MINIATURIZED TWO-PHOTON MICROSCOPES AND THEIR APPLICATIONS

by

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Abstract

Two-photon microscopy (TPM) has been widely accepted owing to its high resolution, 3D imaging capability, resistance to tissue scattering hence deeper penetration, and the ability to simultaneously excite multiple fluorophores with a single source. However, the application of tabletop TPM is hindered by its bulky size. In this dissertation, two different strategies were explored in miniaturizing TPM for their respective applications. One is a two-photon fiberscope for neuroimaging of freely walking rodents, the other is a handheld two-photon rigid probe for optical biopsy.

In case of the two-photon fiberscope, this dissertation reports our twist-free two-photon fiberscope imaging system for enabling neuroimaging on freely rotating/walking mice. The system includes an optoelectrical commutator (OEC) with active rotational tracking and compensation capabilities to allow the animal to rotate and walk in arbitrary patterns during two-photon fluorescence imaging of neural activities. The OEC provides excellent optical coupling stability (<±1% fluctuation during rotation) and an extremely high torque sensitivity (<8 mN·m). In addition, the system is equipped with a custom GRISM (grating and prism) to effectively manage the temporal properties of the femtosecond excitation pulses through the fiber-optic system, which improves neuroimaging signal by ~2X. Furthermore, to allow two-photon fiberscope imaging of deep neurons through GRIN lens implants, we have completely redesigned our fiberscope based on the concept of composite fiber cantilever. Our new fiberscope (type II) is compatible with GRIN lens implants, allowing for the first time two-photon imaging of deep neurons on freely behaving mice. The type II fiberscope also offers a 6X improvement in field of view (FOV), enabling simultaneous imaging of more than 50 cortical neurons on freely behaving mice. The abovementioned technical advancements greatly extend the functionality and robustness of our
fiberscope system, giving the neuroscience community a useful research tool that allows twist-free, high quality, and large FOV imaging of both superficial and deep neurons on freely behaving mice.

This dissertation also reports an advanced biopsy-needle compatible varifocal two-photon handheld rigid probe for depth-resolved optical biopsy of unlabeled biological tissues \textit{in vivo} and \textit{in situ}. The probe is able to perform two-photon autofluorescence (TPAF) imaging near histopathological resolution with a good SNR. It is compatible with the 14-gauge biopsy needle protocol, with a 1.75-mm outer diameter and 15-cm probe length. The probe is also capable of 3D imaging at a maximum speed of 10 FPS, with a 120 μm field of view and a 200 μm focus scanning range. With our rigid probe, depth-resolved optical biopsy of internal organ has been demonstrated on mouse kidney for the first time through a biopsy needle. This rigid probe marks an important step towards clinical translation of TPM.

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**Figure 55. Intensity autocorrelation.** (A) Schematic of the intensity autocorrelation. (B) Phase matching condition in the nonlinear crystal for intensity autocorrelation. \( k_n \) and \( n_n \) are wave vectors and refractive indices of the crystal each beam sees. (C) Photo of the output from an intensity autocorrelator. M: Mirror; BS: Beam Splitter; L: Lens; XTAL: Nonlinear Crystal; SHG: Second Harmonic Generation; SFG: Sum Frequency Generation......................................................................................................................................................................................... 119

**Figure 56. Interferometric autocorrelation.** (A) Schematic of interferometric autocorrelation. (B) Phase matching condition in the nonlinear crystal for interferometric autocorrelation. \( k_n \) and \( n_n \) are wave vectors and refractive indices of the crystal each beam sees. M: Mirror; BS: Beam Splitter; L: Lens; XTAL: Nonlinear Crystal; SHG: Second Harmonic Generation; CF: Color Filter. ..... 122

**Figure 57. Home-built interferometric autocorrelator.** CC: Corner Cube; BS: Beam Splitter; LS: Loudspeaker; BBO: Beta Barium Borate Crystal; CF: Color Filter; CM: Curved Mirror.... 125
Chapter 1  Introduction

1.1  Two-photon microscopy

Two-photon microscopy\(^1\) (TPM) is a nonlinear fluorescence microscopy technique that allows imaging tissue \textit{in vivo} up to \(\sim 1\) mm in thickness\(^2\). Different from traditional (single-photon) fluorescence microscopy, where the emission wavelength is longer than the excitation wavelength, two-photon fluorescence (TPF) process works by simultaneous absorption of two photons with longer (doubled) wavelength and emitting a single fluorescence photon at a shorter wavelength (as shown in Figure 1 below).

![Two-photon fluorescence process](image)

**Figure 1.** Single-photon and two-photon fluorescence processes.

TPF is a nonlinear process and it requires high field intensity. Typically, TPM uses near-infrared (NIR) femtosecond lasers (e.g., Ti:sapphire lasers at \(\sim 800\) nm, Yb:Fiber lasers at 1060 nm) which provides very high instantaneous peak powers for the TPF to occur. The use of NIR excitation light brings the advantage of reduced scattering in tissue, allowing deeper penetration depth for the excitation beam. In addition, the nonlinear nature of TPF also confines the

\[1\]
\[2\]
fluorescence generation in a small volume nearby the excitation beam focus (shown in Figure 2 below), greatly suppressing the out-of-focus background signal.

![Figure 2. Localization of excitation by two-photon excitation](image) (A) Single-photon excitation of fluorescein by focused 488-nm light (0.16 NA). (B) Two-photon excitation using focused (0.16 NA) femtosecond pulses of 960-nm light. Reprinted with permission from Springer Nature.

TPM has the following appealing features compared to other microscopy techniques:

- **Deeper penetration depth:**
  
  Due to the longer excitation wavelength and localized fluorescence generation, TPM naturally exhibits superior penetration depth and brings much higher signal to background ratio (SBR) when imaging deep into tissue.

- **Optical sectioning (3D imaging) capability:**
  
  The excitation of TPF is highly localized, as compared to the case of single-photon fluorescence where an entire focal cone above the focus is excited. Therefore, scanning the excitation beam in 2D already produces an optical section of the tissue, no pinhole is required in the detection path.

- **Enhanced collection efficiency:**
Since no pinhole is used for detection, all the fluorescence photons, no matter how many times scattered in the tissue, will be collected by the detector as long as they fit inside the detection cone of the objective. In addition to the localized excitation and longer excitation wavelength, the pinhole-less detection scheme also makes TPM much more resistant to tissue scattering.

- **Reduced phototoxicity:**
  Both the localized excitation and the longer excitation wavelength reduce photodamage and photobleaching, rendering TPM more appealing for long term in vivo imaging compared to the confocal counterpart.

- **Easy multi-channel and multi-modality integration:**
  Since the two-photon excitation spectra of multiple fluorophores are largely overlapped, simultaneous multi-channel imaging could be easily realized by adding detection channels to the TPM without adding additional excitation sources. In addition, second harmonic generation (SHG) can occur at all the excitation wavelength, this made SHG+TPM a popular multi-modality imaging setup.

For the above reasons, TPM enjoys wide adoptions in the field of neuroscience, and has shown great potential in clinical applications.

### 1.2 TPM for neuroscience

#### 1.2.1 Conventional tabletop TPM

Since its invention about 30 years ago, TPM enjoyed wide adoption in the field of neuroscience. Structural and functional imaging of neurons in vivo using voltage-sensitive dyes with TPM was performed as early as in 1994, only a few years after the invention of the technology. And with the later development of genetically encoded calcium indicators (such as GCaMPs), TPF
imaging of neural activities at synapse resolution had become the standard technique for neuroscience studies.

The past three decades have witnessed numerous efforts in advancing the TPM technique for neuroscience. In pushing for bigger imaging field of view (FOV), centimeter-scale two-photon microscopes were demonstrated by multiple groups\textsuperscript{14-16}, enabling simultaneous imaging of thousands of neurons over multiple brain regions. In pursuing faster imaging speed, kilohertz frame rate two-photon microscopes\textsuperscript{17-19} were built using spatial temporal multiplexing techniques, making real time two-photon voltage imaging possible. The depth limit of TPM imaging in neocortex was well-known to be $<1$ mm\textsuperscript{4}. However, by utilizing the concept of higher order three-photon fluorescence at even longer excitation wavelength (1.3-1.7 μm), a 1.7 mm imaging depth was realized\textsuperscript{20}, which made functional neuroimaging through intact skull\textsuperscript{21} possible. Wavefront shaping techniques\textsuperscript{22,23} were also utilized in TPM for neuroimaging, allowing better signal and resolution when imaging deep in the brain.

1.2.2 Miniaturized TPM for neuroimaging on freely behaving rodents

Another technological trend of optical imaging in neuroscience is the miniaturization of imaging devices to enable real-time neuroimaging of freely behaving rodents\textsuperscript{24,25}. The head-mounted miniaturized single-photon fluorescence microscope had provided a unique capability for imaging neural activities on freely behaving mice and made significant impact on the field. However, unlike TPM, its resolution, particularly depth resolution, is limited in addition to usually sub-optimal signal to background ratio (SBR) caused by tissue scattering.

The idea of making a miniaturized head-mounted two-photon microscope for brain imaging on freely behaving rodents was first proposed almost two decades ago\textsuperscript{26}. However, technical challenges, such as heavy weight (25 g) imposed on the animal, limited the subsequent applications of the technology. Since then, numerous technical efforts had been investigated to address the challenges. Notably, the use of Gradient-index (GRIN) lens for two-photon brain
imaging\textsuperscript{27} drastically reduced the weight of the objective; the introduction of a second collection fiber further reduced the size and weight of the miniature microscope\textsuperscript{28,29}; the advancements in pulse delivery schemes for delivering shorter and cleaner Ti:sapphire laser pulses over optical fiber\textsuperscript{30,31} resulted in stronger signal and better sensitivity of the miniature microscope. Recently, the above advancements were elegantly consolidated and a 2.15-g miniature two-photon microscope was successfully demonstrated on a freely behaving mouse\textsuperscript{32}.

### 1.2.3 Scanning TPF fiberscope technology

Scanning TPF fiberscope technologies\textsuperscript{33-38} represent another promising opportunity for imaging of neural activities on freely behaving mice. The first fully integrated TPF fiberscope used a piezoelectrically actuated resonant fiber-optic scanner and a single double-clad fiber (DCF) for both excitation and detection\textsuperscript{34}, which, unlike in a dual-beam configuration for excitation and emission\textsuperscript{28,29,32}, avoids beam folding and effectively reduces the footprint to ~3 mm in diameter and weight to <1 g.

