Optogenetics: A New Enlightenment Age for Zebrafish Neurobiology

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ABSTRACT: **Zebrafish became a model of choice for neurobiology because of the transparency of its brain and because of its amenability to genetic manipulation. In particular, at early stages of development the intact larva is an ideal system to apply optical techniques for deep imaging in the nervous system, as well as genetically encoded tools for targeting subsets of neurons and monitoring and manipulating their activity. For these applications, new genetically encoded optical tools, fluorescent sensors, and light-gated channels have been generated, creating the field of "optogenetics." It is now possible to monitor and control neuronal activity with minimal perturbation and unprecedented spatio-temporal resolution. We describe here the main achievements that have occurred in the last decade in imaging and manipulating neuronal activity in intact zebrafish larvae. We provide also examples of functional dissection of neuronal circuits achieved with the applications of these techniques in the visual and locomotor systems.** © 2011 Wiley Periodicals, Inc. Develop Neurobiol 72: 404–414, 2012

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INTRODUCTION

The zebrafish model has entered the field of neurobiology with the great promise to be amenable to genetic manipulations and optically accessible at larval stages for *in vivo* imaging studies. In addition, due to its small size all neurons from a defined circuit can be monitored at once under a laser scanning microscope. For instance, in the visual motor response pathway, it is possible to monitor neurons from every layer of processing while visually stimulating the animal, including the retina (Dreosti et al.,

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2009), the tectum (Ramdya et al., 2006; Ramdya and Engert, 2008; Sumbre et al., 2008; Del Bene et al., 2010), and the hindbrain (Orger et al., 2008). Defined neuronal subpopulations can be easily targeted using specific promoters (Higashijima et al., 2000) or transgenic lines derived from gene trap or enhancer trap screens (Davison et al., 2007; Scott et al., 2007; Asakawa et al., 2008; Ogura et al., 2009; Scott and Baier, 2009). The physiology of neurons from diverse anatomical regions can be recorded in patch clamp in the living animal (Saint-Amant and Drapeau, 2001; McLean et al., 2007). Finally, the larvae display robust behaviors amenable to functional and genetic dissection (Gerlai, 2010).

The application of optogenetics to zebrafish neurobiology has also enabled for the first time to functionally test the role of identified neurons in behaviors. The optogenetic revolution has allowed neuroscientists to silence and to activate neuronal circuits while observing the neuronal activity or the behavior of live animals (Zhang et al., 2007a; Luo et al., 2008). Light-gated channels and pumps allow the activation and silencing of neurons, and they are referred as "optogenetic actuators." Fluorescent proteins have been engineered to sense calcium or membrane potential and we will refer to them as "optogenetic sensors." This review presents an updated view of the recent application of actuators and sensors in zebrafish neuronal circuits (Table 1).

CONTROLLING NEURONAL ACTIVITY WITH LIGHT

Currently, two major classes of optogenetic actuators exist: (i) microbial opsins (Ebnet et al., 1999; Nagel et al., 2003; Boyden et al., 2005; Zhang et al., 2007b, 2008; Chow et al., 2010), where the endogenous retinal is bound to the protein core and its light-driven isomerization controls gating of the ion channel or ion pump; (ii) engineered neuronal receptors/channels tethered to a chemical photoswitch, where photoisomerization controls the gating of the ion channel (Banghart et al., 2004; Trauner and Kramer, 2004; Kramer et al., 2005; Chambers et al., 2006; Volgraf et al., 2006; Gorostiza and Isacoff, 2007; Gorostiza et al., 2007; Szobota et al., 2007; Fortin et al., 2008; Gorostiza and Isacoff, 2008b; Isacoff and Smith, 2009; Kramer et al., 2009).

