An ultra-widefield microscope for high-speed, all-optical electrophysiology

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Abstract: Using optogenetics, the ultrawidefield Firefly microscope enables simultaneous stimulation with blue light and transmembrane voltage recordings with red light from >100 neurons with subcellular spatial and millisecond temporal resolution.
OCIS codes: (180.2520) Fluorescence microscopy; (000.1430) Biology and medicine; (110.2945) Illumination design

1. Introduction

Recent decades have seen an explosion in fluorescent sensors for biology[1,2]. These sensors detect varied facets of cellular state and function from enzyme activity[3] to cellular pH[4] to transmembrane voltage[5]. In addition to fluorescent reporters, and array of light-driven actuators of cellular function have been developed[6]. Reporters and actuators can be targeted to sub-cellular locations such as the endoplasmic reticulum or mitochondria, yielding additional insights into cellular processes. Because of the localization of the reporter, often the time dynamics of cellular physiology can be extracted by averaging over the entire cell: fine spatial resolution yields little extra information. In these cases, it is more profitable to image a large field of view at low-magnification to record from many cells simultaneously to sample endemic cellular variability. The Firefly microscope can image a 5 x 5 mm field of view (FOV) with high sensitivity at a 100 Hz frame rate or a 0.5 x 5 mm FOV at a 1 kHz frame rate.

There has been great progress recently on imaging large FOVs on 2-photon microscopes in tissue, e.g. [7]. Here we focus instead on cell culture measurements, where low autofluorescence and scattering make it possible to record at high speeds with a good signal to noise ratio (SNR). We have optimized the microscope for high light-collection efficiency, low autofluorescence background, and reconfigurable patterned illumination for the stimulation of single cells.

The microscope was primarily designed for voltage imaging using Optopatch [5], shown in Fig. 1, which uses a pair of transmembrane rhodopsin proteins. A sensitive, blue-shifted channelrhodopsin CheRiff depolarizes the cell in response to blue light, triggering action potentials in neurons or cardiomyocytes. An optically orthogonal, far-red voltage reporter QuasAr tracks transmembrane potential with millisecond temporal resolution. Figure 1B shows the high correspondence between fluorescence and current-clamp voltage recordings when the cell is stimulated by 470 nm light pulses.

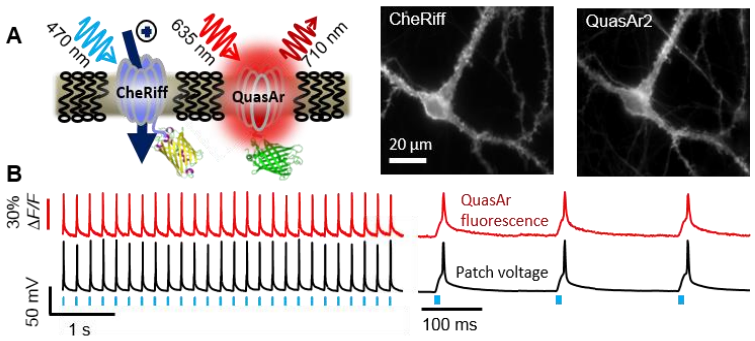

Figure 1 – All-optical electrophysiology with Optopatch. (A) Optopatch is comprised of the blue light-stimulated channelrhodopsin CheRiff (bound to mOrange) and the red light-emitting voltage sensor QuasAr (bound to Citrine). Fluorescence images show the membrane bound proteins in a rat hippocampal neuron. (B) Fluorescence (red) and patch-clamp voltage recordings (black) during a train of stimulus pulses (blue) showing the fidelity of the voltage reporter.

2. Optical design

The Firefly microscope (Fig. 2A) is designed around the Olympus MVPLAPO 2 XC, a 2x objective with a high NA of 0.5 for its very large, >6 mm FOV. Using its sister-objective or a commercial camera lens as the microscope tube lens (Olympus MVPLAPO 1 XC, Zeiss 135mm f/2 Apo Sonnar T* ZF.2), the sample is imaged at 2 or 2.7x onto a

Hamamatsu Orca FLASH scientific cMOS camera, which makes fast frame rates possible. Because of the high excitation intensities required to image QuasAr (~300 W/cm^2) and the large microscope area, ~10 W of 635 nm light must be delivered to the sample. With this much power, autofluorescence in a traditional epifluorescence geometry from the objective itself overwhelms the fluorescent protein signal. Instead, we take advantage of the long objective working distance to place a fused silica prism between the sample and the objective, and sample and prism are coupled by immersion oil (Fig. 2B). The input angle is set just shy of total internal reflection (TIR), so refraction of the transmitted beam at the glass-water interface significantly reduces the beam width, reminiscent of light sheet microscopy[8]. The excitation beam rapidly exits the FOV, minimizing autofluorescence, and increases the light intensity.

