Bioluminescence as a Tool to Monitor Neural Activity in Freely Behaving Animals

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Introduction

To correlate the activation of specific neurons with the execution of specific behaviors, it is necessary to monitor neural activity while an animal behaves. Neural recordings in freely moving animals are possible with electrophysiology, but the available techniques are invasive, often cannot target specific neurons, and are restricted to organisms that can physically transport the required electronics (Wilson and McNaughton, 1994; Fee, 2000; Kralik et al., 2001; Hahnloser et al., 2002; Miller and Wilson, 2008).

Optical techniques, which use genetic strategies to target protein reporters to specific neurons and noninvasively monitor their activity, provide a promising alternative (Denk and Svoboda, 1997; Adelsberger et al., 2005; Deisseroth et al., 2006; Luo et al., 2008). These tools have provided access to neurons in the larval zebrafish brain and allow researchers to monitor and manipulate their activity *in vivo* (O'Malley et al., 1996; Brustein et al., 2003; Niell and Smith, 2005; Douglass et al., 2008; Ramdya and Engert, 2008). In particular, synthetic and genetically encoded fluorescent Ca^{2+}-indicators, which target distinct neural populations, have been used to relate the activity of defined cell types to different stimuli or behaviors (Takahashi et al., 2002; Higashijima et al., 2003; McLean et al., 2007; Orger et al., 2008). However, several drawbacks apply: Fluorescence imaging strategies used to optically detect neural activity are selective for signals arising from a narrow focal plane, and these signals are corrupted when motion causes fluorescently labeled neurons to enter and exit the imaged region (O'Malley et al., 1996; Gahtan et al., 2002). This sensitivity to movement has limited imaging techniques to the study of restrained, paralyzed, or anesthetized animals for which behavior is abolished or severely restricted (Galizia et al., 1999; Briggman et al., 2005; Dombeck et al., 2007; Orger et al., 2008).

In principle, a nonimaging optical technique could allow researchers to monitor neural activity in freely behaving zebrafish. A nonimaging system utilizes a large-area photo detector to collect the light emitted from anywhere within the behavioral arena; as a consequence, optical signals from neurons labeled in the translucent organism would be unaffected by an animal's movement within the collection volume. Although this approach sacrifices spatial resolution, when imaging is used with freely behaving animals, the light is scattered in the intact tissue, and the motion of the labeled neurons outside the focal plane severely degrades the available spatial information. Furthermore, natural behavior requires an arena size substantially larger than the animal. Thus, unless behavior is slow enough to allow the imaging setup to accurately move along with the animal, the entire arena must be imaged at high resolution to gain any significant spatial information while maintaining high frame rates capable of monitoring activity on physiologically relevant time scales.

Imaging Zebrafish Neurons Using a Novel Nonimaging Technique

In our zebrafish assay, a 1 megapixel camera imaging the entire 12.5 mm diameter arena would have pixel sizes >10 μm, larger than most zebrafish neurons. Given the limited spatial information available to an imaging assay of behaving animals, we decided to pursue a nonimaging approach, which is technically straightforward, temporally fast, and potentially more sensitive. That is, it is able to detect a larger portion of emitted light because the lack of intermediate optics allows the detector to be installed close to the behavior arena. Furthermore, in our experimental setup, spatial resolution can be restored indirectly using genetic targeting strategies that spatially restrict the expression of our reporter to neurons of interest (Luo et al., 2008) and provide a powerful new tool for selectively recording from genetically defined neurons during natural behavior.

Unfortunately, two features of the commonly used fluorescent activity reporters preclude their use in such a nonimaging setup:

- First, most fluorescent Ca^{2+} indicators have baseline light emission (when unbound to Ca^{2+}), and, even without this basal fluorescence, autofluorescence would provide a significant background signal. With these sources of background emission, any changes in excitation or detection efficiency caused by motion within the collection volume would produce changes in the detected fluorescence unrelated to neural activity; and
- Second, the use of fluorescent indicators in a nonimaging setup would require an intense visible excitation light to homogenously fill the behavior/collection arena. In addition to the technical challenge, this excitation light would disrupt assays of vision and might confound other behavioral experiments (e.g., studies of sleep).

