Precision Optogenetics: millisecond time resolution optical imaging and control of neuronal circuits

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Abstract

Memory and other information in the brain is thought to be encoded in synaptic strength. Through the biological process of Spike-Timing Dependent Plasticity (STDP), changes in synaptic strength depend sensitively on the time sequence of neuronal firing events. Holographic optogenetics enable targeting of multiple neurons, but are currently limited to either simultaneous firing or large delays (30 ms) between firing. The goal of this project is to generate new optogenetic technologies to study and perturb the activity of in vitro neuronal circuits at short enough timescales to generate large changes in synaptic strength. To achieve this goal we introduce the novel microscope *Sauron,* which images cellular electrical activity at high frame rates and perform fast-switching of light fields for photo-stimulation via an SLM. Preliminary experimental results demonstrate the fast-switching and fast imaging capabilities on Human Embryonic Kidney cells, and a modular analysis framework allows us to extract spiking histories of cells from the fluorescent images. Finally, we outline an experimental procedure to study STDP in neuronal cultures, improvements to the experiment, and a collaboration with the Babadi Group to extract functional network connectivity.

Introduction

Optogenetic tools enable optical control of neuronal activity. Recent advances in optogenetics combined with a two-photon microscope and Spatial Light Modulator (SLM) could enable perturbations of neuronal dynamics at a temporal rate on the order of action potentials (few ms). New fast membrane voltage-sensitive dyes (VSD) combined with an epi-fluorescence path enable imaging of membrane potential dynamics, and with analysis provide a fast and direct way to extract neuronal network dynamics on the order of milliseconds. Functional connectivity tools such as Granger Causality could then be used to study how the structure of the network and plasticity changes under stimulation of individual neurons.

The idea that one could control the action potentials of cells using light was conceptualized in 1999 by Francis Crick at the Kuffler Lectures at UC San Diego. The earliest experiment of optical stimulation of genetically designated neurons was published in 2002 by Boris Zemelman and Gero Miesenboeck, using Drosophila rhodopsin [1]. The three-gene system cHARGe was not used by many groups due to its slow temporal precision on the order of seconds and high variability. Karl Deisseroth and his lab improved on this by demonstrating in 2005 that

Channelrhodopsin-2 (ChR2), a light-activated ion channel naturally occurring and serving as photoreceptors in green algae, could be deployed to control mammalian cells *in vitro* with millisecond temporal precision [2].

These advances made it possible to stimulate neurons with millisecond precision but had some drawbacks: the small single-channel conductance, a single excitation spectrum (peaking at 470nm) and slow recovery time, resulting in rates of stimulation limited to 20-40Hz. Since then, many new channelrhodopsins have been discovered and others genetically engineered from ChR2 for improvement in select characteristics (action spectra, kinetics, and light-sensitivity). In 2014, after de novo sequencing and characterization of opsins from 127 species of algae, the Boyden lab reported on two new channelrhodopsins nicknamed Chrimson (CnChR) and Chronos (ShChR). Chronos had the fastest kinetics to date and a spectral peak around 500 nm, while Chrimson was the most red-shifted opsin, with a spectral peak at 590 nm and sensitive up to 660nm. This work showed that expression of Chrimson and Chronos in two distinct populations of neurons enabled independent optical control of the subpopulation [3].

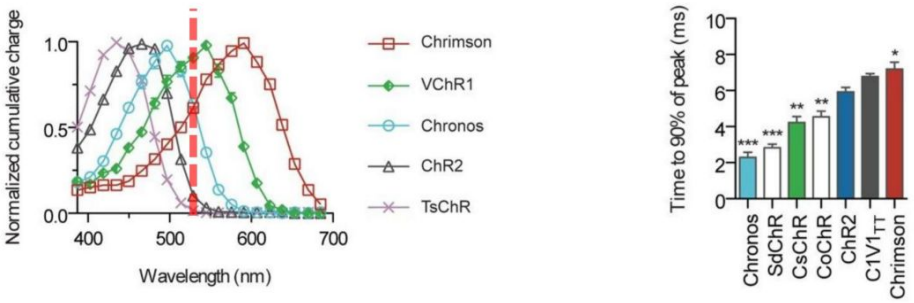

Figure 1: Spectral sensitivity (left) and T_{on}, the time to reach 90% peak current (right) of opsins in this study. Red dotted-line represents $\lambda_{IR}/2$. [figure adapted from Opt. Lett. 33, 440 (2008).]

Neuronal activity is often measured with classical electrophysiology techniques such as patch-clamping and microelectrode arrays or indirectly through calcium imaging. Classical electrophysiology techniques suffer from being invasive and low throughput, while Ca^{2+} imaging is non-invasive and high-throughput but only an indirect indicator of voltage change. Calcium indicators have a slow decay constant and require complex spike reconstruction algorithms to approximate the underlying neuronal activity [4].

More recently, non-invasive high throughput optical techniques for directly measuring voltage have surfaced in the forms of genetically-encoded voltage indicators and voltage sensitive dyes. A recent far-red voltage sensitive dye, the Berkeley Red Sensor of Transmembrane potential (BeRST) is particularly suited for our task due to its speed, reliability and spectrum. BeRST is a photostable membrane-localized dye with high voltage sensitivity and a sub-millisecond

response time. Depolarization of the membrane results in linearly increased fluorescence with a $\Delta F/F = 24\%$ corresponding to a 100 mV change in potential [5].