This dissertation reports a new development that solves the bottle-neck challenge in head-mounted two-photon neuroimaging on live rodents by allowing our two-photon fiberscope to actively track and follow the rotation of the animal. Thus, for the first time, dynamic imaging of neural activities with subcellular resolution become possible on true freely rotating/walking rodents. Our two-photon fiberscope system includes an optoelectrical commutator (OEC) with active rotational tracking and compensation to allow the animal to walk and rotate in arbitrary patterns during two-photon imaging of neural activities. This fiberscope system was able to perform prolonged recordings of dendritic and somatic calcium dynamics with subcellular resolution in the motor cortex of freely behaving mice in a twist-free fashion.

In addition, our fiberscope system was equipped with a GRISM (grating and prism) per-chirper, which improved the quality of femtosecond pulses delivered through fiber and resulted in a 2X increment in neuroimaging signal \textit{in vivo}. Furthermore, to allow two-photon fiberscope
imaging of deep neurons through GRIN lens implants, we have completely redesigned our fiberscope based on the composite fiber cantilever\textsuperscript{39}. Our new fiberscope (type II) was compatible with GRIN lens implants, allowing for the first time two-photon imaging of deep neurons on freely behaving mice. The type II fiberscope also offered a 6X improvement in FOV (area), enabling simultaneous imaging of more than 50 cortical neurons on freely behaving mice.

1.3 TPM for clinical applications

1.3.1 Label-free TPM imaging of tissue

One of the most important features of TPM that made it popular as a potential imaging technology candidate for clinical translation is that it is capable of providing histopathological resolution imaging of tissue \textit{in vivo} in a label-free fashion. Endogenous fluorophores such as reduced nicotinamide adenine dinucleotide (NADH), oxidized flavin adenine dinucleotide (FAD), tryptophan, as well as tyrosine can be easily detected by TPM\textsuperscript{6}. Endogenous structural proteins such as collagen, microtubules, and muscle myosin also exhibit second harmonic generation (SHG) characteristics\textsuperscript{40-42}, allowing them to be imaged by TPM at submicron resolution without labelling.

NADH and FAD are critical metabolic coenzymes in all living cells contributing to the tricarboxylic acid (TCA) cycle. Intensity\textsuperscript{43} and fluorescence lifetime\textsuperscript{44} TPM imaging of NADH and FAD can reveal metabolic functional states of the cell and tissue \textit{in vivo}, making it a popular tool for cancer research\textsuperscript{45}. In addition, studies\textsuperscript{46,47} had shown that TPM provided considerable label-free contrast for skin cancer detection, and it had already been approved for skin imaging in European Union\textsuperscript{48}.

1.3.2 Miniaturized TPM for optical biopsy

Optical biopsy refers to methods of using optical imagining or spectroscopy to evaluate tissue histopathology \textit{in vivo} and \textit{in situ} without tissue excision\textsuperscript{49-53}. Among various potential technologies for optical biopsy such as confocal laser scanning microscopy (CLSM)\textsuperscript{54-56}, optical coherence
tomography (OCT)\textsuperscript{57-62}, and two-photon microscopy (TPM), TPM is of particular interest for its label-free imaging capability.

However, conventional tabletop TPMs are bulky and not suitable for internal organ imaging. Recently, the development of scanning two-photon fibrescope enables label-free, \textit{in vivo}, high-resolution and functional histological assessment of internal organs that was previously impossible\textsuperscript{10,63-70}. An alternative approach for TPM to reach internal organs was to use a rigid probe made of a gradient index (GRIN) relay lens to deliver the excitation light to and collect the fluorescence (or SHG) from tissue\textsuperscript{71-73}. Compared with the flexible fiberscopes, the rigid probe is more desirable in laparoscopic applications or in interfacing with a biopsy probe (i.e., by going through the cannula of a biopsy needle). In addition, the rigid probe can be made smaller since the beam scanning mechanism can be packed outside the probe at its proximal end, offering more flexibility in design and additional functionalities. Nonetheless, all prior efforts enabling TPF imaging of internal organs involved surgical elevation of target organs to reduce motion artifact and most lacked a built-in mechanism for depth scanning\textsuperscript{70,72,74,75}, which fell short of the \textit{in situ} requirement for optical biopsy.

This dissertation also reports a more advanced biopsy-needle compatible varifocal multiphoton handheld rigid probe for depth-resolved optical biopsy of unlabeled biological tissues \textit{in vivo} and \textit{in situ}. The probe could perform two-photon autofluorescence (TPAF) imaging near histopathological resolution with a good SNR. It was compatible with the 14-gauge biopsy needle protocol, with a 1.75-mm outer diameter and 15-cm probe length. The probe was also capable of 3D imaging at a maximum speed of 10 FPS, with a 120 μm field of view and a 200 μm focus scanning range. With our rigid probe, depth-resolved optical biopsy of internal organ was demonstrated on mouse kidney for the first time through a 14-gauge biopsy needle.
1.4 Chapter Overview

In this dissertation, we explore two types of miniature TPM imaging systems (fiberscope and rigid probe) for their respective applications. Chapter 2-4 cover our efforts in enabling the two-photon fiberscope imaging system for imaging of both superficial and deep neurons on freely walking mice. And Chapter 5 covers our hand-held two-photon rigid probe for optical biopsy.

In Chapter 2, the design and construction of our fiberscope imaging system is described in detail. The next chapter (Chapter 3) details the methods and apparatus that enabled our two-photon fiberscope system to perform neuroimaging reliably. In addition, neuroimaging results collected by both our type I and type II fiberscopes are also presented in the chapter. Chapter 4 covers our efforts in developing a SMA-based feedback-controlled depth scanner for fiberscope imaging. In Chapter 5, the engineering details of our advanced two-photon handheld rigid probe is presented. And in the final chapter (Chapter 6), we draw conclusions from our current work, and discuss the future perspectives of our miniaturized TPM technologies with regard to their respective applications.
Chapter 2  Two-photon Fiberscope System

In this chapter, we present our twist-free ultralight two-photon fiberscope system for enabling neuroimaging of freely walking and freely rotating mice. To accommodate the arbitrary turning movement of the mouse, minimize the restraining force exerted to the mouse head, and prevent the optical fiber/electrical wires from being twist-broken, we connected the system to an optoelectrical commutator (OEC) with active rotational tracking and compensation. The OEC provided excellent stability with < ±1% variation in optical coupling efficiency over 360-degree rotation, allowing the fiberscope to operate at any given rotational angle. The active tracking mechanism required minimal torque (<8 mN·m) to be activated, essentially freeing the animal from rotational restrictions. In addition to OEC, the system was also equipped with a custom pre-chirper based on grating and prism (GRISM) for optimizing excitation of genetically encoded calcium indicator, GCaMP, at 920 nm. The GRISM offered an ~50% throughput, and experimentally exhibited ~3 dB improvement on imaging SNR, compared with our previous method. In order to achieve compatibility with GRIN lens implants for deep brain imaging, we have completely redesigned our current (type I) fiberscope. The new fiberscope (type II) was based on the concept of composite fiber cantilever. By paring the composite fiber cantilever with a half-pitch GRIN lens as an objective, the type II fiberscope achieved a much larger 350-μm FOV with a moderate loss of resolution, at the same time being compatible with GRIN lens implants for imaging deep neurons. The software and hardware upgrades made to the imaging system with respect to previous work are also detailed in this chapter.
2.1 Two-photon Fiberscope System

Figure 3. Two-photon fiberscope platform for brain imaging on freely rotating/walking rodents. (A) System schematic including the OEC and GRISM pre-chirper. PM-SMF: Polarization Maintaining Single-mode Fiber; FC: Fiber Collimator; M: Mirror; RM: Roof Mirror; FL: Fiber Launcher; SMF: Single-mode Fiber; KM: Kinematic Mount; DM: Dichroic Mirror; L: Lens; F: Filter; PMT: Photomultiplier Tube; MO: Motor; B: Bearing; BP: Belt and Pulley; SR: Slip Ring; E: Encoder. (B) A representative image of firing neurons acquired by the system on a freely behaving mouse (enhanced by a 10-frame moving average). (C) Photo of the fiberscope attached to the head of a freely behaving mouse expressing GCaMP6m in the primary motor cortex. Inset: photo of a stand-alone two-photon fiberscope.

Figure 3A illustrates the schematic of our two-photon fiberscope system, including the OEC and GRISM pre-chirper. The OEC maintained an excellent fiber core-to-core coupling over rotation, allowing the fiberscope to operate at any angle of rotation. The GRISM pre-chirper enabled optimized delivery of femtosecond pulses to tissue over fiber, resulting in more efficient fluorescence generation and less needed incident power. In our system, the excitation laser pulses
reached tissue through three optical fibers: A Ti:sapphire laser beam (920nm, 120 fs, shown in red) was first coupled into a polarization-maintaining single-mode fiber (PM-SMF, Fiber 1) for spectral broadening, and was then fed to the GRISM pre-chirper and coupled back into a second single-mode fiber (SMF, Fiber 2). Fiber 2 delivered the laser pulses to the OEC, where the pulses were relayed to the DCF core of the fiberscope for imaging. The overall power transmission of the entire system (from input to the distal end of the fiberscope) was measured to be 10%. The fluorescence photons (shown in green) collected by the fiberscope from tissue were first delivered to the OEC through the inner cladding (and the core) of the DCF, and were then steered off-axis with a dichroic mirror (DM) to a photomultiplier tube (PMT) for detection.

The fiberscope had its proximal end connected to the OEC. Two types of fiberscopes were used. Type I fiberscope had a similar design to our previous work\textsuperscript{33}, with a piezoelectrically actuated resonant fiber-optic scanner and a miniature compound objective housed inside a hypodermic tube of a 2.8 mm diameter. The type I fiberscope offered a 0.9 μm×5 μm resolution and 140 μm-wide field of view (FOV), making it suitable for high-resolution imaging of dendritic processes. The type II fiberscope (detailed in section 2.5) was based on our recently developed composite cantilever design\textsuperscript{39}, by reducing and redistribution of the system magnification, a >300 μm FOV was achieved with moderate loss of resolution (1.1 μm×14 μm). The type II probe was used for imaging neuron populations across a larger FOV. Both type I and II fiberscopes weighed 0.6g. And they both offer a sufficient working distance (300 μm for type I, 450 μm for type II) for imaging access to cortical layers 1-2 (and possibly partial layer 3) through a chronical glass imaging window, reducing the invasiveness to the brain. Figure 3B shows a representative image of neurons acquired by the system with type I fiberscope on a freely behaving mouse expressing GCaMP6m in the area M1 through viral infection.
2.2 Optoelectrical commutator (OEC) with active rotational tracking

2.2.1 Design considerations

Ideally, an optoelectrical commutator (OEC) should allow the entire fiberscope to be freely rotatable while maintaining steady optical and electrical connections to the base instrument. Steady electrical connections over rotation can be readily accomplished by using slip rings that are commercially available. However, for steady optical connection, the off-the-shelf fiber-optic rotary joints (single mode) typically offer 10%~20% of throughput variation (Table 1) at the excitation wavelength over rotation. This is not acceptable for two-photon imaging as its fluorescence signal has a quadratic dependence on the incident power. Assuming our target rotation-induced fluorescence fluctuation is less than 10%, the excitation throughput variation shall be maintained less than 3% over rotation. Thus, a fiber-optic rotary joint optimized for two-photon imaging with a minimum (<3%) coupling fluctuation is needed. Another consideration is that the integration of an electrical slip ring and a fiber-optic rotary joint will inevitably result in a bulky construction of the commutator with high inertia. To minimize the restraining force exerted to the animal by the commutator, we adopted a sensitive active rotational tracking mechanism.