In contrast to fluorescent reporters, aequorin, a Ca^{2+}-dependent bioluminescent protein produced by the jellyfish *Aequorea Victoria* (Shimomura, 1995), has no background light emission at basal Ca^{2+} levels and does not require excitation light. Based on these properties alone, we reasoned that aequorin might be well suited for nonimaging assays of neural

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activity in freely moving zebrafish. Furthermore, aequorin has demonstrated excellent characteristics as a genetically encoded Ca^{2+} sensor (Baubet et al., 2000). Upon binding calcium, aequorin (Luciferase) catalyzes the completion of the Luciferase reaction, the oxidation of its substrate (Luciferin) coelenterazine (CLZN), resulting in the production of a blue photon (Daunert and Deo, 2006).

Purified aequorin has been employed as an optical indicator of intracellular Ca^{2+} in many cell types, including neurons (Ashley and Ridgway, 1968; Smith and Zucker, 1980; Ross, 1989). In jellyfish, aequorin naturally occurs as a complex with green fluorescent protein (GFP), and via a process termed chemiluminescence resonance energy transfer (CRET), transfers the energy from CLZN oxidation to GFP, resulting in the emission of a green photon (Hastings and Johnson, 2003).

The efficiency of Ca^{2+}-dependent photoemission from *Aequorin* is enhanced when associated with GFP (from 10% to 90%), which inspired the development of a GFP–aequorin (GA) fusion (Baubet et al., 2000). GA retains the fast kinetics of aequorin (6-30 ms rise time) (Shimomura et al., 1993), and its sensitivity to Ca^{2+} concentrations range from 100 nM to 10 uM (Curie et al., 2007), which is on par with the best synthetic Ca^{2+} sensors. In addition, the associated GFP provides a fluorescent tag that can be imaged using conventional fluorescence methods in order to localize and quantify GA expression. These improved features of GA have fostered new interest in bioluminescence assays for neural Ca^{2+}