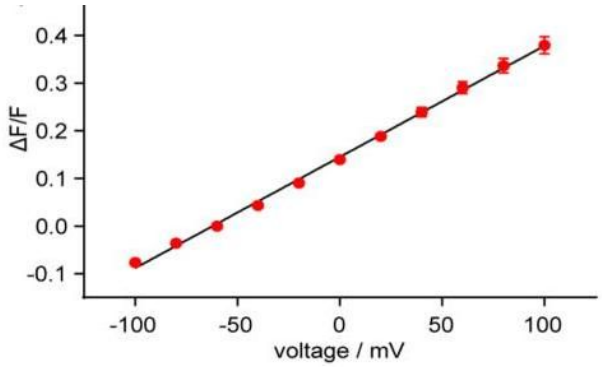

Figure 2: Linear change in fluorescence response with membrane potential (Figure from [5]).

Two-photon excitation microscopy is a frequently used alternative to confocal microscopy due to its increased imaging depth, reduced phototoxicity and natural optical sectioning. The greater imaging depth compared to one-photon excitation microscopy arises from the wavelength-dependent scattering in biological tissue. This wavelength-dependent scattering in biological tissue is complex, but can be approximated as being an intermediate of Rayleigh Scattering and Mie Scattering [6]. Rayleigh Scattering is proportional to $1/\lambda^4$ while Mie Scattering it is proportional $1/\lambda^{0.5}$. Thus, for a 520nm single-photon excitation and 1040nm two-photon excitation, we expect a reduction in scattering of ~30-94%. The two-photon absorption cross-section increases quadratically with intensity, which gives these systems great optical sectioning without the use of a pinhole, since light outside the focal plane will not result in absorption. This cross-section also spares the biological tissue from unnecessary photobleaching and phototoxicity. Two-photon excitation microscopes operate using a pair of resonant-galvo scanners and have great spatial resolution but are limited to 30Hz, making them good for calcium imaging but unable to image on the timescale of action potentials. However, the advantages of this imaging method from the two-photon process transfer to a photo-stimulation method. So while we opt for a fast epifluorescence path for imaging our monolayer cultures, a two-photon stimulation path is designed with direct applications to *in vivo* studies.

We describe here an optical system, nicknamed Sauron, that would best utilize the fast kinetics of blue-green opsin Chronos and far-red voltage sensitive dye BeRST, and be suitable for *in vivo* studies with little modifications. Two-photon stimulation is performed via a 1040nm Discovery Chameleon laser combined with a Boulder Nonlinear Systems Spatial Light Modulator (SLM) utilizing advanced control methods, piped into a Sutter Movable Objective Microscope. This system has the unique ability to perform fast switching of light patterns for targeting individual

(or groups) of neurons sequentially. It does this with great diffraction efficiency (>80%), penetration depth and optical sectioning. An epifluorescence path custom-build onto a Leica DM IRBE inverted microscope performs fast imaging of BeRST's far-red emitted light with a Hamamatsu EMCCD. Our analysis framework in Matlab can then use the resulting fluorescent *TIF* image stacks for segmentation and computation of $\Delta F/F$, enabling us to monitor network dynamics on a cellular level. Experiments are performed on electrically activatable Human Embryonic Kidney cell cultures and future experiments are laid out for stimulation of i*n vitro* neuronal networks with the aim of assessing how quickly changes in synaptic plasticity can be induced.

Methods

A. Cell Cultures

This system's primary purpose is to study in vitro neuronal networks. Embryonic rat cortical and hippocampal neurons are cultured in a growth media and left to grow to form a network. They are transduced with Chronos a week before imaging, and then BeRST is applied about 30 minutes to an hour before experiments. When specified, the media will be changed to Artificial Cerebrospinal Fluid (ACSF), which better matches endogenous Cerebrospinal Fluid and makes firing more likely but is toxic under long exposure. Imaging and experiments are done at various days: 5-25 days *in vitro* (DIV) (dissected at day 18).

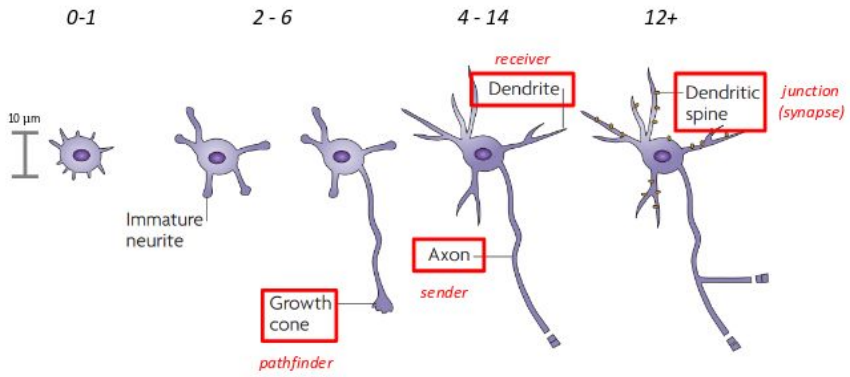

Figure 3: Neuronal morphology can be described as developing in 5 stages (Arimura and Kaibuchi, Nat Rev Neurosci, 2007). Dr Kate O'Neill has noted the number of DIV this corresponds to.