Microbial-like opsins are a class of proteins identified in multiple organisms including in unicellular algae where they mediate phototaxis or photophobic behavior (Beckmann and Hegemann, 1991; Deininger et al., 1995; Hegemann, 1997; Holland et al., 1997; Braun and Hegemann, 1999; Ebnet et al., 1999; Ehlenbeck et al., 2002; Nagel et al., 2003; Kateriya et al., 2004; Nagel et al., 2005b). The best known of these proteins is undoubtedly channelrhodopsin-2 (ChR2), originally isolated from the green algae *Chlamydomonas reinhardti* (Nagel et al., 2003). ChR2 is a blue light-sensitive cationic channel that

Table 1 Optogenetic Tools Successfully Applied in Zebrafish

	Application in Zebrafish	References
Optogenetic actuators		
ChR2 and ChR2-H134R	Activation of somatosensory and hindbrain neurons	(Douglass et al., 2008; Arrenberg et al., 2009; Zhu et al., 2009; Schoonheim et al., 2010)
eNpHR	Silencing of hindbrain neurons	(Arrenberg et al., 2009; Schoonheim et al., 2010)
LiGluR	Activation of spinal cord neurons	(Szobota et al., 2007; Wyart et al., 2009)
Optogenetics sensors		
YC2.1	Detection of Ca^{2+} influx in spinal cord neurons	(Higashijima et al., 2003)
IP	Detection of Ca^{2+} influx in olfactory bulb neurons	(Li et al., 2005a,b)
GCaMP1.6	Detection of Ca^{2+} influx in neurons of the optic tectum neuropil	(Sumbre et al., 2008; Del Bene et al., 2010)
GCaMP3	Detection of Ca^{2+} influx in neurons of the optic tectum	(Del Bene et al., 2010)
GCaMP-HS	Detection of Ca^{2+} influx in spinal cord neurons	(Muto et al., 2011)
SyGCaMP2	Detection of Ca^{2+} influx in presynaptic terminals of tectal neurons ad retina bipolar cells	(Dreosti et al., 2009)
(GFP)-Aequorin	Non-immaging detection of Ca^{2+} influx in HCRT neurons and in pan-neuronal transgenic line	(Naumann et al., 2010)
Mermaid	Detection of transmembrane potential changes in heart muscle cells	(Tsutsui et al., 2010)

opens when illuminated with the proper light (peak absorbance ~460 nm) of even relatively low intensity (on the order of 1 mW/mm^2), leading to cell depolarization. ChR2 is able to operate in the millisecond time-scale allowing a temporally precise control of neuronal activity reaching spiking rates of approximately 40 Hz in neurons (Boyden et al., 2005; Li et al., 2005b; Deisseroth et al., 2006; Zhang et al., 2006; Airan et al., 2007; Arenkiel et al., 2007; Gradinaru et al., 2007; Zhang et al., 2007a,b; Schneider et al., 2008; Gradinaru et al., 2009). Due to its relatively low conductance it requires a high level of expression to efficiently activate in vertebrate neurons. To overcome this limitation, a mutated form of ChR2 has been engineered to increase its conductance introducing the single point mutation H134R (Nagel et al., 2005a). ChR2, like other microbic opsins, uses all-trans retinal as chromophore, therefore it does not need the addition of any exogenous chemicals in vertebrate cells, where retinal is normally present at sufficiently high concentrations. To visualize ChR2 expression *in vivo*, fusion variants with fluorescent proteins are normally used. In zebrafish ChR2 has first been used in transient expression experiments driving its expression in somatosensory neurons mediating escape response (Rohon Beard and trigeminal neurons) (Douglass et al., 2008). Specific cell targeting was achieved using the *isl1* enhancer sequence (Higashijima et al., 2000) and high levels of expression were achieved using the UAS/Gal4 bipartite system (Asakawa and Kawakami, 2008; Halpern et al., 2008; Scott, 2009). The analysis of the responses to photoactivation of 24 h post fertilization transgenic larvae confirmed that *isl1* positive somatosensory neurons triggered escape responses (Douglass et al., 2008). Coupling electrophysiology recording of trigeminal neurons with ChR2 activation, the authors demonstrate that in larval zebrafish somatosensory neurons show extremely low levels of spontaneous activity. In fact, single spikes in single trigeminal or Rohon Beard neurons can result in escape responses and drive action potentials in the hindbrain target Mauthner cells.