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<th>Model</th>
<th>One-way Loss (dB)</th>
<th>Pk-Pk Variation (dB)</th>
<th>Pk-Pk Variation (%)</th>
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<td>0.5</td>
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</tr>
</tbody>
</table>

Table 1. Survey of off-the-shelf single mode fiber-optic rotary joints
2.2.2 Construction and alignment of OEC

Figure 4. OEC schematic. FC: Fiber Collimator; SMF: Single-mode Fiber; KM: Kinematic Mount; DM: Dichroic Mirror; L: Lens; F: Filter; PMT: Photomultiplier Tube; MO: Motor; B: Bearing; BP: Belt and Pulley; SR: Slip Ring; E: Encoder.

Figure 4 shows the schematic of the OEC. Similar to our previous work of a fiber-optic rotary joint for endoscopic optical coherence tomography (OCT), the optical connection over rotation was realized by a pair of fiber-optic collimators (FC), with one mounted stationary and the other mounted on a rotary shaft. On the rotary side, its FC was connected to the rotary shaft with a kinematic mount (KM) for precision alignment of its optical axis to the rotational axis of the shaft. The stationary FC was connected to the baseplate with another KM for colinear alignment between the two FCs. Once the two FCs and the shaft were aligned, the excitation beam from the input fiber (shown in red) could be coupled into the single-mode core of the DCF with minimum power.
fluctuation over rotation. The fluorescence photons (shown in green) collected by the fiberscope was delivered by the outer cladding (and the core) of the DCF, then collimated by FC and steered off-axis with a dichroic mirror (DM) to a photomultiplier tube (PMT) for detection. Different coatings were chosen for the stationary FC (F240APC-850, Thorlabs) and the rotary FC (CFC-8X-A, Thorlabs) to maximize the transmission of the excitation beam (920 nm) and fluorescence photons (~530 nm), respectively.

Mechanically, the OEC was constructed based upon a rotary shaft and two bearings. A tolerance study was performed to guide the selection and configuration of two bearings. The optical coupling efficiency of the FC pair at 920 nm was calculated using ZEMAX as a function of angular and lateral misalignment between the two FCs (Figure 6A), with their spacing set to 100 mm. We concluded that to keep the coupling fluctuation below ±3%, the angular and lateral alignment tolerance over rotation should be kept below ±3.35 mdeg and ±128.4 microns, respectively. This would require two bearings of ABEC-1 specifications (7.5 μm, ISO 492, normal class 6×) to be placed at least 110 mm apart. And in our OEC, two ball bearings (2722T31, McMaster) were placed 127 mm apart and were connected by a hollow-core shaft of a 3/4-inch diameter.

For electrical connections between the fiberscope (rotational) and the base instrument (stationary), a hollow-shaft slip ring (SR) was introduced between the two bearings (B), and the electrical drive wires of the fiberscope went through the core of the shaft, then they were connected to the base instrument via the SR (525-1200, Orbex Group).

We employed a two-step procedure for optical alignment of the FC pair. First, the optical axis of the rotational FC was aligned with the rotary axis of the shaft by adjusting its KM. Specifically, a white light was coupled into the cladding of the DCF from the distal end of the fiberscope, and the collimated white light from the rotational FC was monitored ~0.5 m away with a CCD camera. By rotating the shaft and adjusting the KM, the optical axis of the rotational FC could be aligned to the rotary axis of the shaft with high precision. Next, with the excitation laser
beam turned on and power throughput monitored from the distal end of fibrescope through a pinhole, the kinematic mount of the stationary FC was adjusted with back-and-forth rotations of the shaft, until a maximum steady power throughput was achieved over rotation.

2.2.3 Feedback loop and electronics

The active tracking and following functionality of the OEC was realized by a rotary encoder (E) and a stepper motor (MO) mechanically driving the shaft with a belt and pulley (BP). Figure 5 shows the feedback loop for the active tracking functionality. Once a tiny twist (angular displacement) was detected by the rotary encoder (CUI, AMT103), it sends an electrical pulse to a
microcontroller (Arduino Mega 2560) via the slip ring (Orbex Group, 525-1200). The microcontroller is constantly monitoring the pulsed signal from the encoder induced by angular displacement. Once the number of pulses (angular displacement) passes a certain threshold, the microcontroller then drives the motor (Jinwen, 120027) via the stepper driver (Trinamic, TMCM-1070), which eventually rotates the entire OEC accordingly and releases the torsion in the fiber. Empirically, the threshold was set to 20 pulses which corresponds to 0.88 degrees of tracking sensitivity (at the encoder resolution of 8192 pulses/revolution).

The minimum torque required to activate the tracking mechanism was measured by mounting the fiberscope vertically on a bearing. By winding a thin copper wire on the bearing and pulling the wire with suspended weights through a pulley, a minimum weight of 48 g was recorded to activate the tracking mechanism. By multiplying the force with the radius of the bearing (15.5 mm), the minimum required activation torque was found to be 7.29 mN·m. To overcome the torque, a mouse would only need to generate 12.4 g of force on its front or rear limbs (assuming a 6 cm body length), which is much below the 50 g force an adult mouse can typically generate from its forelimbs. Thus, this <8mN·m activation torque of the OEC corresponded to a sufficiently small torque the mouse needed to overcome.
2.2.4 OEC performance

Figure 6. OEC performance. (A) Tolerance study of the OEC. For maintaining the throughput fluctuation of the excitation light lower than 3%, the lateral and angular misalignment should be kept within 128.4 μm and 3.35 mdeg, respectively. (B) The measured normalized throughput fluctuation over 360 degrees of rotation. Results showed the throughput variation of the OEC was below 1%. (C) Mouse heading orientation vs. OEC tracking angle. The mouse made more than 3 full rotations within 100 seconds and the OEC was able to track the rotation with a 19.3° mean tracking error.

The laser power throughput of the OEC (from the exit of Fiber 2 to the distal end of the fiberscope) was measured to be 57%, with its relative fluctuation over rotation kept below ±1% (Figure 6B). This throughput fluctuation would translate to a maximum of 3.2% rotation-induced change in the
measured ΔF/F during two-photon neuroimaging, which is considered negligible for neuronal calcium dynamics under TPM (typically with ΔF/F > 100%). The active tracking mechanism was experimentally tested on a freely behaving mouse. Figure 6C shows the mouse heading angle curve inferred from behavioral camera footage as well as the simultaneously recorded OEC angle. Over the course of 100 seconds, the mouse made more than three full turns and the OEC closely followed the turning motion of the mouse (Figure 6C) with a 19.3°+/−15.7° (mean+/− SD) tracking error. Thus, our OEC can efficiently sustain steady optical and electrical connections of the fiberscope to the base instrument while timely compensating the torque generated by the rotational movement of the animal.

2.3 GRISM-based temporal pulse management and in vivo fluorescence enhancement

2.3.1 Motivation

Fiber delivery of femtosecond laser pulses from a Ti:sapphire laser has been a challenge. Material dispersion and nonlinear effects in optical fibers require careful management, which would otherwise lead to severe temporal broadening of the laser pulses and thus dramatically reduce the fluorescence yield for two-photon microscopy. Material dispersion alone of a 1-meter single-mode fiber (27000 fs² at 920 nm) is already beyond the capabilities of most built-in dispersion management units of Ti:sapphire lasers, and therefore customized solutions are needed. Our previous grating-based method worked well in counteracting the nonlinearity and group velocity dispersion (GVD) of the optical fibers. Still, the residual third-order dispersion (TOD) was unaccounted for, which introduced side lobes in the pulse profile and thus led to a reduced peak power as well as fluorescence yield. A GRISM is a device well-known for simultaneous compensation of both material GVD and TOD, and a reflection-type GRISM had been adopted in two-photon microscopy at 800 nm. However, the reflection-type GRISM presented limited
wavelength tunability and sub-optimal power throughput, which hindered its application in neuroimaging at 920 nm. Neuroimaging at 920 nm calls for a new GRISM design that can provide a good tenability and transmission throughput. Here we will adopt transmission-type gratings into our GRISM design.

2.3.2 Theory of transmission-type GRISM

Figure 7. Schematic of a transmission-type GRISM. The beam was launched from O and ended at M. A and A’ are both midpoints of the gratings, $\overline{AO} = \overline{A'O'}$ and $\overline{AO}/\overline{A'O'}$. MO’ is perpendicular to the beam DM.