These neurons are postmitotic primary cells, so they cannot divide. While useful for simulating the behavior of *in vivo* neuronal networks, primary neurons are time consuming to procure and culture and also, frequently become unhealthy or die under certain steps in culturing such as transfection. Thus, it is exceedingly useful to have an immortalized cell line that mimics neurons and can be used for testing purposes when neurons are not available. The immortalized human

embryonic kidney (HEK-293) cell line has been engineered to be electrically excitable through stable expression of an inward rectifier potassium channel K_{ir} 2.1 and a voltage-gated sodium channel Na_V 1.5 [7]. We use these engineered cells in its two forms: NK-HEKs which are described above, and OS-HEKs which are identical but also express the blue channelrhodopsin CheRiff and far-red voltage indicator QuAsar2. Expressing CheRiff means they are optically stimulatable without modification, although we will use Chronos instead. HEK cells naturally have connexins, enabling bidirectional flow of ions and thus propagation of electrical signal to nearest neighbors.

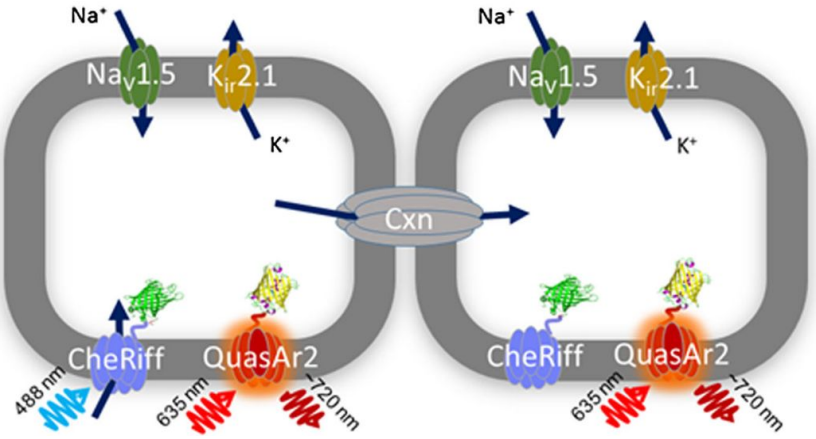

Figure 4: Functional components of Optopatch Spiking-Human Embryonic Kidney (OS-HEK) cells, used in our experiments.

B. Sauron Optical System

Sauron is a new optical system consisting of an epi-fluorescence path for optically measuring voltage and a two-photon photo-stimulation path utilizing an SLM. A sampler path is added for calibration of the SLM with the imaged field of view. The optical diagram is shown in Figure 5.

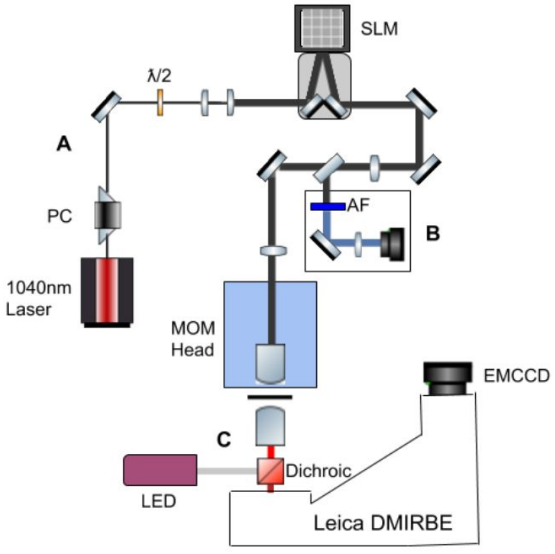

Figure 5: Optical diagram showing photo-stimulation path (A), sampling path (B), and epi-fluorescence path (C).

B1. Optical Voltage Sensing

Membrane potential is optically measured using the voltage-sensitive dye BeRST, applied to cells via a bath for 1 hour prior to imaging. The epi-fluorescence path uses a Hamamatsu ImagEM X2 EMCCD on an inverted Leica DM IRBE microscope, with illumination provided by a Thorlabs 440mW MCWHL5-C1 LED with a far-red FISH filter cube (630/20x and 667/30m). BeRST has an excitation peak at 658 nm and emission peak at 683 nm, so this filter cube is not ideal but will still provide substantial signal. The Hamamatsu's frame rate ranges from 70 fps (full-field of view and 1x1 binning) to 500+ fps (cropped field with 4x4 binning).

The dye is very photostable with a bleaching half-life of 5 minutes (I=1.62 W/mm^2, λ = 631 nm), so bleaching should not be an issue on the timescale of our imaging sessions. BeRST has been shown to have a sub-ms response time, indistinguishable from patch-clamping within 0.56 ms with a $\Delta F/F$ for cultured neuron action potentials of 9.5% +/- 1.2% [5].