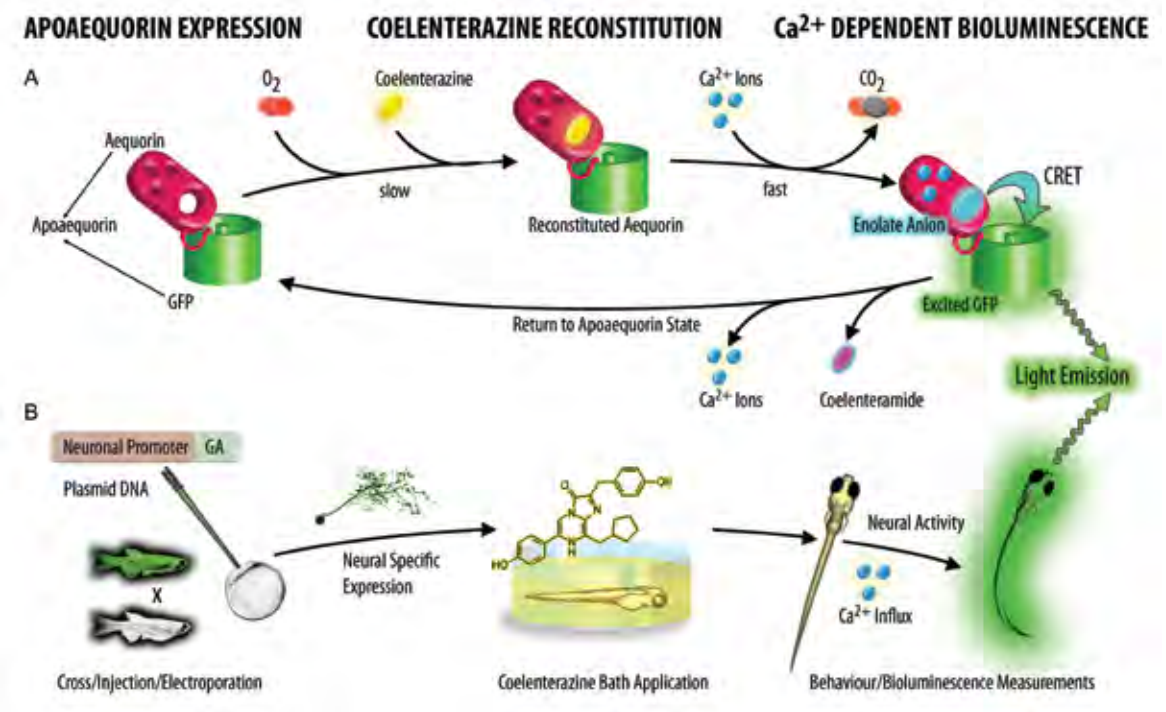


Figure 1. Neuroluminescence in zebrafish: mechanism and method. The upper diagram (***A***) schematizes the molecular reaction underlying the Ca^{2+}-dependent light emission of GA, and the lower diagram (***B***) shows how GA was employed as a bioluminescence reporter of neural activity (neuroluminescence) in freely swimming zebrafish. ***A***, Light emission requires the reaction of GFP-Apoaequorin (Ga) with CLZN. Followed by its reaction with oxygen to form a stable peroxide intermediate, one CLZN molecule will stably, but not covalently, bind to Ga to form GA. Only then can the binding of Ca^{2+} ions induce a conformational change in GA that initiates the oxidative decarboxylation of CLZN. This change releases CO_2 and produces an excited enolate anion that transfers its energy by a process known as nonradioactive CRET, which excites GFP and eventually results in the emission of a green photon. After photon emission, both the coelenteramide and Ca^{2+} are released and Ga is again available for reconstitution. ***B***, Neural expression of Ga is achieved by microinjecting embryos at the single-cell stage with plasmid DNA encoding Ga under the control of a neuron-specific promoter. Generation of stable transgenic fish or other expression targeting methods (e.g., electroporation) can also be used to introduce Ga into the neurons of interest. Neuronal Ga reacts with coelenterazine *in vivo* to form the luminescent GA by immersing the entire zebrafish larva in embryo medium containing CLZN dissolved in cyclodextrin. After an incubation time, neural Ca^{2+}-dependent light emission (neuroluminescence) can be detected during unrestrained behavior.

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signals, and GA has been successfully employed to monitor pharmacologically evoked activity in a variety of experiments: to study neural populations of restrained flies (Martin, 2007), detect mitochondrial Ca^{2+} in the muscles of behaving mice (Rogers, 2007), and image the bioluminescent signals from individual neurons in disassociated cell cultures and slice preparations (Drobac, 2009).

Here we describe the implementation of a novel technique that fundamentally extends these approaches. It comprises a nonimaging setup for noninvasively monitoring the GA bioluminescence resulting from the neural activity of genetically specified neurons in larval zebrafish during natural, unrestrained behavior. Although initially limited to behaviors naturally occurring in darkness, we also describe and implement a novel bioluminescence detection strategy that uses stroboscopic illumination to reproduce natural visual illumination, and thus extends this technique to the investigation of visually driven behaviors.

Neuroluminescence reports neural activity in a freely behaving zebrafish

The mechanism of Ca^{2+}-dependent bioluminescence from GA and the steps taken to use GA as a neural activity reporter are schematized in Figure 1. Neuron-specific expression of GFP-Apoaequorin (Ga) was achieved by injecting single-cell embryos with plasmid encoding Ga downstream of a neural promoter. Following a 24 h exposure to CLZN, transgenic panneuronal GA zebrafish (Fig. 2*A*) were placed into the recording device (Fig. 2*B*), where they swam freely within a behavior chamber positioned directly beneath a large-area photomultiplier tube (PMT). While the PMT detected single photons emitted within the arena, an infrared (IR) charge-coupled device (CCD) camera tracked fish movement.