Channelrhodopsin stimulation is implemented with an intense 470 nm LED reflected off a digital micromirror device (DMD) for patterned stimulation (Fig. 2B). For excitability measurements, synaptic blockers are added to uncouple intercellular communication, and all cells are stimulated and recorded from simultaneously. For synaptic measurements, cells can instead be stimulated one at a time using the DMD. As each cell is stimulated, the post-synaptic potentials are recorded from all other cells in the dish.

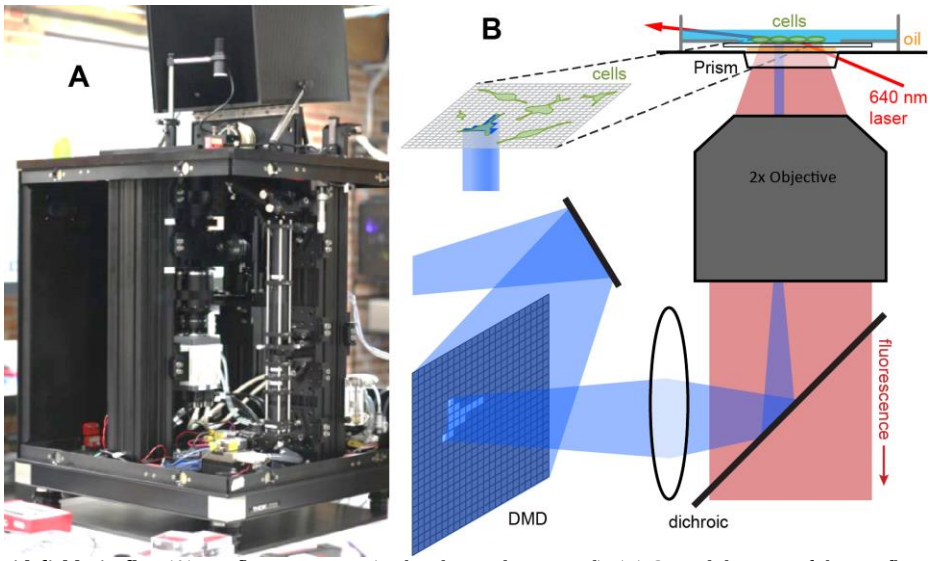

Figure 2 – Ultra-widefield Firefly. (A) Firefly microscope (with side-panels removed). (B) Optical diagram of the Firefly. Through-prism red excitation yields high intensities with low background. The DMD enables high-speed, targeted stimulation of single neurons or sub-cellular features.

3. Results

The low-magnification images enable electrophysiology recordings from roughly 100 neurons in parallel, maintaining the richness of patch-clamp recordings but at a drastically increased throughput. Figure 3 steps through the analysis flow for a pair of movies from one FOV of the Firefly microscope, pre- and post-drug addition. The movie is divided up into around 50 sub-regions using a watershed algorithm, isolating cells or small clusters of cells. Using principle component analysis and independent component analysis (PCA/ICA), the program automatically identifies each active neuron using its unique spiking pattern and merges the sub-regions to connect any cells crossing a boundary. The outputs are pixel weights (Fig. 3A) and voltage traces (examples in Fig. 3B) for each cell. To probe cell behavior in different conditions, we use a variety of blue light stimuli: blue steps of different intensities to drive different baseline depolarizations, pulse trains of different frequencies to probe maximal spike rates, and an intensity ramp to study the initiation and evolution of spiking. The action potentials are identified within the time trace for each cell, and the spike times for each neuron are compiled in a raster plot to show spike patterns (Fig. 3C) and average population spike rates (Fig. 3D). Spike shape parameters are tabulated for each spike (Fig. 3E), and more complex spike timing and adaptation parameters are calculated (Fig. 3F & G). Results are automatically compiled into histograms, and any statistically significant effects (i.e. phenotypic differences between healthy and disease or differences after drug addition) are automatically flagged for closer scrutiny. Figure 3H shows the effect of 1 μM ML213, a K_V 7.x opener that strongly suppresses neuronal excitability by hyperolarizing the neurons. We robustly see effects from sodium, calcium, and potassium channel modulators as well as inflammation cocktails that increase excitability.

