Next generation high-throughput random access imaging in vivo

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Our team is developing a next-generation high-throughput random-access imaging system for real-time monitoring of sensory-driven synaptic activity. This monitoring takes place across all inputs to single living neurons in the context of the intact cerebral cortex, in order to better understand how these synaptic signals are integrated and processed. Our first target is to monitor calcium signals from approximately 10,000 locations corresponding to all excitatory synapses of a single neuron with 100 ms temporal resolution. While calcium imaging with GCaMP is well-established, the RCaMP excitation wavelength at about 1050 nm is more compatible with high power Yb-fiber based femtosecond laser sources.

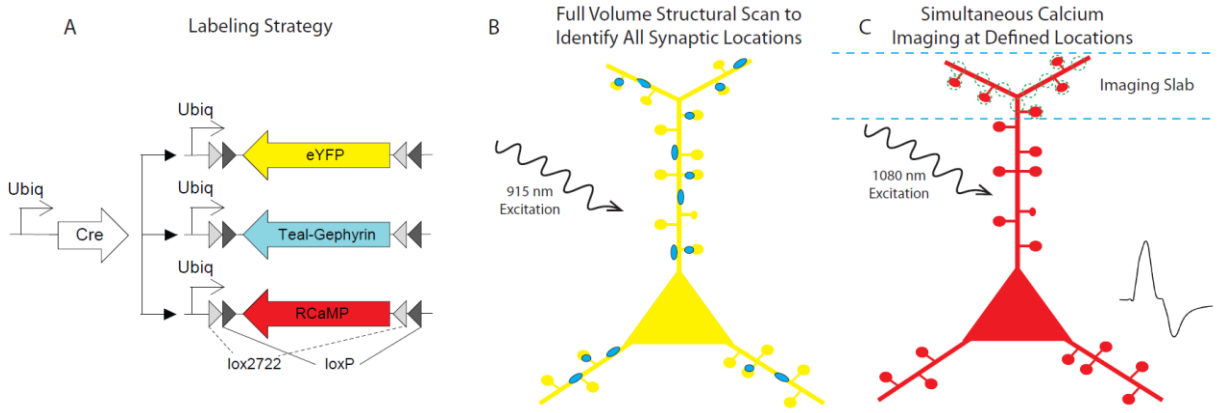

Figure 1: Labeling strategy for RCaMP & structural imaging. eYFP is used as a cell fill, TealFP-Gephyrin as a synaptic marker, and RCaMP as an indirect measure of local potential.

We are testing new RCaMP variants, expressed both in neuron cultures and in the mouse brain, to evaluate signal intensity, as well as spectral compatibility with other fluorophores used as a cell-fill and as synaptic markers (Fig. 1A). In addition to RCaMP, sparsely labeled neurons each express a fluorescent cell fill to delineate their structure, including dendritic spines, the sites of excitatory synapse. In addition, a second fluorophore, fused to the inhibitory postsynaptic marker gephyrin, labels all inhibitory synaptic sites on the same neuron. These neurons are first scanned using high volume, high-resolution dual-color two-photon microscopy to provide a coordinate map of all synapses on the labeled neuron (Fig. 1B). Since all the relevant synaptic locations can be initially determined by low speed structural imaging, the sampling of the calcium signals from these known locations is most efficiently accomplished by selective excitation.

The design of the selective excitation system is based on 3D holographic patterning, where a neuron spanning approximately 150 µm in depth is divided into 10 “slabs” with a

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lateral extent of 300x300 µm^2. Approximately 1000 synaptic locations lie within each slab (Fig. 1C). These locations can be simultaneously excited by holographically generating a set of 3D-distributed near-diffraction-limited spots using a spatial light modulator. As a pilot, we have demonstrated that we can generate the required density of spots to illuminate a 100x100x15 µm^3 volume. We are also evaluating a complete system design that includes an electro-fluidic lens that will translate the 15 µm excitation slab through a 150 µm extent of a neuron's depth *in vivo* (Fig. 2).

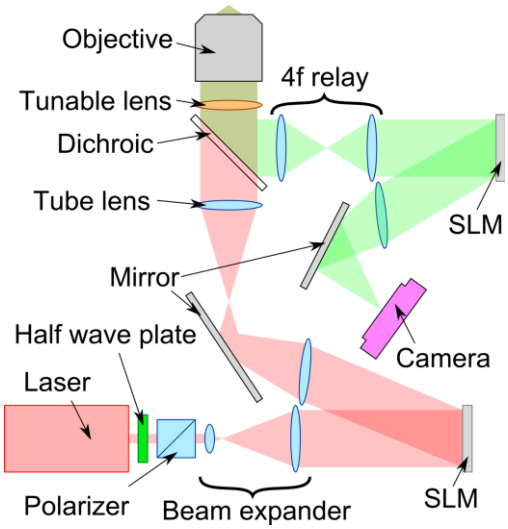

Figure 2: Instrument optical diagram

Holographic excitation is a well-established low-risk technique for maximizing the system power efficiency [1]. System throughput is maximized by use of an Ytterbium fiber-based femtosecond laser, with a fixed wavelength of 1030nm. These lasers can achieve 200fs pulse durations with 1MHz repetition rates and average power in excess of 20W, far in excess of comparable Titanium-Sapphire lasers, and highly compatible with high-throughput imaging. In order to distinguish synapses located above one another on a dendrite, a Laguerre- Gaussian phase plate is used to modify the point-spread function so that it rotates a function of depth [2]. By knowing the excitation locations, the emission from each location can be recovered by linear unmixing. Preliminary results show that multiple locations (400 spots in current tests) can be patterned in a 100x100x40 µm^3 volume simultaneously (Fig. 3; the change in scale bar is a consequence of using a tunable lens to change the focal plane, which results in a simultaneous change in magnification).