Figure 7 shows the schematic of a transmission-type GRISM consists of a pair of 45° right-angle prisms with refractive index of n, sandwiched between two transmission-type gratings (with grating
periodicity $\delta$). The side dimension of the prism is $l$, and they are spaced at distance $d$. The -1 order (optimized for maximum efficiency) of the diffraction grating is used. A beam of wavelength $\lambda$ (in air or vacuum) is launched from O to M. $\overline{AO}$ is set to a constant distance $c$. Based on the grating equation and Snell’s law, we can have:

$$ r = \sin^{-1}\left(\frac{\lambda \sin i}{n}\right), \quad \text{Eq. 1} $$

$$ \beta = \sin^{-1}\left(n \sin\left(\frac{\pi}{4} - r\right)\right). \quad \text{Eq. 2} $$

And based on the geometry we can write the length of each beam segment as a function of $r/\beta$ and $d$:

$$ \overline{BC} = \frac{d}{\cos \beta}, \quad \text{Eq. 3} $$

$$ \overline{AB} = -\frac{\frac{1}{2} \tan r}{\sin r(1+\tan r)} = \frac{l}{2} \frac{1}{\sin r \cos r}, \quad \text{Eq. 4} $$

$$ \overline{CK} = \sqrt{2}l \left(1 - \frac{\frac{1}{2}}{1+\tan r}\right) - d(\tan r - 1), \quad \text{Eq. 5} $$

$$ \overline{CD} = \frac{\overline{CK}}{\sqrt{2} \cos r}, \quad \text{Eq. 6} $$

$$ \Delta y = \frac{\overline{CK}(1+\tan r)}{\sqrt{2}} - l/2, \quad \text{Eq. 7} $$

$$ \overline{DM} = \frac{c(\frac{c}{\sin i} + \Delta y)}{\frac{c}{\sin i}} = c + \Delta y \sin i. \quad \text{Eq. 8} $$

And the single-pass optical path length (OPL) from O to M, adding a grating phase correction term, $-\Delta y \frac{\lambda}{\delta}$ (for maintaining the flat wavefront for the collimated beams at input/output $^{82,83}$) is:

$$ OPL(\lambda) = c + n\overline{AB} + \overline{BC} + n\overline{CD} + \overline{DM} - \Delta y \frac{\lambda}{\delta}. \quad \text{Eq. 9} $$
The single-path optical phase delay is:

$$\phi(\omega) = 2\pi \frac{OPL(\omega)}{\lambda} = \frac{OPL(\omega)\omega}{c}. \quad \text{Eq. 10}$$

The total single-pass 2\textsuperscript{nd} and 3\textsuperscript{rd} order dispersion of GRISM is defined as $D_2 = \frac{d^2\phi}{d\omega^2}$, $D_3 = \frac{d^3\phi}{d\omega^3}$. And based on Eq. 1-10, the total dispersion of GRISM as function of incident angle $i$ and spacing $d$ can be calculated analytically.

2.3.3 Using ZEMAX for GRISM design

![A realistic GRISM Layout in ZEMAX](image)

**Merit Function Editor**

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<th>Hy</th>
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</tbody>
</table>

**Figure 8. Using ZEMAX for GRISM design.** (Upper) A realist GRISM layout in ZEMAX. (Lower) Merit function editor showing two target value as well as the calculated optical path length (OPL)
Note the derivation in 2.3.2 incorporated an ideal thin transmission-type grating with zero thickness and was derived based on right-angle prisms. In practice, transmission gratings have finite thicknesses, and prisms can have arbitrary apical angles, which would render our derivations inaccurate. A better design approach is to build the GRISM model inside commercial ray tracing software such as ZEMAX, and numerically compute its dispersion parameters. Here, ZEMAX OpticStudio 18 was used for this GRISM design (Figure 8). Once the GRISM model was created, its total OPL at the current wavelength could be easily calculated using the PELN operand in the merit function editor. Based on the OPL, a customized ZPL script (see Appendix A) was created to convert the OPL to dispersion parameters using finite difference. Here the target dispersion parameters were the total 2\textsuperscript{nd} order dispersion (single pass) of the GRISM, as well as the ratio between its 2\textsuperscript{nd} and 3\textsuperscript{rd} order dispersion. To compensate for an optical fiber total of 3.5 m long at 920 nm, the GRISM needs to generate \(-47000\) fs\(^2\) total dispersion at a ratio \(D_3/D_2\) of 1.223 fs. These target values were then input into the merit function editor in ZEMAX, and with the beam incident angle and GRISM separation set as variables, the built-in ZEMAX optimization could begin. Once the optimizer converges, the best combination of beam incident angle and GRISM separation could be figured out for a particular GRISM design. With this method, different prisms as well as gratings could be tried out, and the combination with the best transmission could be selected for use.
2.3.4 GRISM pre-chirper construction

Figure 9. Transmission-type GRISM pre-chirper. PM-SMF: Polarization Maintaining Single-mode Fiber; FC: Fiber Collimator; M: Mirror; RM: Roof Mirror; FL: Fiber Launcher.

Using the abovementioned method, we arrived at the design shown in inset of Figure 9. It consisted of a pair of transmission gratings (900 l/mm @ 930 nm, 25.4 mm dia. Wasatch Photonics) sandwiching a pair of N-SF11 right-angle prisms (#45-950, Edmund Optics). The higher index provided by the N-SF11 glass led to a larger incident angle on the grating. This ensured the
GRISM’s desired incident angle for dispersion compensation is near its angle of maximum power transmission, allowing a 46% experimental power throughput (double pass).

By design, the GRISM can tune its GVD by varying its separation $d$ and tune the TOD/GVD ratio by changing the beam incident angle $i$ (see Figure 7). To compensate for both the GVD and TOD of optical fibers at 920 nm (with a ratio TOD/GVD $\sim$1.22 fs), the incident angle of GRISM should be set to 21.2° so that it can provide a ratio TOD/GVD of 1.22 fs to compensate the fiber dispersion. At the 21.2° incident angle, all the refractions at air-prism interfaces are close to Brewster’s angle for p-polarized light, attributing to only about 13% Fresnel reflection loss for a single pass through the GRISM. The 21.2° incident angle is also close to the grating’s maximum diffraction angle at 24° (90% efficiency), further contributing to a better efficiency of our GRISM. The theoretical double-pass power transmission of the GRISM was predicted to be $(0.87 \times 0.92)^2 \approx 50\%$, which is close to our 46% experimental measurement.

The grating and prism were held together with custom-machined mounts. The tunability of the GRISM separation was provided by a pair of linear translational stage with each half of the GRISM attached. The entire GRISM was mounted on a rotary stage for precise adjustment of the beam incident angle. The angle and separation of the GRISM were optimized via imaging test of a green fluorescent reference slide (2273, Ted Pella Inc.), during which the fluorescence signal was maximized by fine adjustment of both the angle and separation of the GRISM.

In the entire imaging system, the pulses were delivered through a total of 3 meters of optical fibers, including a 0.5-meter PM-SMF (Fiber 1, pre-compensation), a 1.5-meter SMF (Fiber 2, delivery) and a 1-meter DCF in the fiberscope (Fiber 3, delivery). With its angle and separation optimized (with $d=\sim$1.5 mm), the GRISM enabled the delivery of 76 fs pulses to tissue over 3 meters of fiber at 920 nm.
GRISM performance characterization

Figure 10. Temporal pulse characteristics and *in vivo* fluorescence enhancement using GRISM. (A) Autocorrelation traces of pulses delivered to the tip of the fiberscope using GRISM (blue, 76 fs) and grating pair (black, 111 fs). (B) Signal comparison between grating pair and GRISM when imaging a reference fluorescence slide using the same excitation power (11 mW), results showed an ~2X enhancement when using GRISM. (C) Raw fluorescence traces of 2 neurons imaged using GRISM and grating pair for pulse delivery. (D) Peak signal comparison of 12 neurons imaged using grating and GRISM under the same incident power. The mean peak signal of the 12 neurons showed a >2X enhancement when using GRISM.
For comparison purpose, a grating pair-based pre-chirper was step up alongside with the GRISM and was designed to share the same Fibers 1 and 2 with the GRISM. For pulse width measurement, the excitation beam from the distal end of fibrescope was first collimated, and then directed to a home-built interferometric autocorrelator. The interferometric autocorrelation trace showed a 118 fs/170 fs FWHM for GRISM/grating (Figure 10A), which corresponded to 76 fs/111 fs for the temporal pulse width (assuming a $sech^2$ shape). And when imaging a fluorescence reference slide under the same excitation power (11 mW), the GRISM-based method showed an ~2X increase in imaging signal as compared with the grating pair-based method (Figure 10B).

The *in vivo* signal comparison between GRISM and grating was performed by imaging a head-restraint GCaMP6m mouse over the same FOV with the same average power (16.5 mW) with a type II fibrescope, but different pulse delivery schemes. Specifically, the imaging FOV on the head-restraint was first identified with the system using GRISM, then the spontaneous neural activity in the FOV was recorded for ~5 minutes (1000 frames). Next, the system was quickly switched to the grating pair without touching the fibrescope, and with the power adjusted to the same level, another 1000 frames were saved recording the spontaneous activities with the grating pair. In the 2000-frame dataset, 12 regions of interest (ROIs) containing individual neurons were identified and the raw intensities in the ROIs were averaged to get the raw fluorescence signals from the neurons, with 2 representatives traces shown in Figure 10C. Quantitative analysis of the data from the 12 neurons showed that the GRISM improved their peak intensities by >2X on average (Figure 10D), which is consistent with the fluorescence slide result. This also meant reduced excitation power needed for *in vivo* imaging (from 40-80 mW down to 20-40 mW) as well as the reduced chance of photodamage during *in vivo* experiments.
2.4 Type I Fiberscope

Figure 11. Type I fiberscope. (A) Schematic of the Type I fiberscope showing the scanner fiber cantilever and the micro-objective. (B) The piezoelectric scanner and its spiral scanning pattern.

Figure 11 shows the type I fiberscope based on our legacy design detailed elsewhere. It featured a piezoelectric fiber scanner and a high-NA micro-objective. The piezoelectric fiber scanner consisted of a piezoelectric tube (PZT) with a double-clad fiber (DCF) glued in the center. The DCF can be driven as a cantilever by applying sinusoidal voltages matching the resonance frequency of the cantilever. By controlling the relative phase of the driving voltage applied to the X and Y electrodes pairs of the PZT, the DCF tip could be driven in a spiral pattern (Figure 11B).

The micro-objective consisted of a plano-convex lens and two GRIN lenses sandwiching a diffractive phase mask. The GRIN lens close to the fiber was of 0.2NA and ~1/4 pitch, and the
GRIN lens close to the sample was of 0.5NA and <1/4 pitch. Another plano-convex lens was placed in between the GRIN lens and the sample, contributing the 0.8NA of the objective. The diffractive mask was introduced for counteracting the chromatic focal shift of the GRIN lens, enabling an achromatic design. Here, a scaled-up version of the objective detailed in previous work was used for type I fiberscope, which allowed a 300-μm working distance (WD) and 140-μm field of view (FOV). The imaging resolution of type I fiberscope was 0.9 μm × 5.0 μm (lateral × axial). Paired with OEC and GRISM, our type I fiberscope was capable of resolving dendritic spines on freely walking mice (see Section 3.5).