More recently, cell-specific, temporally controlled, and high-level expression of ChR2 has been achieved in zebrafish using viral gene delivery and the iTet-Off system (Zhu et al., 2009). The use of the bipartite iTetOff system in particular seems quite promising because it offers an alternative to the more widely applied UAS/Gal4 expression system, for which many transgenic lines are available (Davison et al., 2007; Scott et al., 2007; Asakawa and Kawakami, 2008; Ogura et al., 2009; Scott and Baier, 2009). Combining the two systems in the same animal offers the exciting possibility to express different transgenes in two separate and genetically defined neuronal populations. Moreover, the Tet system can be modulated temporally by doxycycline administration. Similar to the UAS/Gal4 system, this approach has the tendency to create transgenic lines showing expression in distinct, sparse, and stable populations of neurons that appeared to be subsets of the neurons targeted by the promoter driving the Tet-activator (Zhu et al., 2009). This is probably due to epigenetic silencing mechanisms (Goll et al., 2009; Akitake et al., 2011). Nevertheless, this property can also be used to create more discrete expression patterns that may reveal novel neuronal circuits. Using this rationale and the virtually pan-neuronal promoter HuC (Park et al., 2000), transgenic lines with sparse transgene expression have been generated (Zhu et al., 2009). In one of these lines, ChR2 expression and activation with low intensity blue light induced backward swimming. This behavior is never observed during spontaneous swimming and it will be of great interest to dissect the neuronal circuit that is responsible for it.

Microbial opsins provided neurobiologists with optogenetic tools that are also able to hyperpolarize membrane potential and therefore silence neurons. The first of these tools was the chloride pump halorhodopsin derived from the halobacterium *Nathomonas pharaonis* (NpHR) (Hegemann et al., 1985; Oesterhelt et al., 1985). NpHR when activated by green/yellow light (peak absorbance ~570 nm) moves chloride ions inside the cell, hyperpolarizing the neurons, and inhibiting spiking. Since the original NpHR has been observed to poorly translocate to the plasma membrane, leading to toxic intracellular aggregates, an improved version with enhanced membrane localization has been developed (eNpHR) (Zhao et al., 2008). In zebrafish, eNpHR has been used to efficiently suppress neuronal activity *in vivo* and, because of the different light activation wavelength, the combination of NpHR silencing with ChR2-mediated excitation has been achieved in the same animal (Arrenberg et al., 2009). Using this versatile system a small region of the caudal hindbrain, just rostral to the *commissura infima Halleri*, was identified to be sufficient to initiate a locomotor command. In this work, high spatial resolution was achieved using fine optical fibers to deliver light to very restricted neuronal populations. Taking advantage of the rebound neuronal activity observed in neurons after prolonged eNpHR silencing, it has been possible to dissect the kinetics of this rebound induced swimming response that occurs in less than 300 ms from the light offset. Most of the latency of this response is due to an intra-hindbrain circuitry ac-

tivity, while spinal circuits and muscle respond very fast to descending inputs.

Recent work, using a similar combination of ChR2 and NpHR co-expression, and optic fiber light stimulation, has made it possible to identify in the zebrafish hindbrain the location of the neurons that, when activated, are sufficient for the generation of eye saccades (Schoonheim et al., 2010). The observed latency between ChR2 activation and initial eye movement was 28 ms only. No other behavior was observed, such as tail movements or escapes, confirming the specificity of the induced response. This group of neurons located in rhombomere 5 has been proposed to be the burst generator present in the larval zebrafish and playing an analogous function to the saccadic burst generator engaged during free viewing in primates (Scudder et al., 2002). In this study, ChR2 has been also used for the first time to rescue a behavioral phenotype in the zebrafish mutant *didy* (Schoonheim et al., 2010). In these larvae, a mutation in the voltage-gated sodium channel NaV1.1lb causes a selective defect in the sustenance of saccadic eye movements while other behaviors appear normal. This phenotype was rescued by optical stimulation of ChR2 expressing burst-generating neurons in the hindbrain. It is likely that the sodium currents added by activated ChR2 depolarized the affected neurons and thus helped them surpass the firing threshold in the absence of functional NaV1.1lb. The remaining endogenous voltage-gated sodium channels were then most likely sufficient to carry the subsequent spikes.

