A Coherent Whitepaper

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Optogenetics: Ultrafast Fiber Laser Enables Two-Photon Studies Using Chrimson Photoactivator

The unique combination of high pulse energy and short pulsewidth at longer wavelengths from a Fidelity ultrafast laser enables all-optical physiology studies of neural circuits of Drosophila melanogaster at single neuron resolution.

Introduction

In optogenetics, a common challenge with all-optical physiology experiments is to minimize potential crosstalk between photoactivation and calcium ion imaging. A popular approach is to use some type of wavelength separation. In the laboratory of Professor David Anderson at Caltech, graduate student Yonil Jung has exploited this method by using two-photon excitation of a newer long wavelength opsin for photo stimulation – Chrimson – enabling data of neural circuits of *D. Melanogaster* with single cell spatial discrimination. The high power (> 2 watts) and short pulsewidth (55 fs) from an advanced femtosecond fiber laser (Coherent Fidelity-2) were critical in this first successful use of two-photon activation of Chrimson.

Frontis. The Fidelity-2 is an advanced, compact and rugged Yb-fiber femtosecond laser.

All-Optical Physiology

Over the past decade, from the activation (or inhibition) of neurons with genetically encoded probes called opsins has emerged the diverse and fast growing field of Optogenetics. All-optical physiology combined with two-photon microscopy is a powerful technique used in many optogenetic studies as it enables researchers to determine the functional connectivity of a

neuron population with exquisite temporal and spatial (single neuron) precision. In these typically *in-vivo* microscopy experiments, light is used both to photoactivate one or more targeted neurons, as well as to image any resultant activity in nearby neurons. This functional imaging utilizes some type of calcium ion (Ca^{2+}) indicator or probe, whose fluorescence intensity increases in the presence of higher concentration of these ions, indicating neural activity – like action potentials. These types of studies often benefit from using excitation light at two different wavelengths: one to activate or to inhibit the targeted neurons and another one to measure the calcium ion concentration.

Newer genetically encoded Ca^{2+} indicators (GECI) such as the GCaMP family have opened the path to using model animals expressing all these functional probes via genetic coding. Genetic expression is important because it allows tailoring colonies of animals ready for specific experiments and without the potential toxicity and other perturbations that may result from using exogenous, chemical probes.

Two-Photon Excitation with Ultrafast Lasers

Optogenetics experiments can be conducted using various types of light sources: LEDs, continuous wave (CW) lasers, and femtosecond lasers. The first two approaches typically use blue and yellow sources to activate and inhibit neurons. While the use of LEDs has sometimes proved convenient and economical for behavioral studies, all-optical physiology experiments predominantly rely on lasers. The reason is spatial brightness and spatial resolution. Specifically, a LED can be efficient for indiscriminately exciting a large area of brain tissue, but a laser is critical to image the Ca^{2+} concentration in individual neurons or to activate a small population of neurons with the highest temporal and spatial precision, rather than an indiscriminately larger area.

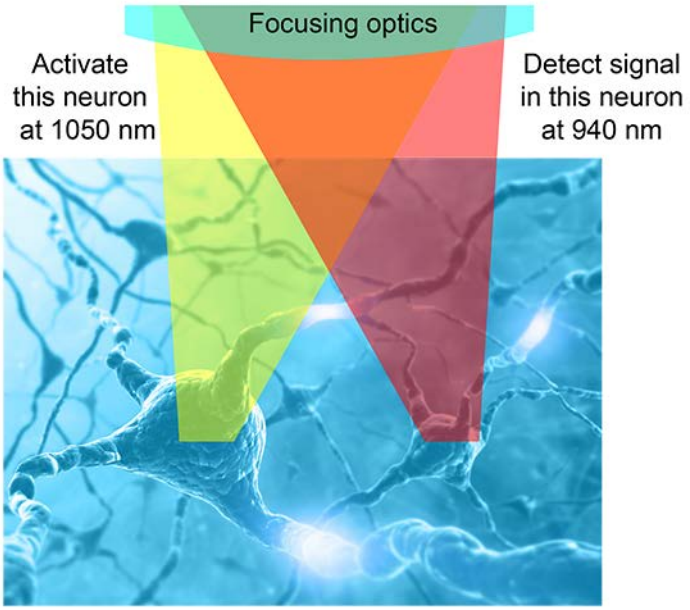

Figure 1. In all optical physiology experiments with multiphoton lasers, neural activity can be manipulated and monitored at single neuron resolution. The use of two separate wavelengths for photoactivation and detection simplifies data analysis.