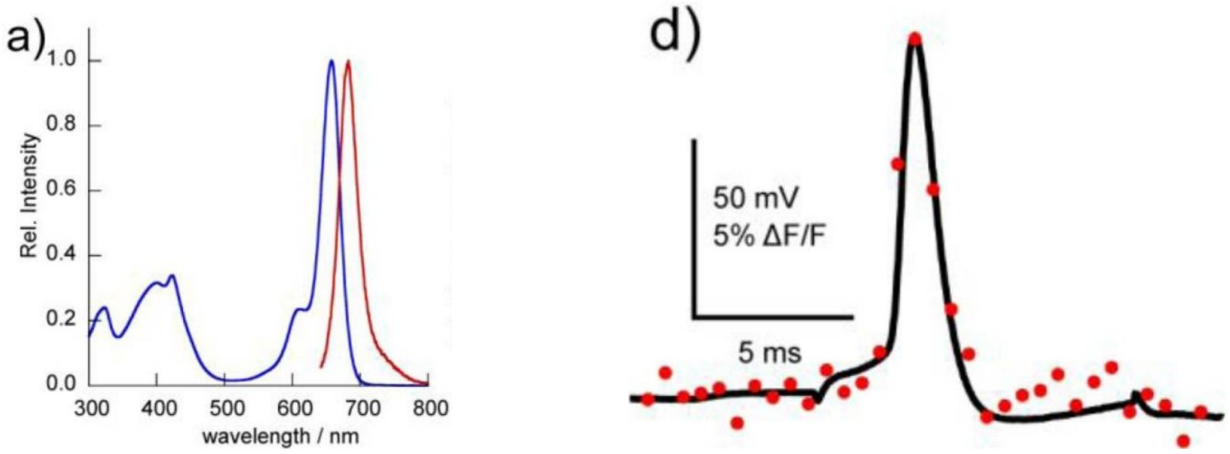

Figure 6 : BeRST excitation and emission spectra (left). BeRST compared to patch clamping (right). (Figures adapted from [5])

B2. Photostimulation

HEK cells and neurons are made photostimulatable by expression of the channelrhodopsin Chronos (ShChR) obtained from the Boyden Lab. As discussed, Chronos has extremely fast kinetics with a T_{on} = 2.3 ms and a T_{off} = 3.6ms and blue-green sensitivity [3]. Its spectral sensitivity is essential for no cross-talk to occur with the far-red channel used for imaging voltage. Two-photon photo-stimulation uses the 1040nm beam from the fixed output channel of the Chameleon Discovery with nominal power of 1.7W. A pockels cell and half-wave plate are used to shutter and modulate the power of the beam, after which is expanded and reflected off of the SLM, before being focused onto the back aperture of the Olympus 20x objective in the Sutter MOM head.

SLM Method

To target individual neurons and quickly switch between different targets, a 256x256 nematic liquid crystal SLM from Boulder Nonlinear Systems (HSP256-1064) is emplyedalong with the Ritsch-Marte group's advanced control software [8]. The SLM modulates the phase of the incoming light at each pixel by controlling the electric field applied to the liquid crystals at each pixel. This electric field drives a change in the tilt of the liquid crystal molecules, changing the refractive index at that pixel.

The phase mask applied at the SLM directly controls the intensity pattern at the focal plane, and iterative algorithms such as the Gerchberg-Saxton algorithm enable retrieval of the phase mask using the desired intensity pattern at the optical plane. Thus we can target individual (or groups) of neurons, limited only by the rate at which we can update the electric field at each pixel of the SLM. Herein lies the issue, as most high-resolution commercial SLMs are too slow with the highest speed SLMs having out of the box phase mask update times limited to 10 ms.

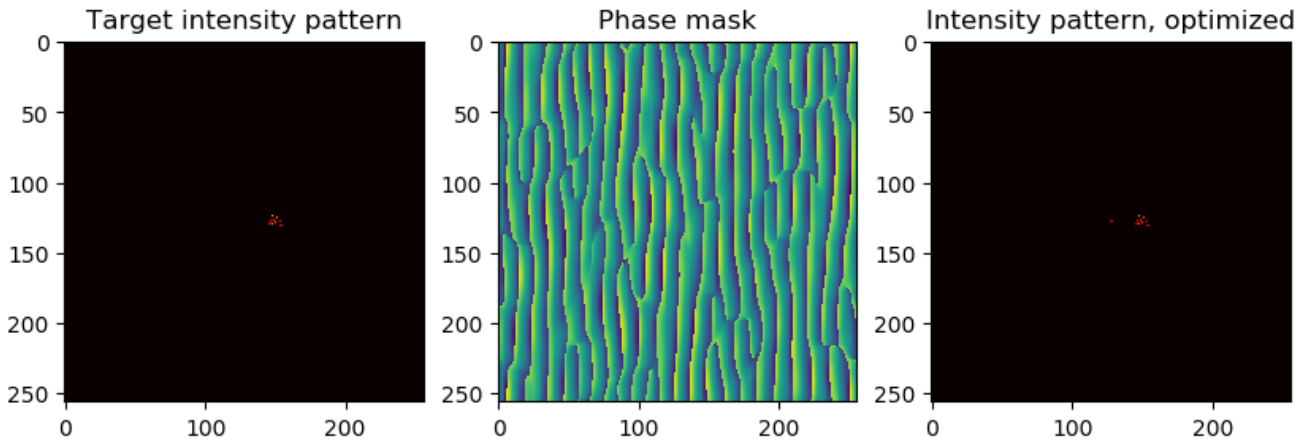

Figure 7 : A target intensity pattern, the phase mask that the modified Gerchberg-Saxton converged to, and the actual intensity pattern resulting from this phase mask.