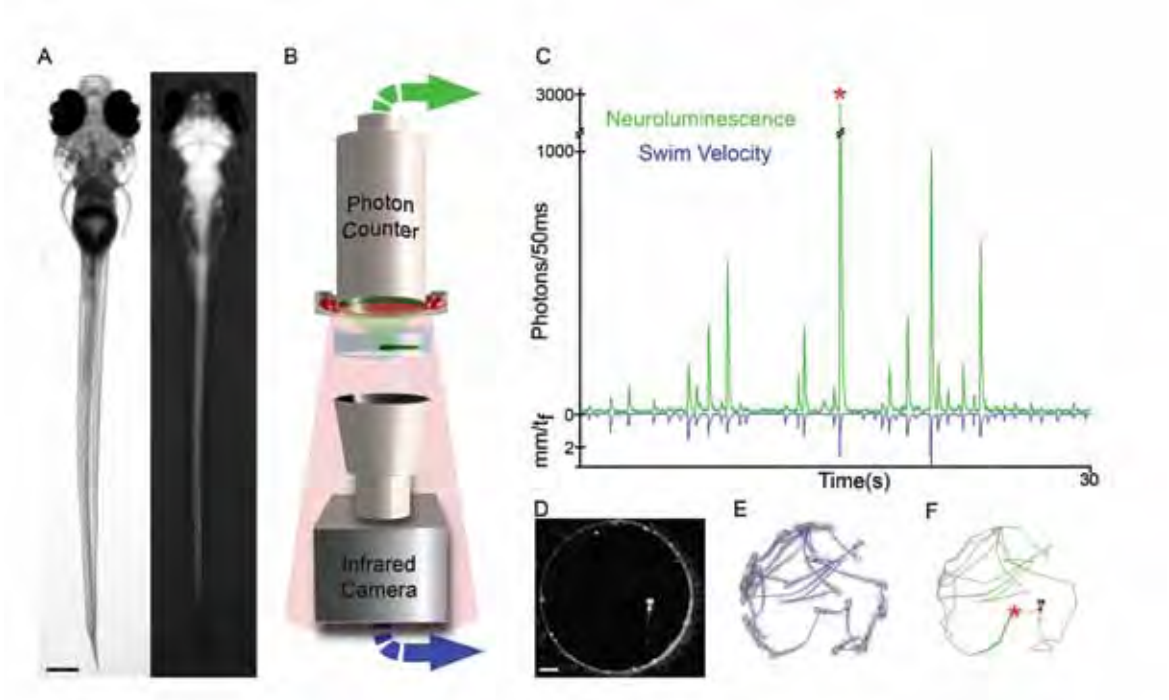


Figure 2. Monitoring the neural activity of freely behaving zebrafish. ***A***, Bright-field (left) and fluorescence (right) micrographs of a 7 dpf *Nbt:GA* transgenic, *nacre*(–/–) zebrafish larva. ***B***, Neuroluminescence setup: A large-area (25 mm diameter) photon-counting PMT is situated above a transparent behavior chamber (12.5 mm diameter, not drawn to scale). The highly sensitive light detector is protected by an IR-blocking filter so that a ring of IR LEDs can be used to illuminate the behavioral chamber. Fish are detected by an IR-sensitive CCD camera positioned below the chamber. The spectral separation between bioluminescence and illumination allows for the simultaneous recording of neuroluminescence signals and the behavior of freely swimming zebrafish larvae. ***C***, Exemplary neuroluminescence recording of a 7 dpf *Nbt:GA* transgenic zebrafish larva previously exposed to CLZN. Photon emission and behavior (swim speed in millimeter moved per frame period [mm/t_f]) is shown for a 30 s recording. A mechanical stimulus was delivered at 15 s (red asterisk), inducing a fast startle response and an increase in neuroluminescence. ***D***, Raw image acquired by the IR CCD camera during simultaneous neuroluminescence recording. ***E***, Superposition (inverted grayscale) of all frames acquired during the 30 s recording period shown in ***C***, with the fish position trajectory shown as a blue color overlay. ***F***, The fish trajectory shown in ***D*** is overlaid with a green line displaying neuroluminescence amplitude as line-width; (*) indicates the time of the mechanical stimulus.