Although CW lasers are used in many experiments, and in a confocal configuration provide sectioning capabilities, cutting-edge microscopy

experiments studying multiple neurons at single neuron resolution frequently use femtosecond lasers for one or even both of the excitation tasks: photoactivation and calcium imaging. This is because two-photon excitation with femtosecond lasers is well documented in delivering an excellent combination of advantages. First, the use of 2x longer wavelengths limits any photodamage in the living sample. Second, and just as important, scattering losses in living tissue decrease at longer wavelengths, which therefore provide deeper penetration and imaging ability. Consequently, the majority of studies involving in vivo functionality of small neuron populations across a cortex are performed using these femtosecond tunable lasers.

Key Experimental Challenges

Two typical obstacles that researchers like Jung encounter with the two-photon approach are the relative difficulty to activate opsins, and the cross-talk between opsins and Ca^{2+} indicator. Compared to exciting fluorescence for conventional imaging, activating a neuron, i.e. triggering an "action potential" takes considerably more laser excitation, especially with two-photon excitation. One frequently used solution to address this challenge was introduced by David Tank at Princeton University. His method consists of scanning the focused laser beam in a spiral pattern, progressively covering the entire neuron body or soma until the action potential is produced. This may take something on the order of 30 ms for each neuron, depending on the temporal dynamics of the particular opsin and its distribution density in the targeted neuron(s). This spiral scanning works very well with relatively small populations and Jung adopted this proven approach in the study described here.

The issue of crosstalk between photoactivation and calcium imaging has come more to the forefront as two-photon all-optical physiology has expanded in the last 2-3 years. The problem is that the two-photon spectra of most probes are quite broad (as much as 50-100 nm or more in the case of quantum dots) and generally wider than the corresponding one-photon spectra. Even with the rapidly expanding choices of GECIs and Opsins it is not trivial to separate optogenetics activation from Ca^{2+} detection. In a worst case scenario, activating a neuron may result in spurious Ca^{2+} signals or vice versa.

Several teams are working to solve this crosstalk problem by developing opsins and Ca^{2+} indicators that are better separated in excitation wavelengths – see figure 1. One approach is to develop red-shifted probes that exhibit minimal cross talk when used in conjunction with more conventional shorter wavelength probes. These new red-shifted genetically encoded tools include photactivators (opsins) such as C1V1 and ReaCh, as well as calcium indicators such as the RCaMP family. These are well separated spectrally from well-established shorter wavelength opsins like ChR2 and calcium probes such as the GCaMP family. It's important to

note that in addition to providing wavelength separation, the development of red-shifted probes enables deeper penetration and lower damage in the brain tissue, as noted above.

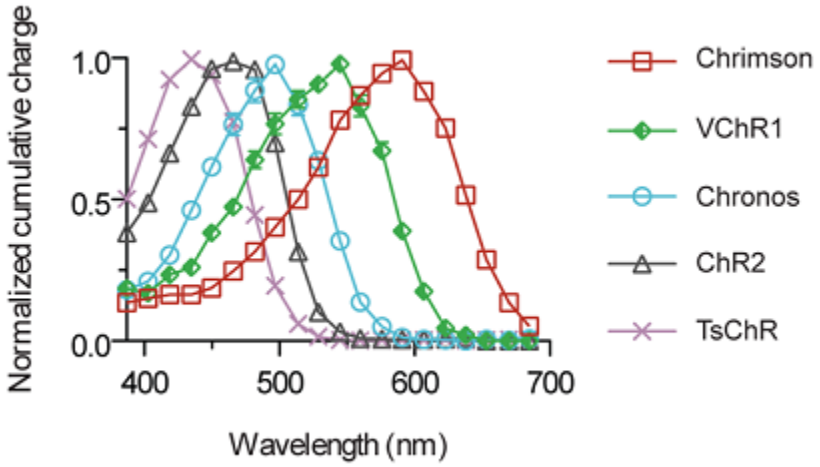

Figure 2. The single photon absorption spectrum of Chrimson is significantly red shifted compared to other opsins and several commonly used Ca^{2+} indicators. Graph courtesy of Professor Edward Boyden, Massachusetts Institute of Technology.