The 256x256 BNS SLM we use has nominal speeds of only 30Hz. To sequentially stimulate two neurons on the scale of action potentials requires an SLM that can operate at a near kHz rate. The control software developed by Gregor Thalhammer and others in the Ritsch-Marte group enables significant speed enhancement, down to 2ms transition between two patterns, by making use of the techniques of ***overdrive*** and ***phase change reduction*** [8].

The ***overdrive*** method is already relatively well known, and takes advantage of the transient response of the pixels when a voltage is applied. When a voltage U_1 is applied to a pixel, the phase φ_1 is reached after some time. This response is exponential and approximately given by

$$\varphi_1 = \varphi_0 + (\varphi_1 - \varphi_0)(1 - e^{(t-t_o)/\tau}),$$

where t_o is the time at which we applied the voltage U_1 and τ is the characteristic time constant. Thus one can instead apply the maximum voltage U_{max} and the switch to the voltage U_1 once the desired phase φ_1 is reached to switch the phase much more quickly. If the phase φ_1 is less than the original phase, the minimum voltage is applied instead (U_{min}= 0).

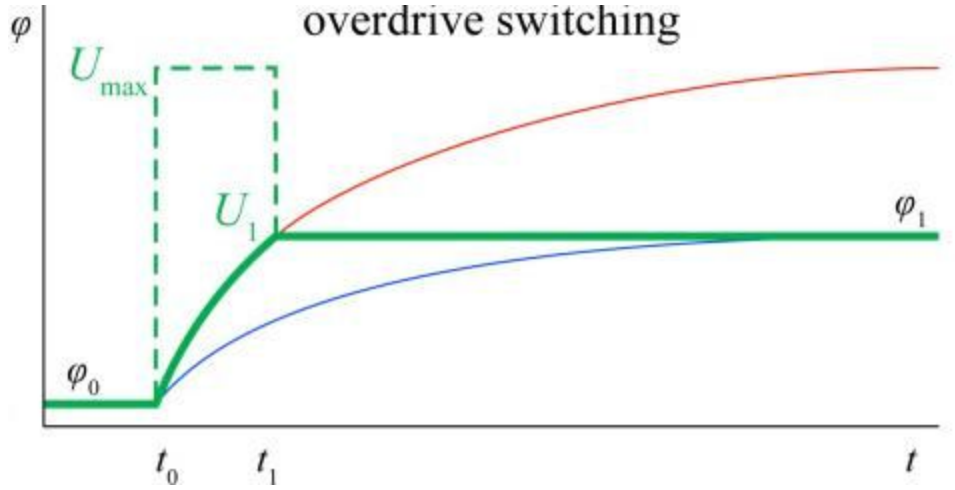

Figure 8: Illustration of the overdrive method, the red line is the typical response when applying voltage U_1 for desired phase φ_1. [G Thalhammer et al. 2013, 8]

The second technique, ***phase change reduction***, exploits the extended phase stroke that many SLMs have. This requires the SLM to have a phase stroke greater than 2π at each pixel. Since the generated diffraction pattern is equivalent for a pixel's phase modulo 2π (i.e. 2.1π and 0.1π result in the same diffraction patterns), we can choose the one that is closer to the current pixel value to minimize transition time.

C. Analysis code

A modular image processing and analysis framework was developed in Matlab to infer and visualize electrical activity of cells from fluorescence imaging. This framework takes in a *TIF* stack of fluorescent images and uses only four other parameters to transform this image stack into ΔF/F traces on a cell-by-cell basis and from this, spike traces for each cell. These parameters are minimum (A_{min}) and maximum size in pixels of a cell (A_{max}), threshold for binarization (t_b), and percentage of frames to be used for preprocessing (p). A high-level explanation is provided below, where numbers in parenthesis correspond to specific scripts in the order they are run.

First, the image stack is pre-processed (1) to obtain an image of the average "firing" event by averaging over the frames consisting of the top p percent in average intensity per pixel. This

image is passed to the segmentation function (2), which uses adaptive thresholding to binarize using t_b, then various filters including skeletonize and sharpening to obtain thin boundaries for cells. This image is then inverted and labeled to get contiguous blobs of individual cells, filtered by area before extracting the membranes and background areas which are returned as label masks. Background average fluorescence is computed at each frame, then ΔF/F is computed on a cell-by-cell basis (3) using moving median (window is 10% of total frames), to control for photobleaching and stored in a *csv*. This *csv* is used to identify the most electrically active cells (4) and then, a z-score of 1.5σ is used as a threshold for spiking and a raster plot of all cell spiking history is displayed and saved (5).