2.5 Type II Fiberscope

2.5.1 Motivation

The abovementioned type I fiberscope performed well in high-resolution imaging applications. However, its application in imaging neuron populations was limited due to its small FOV (140 μm), which only allowed imaging 20-30 neurons at a time. In addition, the objective of type I fiberscope was incompatible with implantable GRIN lens, excluding its application in deep brain imaging in areas such as hippocampus, entorhinal cortex, thalamus, hypothalamus, and medical prefrontal cortex (mPFC). For these reasons, we have completely re-designed our fiberscope, and came up with our new type-II fiberscope. Our type II fiberscope was compatible with GRIN lens implants, at the same time providing a much larger imaging FOV (350 μm), greatly extended the applications of our fiberscope in both deep brain imaging and population-wise neuroimaging.

2.5.2 GRIN implant lens compatibility

Prior study suggested that due to the much shorter wavelength of fluorescence (530 nm) compared to excitation (920 nm), the back-traced fluorescence beam would come to a premature focus before the fiber scanning plane due to chromatic focal shift of the objective. This would degrade the fluorescence collection efficiency, hence the SNR of the fiberscope. This is one of the
major reasons that a phase diffractive grating was introduced in the objective of type I fiberscope to counteract the effect. The type I fiberscope worked well stand alone, however, it cannot image through a GRIN implant lens due to the magnified focal shift of the GRIN implant lens.

Consider a GRIN lens \( L_2 \), half pitch was put in front of a fiberscope objective \( L_1 \) (ideal lens) as shown below. The focal length of \( L_2 \) at 530 nm is shorter by \( \Delta f \) as compared to its focal length at 920 nm \( (f'_2 = f_2 - \Delta f, \Delta f \) is small).

![Diagram](image)

**Figure 12. Fiberscope imaging through a lens with chromatic focal shift.**

Here we define the magnifications \( M_1 \) and \( M_2 \) of \( L_1 \) and \( L_2 \) as:

\[
M_1 = \left| \frac{v_1}{u_1} \right|, \quad \text{Eq. 11}
\]

\[
M_2 = \left| \frac{v_2}{u_2} \right| = 1. \quad \text{Eq. 12}
\]
The unit magnification of $L_2$ is assumed because the GRIN implant lens is of 0.5 pitch, serving as a pure imaging relay. At fluorescence wavelength (530 nm), using the lens equation and apply the 1st order Taylor expansion:

$$u'_2 = \frac{f_2v_2}{v_2-f_2} = \frac{2f_2(f_2-\Delta f)}{f_2+\Delta f} \approx u_2 - 4\Delta f,$$

Eq. 13

$$v'_1 = (v_1 + u_2) - u'_2 = v_1 + 4\Delta f,$$

Eq. 14

$$u'_1 = \frac{f_1v'_1}{v'_1-f_1} = \frac{u_1\frac{M_2}{M_1+1}(M_1u_1+4\Delta f)}{M_2u_1-u_1\frac{M_1}{M_1+1}-4\Delta f} \approx u_1 - \frac{4\Delta f}{M_1^2}.$$

Eq. 15

As a result, the chromatic focal shift of GRIN lens $L_2$ ($4\Delta f$) is magnified by $\frac{1}{M_1^2}$ times by the ideal objective $L_1$. And in reality, the chromatic focal shift of objective $L_1$ will be further added to Eq. 15, although being a small contribution compared to the $4\Delta f / M_1^2$ term.

1-mm Half-pitch GRIN lens

Excitation: 920 nm
Fluorescence: 530 nm
Focal Shift: 90μm

Figure 13. Chromatic focal shift of a GRIN implant lens

As shown in Figure 13, a realistic 1-mm half-pitch GRIN implant lens for deep brain imaging have 90 μm of focal shift ($4\Delta f$). If put in front of the type I objective ($M_1 = \frac{1}{4.8}$), it will
lead to a focal shift at the fiber scanning plane of 2076.6 μm. This diminishes any fluorescence signal, making it impossible for the type I fiberscope to image through GRIN implant lenses.

2.5.3 Type II fiberscope design

To enable neuroimaging through GRIN implant lens, the type I fiberscope needs to be redesigned. And since the chromatic focal shift of the GRIN implant lens will be magnified by the fiberscope objective, an objective of unity magnification is preferred. For the new type II fiberscope design, we have replaced the objective with a GRIN rod lens of 0.5 pitch. However, this approach inevitably would reduce both the excitation and collection NA, sacrificing both resolution and sensitivity of the fiberscope. And to mitigate the effect, the composite fiber cantilever\textsuperscript{19} was introduced. This would allow GRIN implant lens compatibility, as well as a much bigger imaging FOV thanks to the much-reduced magnification factor.

Figure 14. Schematic of the type II fiberscope. The fiberscope consisted of a composite fiber cantilever scanner and a GRIN objective (GL2), and it is coupled to the GRIN implant lens via microscopy oil. The composite fiber cantilever was constructed from a double-clad fiber (DCF), a short piece of coreless fiber
(CF) and a small GRIN lens (GL1). Inset: Excitation light path of the fiberscope. PZT: Piezoelectric Tube; DCF: Double-clad Fiber; CF: Coreless Fiber; GL: GRIN Lens.

Figure 14 illustrates the schematic of the type II fiberscope imaging through an implanted GRIN lens (1 mm). Type-B microscopy oil was used to couple the fiberscope to the GRIN implant for better transmission of both excitation and fluorescence. The type II fiberscope was consisted of a composite fiber cantilever scanner and a GRIN objective (GL2, NEM-050-25-10-860-S-0.5p, GRINTech). The composite fiber cantilever was constructed upon a short (1.25 mm, maximum length allowed before beam clipping in GL1) piece of coreless fiber (CF, FG250LA, Thorlabs) spliced to the double-clad fiber (DCF), with a small GRIN lens (GL1, GT-IFRL-035-005-50-CC, GRINTech) UV-epoxied to the tip of the CF. The composite fiber cantilever was glued to a piezoelectric tube (PZT) so that the cantilever could be driven in a spiral scanning pattern. Excitation light from the core of the DCF (NA=0.13) propagates through the CF, gets focused by GL1, then gets relayed by GL2 to imaging plane I (NA=0.33), where superficial cortical neurons can be imaged directly. For deep neuron imaging, beam at imaging plane I gets relayed again by the GRIN implant and re-focused to imaging plane II. This fiberscope design reduces and moves the system magnification to the scanning fiber cantilever, enabling the compatibility with the implanted GRIN lens for deep brain imaging, at the same time allowing much larger imaging FOV. The entire fiberscope was housed and protected inside a gauge-11 hypodermic tube of 0.12” (3 mm dia.) with 3D-printed support structures (shown in green).
2.5.4 Type II fiberscope construction

![Figure 15. Customized assembly tooling for type II fiberscope.](image)

(A) Photo of a customized alignment station with 8 degrees of freedom and inspection cameras. (B) Customized spring-loaded holding tool for the 350 μm GRIN lens of the composite fiber cantilever.

The construction of the type II fiberscope started by making of the composite fiber cantilever. Firstly, our double-clad fiber (DCF) was stripped and cleaved. With the cleaved facet inspected under a microscope, the DCF was then spliced to a piece of coreless fiber (CF, FG250LA, Thorlabs) of the same 250-μm diameter. Next, the spliced fibers (DCF+CF) were loaded again on the fiber cleaver and cleaved at 1.25 mm away from the splice point on the coreless fiber, leaving the DCF end-capped by a short piece of coreless fiber (1.25 mm long). The end capped DCF was then loaded on the 8D alignment station shown in Figure 15A, with another piece of GRIN lens (GL1, 350 μm in diameter) mounted on the other side on a customized spring-loaded holding tool (Figure 15B). The end capped DCF and the GRIN lens were aligned actively under inspection cameras. Once the
alignment was optimized, UV epoxy was applied between the two parts and a UV lamp was use for curing of the epoxy. Finally, the finished composite fiber tip was then glued to a piezoelectric tube (PZT) for later assembly.

Once a PZT fiber scanner with the composite cantilever was completed, it was assembled with a gaue-11 hypodermic housing on the same alignment station (Figure 15A). After the housing was aligned and glued to the scanner, the GRIN objective (GL2) with the 3D-printed housing was assembled to the hypodermic housing using the same procedure, with the spacing between the GL1 and GL2 controlled precisely at 80 μm under cameras.
2.5.5 Type II fiberscope performance characterizations

![Image of graphs and images showing resolution and FOV of the type II fiberscope.](image)

**Figure 16. Resolution and FOV of the type II fiberscope.** (A) Type II fiberscope imaging resolution measurement results measured both in imaging plane I (blue) and II (black). (B) Field of view (FOV) validation using the fiberscope imaging cortical V1 neurons, with the type II fiberscope enabling 350 μm FOV as compared to the type I fiberscope (140 μm).

The imaging resolution of the type II fiberscope was measured using 0.2 μm sub-resolution fluorescence beads in both imaging plane I (for superficial cortex imaging, Figure 14) and II (for
deep brain imaging, Figure 14). Lateral resolution was measured directly via imaging individual fluorescence beads, with the upper plot of Figure 16A showing the measurement results at both imaging planes (average of 3 measurements). Both planes yielded ~1.1 μm lateral imaging resolution. Axial resolution was measured by physically translating the fluorescence beads sample axially using a translational stage at 2 μm intervals. The lower plot of the Figure 16A shows the axial resolution measurement results in both planes, with imaging plane I yielded a 14.4 μm axial resolution, and plane II yielded a slightly degraded 15.3 μm resolution due to added aberration in the GRIN implant lens.