In parallel to the generation of optogenetic tools derived by intrinsically light sensitive proteins, an alternative approach to achieve optical control of neuronal activity has been developed. This approach lies in genetically engineering existing target proteins, channels or receptors, and binding them *in vivo* to an exogenous chemical photoswitch (Kramer et al., 2005; Gorostiza and Isacoff, 2007; Fortin et al., 2008; Gorostiza and Isacoff, 2008a; Isacoff and Smith, 2009; Kramer et al., 2009). The core of the used phostoswitch consists of an azobenzene functional group that isomerizes when illuminated with UV and green light. A maleiamide group at one end of the chemical photoswitch reacts with the target protein binding to an introduced cystein. At the other end of the chemical group, a ligand can function either as an agonist or an antagonist of the protein. UV and green light induces photoisomerization of the azobenzene group to the *cis* and *trans* conformations, respectively. This, in turn, results in the movement of ligand back and forth controlling the protein function.

Using this approach, the light-gated glutamate receptor LiGluR was obtained, mutating the glutamate receptor iGluR6 through the addition of a single cysteine near the binding site of glutamate. When this cysteine was bound *in vivo* to the photoswitch MAG (Maleamide Azobenzene Glutamate), the ion cannel could be rapidly opened and closed with UV and green light pulses, leading to fast and precise control of neuronal firing (Szobota et al., 2007). LiGluR was the first type of these "chemical optogenetic" tools to be used in zebrafish where the *in situ* labeling of the genetically engineered protein with MAG is achieved by simple bath application in the 5-day-old larvae (Szobota et al., 2007). LiGluR was used in zebrafish to analyze motor behavior and to identify the neuronal type in the spinal cord that is sufficient to induce swing behavior (Wyart et al., 2009). Using a novel approach named "intersectional optogenetic" several Gal4 lines were used to express LiGluR in specific spinal cord neurons and the motor behavior output following their activation was tested. This approach led to the identification of a specific group of cerebrospinal fluid contacting neurons that are the spinal input to the central pattern generator (CPG) underlying spontaneous locomotion (see Fig. 1). These neurons were identified as the GABAergic ipsilateral ascending Kolmer Agduhr (KA) cells. The KA-evoked swim was distinct from the asymmetric touch-escape response, which could be induced by photo-stimulation of sensory Rohon-Beard cells with LiGluR. In larval zebrafish, when GABAergic transmission is excitatory (Brustein et al., 2003), KA neurons are necessary for the normal frequency of spontaneous swimming and seem sufficient to drive the CPG.

MONITORING NEURONAL AND SYNAPTIC ACTIVITY WITH LIGHT

Neuronal activity can be monitored *in vivo* through the indirect flux of Ca^{2+} through the membrane leading to an increase in Ca^{2+} concentration (Denk et al., 1996; Yasuda et al., 2004). The groups of Tsien (Baird et al., 1999; Miyawaki et al., 1999), Myazaki (Nagai et al., 2001), Looger (Tian et al., 2009), Nakai (Nakai et al., 2001; Ohkura et al., 2005), and others have generated a large number of genetically encoded indicators with diverse 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). Genetically encoded calcium indicators (GECIs) can be targeted to specific, genetically defined cell