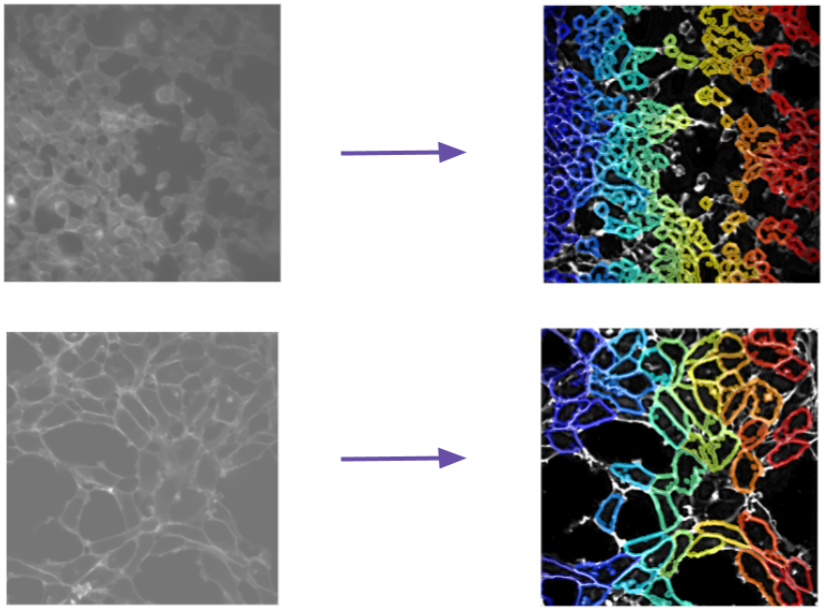

Figure 9 : Label masks from segmentation (step 2) overlain on the average firing images from two image stacks of HEK cells, taken on different microscopes and of different levels of confluency.

The modularity stems from the fact that, whenever possible, each task was separated into a script which takes only the necessary parameters for that task. Care was taken to ensure that the inputs and outputs at each script make this possible. So, while a basic segmentation script was implemented, a user could easily replace that step with a call to their own segmentation code, as long as it returns two label masks of background and foreground. Similarly, any post-processing. The Matlab code in its entirety is located at https://github.com/LucDoh/cellSegmentation-Analysis.

Results

Preliminary experiments to test the fast-switching using the BNS SLM system were done in July 2018 during a visit from Dr. Gregor Thalhammer. A fixed sample of neurons was imaged using

a 60x objective, dichroic cube and a Basler ac640-750um with a maximum frame rate of 750 fps. A pair of neurons were found in the FOV, The intensity patterns for photo-stimulation were simply a collection of 10 points which would fill the soma. An implementation of the Gerchberg-Saxton algorithm was used to produce the two phase masks and Dr. Thalhammer's software would create and send a series of frames to the SLM that minimized the transition rate between the masks.

We create two desired intensity patterns that would fit inside the soma and computed their corresponding phase masks. We were able to switch between these phase masks within 2.5 ms, thus confirming that the photostimulation at the timescales desired was feasible using this system.

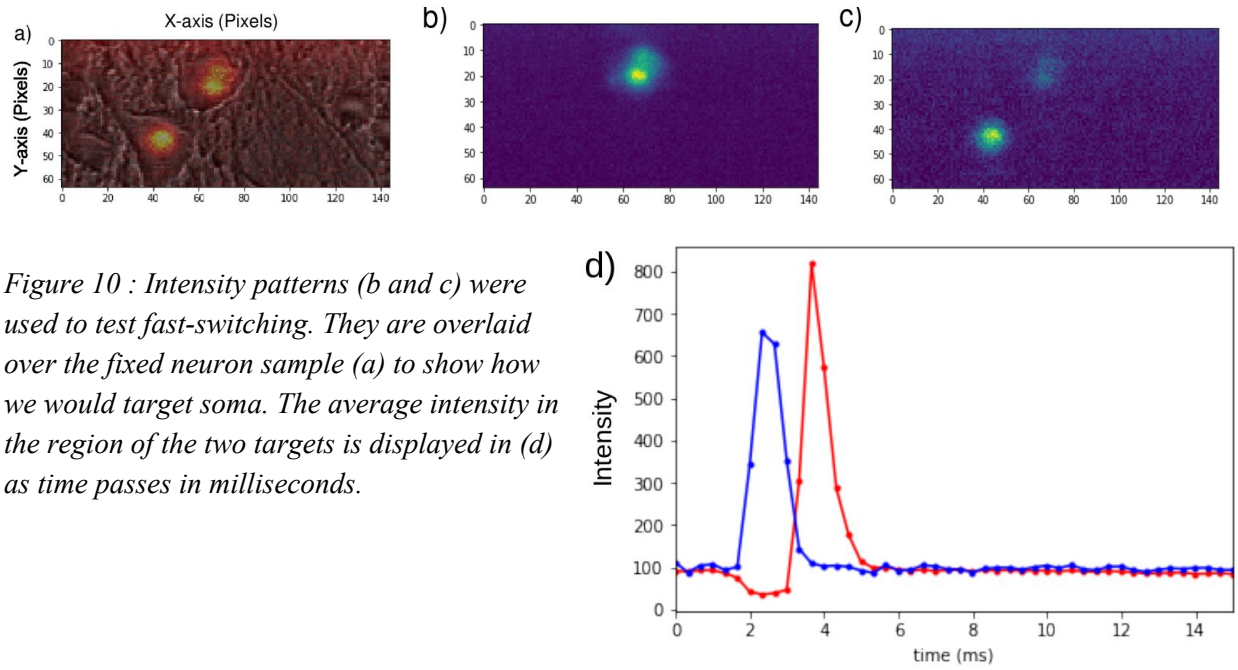

Figure 10 : Intensity patterns (b and c) were used to test fast-switching. They are overlaid over the fixed neuron sample (a) to show how we would target soma. The average intensity in the region of the two targets is displayed in (d) as time passes in milliseconds.