For the sake of FOV comparison with the fiberscope of previous generation, neurons of visual cortex (V1) were imaged using two generations of fiberscopes. Figure 16B shows the representative frames of firing V1 neurons acquired by the two fiberscopes, the current fiberscope was able to image a FOV of 350 μm, increasing the imaging area by 6.25X compared to the previous 140-μm FOV.
2.5.6 Comparison with single-photon wide-field microscopy in imaging deep neurons

Figure 17. Fluorescence signal of type II fiberscope compared to wide-field microscopy in deep neuron imaging. (A) Fluorescence signal comparison between single-photon (1P) wide-filed fluorescence microscope and the two-photon (2P) fiberscope. With the same region of interest (ROI) in mPFC of the same mouse identified in both modalities, the fluorescence (ΔF/F) of 3 representative neurons plus one background ROI were plotted for both modalities. Scale bar: 10 μm. (B) Scatter plot of peak ΔF/F of 21 neurons identified under both imaging modalities, with a linear fitting showing a 9.2X improvement in ΔF/F of our 2P fiberscope on average compared to the 1P counterpart. [Note: the dot-array appearance in the 2P image in (A) was due to resampling artifact when rotating this image.]
The calcium dynamics of medial prefrontal cortex (mPFC) neurons in a head-restraint mouse were imaged through its implanted GRIN lens using a home-built single-photon (1P) wide-field fluorescence microscope and our two-photon (2P) type II fiberscope consecutively. The imaging results were compared and ROIs containing the same neuron were identified. Figure 17A shows ROIs from two imaging modalities with identical neurons. The raw ΔF/F of the three representative neurons and a background region were derived and plotted by dividing the raw signal by its corresponding background (F). The background (F) of each trace was computed by sorting the raw values and finding the value at the lowest 20%. Compared to 1P results, the 2P ΔF/F showed much cleaner background and a factor of ~10X higher in intensity. A total of 21 neurons were identified using the two modalities, and their peak ΔF/F were plotted in Figure 17B with x-axis and y-axis showing their respective 1P and 2P peak ΔF/F values. A linear fitting was performed on the data, and the slope of the fitting showed an average of 9.2X improvement in ΔF/F of our 2P fiberscope compared to the 1P counterpart, consistent to previous finding in cortical V1 neurons.32
2.6 Two-photon fiberscope system implementation details

2.6.1 Control system architecture

![Control system architecture of fiberscope imaging system](image)

*Figure 18. Control system architecture of fiberscope imaging system.* Blue arrow shows digital signal routes and green arrow shows analog signal routes. MCU: Microcontroller Unit; PMT: Photomultiplier Unit; HV: High Voltage; PreAmp: Pre-amplifier; PZT: Piezoelectric Tube; DAQ: Data Acquisition Card; Coax: Coaxial Cable; USB: Universal Serial Bus; PC: Personal Computer.

Figure 18 shows the control system architecture of the fiberscope imaging system. The entire system is controlled by a custom software running on a PC. The fiberscope imaging started by the software controlling the DAQ card generating the X and Y scanning waveforms for the PZT scanner. Next, the DAQ waveforms were amplified by the PZT driver and finally fed through the slip ring to reach the PZT inside the fiberscope. The time-varying fluorescent photons were detected by the stationary PMT mounted on the breadboard of OEC, then converted to electrical current. The signal current from the PMT were fed to the pre-amplifier (PreAmp) then converted to voltage signals. The pre-amplifier included a transimpedance amplifier for current-to-voltage conversion.
and a 4th order Butterworth low pass filter. The voltage signal from the pre-amplifier were sent to the analog input port for the DAQ card for digitization. The digitized voltage signals were then given spatial assignments according to the scanning waveform and painted on the computer screen by the control software for real time image display.

The OEC had its own feedback-controlled loop. The twist on the fiber was firstly sensed by the rotary encoder and sent to the MCU as digital voltage pulses. The MCU had a custom firmware running, which monitors the pulses coming in from the encoder. Once the number of pulses reached a preset threshold, pulses were sent to the motor driver which rotated the motor in the same direction to reverse the twist in the fiber. This reverse of the twist was also sense by the encoder and then the MCU, which stopped the motor once the twist was released. In this way, the fiber twist can be tracked and followed in real time mechanically.

The control software communicated to the MCU via USB connection using the ‘serial over USB’ protocol, which parsed the real time angular data send from the MCU and displayed it on the screen as well as saved them to the metadata of the imaging frames while performing fiberscope imaging. The tracking function of the OEC could also be enabled/disabled form the software by sending control strings to the MCU using a customized protocol. The behavior camera was also connected to the PC via USB connection. The control software had built-in support for the behavior camera, which streamed the video from the camera, displayed it on the screen and saved the video to disk in synchronization with the saving of the fiberscope imaging data.

Both the MCU and the behavior camera were powered by the 5V USB power from the PC. The motor driver was powered by a 12VDC power supply. The PMT was powered by a custom high-voltage (HV) supply that can generate variable high voltage between -300V to -1000V. The HV supply was powered by another stand-alone 12VDC power supply to avoid noise coupling from the motor. The pre-amplifier was powered by pair of rechargeable batteries which provided ±7V when fully charged. The PZT driver was powered by a pair of DC power supply that generated ±75V.
2.6.2 OEC MCU firmware and communication protocol

Here an Arduino Mega 2560 microcontroller was used as the MCU for controlling the OEC tracking functionality. Figure 19 shows the program flow chart of the firmware running on the MCU. The source code of the firmware is available in Appendix B.

Figure 19. Program flow chart of the MCU firmware controlling the OEC. OPs: Operations.
The closed-loop control was implemented with interrupt service routine (ISR) that was evoked every 1 millisecond by a hardware timer. As shown in the left flow chart of Figure 19, the ISR started by reading the encoder angle, and then compare it with a pre-set threshold. If the encoder angle exceeded the threshold, the motor driver signal would be generated to rotate the OEC. And once the encoder angle was reduced below the threshold, no motor driving signal would be generated and the OEC would stay still.

Table 2. List of command strings to control the OEC.

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<tr>
<th>Command</th>
<th>Function</th>
<th>Example</th>
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<tr>
<td>“F”</td>
<td>Enable tracking</td>
<td></td>
</tr>
<tr>
<td>“J”</td>
<td>Jog Motor</td>
<td>“J-500”; “J500”; “J0”</td>
</tr>
<tr>
<td>“S”</td>
<td>Save Parameter to EEPROM</td>
<td></td>
</tr>
<tr>
<td>“R”</td>
<td>Read Parameter to EEPROM</td>
<td></td>
</tr>
</tbody>
</table>

The main() loop of the firmware handled the initialization of the ISR, then checked if any control command was sent from the PC. Next it performed the corresponding control operation listed in Table 2, and finally sent back the current status of the OEC back to the PC for monitoring. The OEC tracking function could be enabled by sending “F” command to the MCU and could be stopped by sending the “J0” command. To jog the stepper motor for specific number of steps, the “J” command could be used. For example, command “J-500” moves the motor 500 steps backward, and “J500” moves the motor 500 steps forward. To save the current motor position to the non-volatile memory (EEPROM) of the MCU, the “S” command could be used. And to load the saved data from the non-volatile memory, “R” command could be used. An example usage of “R/S” command is that, before powering off the OEC, “S” could be sent to memorize the current motor
position. And next time right after powering on the OEC, the “R” command could be used to load the saved motor position. In this way, the absolute position of the motor could be transferred seamlessly across experimental sessions, even between power cycles.

### 2.6.3 Multi-channel two-photon signal pre-amplifier

![Schematic of a single-channel two-photon pre-amplifier.](image)

To satisfy the need for simultaneous multi-color neuroimaging, a multi-channel two-photon signal pre-amplifier was developed. It was based on the single-channel pre-amplifier previously developed (Figure 20). Briefly, a single channel of the pre-amplifier consisted of a transimpedance amplifier (TIA) and a low pass filter (LPF). The TIA was based on an OPA657 operational amplifier (op-amp) and had a 250K Ohm transimpedance. The LPF was a 4th order Butterworth filter in a Sallen-key configuration, implemented by two OPA2211 op-amps. The LPF had a non-unit gain of $4\times$ and a 3dB bandwidth of 1MHz. In this way, a single channel pre-amplifier was capable of converting the photo current of the PMT to voltage, then low pass filtered (integrated) for digitization.
Figure 21. Print Circuit Board (PCB) of the multi-channel pre-amplifier. (A) The PCB design layout of the multi-channel pre-amplifier. (B) Photo of the multi-channel pre-amplifier.

The multi-channel pre-amplifier print circuit board (PCB) was based on our previous group member Dr. Kartikya Murari’s design but re-arranged in a modular layout. As shown in Figure 21A, each signal channel was based on the same channel layout, with self-contained power supply chips for independent operations. Each channel was also equipped with sharable power buses that could deliver/share power to/from adjacent channels using the power-sharing jumpers (Figure 21B). In this way, the channel count of the pre-amplifier could be easily expanded in the future without significant modifications to the circuit layout. Here, all the circuit design and layout were performed in Altium Designer 15. The PCB was manufactured by JLC.com, and common components (resistor/capacitor of common package and values) were assembled in factory by their pick and place assembly service. The op-amps and power supply chips were soldered by hand. This multi-channel pre-amplifier was tested to have 1MHz 3dB bandwidth for each channel, and a more than -60dB channel-to-channel signal isolation ratio.
2.6.4 System upgrades to support 5MHz sample rate

The type II fiberscope offered an ~2X boost in FOV radius, at the edge of the FOV, the scanning speed of the beam would move 2X faster than before (assuming same scanning frequency). At a FOV diameter of 300 μm, with the imaging spatial resolution of 1 μm, ~1900 points were needed to be sampled at the outer ring of the FOV to suffice Nyquist criteria. If the scanner has a nominal frequency of 2.5kHz, this requires the digitizer to operate at least at 4.7MHz. Our previous type I fiberscope imaging system used DAQ cards with maximum sampling speed of 1.25MHz (Table 3), which fall short of the requirement derived above (4.7MHz). Here, a faster DAQ card with 5MHz sampling rate (NI PCI-6111) was used, and corresponding upgrades were performed to the electronics/software in order to support the higher sampling rate.

Table 3. Different DAQ cards used for different fiberscopes.

<table>
<thead>
<tr>
<th>Fiberscope Type</th>
<th>DAQ Card</th>
<th>Maximum Sampling Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>NI PXI-6259</td>
<td>1.25 MHz @16Bit</td>
</tr>
<tr>
<td>I</td>
<td>NI PXI-6251</td>
<td>1.25 MHz @16Bit</td>
</tr>
<tr>
<td>I</td>
<td>NI PCIe-6351</td>
<td>1.25 MHz @16Bit</td>
</tr>
<tr>
<td>II</td>
<td>NI PCI-6111</td>
<td>5 MHz @12Bit</td>
</tr>
</tbody>
</table>

Table 4. Components swap table for pre-amplifier 2.5 MHz bandwidth upgrade.

<table>
<thead>
<tr>
<th>Designator</th>
<th>Old Value</th>
<th>New Value</th>
<th>Designator</th>
<th>Old Value</th>
<th>New Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>680 Ω</td>
<td>68 Ω</td>
<td>R5</td>
<td>470 Ω</td>
<td>100 Ω</td>
</tr>
</tbody>
</table>
To support the 5MHz sample rate, the bandwidth of the pre-amplifier needed to be increased to 2.5MHz, which required a redesign of the LPF in the pre-amplifier. With the help from the Analog Filter Wizard\textsuperscript{86}, a new set of values for the resistors/capacitors were computed and listed in Table 4. This new design preserved the original 4th-order Butterworth filter in the Sallen-key configuration, allowing the multi-channel pre-amplifier PCB to be reused. And by swapping the 11
components (C2-C4, R1-R8) with new ones, the bandwidth of the pre-amplifier was increased to 2.5MHz, matching the 5MHz sample rate (Figure 22).