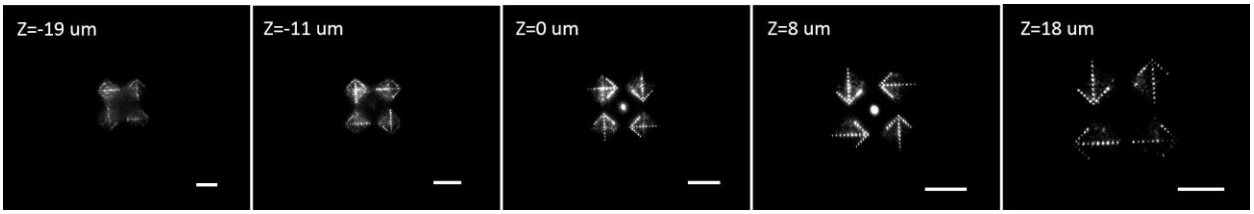

Figure 3: Experiment data of two-photon excited multiple spots controlled distribution in 3D simultaneously. Each arrow contains 20 spots. The scale bar is 50 µm.

The 3D Excitation Point-Spread Function (PSF) of the system has been measured, and found to be near the diffraction limit (Fig. 4 A-B). The intensity histogram of the spots shows a non-uniform intensity distribution (Fig. 4C). This non-uniformity is a function of both the sample inhomogeneity as well as the differing excitation power inherent to using phase-only holographic excitation.

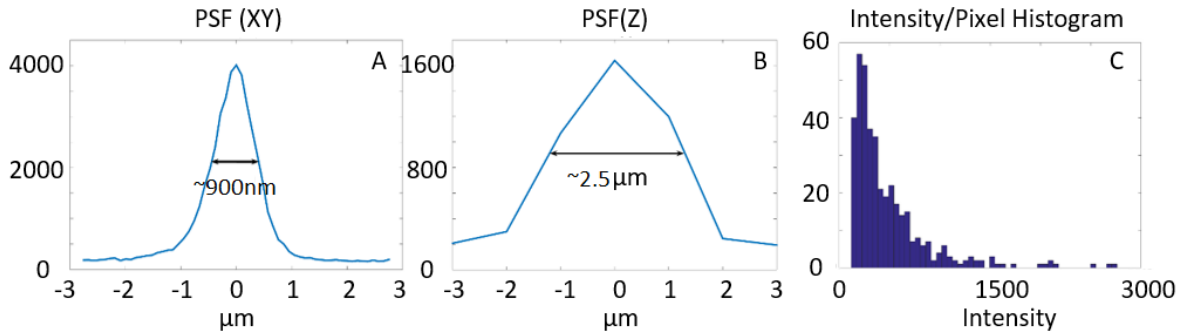

Figure 4: Quantitative analysis of multiple spots in 3D. (A) Lateral PSF (B) Longitudinal PSF (C) Intensity distribution of 400 spots.

Since the depth-of-field of a typical high numerical aperture objective is approximately one micron, a Gaussian-Laguerre (GL) element is inserted at the Fourier plane of the detector. With the GL element, point sources at different depths are imaged as pairs of spots with depth-dependent rotation angles. We have demonstrated that the depth-of field can be extended, and that the axial locations of excitation spots at different depths can be simultaneously identified (Fig. 5).

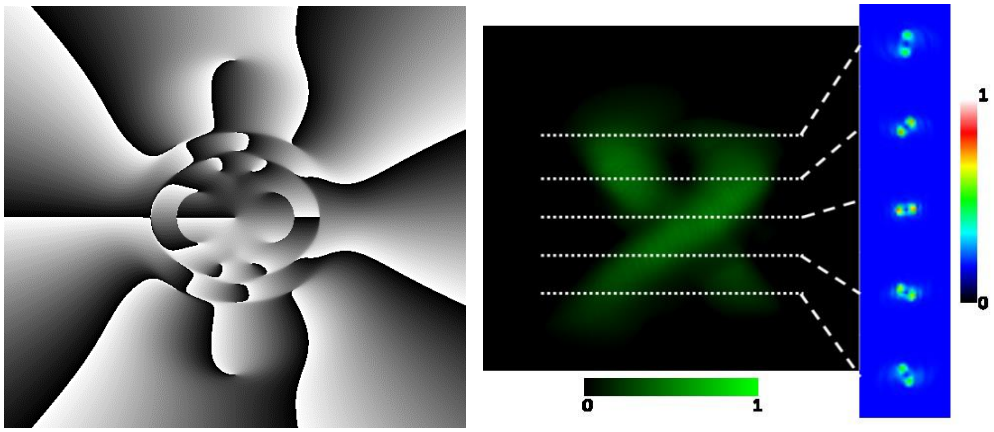

Figure 5: Optical transfer function of a rotating beam, constructed using a Laguerre-Gaussian phase plate. (A) calculated phase plate design, in accordance with the design process outlined in [2]. (B) A 3D rendering of the point-spread function as a function of depth. X-Y slices taken at different depths are shown on right.

In summary, we present a custom-designed optical system to perform functional imaging of all synapses in a single neuron. Development of the optical system is almost complete, and significant progress has been made in co-expressing the three required fluorescent proteins. In the next step, we will monitor the Calcium signal of multiple synapses *in vivo*, which will potentially reveal the communication network among synapses of a single neuron.

References

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