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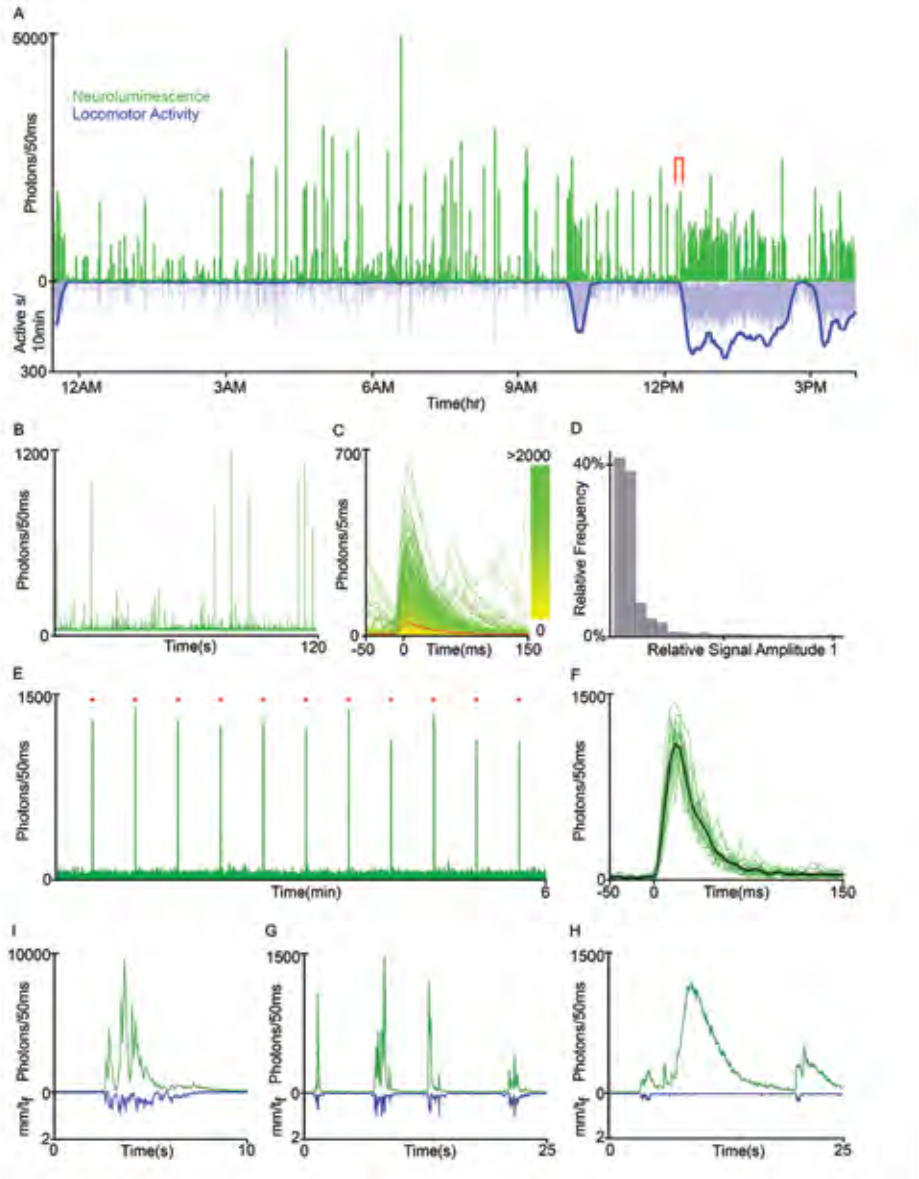