OS-HEKs cells were found to undergo spontaneous firing when cultured at high confluency and placed in ACSF (08/31/18). These cells were imaged at 70 fps using a confocal path at 70 fps after being dyed with BeRST on a homemade microscope named the Multiscale Microscope (not discussed further here). A 40x Nikon objective was used in conjunction with the Hamamatsu EMCCD, now on Sauron. We demonstrate the effectiveness of our analysis code on fluorescent images of cells stained with BeRST. It is able to accurately identify the large majority of cells (Figure 9, top), extract their membranes and then infer their spiking activity on a cell-by-cell basis. A z-score of 2.0 was used as a threshold for spiking, from which we can plot the spiking history of cells (Figure 12).

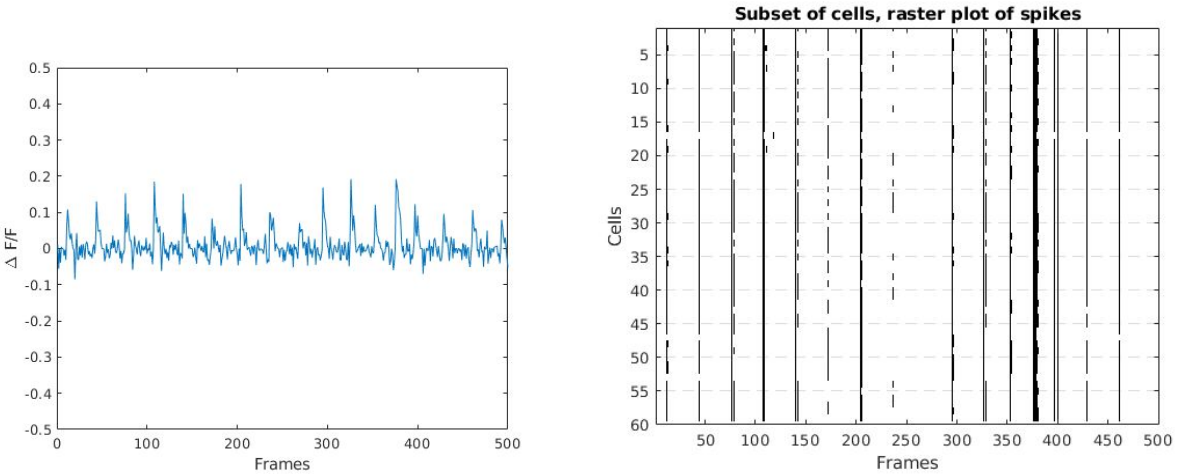

Figure 11 : ΔF/F trace of a single cell (left) and the raster plot for spiking of ⅓ of all segmented cells (right).

A set of experiments with OS-HEKs were done (04/25/19) on Sauron to replicate the results shown on the Multiscale Microscope and demonstrate that it can be done with the simpler and faster epi-fluorescence path utilizing the same EMCCD camera at 200 fps. The cells in this case did not spontaneously fire when put in ACSF, perhaps due to less confluency or a difference in media. They were made to fire using two short (~250ms) pulses of DC electric field stimulation of 5V/cm.

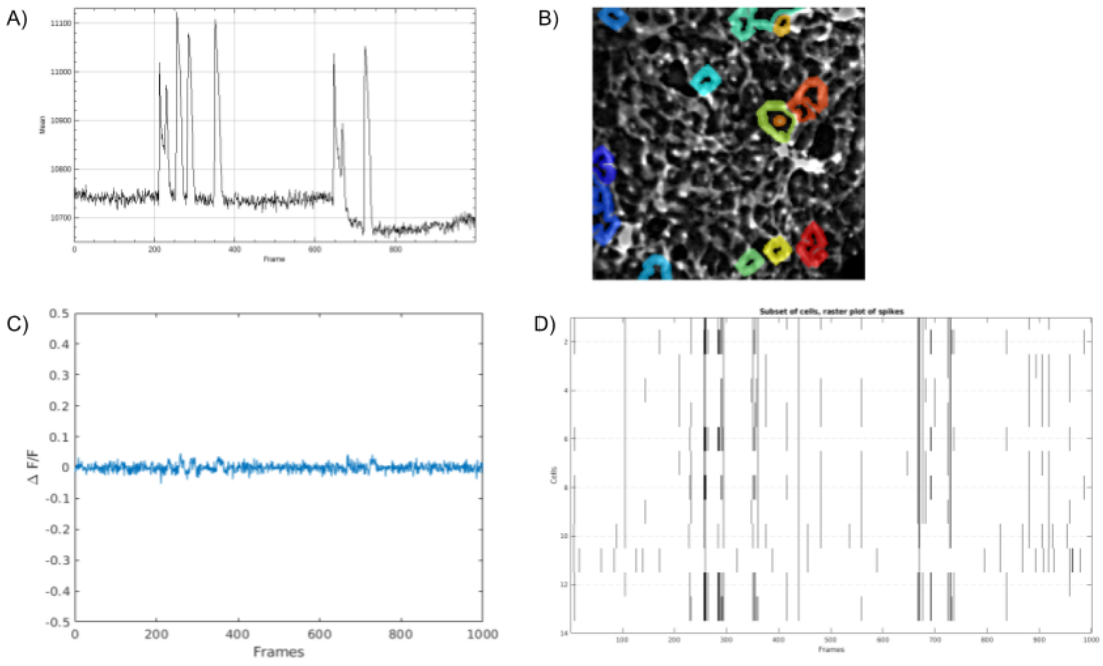

Figure 12 : DC field stimulation of HEK cells, imaging via epifluorescence path on Sauron.