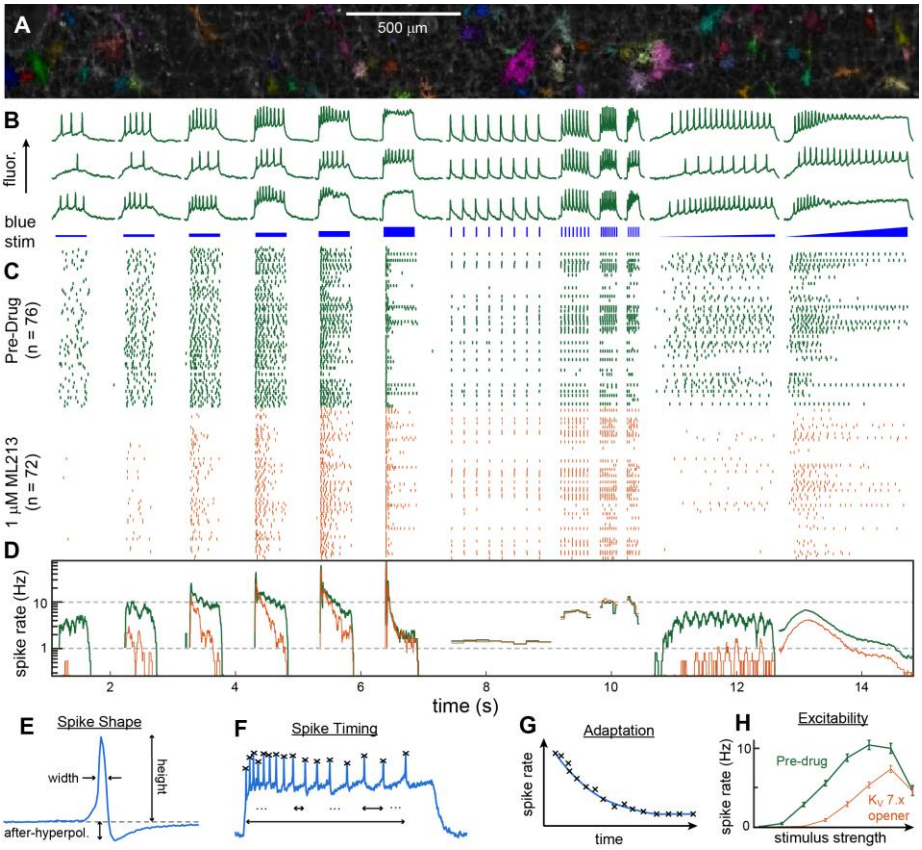

Figure 3 – Data analysis pipeline. (A) Firefly image with overlay (colored regions) of hiPS cell-derived motor neurons identified by automated analysis. (B) Example voltage recordings from selected cells, and the blue stimulus used to evoke firing: steps, pulse trains, and ramps. Time scale in D. (C) Raster plot where each point is an identified action potential and each row is a neuron from a single FOV. Before (green) and after (orange) addition of ML213 at 1 μM, a potassium channel opener that lowers resting potential and suppresses firing. (D) The spike rate averaged over cells. (E) Spike shape, (F) spike timing properties, and (G) adaptation are automatically extracted for each cell. (H) The excitability extracted from the staircase in D.

In addition to pharmacology, we have observed phenotypic differences in rodent and human induced pluripotent stem (iPS) cell derived neuronal disease models including ALS, epilepsy, and schizophrenia: cells derived from patients fire differently than those from healthy controls. We have found that the large, natural variability in neuronal spiking behavior tends to obscure the effects of genotype or drug, and the large number of neurons measured on Firefly is critical to reach meaningful conclusions. We hope the Firefly microscope will spur progress in *in vitro* neurological disease modeling.

4. References

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