium sodomense*, and Mac from *Leptosphaeria maculans*. These proton pumps show larger currents under lower light power (<10 mW/mm$^2$, green-yellow for Arch and blue-green for Mac) and fast recovery following light-dependent inactivation

*in vitro* and in awake, behaving mice (Chow et al., 2010). Their application in zebrafish should allow an efficient silencing of neurons enabling to test for sufficiency of neurons in circuits.

### Engineering of light-responsive neuronal receptors and channels

An alternative approach to achieve optical control of neuronal activity lies in coupling existing target proteins, channels, or receptors to an exogenous chemical photoswitch (Fortin et al., 2008; Gorostiza and Isacoff, 2008). The photoswitch core consists of an azobenzene functional group which reversibly isomerizes with UV and green light. A maleimide group at one end reacts with an introduced cysteine on the target protein surface, whereas on the other end a ligand functions either as an agonist, antagonist, or blocker of the protein. Photoisomerization in response to UV and green light moves the ligand back and forth in its binding site and therefore controls protein function.

So far, light-gated glutamate receptors (Volgraf et al., 2006; Gorostiza et al., 2007; Szobota et al., 2007) and potassium channels (Banghart et al., 2004; Chambers et al., 2006; Fortin et al., 2008) have been engineered by these means. In particular, the light-gated glutamate receptor LiGluR was obtained by mutating iGluR6 through the addition of a single cysteine near the binding site of glutamate. The photoswitch MAG consists sequentially of a cysteine-reactive group (maleimide), a switch (azobenzene), and the ligand glutamate. With UV light, the azobenzene bends into the *cis* configuration, and thereby bringing the glutamate into the binding site of the receptor, gating the channel and leading to an entry of cations into the cell (Volgraf et al., 2006; Gorostiza et al., 2007). Green light extends the azobenzene group into the *trans*-configuration and moves the glutamate out of the binding site, closing the channel. This process can occur extremely fast and reversibly, leading to remarkable control of neuronal firing (Szobota et al., 2007).

A similar engineering strategy can be extended to a wide variety of receptors and channels that are expressed in neurons. The major advantage lies in the fact that those channels are expressed in neurons, with optimal targeting to the plasma membrane and a conductance much larger than typical microbial opsins. Moreover, because we have accumulated vast knowledge about these neuronal proteins, it is possible to optimize light-gated forms for their permeability to ions, voltage dependence, kinetics, and affinity for endogenous ligands, as well as their intracellular targeting. The major drawback of applying this approach *in vivo* is the efficient labeling of the engineered protein *in situ* with the exogenous photoswitch. However, the application of photoswitches can be effective in zebrafish larvae owing to their high permeability to small molecules. For example, injection of MAG into the spinal cord of zebrafish larvae allowed the efficiently labeling of LiGluR in this neuronal population. Remarkably, the simple bath application of the photoswitch MAG enables the compound to diffuse broadly through the blood-brain barrier into the brain of 5-day-old larvae (Szobota et al., 2007; Wyart et al., 2009). This easy labeling technique was used to demonstrate that LiGluR-dependent activation of Kolmer Agduhr neurons modulates locomotion at early stages of development (Wyart et al., 2009).

## Genetic targeting of neuronal populations in zebrafish

Optogenetics relies on molecular genetic methods to target the expression of sensors and actuators in specific cell populations. In fact, the power of this approach depends on the restricted gene expression that can be achieved. In zebrafish, non-invasive genetic targeting typically requires the creation of stable transgenic lines that are produced by injection of a DNA construct into one-cell stage embryos (Westerfield, 2000; Nusslein-Volhard and Dahm, 2002). This technique has become much more efficient owing to the use of Tol2 transposition (Kawakami, 2004, 2007) or I-SceI meganuclease (Grabher and Wittbrodt, 2008) to increase the rate of injected DNA integration in the genome and the generation of founder fish that carry the transgene integrated in their germline. If a gene with the desired expression pattern is known, its endogenous regulatory sequences can be cloned and used to drive expression of any gene of choice. This approach has the advantage of recapitulating the cell type-specific pattern of expression, but can also lead to frustrating results when not all of the regulatory elements are present in the injected DNA construct, causing incomplete or ectopic expression. To overcome this problem, bacterial artificial chromosomes (BACs) engineered by homologous recombination have been successfully used to create the desired transgenic construct to inject into embryos (Jessen et al., 1999). BACs allow the use of longer sequences of genomic DNA (usually around 100–150 kbp) that are more likely to contain all the required regulatory sequences to recapitulate endogenous gene expression accurately in transgenic animals (Yang et al., 2006). Recently, it has been shown that the Tol2 system can be applied to BAC transgenesis, significantly improving the efficiency of this technique (Suster et al., 2009).

An alternative approach to drive the expression of transgenes in specific neuronal populations relies on the creation of enhancer-trapping or gene-trapping libraries of transgenic lines. Such libraries have been already created in several screens and continue to be generated by the zebrafish community in increasing number (Scott et al., 2007; Davison et al., 2007; Asakawa et al., 2008; Scott and Baier, 2009; Ogura et al., 2009). In these transgenic fish, expression of a marker gene (usually the Gal4 transcription factor) is due to its random insertion close to endogenous (and often unknown) regulatory sequences.