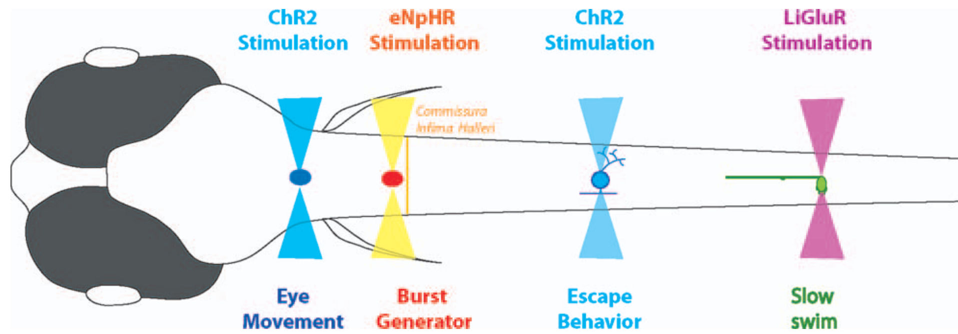

Figure 1 Schematic of the locomotor functions probed using optogenetics in the zebrafish larva central nervous system. A nucleus controlling eye saccade generation was identified in rhombomere 5 using ChR2 stimulation (Schoonheim et al., 2010). A nucleus generating burst swimming was identified in a small region of the caudal hindbrain, rostral to the *commissural infima Halleri* (Arrenberg et al., 2009). The escape behavior was triggered by single Rohon Beard cell activation (Douglass et al., 2008). Remote activation of Kolmer-Agduhr cells lead to a slow swim response (Wyart et al., 2009). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

types or targeted to particular subcellular regions (Miyawaki et al., 1997). GECIs consist of engineered 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 include both single fluorescent proteins, typically consisting of circularly permutated fluorescent proteins whose fluorescence properties are modified in response to Ca^{2+} binding to Ca^{2+} recognition elements (Baird et al., 1999; Nagai et al., 2001; Nakai et al., 2001) and fluorescent protein pairs that mediate Förster resonance energy transfer (FRET) where conformational changes driven by Ca^{2+} binding modulate the FRET efficiency altering emission intensity of both FRET donor and acceptor fluorophores (Miyawaki et al., 1997). The Ca^{2+} binding domains of troponin-C (TnC) (Heim and Griesbeck, 2004) and calmodulin (CaM) (Miyawaki et al., 1997; Nakai et al., 2001) have been used and modified to optimize the conformational changed induced by Ca^{2+} binding. The first demonstration of the use of GECIs in defined population comes from a study in the spinal cord. The pioneering work of Fetcho and colleagues demonstrated the use of GECI to detect neuronal activity in single neuron in the living larva (Higashijima et al., 2003). They used the FRET based calcium sensor yellow cameleon 2.1 (YC2.1) (Miyawaki et al., 1999) to measure activity in spinal cord neurons of larvae by confocal microscopy. Cameleon is a hybrid protein in which cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) are linked by calmodulin and an M13 calmodulin-binding domain. In response to an increase in calcium concentration, calmodulin binds calcium and interacts with M13. The conformational change of the protein increases the efficiency of fluorescence resonance energy transfer from CFP to YFP. Therefore, on CFP excitation, an increase in calcium concentration causes an increase in the YFP/CFP fluorescence intensity ratio. Cameleon can therefore be used as a ratiometric calcium indicator. The YC2.1 protein was expressed transiently or in a stable manner in the cytoplasm using the general neuronal promoter Islet-1 targeting Rohon-Beard neurons (Higashijima et al. 2000) or the α-tubulin promoter to target motoneurons and interneurons (Higashijima, 2003). Electrical stimulation and touch-escape responses elicited spikes in Rohon Beard sensory neurons, primary motoneurons, and CiD interneurons in the spinal cord (Higashijima et al., 2003). Due to the ratiometric response of the YC2.1 indicator, small motion artifacts were minimal in a first approximation and the authors could image neuronal activity during an escape response in partially restrained animals. In Rohon-Beard neurons, electrical stimulation that elicited a single spike could be detected by YC2.1. This study was the first proof of principle of the targeted expression and use of GECIs for detecting neuronal activity in zebrafish larvae. Due to the small signals that cameleon proteins show *in vivo* compared to their *in vitro* performance, later studies in zebrafish have focused on the use of other sensors: the inverse pericam (IP) (Nagai et al., 2001), GCaMP1.6 (Ohkura et al., 2005), and GCaMP2 (Tallini et al., 2006), which are single-wavelength indicators lacking the benefits of ratiometric approaches.