The legacy software detailed in\(^1^0\) used a hardware TTL signal for pausing/enabling the analog to digital convertor (ADC) of the DAQ card. In this way, the ADC of the DAQ card was only enabled during the open phase of the scanning, reducing the data generated for post-processing. This scheme worked well for first 3 DAQ cards listed in Table 3. However, the abovementioned triggering scheme was incompatible with the 5MHz DAQ card (NI PCI-6111), and as result, a new triggering scheme was adopted.

Instead of using a hardware TTL signal for pausing/enabling the ADC, in the new scheme, the ADC is always running. Here, the DAC (producing the PZT driving signal) and the ADC (data acquisition) are working simultaneously on the same card using the same on-board clock, and they are thus implicitly synchronized. The number of ‘open/close phase’ samples \(N_1/N_2\) to acquire in each frame cycle is pre-determined before each imaging session. Every time \(N_1+N_2\) fresh frame samples arrive in the computer memory, the software saves the first ‘open phase’ \(N_1\) samples and discards the rest ‘close phase’ \(N_2\) samples. In this way, data can be synchronized without relying on a hardware trigger. The new triggering scheme had been tested with all the DAQ cards listed in Table 3, and should be compatible with future hardware upgrades.

2.7 Chapter summary

In this chapter, the construction of our fiberscope imaging system was described in detail. The chapter started with Section 2.1 describing the overview of the two-photon fiberscope imaging system, then moved on to Section 2.2 for a detailed description of the opto-electrical commutator (OEC). Section 2.3 covered the theory and construction of the GRISM pre-chirper, and Section 2.4 and 2.5 reported the design and construction of the type I and II fiberscopes. And finally, the implementation details of the imaging systems were described in Section 2.6.
2.8 Contributions

The work presented in this chapter, except for the legacy type I fiberscope described in Section 2.4 and the PZT fiber scanner in the type II fiber scope which were built by Dr. Hyeon-Cheol Park in our group, was solely contributed by the author. The mouse model used in deep brain imaging SBR test was provided by Dr. Hui Lu’s group at the George Washington University.
Chapter 3  Two-photon Fiberscope

Neuroimaging on Freely Behaving Mice

With the two-photon fiberscope imaging system detailed in Chapter 2, we successfully performed two-photon imaging of calcium dynamics in both superficial cortex and deep brain regions on freely behaving mice. This chapter details the methods and apparatus that enabled our two-photon fiberscope system to perform neuroimaging reliably, including the animal surgery protocol, head mounting hardware and procedure, data processing methods, and our efforts in \textit{in vivo} cerebral vasculature imaging for system performance validation. Next, neuroimaging results collected by both our type I and type II fiberscopes are presented, with type I fiberscope data focused on high resolution imaging of somatic and dendritic calcium dynamics, and type II fiberscope data focused on neuron populational imaging over a larger FOV as well as deep brain imaging.

3.1  Animal model preparations

3.1.1  Mouse model for superficial cortex imaging

A standard approach for two-photon neuroimaging in superficial cortices is to surgically open the skull, perform adeno-associated virus (AAV) injection to the target cortex, and then seal the skull with glass windows as chronical implants. Here, two different strain of male mice pared with corresponding AAV were used throughout the study (Table 5).
Table 5. Mouse strain and corresponding AAV.

<table>
<thead>
<tr>
<th>Name</th>
<th>Vendor (Strain)</th>
<th>Vendor Stock #</th>
<th>AAV (Vendor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camk2-Cre</td>
<td>JAX (T29-1)</td>
<td>005359</td>
<td>AAV/DJ-flex-GCaMP6m (Baylor College of Medicine)</td>
</tr>
<tr>
<td>Wild type</td>
<td>JAX (C57BL/6J)</td>
<td>000664</td>
<td>pAAV.Syn.GCaMP6m.WPRE.SV40 (Penn Vector Core/Addgene)</td>
</tr>
</tbody>
</table>

At the age of 4 weeks old, male mice were deep anesthetized then locked onto the stereotaxic equipment. After prepping, a 4 mm-wide craniotomy was drilled over the motor cortex using a high-performance surgical drill. The center of craniotomy for different target cortices were listed in Table 6 below:

Table 6. Stereotactic coordinates of motor, visual and somatosensory cortices in mice

<table>
<thead>
<tr>
<th>Cortex</th>
<th>AP (Bregma)</th>
<th>ML (Bregma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motor M1</td>
<td>0.3 mm</td>
<td>±1.6 mm</td>
</tr>
<tr>
<td>Visual V1</td>
<td>-3.8 mm</td>
<td>±2.2 mm</td>
</tr>
<tr>
<td>Somatosensory S1</td>
<td>-0.5 mm</td>
<td>±2.0 mm</td>
</tr>
</tbody>
</table>

About 300-500 nL of corresponding AAV was injected into the exposed cortex at 300 µm in depth via a 1.0 mm O.D. glass microneedle. The microneedle had a 10-20 µm diameter tip and was attached to a Nanoject microinjector pump (Nanoject II, Drummond) with its injection speed set to 100 nL/min. A prefabricated stainless steel cannular (3.7 mm in diameter and 1 mm long)
with a 100 µm-thick (#0) glass coverslip was then placed over the exposed brain and sealed into the skull. The surgical site was then closed using vet glue. A 1-gram titanium head restraining bar was attached to the head with a "cold cure" denture material for later attachment of the fiberscope. The transgene expression was checked 3 weeks after the initial surgery with a tabletop two-photon microscope.

3.1.2 Mouse model for deep brain imaging

For imaging neurons deep in the brain that preclude direct imaging access, GRIN rod lenses were implanted in the brain to relay the excitation beam/fluorescence photons to and from the tissue. In this work, we chose medial prefrontal cortex mPFC as the target brain area. The surgery included AAV injection in the target brain area (mPFC) followed by a slow implantation of the GRIN lens:

Camk2-cre mice (JAX#005359) of ~4-month-old were anaesthetized and placed in a stereotaxic frame (Neurostar, Tübingen, Germany), then a 1.1 mm-diameter craniotomy (AP: +1.95 mm, M/L: -0.5 mm) was made with a high-speed rotary stereotaxic drill (Model 1474, AgnTho's AB, Lidingö, Sweden). The AAV/DJ-flex-GCaMP6m virus (Baylor College of Medicine) was injected unilaterally (Nanojector II, Drummond Scientific) into the left region of mouse prelimbic cortex, with the stereotaxic coordinates from bregma: +1.95 mm anterior-posterior (AP), -0.35 mm medial-lateral (ML), -2.3~2.5 dorsal-ventral (DV) following a high-resolution atlas. A total of 600 nL virus (diluted with 600 nL PBS) were injected at the rate of 75 nL/min at two injection sites and the needle was left in place for an additional 5 min each time after injection.

Then, a 1-mm diameter GRIN lens (~4mm length, Inscopix, Palo Alto, CA) was lowered into left prelimbic region (AP: +1.95 mm; ML: ± 0.35 mm; DV: -2.1~2.3 mm), 0.2 mm above virus injection site, at the speed of 50 µm/min, and then cemented in place (Metabond S380, Parkell). Mice were allowed to recover on a heat pad and thereafter monitored for 7 days closely during which they received injection of analgesic.
Three to four weeks after surgery, the virus expression in the anesthetized mouse was checked with a home-built single photon fluorescence microscope. If GCaMP+ neurons were visible and clear, the 1-gram titanium head restraining bar was then cemented to the skull and would serve as the mounting platform for fiberscope imaging.

### 3.2 Fiberscope head mounting procedure

![Fiberscope head mounting procedure diagram](image)

Figure 23. Fiberscope head mounting procedure.
For superficial cortex imaging, after confirmation of the GCaMP signal with a tabletop microscope, the mouse was anesthetized (2% Isoflurane + 1.5 L/min oxygen) and secured by its head restraining bar (Figure 23). We then mounted the fiberscope on a 3D translational stage with an attached customized head mounting adapter. Next, the fiberscope was subsequently carefully positioned and lowered towards the imaging window by a 3D stage while imaging. Once a good field of view (FOV) was identified, the mounting adapter was then lowered towards the head restraining bar and secured to the fiberscope with a set screw. Dental cement was then applied between the mounting adapter and the head restraining bar. After the curation of the dental cement (~10 min), the 3D stage was removed and the mouse with the attached fiberscope was relocated to a behavior recording chamber (10” ×10” in size with a behavior camera). Finally, after the mouse fully recovered from anesthesia, freely behaving brain imaging experiments would proceed.

Similar procedure was adopted for mounting fiberscope on the head of mouse with GRIN lens implant for deep brain imaging, with the only exception that type-B microscopy oil was added as coupling fluid between the fiberscope and the GRIN implant.
3.3 Data processing

3.3.1 Motion correction

![Figure 24. NoRMCorre-based motion correction on both type I and II neuroimaging data. (Top row)](image)

Average of a 1000-frame image stack collected by the type I fiberscope before and after motion correction.

(Bottom row) Average of a 1000-frame image stack collected by the type II fiberscope before and after motion correction.

During the freely walking imaging sessions, the mouse brain would deform due to rapid acceleration and deceleration, and this would inevitably lead to the constant changing of shape and location of the neurons in a FOV, making it challenging for extracting the calcium dynamics. Here, we employed an established non-rigid registration routine NoRMCorre\(^8\) for correction of motion artifacts in both the type I and type II fiberscope images. Figure 24 shows the motion correction
results for both fiberscopes. By comparison, the averaged image after motion correction showed much better sharpness and contrast for both type I and type II fiberscopes. The parameters (non-default) best tuned for motion correction of fiberscope neuroimaging data are tabulated below in Table 7. The MATLAB version of the NoRMCorre routine was used in this work and all the motion correction-related processing was performed in MATLAB R2018a on Windows 10.

**Table 7. NoRMCorre motion correction parameters for fiberscope neuroimaging.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>d1, d2</td>
<td>256,256</td>
<td>Image dimension (x, y)</td>
</tr>
<tr>
<td>grid_size</td>
<td>[32,32]</td>
<td>Patch size</td>
</tr>
<tr>
<td>max_shift</td>
<td>20</td>
<td>Maximum patch shift in pixel</td>
</tr>
<tr>
<td>us_fac</td>
<td>50</td>
<td>Up-sampling factor</td>
</tr>
</tbody>
</table>
3.3.2 Neuron segmentation

Figure 25. Neuron segmentation results using CaImAn\textsuperscript{89} on both type I and type II fiberscope data.