The increased popularity of red-shifted optogenetic probes has thus driven a need for femtosecond lasers emitting in the 1,040 nm-1,150 nm range. These wavelengths are at the limit or even outside the gain curve of Ti:Sapphire lasers such as the Coherent Chameleon that have been the workhorses of multiphoton microscopy for the last 15 years. To make things worse, one of the most important trends in all-optical physiology is to simultaneously study larger and larger populations of interconnected neurons. The simultaneous excitation of multiple neurons requires more laser power, right at the edge of the Ti:Sapphire gain curve where only limited power is available.

In response, laser manufacturers like Coherent have developed a new type of femtosecond laser based on Ytterbium fiber that can provide several watts or even tens of watts at 1,030-1,070 nm, depending on specifics of the laser architecture. However, the first ytterbium fiber lasers encountered a well-known laser physics problem limiting multi-watt operation to pulsewidths typically longer than 200fs. (In technical terms the problem is due to a trade-off between bandwidth narrowing due to amplification and self-phase modulation in the fiber.) But fortunately, engineers at Coherent could draw on colleagues' long experience designing and building ultrafast fiber lasers for industrial applications. This enabled a proprietary solution, now embodied in the Fidelity family of lasers that provide pulsewidths as short as 55 fs.

Excitation of Chrimson at 1070 nm

In his all-optical physiology experiments, Jung chose to use a newer opsin, called Chrimson. The spectrum of Chrimson is more red-shifted than other opsins; the single photon excitation spectrum of Chrimson is characterized by a peak around 600 nm – see figure 2. So using Chrimson results in less spectral overlap with the GCaMP Ca^{2+} indicators. An additional advantage of Chrimson over other opsins, such as ReaCh, is its higher sensitivity to stimulation. Both of these advantages are well established with one-photon excitation however no one had previously reported successful optogenetic studies stimulating Chrimson via two-photon excitation. There were not even any published two-photon action spectra for this opsin.

Initial attempts at two-photon excitation of Chrimson-expressing neurons with a conventional femtosecond Ti:Sapphire lasers did not result in any reliable activation of the target neurons. Jung then decided to try a Coherent Fidelity-2 laser that produces greater than 2 watts at a nominal center wavelength of 1,070 nm. This is 5-10 times higher than the power from a tunable Ti:Sapphire operating at this wavelength. In addition, the built-in pre-chirp control permits users like Jung to minimize pulse duration at the sample by compensating for the dispersion of the microscope and other optical elements. Imaging and measurement of the Ca^{2+} signals were performed with a Coherent Chameleon Vision, tuned to 920 nm to minimize crosstalk and optimize GCaMP6s fluorescence. Jung used the output of these lasers with a commercial two-photon microscope equipped with two channels: one channel with a pair of galvos employed in the usual raster scanning pattern and powered by Chameleon was used for structural and calcium imaging; the other channel was used to photoactivate Chrimson via the Fidelity-2 scanned in a spiral pattern.

Some Sample Data

The most interesting of the initial results are shown in *figure 3*, where the connectivity of four neurons is assessed with single-cell resolution. Preliminary experiments (b) were performed using wide area stimulation with a LED emitting at a nominal 655 nm and wide field imaging. The neurons were expressing UAS-Chrimson-tdTomato for optogenetics activation and UAS GCaMP6s for measuring Ca^{2+} signals. All four neurons exhibit an elevated Ca^{2+} transient signal, as expected with simultaneous neuronal activation. Then Chameleon and Fidelity-2 were used in place of the LED. The results in (c-e) show the same Ca^{2+} spikes measured in the four neurons now with two-photon laser activation targeting a different single cell among the four neuron ensemble. (f) shows the functional connectivity as resolved by this two-photon all-optical interrogation of the neural circuit. Clearly, this detailed analysis was only possible because the two-photon laser technique allowed photoactivation of individual neurons in this way, with no crosstalk issues with the Ca^{2+} activity signals.