The group of Rainer Friedrich studying neuronal circuits underlying olfaction used IP under the HuC

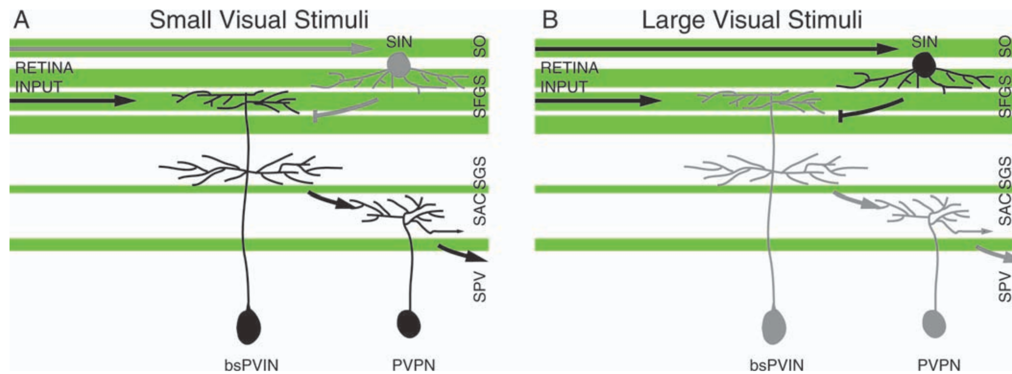

Figure 2 Model of the tectal micro-circuit for size selectivity (Del Bene et al., 2010). A: Small visual stimuli (spots or moving bars) induce robust responses in periventricular interneurons including bi-stratified periventricular interneurons (bsPVIN) and periventricular projector neurons (PVPN). B) Presentation of a large stimulus activates a large number of retinal inputs, which leads to activation of the superficial inhibitory neurons (SIN). As a consequence of this inhibitory transmission, the periventricular neurons are not responding. SO, stratum opticum; SFGS, stratum fibrosum et griseum superficiale; SGS, stratum griseum centrale; SAC, stratum album centrale; SPV, stratum periventriculare. In green is indicated the location of the retinal afferent axons. Black (arrows and neurons) indicates active neuronal pathways, while gray color indicates silent ones. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

promoter to detect [Ca^{2+}] variations in olfactory bulb neurons in intact larvae from 2–6 days post fertilization, as well as in the dissected adult olfactory bulb in response to odor stimulation (Li et al., 2005b). In contrast to IP, YC2.1 failed at showing signal variation under similar conditions. Given that both sensors were expressed under the same pan-neuronal promoter, 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,b).

Regarding the functional dissection of the visual pathways, neuronal activity of retinal ganglion cells has been monitored using GCaMP1.6 (Sumbre et al., 2008; Del Bene et al., 2010). Recently in the group of Herwig Baier, a study further dissected the circuits of the optic tectum, the primary retino-recipient brain region in non-mammalian vertebrates. As in its mammalian counterpart, the superior colliculus, the vast majority of tectal neurons are maximally responsive to stimuli that are smaller than their receptive fields and exhibit smaller responses to larger stimuli. To functionally identify the neural substrate of this spatial selectivity, Baier and colleagues monitored visually-evoked neural activity in genetically defined subpopulations of tectal neurons in zebrafish larvae (Del Bene et al., 2010). Imaging of calcium responses in the neuropil region of the tectum with targeted expression of the sensor GCaMP1.6 revealed that large visual stimuli evoke calcium responses that are largely restricted to the superficial layers of the tectum, whereas small visual stimuli additionally recruit deeper layers of the tectal neuropil. This study used a promising new sensor GCaMP3, the latest member of the GCaMP family of calcium sensors (Tian et al., 2009). It has been reported to show increased baseline fluorescence, three-fold greater dynamic range and higher affinity for calcium. GCaMP3 performed better than any other GECIs tested in pyramidal cell dendrites and was capable of detecting a single action potential (Tian et al., 2009). While single projection neurons expressing transiently GCaMP3 under the *dlx*5/6 promoter responded exclusively to small moving stimuli, a class of inhibitory GABAergic interneurons located superficially in the tectal neuropil responded maximally to large stimuli. These neurons are selectively responsive to large visual stimuli. The confined localization of activity to superficial layers of the tectum for a wide stimulus relies on the recruitment of these cells, since their pharmacological silencing or selective ablation results in a loss of this confinement (Del Bene et al., 2010). Together, these findings identify a novel feed-forward inhibition module operating within the tectum to achieve visual information selectivity (Nevin et al., 2010) (see Fig. 2).