Figure 3. Neuroluminescence and behavior of panneuronal GA-expressing zebrafish. ***A***, Neuroluminescence signals and behavior can be monitored continuously for several days. Shown here is a 16 h excerpt of the recording from a 6 dpf *Nbt:GA* transgenic zebrafish, following 24 h of exposure to CLZN. Despite the constant dark conditions of the assay, much can be observed: an increase in locomotor activity (light blue trace), measured as the number of active seconds in a 10 min sliding window (dark blue trace), and a corresponding increase in neuroluminescent events (green trace) soon after the previously experienced light-on time (9 AM) of the zebrafish light/dark rearing cycle (as is expected, given their circadian modulation of spontaneous swimming). ***B***, Expanding the red bracketed region indicated in ***A*** reveals the range of neuroluminescence signal amplitudes that occur during spontaneous behavior. ***C***, We aligned all the signals detected during the 16 h recording to the signal onset time and color coded each event by the number of photons arriving in a 50 ms window (0 to >2000; see color bar). We found that neuroluminescence events consist of a fast rise and slower decay in light emission, with a large range of peak amplitudes. ***D***, The histogram of signal amplitudes observed from panneuronal GA zebrafish (n = 6 fish, 3125 events), normalized to the maximum signal detected from each individual, demonstrates the frequent occurrence of small amplitude events and a long tail of the distribution populated by increasingly large and rare events. ***E***, A mechanical stimulus delivered by tapping the recording chamber (indicated by red dots) resulted in large neuroluminescence signals coincident with the evoked startle response. ***F***, For the fish shown in ***E***, which had low spontaneous swimming and neuroluminescence activity, the evoked signals were aligned to demonstrate their consistent amplitude, and the average signal (thick line) was used to estimate the kinetics of the evoked neuroluminescence event: time-to-peak: 5-10 ms; decay τ ~30 ms. ***G***, PTZ-induced epileptic behavior, characterized by uncoordinated muscle contraction, is associated with large, sustained bursts of neuroluminescence, which is consistent with the strong neural activation expected during a seizure (t_0 = 1 min after PTZ exposure). ***H***, Continued exposure to PTZ induces periodic seizures associated with strong neuroluminescence (t_0 >10 min). ***I***, Following extended exposure to PTZ, sustained neuroluminescence events are often independent of motor activity (t_0 >17 min).

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Bouts of spontaneous swimming coincided with the emission of large flashes of green light (Figs. 2*C–F*). Neuroluminescence signals were large (signal-to-noise ratio >>100), stable for long periods of time (>24 h), and coincident with spontaneous and evoked swim events (Figs. 2*C*, 3). Spontaneous signals detected from individual zebrafish spanned a range of sizes (Fig. 3*B–C*), exhibiting a smooth amplitude distribution, with a high frequency of small events phasing into a long tail of increasingly large and rare events (Fig. 3*D*). Transient neuroluminescence events were almost always associated with spontaneous swim bouts (Fig. 2*C*), reflecting the neural activation underlying the generation of spontaneous behavior.

In order to measure neuroluminescence signals produced during stimulus-evoked behaviors, we delivered a mechanical tap to the swim chamber to elicit a startle response (Bhatt et al., 2007; Burgess and Granato, 2007). Repeatedly evoked neuroluminescence signals were fast and consistently similar in amplitude (Fig. 3*E*). After aligning the evoked signals from an individual zebrafish, we estimated a time to peak of 5-10 ms and a slower decay time of ~25 ms (24.5 ± 3.9 ms) (Fig. 3*F*), consistent with expectations for aequorin and comparable with popular synthetic Ca^{2+} indicators (Shimomura et al., 1993).

To further investigate the origins of neuroluminescence, we exposed zebrafish to pentylenetetrazole (PTZ), an inhibitor of $GABA_A$ receptors (Leweke et al., 1990) that induces epileptic-like neuronal discharges in humans, rodents, and zebrafish (Baraban et al., 2005; Baraban, 2007). Thirty seconds after bath application of PTZ, zebrafish exhibited sustained periods of uncoordinated swimming accompanied by very large and sustained waves of neuroluminescence (Fig. 3*I*). These early episodes (~3-5 min) were followed by periodic bouts of clonus-like convulsions and smaller waves of neuroluminescence (Fig. 3G). After prolonged exposure to PTZ (~10-20 min), slow waves of neuroluminescence occurred independently of swimming or muscle contraction (Fig. 3*H*). The results obtained with panneuronal GA indicate that neuroluminescence enables the noninvasive, long-term recording of population activity from freely behaving zebrafish larvae.

Monitoring neuroluminescence during visual behavior

Using existing detection methods, bioluminescence experiments have been limited to behaviors that occur naturally in darkness. Imperfections in spectral filters, particularly their inability to adequately reject the high-angle (>45°) incident light collected by the detector, have necessitated that the photon-counting sensor be protected from all but IR illumination (to which most sensors are largely insensitive). To overcome this problem, we have designed a novel detection system that uses a cathode-gated channel photon multiplier (CPM) in a time-gating strategy that allows us to shine a fast flickering illumination light during the coincident detection of the bioluminescence signal.