Segmentation is clearly more difficult on image stacks at higher frame rates due to the accompanied lower resolution, and only 13 cells are identified (one of which appears incorrect). We do see that electrical activity is induced due to DC field stimulation by averaging the intensity by frame (using ImageJ) in Figure 12B. The computed $\Delta F/F$ for spikes is ~2%, which is small compared to previous results, suggesting that these may be sub-threshold action potentials. A raster plot of spiking histories for the 13 identified cells using a z-threshold of 2 is also shown.

On May 2nd 2019, a neuronal imaging session was conducted on Sauron, but BeRST stuck to the poly-d-lysine (PDL) coating used to adhere the cells to the plates. This made it difficult to distinguish cell membranes from this PDL coating when imaging in the far-red. Thus while we could see neurons using bright-field imaging, we were unable to see and stimulate electrical activity in them. Different coatings are being tested which BeRST will not attach to.

Future goals

The system we are developing has a unique ability to investigate how synapses strengthen and weaken due to close and correlated spiking of presynaptic and postsynaptic neurons. A known asymmetric form of "Hebbian learning" called Spike-timing dependent plasticity (STDP) is thought to play a significant role in learning and memory in the brain. In STDP, the temporal order of spiking of presynaptic (A) and postsynaptic (B) neurons will cause either Long-term Potentiation(if A fires before B) or Long-term Depression (if B fires before A) of synapses, and the strength of the change is inversely proportional to the time between the spikes.

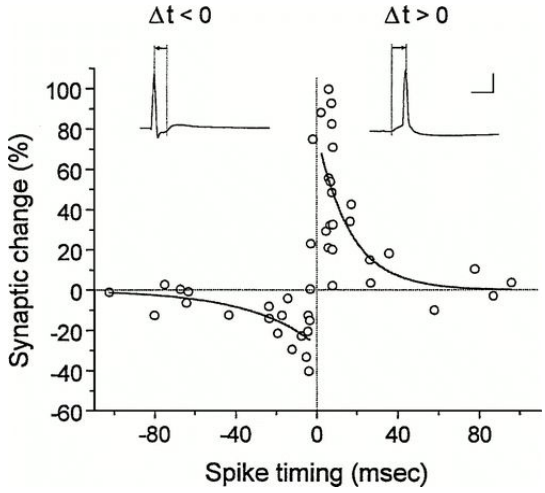

Figure 13 : Spike-timing dependent plasticity, characterized by Bi and Poo using repetitive correlated stimulation at 1 Hz.

The next planned experiment consist of repeated optical stimulation of two neurons in the same field of view in tight succession. The objective will be to demonstrate activity-based modification of neural circuits, as in STDP. We first find two optically stimulatable neurons, which we'll call A and B, in the same field of view. The procedure is then as follows:

i) Stimulate A n times, record activity of neurons through BeRST imaging
ii) Stimulate B n times, record activity of neurons through BeRST imaging
iii) Stimulate A then B with a time delay of 2ms. Do this m times and record activity
iv) Stimulate A n times, record activity of neurons through BeRST imaging
v) Stimulate B n times, record activity of neurons through BeRST imaging

Typical STDP protocols (Markram and Sakmann, 1997; Bi and Poo, 1998; Sjostrom et al.,2001) suggest that the number of pairwise stimulations, m, should be between 50-100. More freedom is available in selecting the number n. It need only be large enough to sample with statistical significance the behaviors of neurons A and B when they are stimulated individually.

While it is difficult to assess whether these two neurons are synaptically connected before the experiment, after this procedure, we can see whether the resulting activity of neuron B has changed significantly when stimulating A as compared to before the pairwise stimulation (step iii). If A and B are connected at all, we expect to see an increase or decrease in the firing rate of B when A is stimulated due to LTP or LTD. Whether this optically driven activity results in LTP or LTD depends on which of the neurons is presynaptic and postsynaptic.

In future work, we will collaborate with the Babadi group to look at functional connectivity in these neuronal cultures from spiking histories through adaptive Granger Causality (AGC) analysis. The Babadi group has successfully used their statistical inference framework to examine spontaneous activity in the ferret auditory and prefrontal cortex during attentive behavior, and extract top-down and bottom-up functional dynamics that varied as a function of task [9]. Their statistically robust techniques will help us to examine changes in synaptic plasticity through changes in the observed spiking history during our experiments.

Some minor improvements to the experiment are also planned. The current dichroic cube used is not red-shifted enough to capture most of the signal from BeRST. Specifically, changing the emission filter should result in a substantial gain in signal. The analysis code currently takes in parameters for maximum and minimum pixel size of cells and while this works reasonably well, some incorrect cells are also identified. A step that checks and removes for anomaly cells before analysis and visualization should be implemented. Additionally, wave propagation of electrical signals in HEK cells can be studied by adding a feature to save the locations in space of each cell, and then adding specialized analysis and visualization on top of the framework.

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