The fusion of to the presynaptic protein synaptophysin to the GCaMP2 protein enabled the group of Lagnado to monitor presynaptic terminals activity *in vivo* (Tallini et al., 2006; Dreosti et al., 2009). The resulting reporter localized to presynaptic terminals, enabling their visualization *in vivo* and it had a linear

response over a wide range of spiking frequencies because of the specific localization of the GCaMP2 protein optimized to sense brief calcium transient in the presynaptic compartment rather than changes in the bulk calcium concentration in the cytoplasm. The fusion was expressed in both tectal neurons and in retina bipolar cells, 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). Recently, Akira Muto in the groups of Nakai and Kawakami observed calcium transients at early stages of development in CaP motoneurons using a novel sensor named GCaMP-HS *in vivo* (Muto et al., 2011).

A complementary way to monitor intracellular [Ca^{2+}] variations and neuronal activity has been recently developed and successfully applied in zebrafish using bioluminescent signal recording in freely behaving animals (Naumann et al., 2010). This method does not require incident light and does not provide any spatial information on the emitted light signal, but it relies on the specific genetic targeting of defined neuronal populations. Neuronal activity is instead detected as bioluminescent signal emitted by transgenic animals whose neurons express the Ca^{2+}-sensitive photoprotein (GFP)-Aequorin (Baubet et al., 2000). Aequorin has no basal activity at resting [Ca^{2+}], while emits photon when [Ca^{2+}] increase as result of neuronal activity. To emit photons upon binding calcium, this protein needs to oxidize a chemical substrate (provided externally in the zebrafish preparation) known as coelenterazine (CLZN). Freely swimming transgenic zebrafish larvae expressing (GFP)-Aequorin can be monitored in non-imaging assays for neuronal activity using large area photomultiplier in a light-proof enclosure. Using this set up, neuronal activity has been recorded in transgenic animals expressing (GFP)-Aequorin in most neurons over many days. This activity was correlated with locomotor behaviors like spontaneous swimming or startle response to mechanical tap (Naumann et al., 2010). Furthermore in this study, the authors expressed (GFP)-Aequorin in a small group of hypocretin-positive (HCRT) neurons of the hypothalamus that have been shown to control arousal in mammals and fish (Prober et al., 2006; Sakurai, 2007; Yokogawa et al., 2007). In these transgenic larvae HCRT neuronal activity was shown to be specifically associated with periods of consolidated locomotor activity, consistent with the hypothesis that HCRT promotes wakefulness and inhibits rest in zebrafish larvae (Prober et al., 2006). This technique was also showed to be sensitive enough to detect activity from a single (GFP)-Aequorin expressing neuron in transient transgenic animals and, with a stroboscopic illumination and with a gated photomultiplier detector, it could be adapted to record visually evoked behaviors (Naumann et al., 2010).