Because the creation of a transgenic line remains a laborious process and expression patterns might not be accurately reproduced following random insertional events, a combinatorial system that allows controlled expression of transgenes by crossing independent activator and responder lines is highly desirable. The bipartite Gal4/UAS system offers this possibility and it has been successfully applied in zebrafish

(Asakawa and Kawakami, 2008; Halpern et al., 2008). This expression system uses the yeast transcription factor Gal4, which activates the transcription of a reporter gene of interest by binding to the UAS *in trans*. This binary system allows the expression of any reporter gene (such as an optogenetic probe) under the control of the UAS to be transcribed in any pattern where Gal4 is active. Other two-component systems are currently used in zebrafish, further expanding the possibilities to express multiple reporter genes in distinct patterns in the same animal. These include the LexPR/LexA (Emelyanov and Parinov, 2008), the Cre/LoxP (Langenau et al., 2005; Pan et al., 2005; Thummel et al., 2005; Liu et al., 2007, 2008; Le et al., 2007; Yoshikawa et al., 2008), the EcR (Esengil et al., 2007), and the Tet (Huang et al., 2005; Zhu et al., 2009) systems. A bipartite system offers a further advantage compared to the direct expression of a reporter gene of an amplification step that produces high expression levels, which are required for effective optogenetic manipulations. Some of these regulatory systems (LexPR/LexA, EcR, and Tet) can be modulated by the addition of exogenous small molecules offering an additional temporal control of transgene expression (Esengil et al., 2007; Emelyanov and Parinov, 2008; Zhu et al., 2009).

Sometimes stable transgenesis is not required, for instance, when single neurons are studied and sparse labeling of a particular cell type is preferable. In this case, foreign DNA can be easily delivered in zebrafish to express a favorite optogenetic tool, by electroporation (Cerda et al., 2006; Hendricks and Jesuthasan, 2007) or by injection in 1–4 cells stage embryos (Koster and Fraser, 2001; Downes et al., 2002; Yoshida and Mishina, 2003; Sato et al., 2007). More recently, a well-designed and versatile method has been developed to generate marked clones of cells that express any desired gene product. This tool relies on both Cre recombinase and UAS/Gal4 systems (Collins et al., 2010). In a fish line containing a transgene called mosaic analysis in zebrafish (MAZe), heat shock induces Cre expression from a heat shock promoter (Halloran et al., 2000). Recombinase-mediated recombination in a random subset of cells is thus triggered, bringing the transcriptional activator Gal4 under control of a ubiquitous promoter. Gal4-VP16 then activates expression of any protein from UAS present on another transgene. Clones of cells expressing any gene product can be generated by crossing MAZe fish with other lines containing UAS-driven transgenes. Heat shock to induce Cre expression can be performed at different developmental points and with different strengths to modulate marked cell clones size.

Another promising option for zebrafish, although not yet as widely used as in mammals, is viral delivery of exogenous DNA. Baculoviruses, sindbis, and rabies viruses can achieve high levels of expression of delivered genes in zebrafish neurons (Wagle and Jesuthasan, 2003; Zhu et al., 2009).

## Illumination strategies for spatiotemporal control of actuators

Optical control of neuronal activity can be achieved at the millisecond resolution by using a wide diversity of light sources in the UV and visible range. The power required for photoswitching is typically between 0.5 mW/mm$^2$ and 500 mW/mm$^2$ depending on the actuator photoswitching efficiency and expression level. As a light source, we can currently use monochromators with a galvanometer driven grating (Volgraf et al., 2006), fast switching light sources equipped with galvanometers (Wyart et al., 2009), bright light-emitting diodes (Campagnola et al., 2008), and lasers (Arrenberg et al., 2009).

As mentioned above, spatial resolution can be achieved genetically with mosaic expression and a broad illumination source (such as a super bright light-emitting diode placed near the animal) (Zhu et al., 2009). However, light pulses in the UV and visible range that act on the retina at sufficient power for isomerization of retinal or chemical switches *in vivo* (>0.1 mW/mm$^2$) will affect the behavioral readout in wild-type animals. Unless activation persists following a light pulse (such as in the case of bistable opsins or of LiGluR after a pulse of UV light followed by no light), confining the field of illumination is often a prerequisite for experiments on awake animals, even when expression of the actuator is specifically targeted to a neuronal population.

A simple and low-cost approach is the closure of the filled aperture in a classical epifluorescence microscope scope to reach single cell ChR2-dependant activation (Douglass et al., 2008). Recently, a laser coupled with an optical fiber of small diameter (50–200 μm) was used to restrain the spread of excitation to single rhombomeres in zebrafish larvae (Arrenberg et al., 2009; Schoonheim et al., 2010). The application of a digital mirror device chip from Texas Instruments (Wang et al., 2007; Wyart et al., 2009) allowed light patterning and activation of either the left or right side of spinal cord segments.

Until now, the spatial resolution of photostimulation was limited in 3D by the cone of the illumination light. Two photon (2P) activation of ChR2 was originally achieved in cultures of mammalian neurons with spiral scanning (Rickgauer and Tank, 2009). In zebrafish, Zhu and colleagues recently activated ChR2 with classical 2P scanning of neurons expressing eChR2 at high levels (Zhu et al., 2009). The use of adaptive optics is another innovative approach which has been successful for generating flexible patterns optical manipulation in 3D. The coupling of spatial light modulators (Papagiakoumou et al., 2008; Zahid et al., 2010) with diffractive optical elements can indeed shape the wavefront of a beam to produce 3D optical sectioning and manipulation with 2P excitation of ChR2 (Andrasfalvy et al., 2010). This method should provide a solution for obtaining spatial confinement of actuator activation and ultrafast switching to probe neuronal circuits in the awake and behaving zebrafish.

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