In our design, computer-controlled gating signals regulate the cathode voltage (gain) of the CPM, a visible light-emitting diode (LED), and the IR illumination light source for a behavior-monitoring camera. During each 10 ms cycle, the CPM is gated "ON" for 9 ms and is able to detect individual

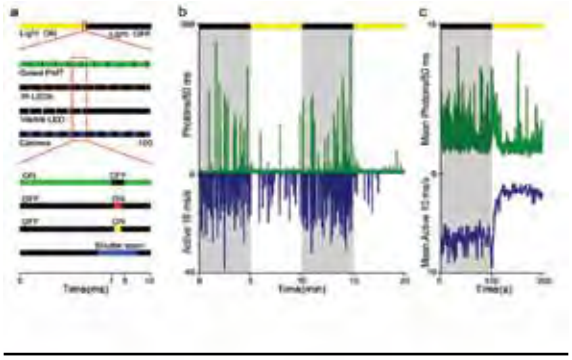


Figure 4. Temporal gating allows the monitoring of neuroluminescence during visual stimulation. ***a***, Schematic diagram of timing protocol of visual stimulation and gating of a CPM. From above: 2 cycles of 5 min "light ON" and "light OFF" periods are shown as they appears to the eye. Below, a zoom-in of the transition time shows 100 ms of the activation states of CPM, IR LEDs, visible LED, and CCD camera. The CPM is continuously gated with a 90% duty cycle, and the IR is switched on a 10% duty cycle. When the CPM is off-gated, the visible LED is switched on for the "light ON" period and switched off completely in the "light OFF" period. The CCD camera shutter is open during the period the IR LED is switched on, and one frame per cycle is acquired. A further zoom-in shows the exact timing of the gating for one cycle during the "light ON" period: CPM, IR LED, visible LED, and CCD camera are synchronized to run at 100 Hz. The CPM records green bioluminescence photons for 9 ms, and the IR LEDs illuminate the fish for 1 ms while the CCD camera acquires a frame. When visual stimulation is required, the visible LED is also switched on during the 1 ms the CPM is off-gated. ***b***, Exemplary neuroluminescence recording (100 Hz) with the gated CPM of a 6dpf *Nbt:GA* transgenic zebrafish larva previously exposed to CLZN. Photon emission and behavior (activity per second) during alternating "light ON" and "light OFF" periods of 5 min each. ***c***, Averages of photon emission and behavior of 5 repetitions of "light ON" and "light OFF" cycles from 30 experiments show increased activity during the light OFF period and corresponding photon emission. This general behavioral change and a light stimulus–locked peak in neuroluminescence and behavior (startle response) indicate that the light has evoked the behavioral change.

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bioluminescence photons. In the final 1 ms, the CPM is rapidly gated "OFF," and both the visible LED and IR illumination are briefly activated, sending ~10^{12} visible photons towards the now insensitive detector for 1 ms (~1 mW LED power). In the next cycle, the CPM is again gated "ON" and able to count single bioluminescent photons. With this 100 Hz repetition rate and a 90% duty cycle, natural illumination conditions can be simulated while 9 of 10 emitted bioluminescent photons can potentially be detected (Fig. 4*a*).

We have tested our time-gated detection/illumination strategy by exposing groups of panneuronal GA zebrafish to short visible light/dark cycles: 5 min ON, 5 min OFF. Zebrafish respond to transient decreases in illumination by increasing their swim activity, whereas an elevation in light levels is followed by a period of suppressed activity (Figs. 4*b*,*c*). We were able to observe these visual behavior patterns while recording neuroluminescence signals, thereby establishing a proof-of-principle that this technique can be expanded to experiments that use natural lighting conditions.

Conclusion

We believe that the fast, stable properties of GA's report of neural activity, along with nonimaging detection strategies, can provide a useful, easily implemented tool for monitoring the activity of genetically specified cell types during natural behavior. GA presents an attractive alternative to other, more technically challenging imaging approaches currently being pursued.

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