Since variation in [Ca^{2+}] remains an indirect way to assess neuronal function, another line of research aims to improve genetically encoded membrane potential sensors (Baker et al., 2008; Siegel and Isacoff, 2010). Recently new improved FRET-based voltage sensor were described (Tsutsui et al., 2008) based on the voltage sensing domain of the *Ciona intestinalis* voltage-containing phosphatase (Ci-VSP) (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 allows the recording of spikes comparable to action potentials with appreciable changes (2–7%) in emission ratio. One of these voltage sensors, named Mermaid, has been used in zebrafish to monitor voltage changes in the developing heart (Tsutsui et al., 2010). These sensors used in mouse brain preparations (Akemann et al., 2010) are highly promising in the intact zebrafish larval brain.

A Bright Future Ahead

It is clear from these few examples how the application of optogenetics to zebrafish offers great promises for the future. New tools are developed at high speed and many of them wait to be applied in zebrafish. For instance, a mutation in site C128 of ChR2 has generated a bi-stable forms of this protein (Berndt et al., 2009; Schoenenberger et al., 2009; Stehfest et al., 2010) that convert a brief pulse of light into a stable step in membrane potential due to their extended open time. These forms retain precise temporal control and can be tightly controlled with pulses of light of different wavelength (Berndt et al., 2009). Moreover another microbial opsin, VChR1, with similar characteristics to ChR2 but red shifted excitation wavelength, has been identified in the colonial green alga *Volvox carteri* (Ebnet et al., 1999; Zhang et al., 2008). The different excitation spectra of VChR1 and ChR2 offer the possibility to combine these tools in the same animal and to control different cell types with different light wavelengths. Improvements in the kinetic control of Chr2 have also been made. New forms optimized for fast kinetics were generated by fusing different parts of ChR2 and ChR1 to create the light gated channels named "ChIEF" and "ChEF"

(Lin et al., 2009). These variants show reduced inactivation upon prolonged stimulation compared to ChR2 and improves the kinetics of the channel by enhancing the rate of channel closure after stimulation allowing for a more precise temporal control of neuronal stimulation. In addition, an ultra-fast variant introducing the point mutation in the site E123T of ChR2 has also been published. This variant with faster off kinetics, termed "ChETA" (from ChR2-E123T accelerated), enables reliable firing up to 200 Hz in mammalian neurons (Gunaydin et al., 2010).

In the field of optogenetic tools for inhibit neuronal activity, novel microbial opsins, Arch from *Halorubrum sodomense* and Mac from *Leptosphaeria maculans,* have been reported (Chow et al., 2010). These proteins act as protonic pumps hyperpolarizating the plasma membrane by an efflux of H^+ ions. 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 than NpHR. Furthermore, Arch spontaneously recovers from light-dependent inactivation, unlike NpHR that enter long-lasting inactive states in response to light. pH changes created by these protonic pumps are well tolerated by neurons probably because they are minimized by self-limiting mechanisms providing for a safe and naturalistic form of neural silencing.

Perhaps, among the "chemical optogenetics" tools developed for neurosciences, the most innovative developments will be observed in the future. So far the groups of Ehud Isacoff, Richard Kramer, and Dirk Trauner have engineered light-gated glutamate receptors (LiGluR) and potassium channels (Banghart et al., 2004; Chambers et al., 2006; Fortin et al., 2008; Janovjak et al., 2010). A similar engineering strategy can be extended to a wide variety of target proteins including ionotropic and metabotropic neurotransmitter receptors, allowing the dissection of specific pathway mediated by the different receptor forms. The major advantages of this approach are that these membrane proteins are normally expressed in neurons, with optimal targeting to the plasma membrane. Moreover, ionotropic typical neurotransmitter receptors have a conductance much larger than microbial opsins. Since we have accumulated vast structural and biochemical knowledge about these membrane proteins, it is possible to optimize light-gated forms for their permeability to ions, voltage dependence, kinetics, and affinity for endogenous ligands. The major drawback of applying this approach *in vivo* is the efficient labeling of the engineered protein *in situ* with the exogenous photoswitch. However, in zebrafish larvae the application of photoswitches can be easily achieved due to their high permeability to small molecules as shown by the LiGluR example (Szobota et al., 2007; Wyart et al., 2009).

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