Summary

In summary, Prof. Anderson's student Yonil Jung has demonstrated the first all-optical physiology studies in a fruit fly using two-photon excitation of the red-shifted photoactivator Chrimson. Its long wavelength excitation spectrum means that Chrimson can be activated in this way with no crosstalk with popular Ca^{2+} indicators and even some of the shorter wavelength opsins. To the best of our knowledge this study is the first time Chrimson has been activated in a two-photon modality. It illustrates how powerful, short-pulse next generation Ytterbium fiber lasers such as the Fidelity family are a perfect match for activation of red-shifted functional and structural probes increasingly used in optogenetics.

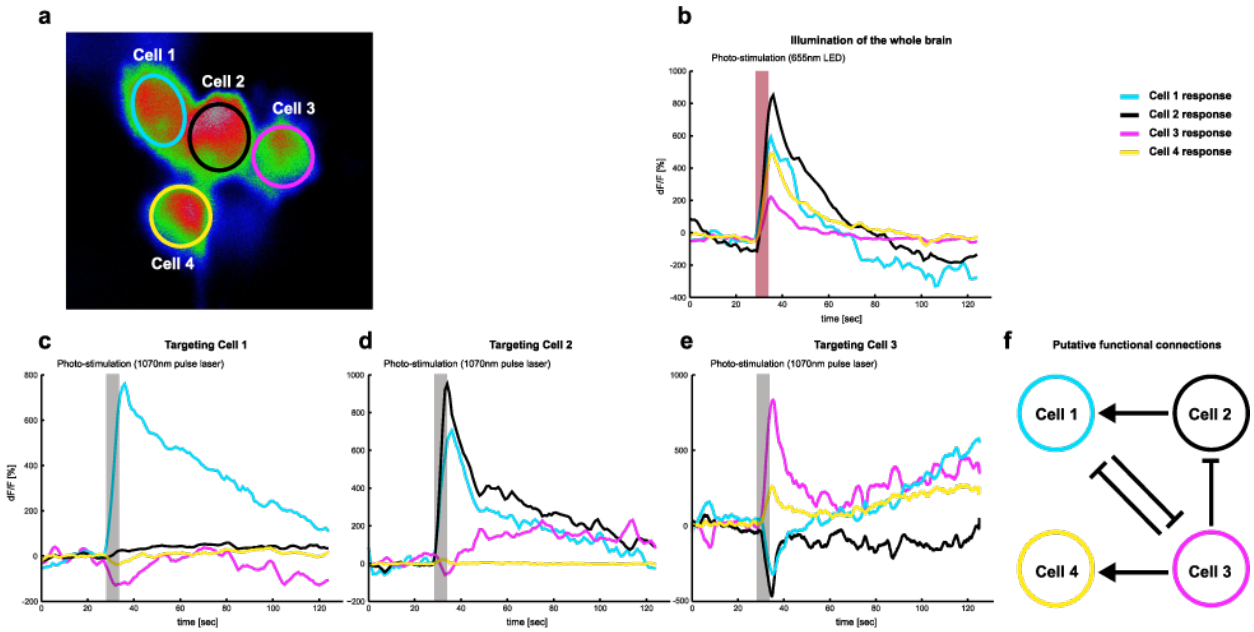

Figure 3. All-optical interrogation of functional neuronal connectivity with single-cell resolution. (a) Cluster of 4 neurons in an adult fruit fly brain. The neurons express both UAS-Chrimson-tdTomato and UAS-GCaMP6s. Ovals defining the regions-of-interest (ROIs) around each cell correspond to the color of the traces in (b-f). (b) GCaMP6s responses evoked by whole-brain photoactivation of Chrimson using a 655 nm LED. Orange bar indicates period of photostimulation. Calcium transients are exhibited by all four neurons. (c-e) GCaMP6s responses of individual cells in the cluster to 2-photon raster-scanning-based photostimulation of the single cells indicated above the panels (c, Cell 1; d, Cell 2; e, Cell 3). Note the different responses evoked by photostimulation of each of the three cells. (f) Functional connectivity between the cells suggested by the experiment. Cell 4 did not exhibit a strong autonomous response to single-cell photostimulation. Data courtesy of Yonil Jung and David J Anderson, California Institute of Technology.