After motion correction, the registered image stack was fed into the CaImAn\textsuperscript{89} pipeline for segmentation of the somata. The CaImAn pipeline is based on constraint non-negative matrix factorization (CNMF) for separation of spatial and temporal components in the three-dimensional (x, y, t) neuroimaging data. It also features a pre-trained machine learning-based classifier to identify and filter out the false detections. Figure 25 shows the neuron segmentation results powered by CaImAn, with the top (bottom) row showing a raw frame of the type I (II) dataset and its corresponding neuron segmentation map. With the neuron segmentation map, the raw ΔF/F of neurons could be easily derived by taking the spatial average of the pixel values inside the segmentation masks. The parameters (non-default) empirically tuned for neuron segmentation are tabulated in Table 8 for both type I and type II fiberscope data. Again, the MATLAB version of the
CaImAn routine was used in this work, and all the processing was performed in MATLAB R2018a on Windows 10.

**Table 8. CaImAn parameters for neuron segmentation**

<table>
<thead>
<tr>
<th>Fiberscope Type</th>
<th>Name</th>
<th>Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I and II</td>
<td>d1, d2</td>
<td>256,256</td>
<td>Image dimension</td>
</tr>
<tr>
<td>I</td>
<td>K</td>
<td>40</td>
<td>Seed size</td>
</tr>
<tr>
<td></td>
<td>gSig</td>
<td>8</td>
<td>Half neuron size</td>
</tr>
<tr>
<td></td>
<td>merge_thr</td>
<td>0.4</td>
<td>Merge threshold</td>
</tr>
<tr>
<td></td>
<td>space_thresh</td>
<td>0.5</td>
<td>r-value threshold</td>
</tr>
<tr>
<td></td>
<td>cnn_thr</td>
<td>0.3</td>
<td>Classifier threshold</td>
</tr>
<tr>
<td></td>
<td>min_SNR</td>
<td>5.0</td>
<td>Minimal SNR</td>
</tr>
<tr>
<td>II</td>
<td>K</td>
<td>80</td>
<td>Seed size</td>
</tr>
<tr>
<td></td>
<td>gSig</td>
<td>6</td>
<td>Half neuron size</td>
</tr>
<tr>
<td></td>
<td>merge_thr</td>
<td>0.5</td>
<td>Merge threshold</td>
</tr>
<tr>
<td></td>
<td>space_thresh</td>
<td>0.5</td>
<td>r-value threshold</td>
</tr>
<tr>
<td></td>
<td>cnn_thr</td>
<td>0.6</td>
<td>Classifier threshold</td>
</tr>
<tr>
<td></td>
<td>min_SNR</td>
<td>3</td>
<td>Minimal SNR</td>
</tr>
</tbody>
</table>
3.3.3 Animal behavior tracking

For animal behavior tracking, we used DeepLabCut\textsuperscript{90} in processing mouse behavior footages recorded by our camera. Figure 26A shows the workflow for using DeepLabCut for animal behavior tracking. Briefly, key video frames were initially identified by the program and then manually labeled (Figure 26B). Next, the initial manual labels were used to train a ResNet convolutional neural network (CNN). The trained CNN was then evaluated using the entire video, and the outlier frames in the output were identified and manually refined to expand the training set.

**Figure 26. Animal behavior tracking.** (A) Workflow using DeepLabCut\textsuperscript{90} for animal behavior tracking. CNN: convolutional neural network. (B) Example of a single labelled frame with the body and head of the mouse marked by the blue and red dots. (C) Trajectories of the two tracking points on the mouse in an experiment session.
This new training set was used for the next round of training and refinement, until reaching the desired tracking outcome. Once the final CNN was trained, it was used to generate the behavior trajectory (Figure 26C) based on the video footage. For our mouse behavior tracking application, we used two labels for its head and body. In this way, the location and orientation of the mouse could be tracked on each video frame. Empirically, three rounds of training and refinement would usually yield a CNN that can track the mouse with >99% accuracy. Here all the behavior tracking was performed with Python 3.6 (Anناconda) on Windows 10 with a PC equipped with Nvidia GTX 1080 graphics card. The Demo_yourowndata.ipynb Jupyter Notebook script in the DeepLabCut package was used for the entire workflow.

3.4 Cerebral blood vessel imaging on freely behaving mice with type I fiberscope

![Figure 27. Cerebral blood vessel images on a freely behaving mouse. BV: blood vessel; BC: blood cell; Scale bar: 20 microns.](image)
Before moving on to functional neural imaging experiments, the efficacy of the entire imaging system (with type I fiberscope) was first evaluated with cerebral blood vessel imaging experiments on freely walking mice. Here wild-type (C57BL/6J) mice were used, and surgeries were performed to fit a glass window atop their cerebral cortex. FITC-dextran (FD70S, Sigma Aldrich) was intravenously (IV) injected before imaging sessions to provide contrast for blood vessel. After the injection, the type I fiberscope was attached on the head of the mice, and the mice were then set free in a 10\'×10\' behavior arena. Figure 27 shows representative images of a cerebral blood vessel captured in the freely walking brain imaging experiment. With FITC-dextran injection, the blood vessels show up bright in the field of view and blood cells can be identified as dark spots in the vessel. In Figure 27, a big branching blood vessel (BV) can be identified in the center, and movements of a single blood cell (BC) can be tracked over time. The circular ring pattern was induced by the spiral scanning scheme and the particles moving inside the vessel. All the images were acquired with 14mW of average optical power on tissue and 1.5 frames per second imaging speed. The circular groves in the blood vessel region are motion artifact resulted from the fast-moving particles in the vessel and the spiral imaging scanning scheme employed by our fiberscope.
3.5 Imaging dendritic calcium dynamics in the motor cortex of freely behaving mice with type I fiberscope

Figure 28. Representative dendritic images collected by type I fiberscope. (A) A representative image of firing dendrites of a freely walking mouse, with dendritic spines clearly resolved. (B) Dendritic activities recorded 1.3 seconds after (A). Scale bars: 20 μm.

To demonstrate the high-resolution imaging capability of our type I fiberscope on freely behaving mice, we performed dendritic calcium imaging on a freely behaving mouse. A type I fiberscope was attached to a mouse with GCaMP-expressing neurons in the motor cortex using the head mounting procedure described previously. Dendritic activities were assessed with the type I fiberscope focused on ~30 μm below the meninges with an average incident power of ~30 mW. With the fiberscope focused on the dendrites and fixed to the mouse head using our head mounting procedure previously described, the mouse was set free and dendritic calcium dynamics were recorded at ~2 frames/sec. Figure 28A-B show two representative images captured 1.3 seconds apart, showing several activated dendrites with their dendritic spines clearly visible.
Figure 29. Dendritic activities captured during freely behaving imaging experiment. (A) Extracted ΔF/F of the six dendrites along with the angular velocity trace of the mouse. (B) Six dendrites identified and segmented by a post-processing pipeline. (C) Absolute Pearson correlation coefficient (PCC) of each dendritic calcium dynamics with angular motion. Scale bar in (B): 20 μm.

The recorded time-series images of dendritic activities were fed to CaImAn processing pipeline, and six dendrites were identified and segmented (Figure 29B). Figure 29A shows the calcium dynamics of the six dendrites over 165 seconds, along with the angular velocity trace of the mouse recorded by the OEC. Pearson correlation coefficients were computed between the angular velocity and each individual dendritic calcium dynamics (Figure 29C). The results showed dendrite #2 held the highest correlation to angular motion among the six dendrites.
3.6 Imaging somatic calcium dynamics in the motor cortex of freely behaving mice with type I fiberscope

Figure 30. Representative somatic images collected by type I fiberscope. (A)-(C) Representative time-series images showing different neurons and their processes activated at different times. (D) Segmentation masks of the 20 neurons overlaid on the max intensity projection of the dataset. Scale bar in (A-D): 20 μm.

Using the same mouse model and procedures as in section 3.5, but with the type I fiberscope focused to >130 μm below the meninges of the motor cortex, somatic calcium dynamics were recorded at ~2 frames/sec with an average incident power of ~40 mW. Figure 30A-C show representative time-series images where different neurons were activated at different times with their processes clearly detectable.
With over 26 minutes of consecutive recordings and 3,000 image frames, our post-processing pipeline identified and segmented 20 neurons, including spatially overlapping ones (neurons #2 and #9) as shown in Figure 30D. The ΔF/F trace of each neuron was calculated based on the segmentation masks and plotted in Figure 31. The behavior of the mouse was also recorded by a video camera, from which time periods of locomoting, grooming and resting were extracted, labeled, and overlaid on the calcium dynamic traces.
Figure 32. Somatic fluorescence statistics. (A) Fluorescence rates of the 20 neurons during periods of locomotion and resting. (B) Mean fluorescence rates of the 20-neuron-ensemble during periods of locomotion and resting. Error bar: standard deviation of the fluorescence rates. (C) Pearson correlation coefficient (PCC) of each ΔF/F trace with angular motion.

Fluorescence rates of the 20 neurons during periods of locomotion and resting were calculated and plotted in Figure 32A, with 11 out of 20 neurons showing increased fluorescence rate during locomotion. The entire 20-neuron-ensemble also showed increased average fluorescence rate during periods of locomotion (as compared to resting periods, Figure 32B). Pearson correlation coefficients were computed between the angular velocity and each ΔF/F trace (Figure 32C), with neuron #3 within the given field of view showing the highest correlation with angular motion, and neuron #20 showing the least correlation.
3.7 Imaging neuron populations in the motor cortex of freely behaving mice with type II fiberscope

Although the type II fiberscope was originally designed for imaging deep neurons through GRIN implant lens, its drastically improved FOV also enabled imaging neuron populations in the motor cortex of freely behaving mice. With our new type II fiberscope, we were able to reliably image the calcium dynamics of more than 50 neurons in the motor cortices of freely behaving mice. Beside neuron populational imaging, the impact of rotational freedom enabled by the OEC was also investigated with our imaging system paired with a type II fiberscope. In addition, the connection between the motor cortex neuron activities and behavior was further investigated based on the type II fiberscope imaging data.

3.7.1 Rotational freedom and freely behaving neuroimaging on mice

To investigate the impact of rotational freedom enabled by the OEC, a type II fiberscope was used for imaging neuron populations in the motor cortex of freely behaving mice while the OEC tracking was turned on or off. The type II fiberscope could image calcium dynamics at 3 frames/sec speed over a 300-µm FOV (larger than the type I fiberscope and thus covering more neurons). A mouse (#1) mounted with the type II fiberscope over its motor cortex was set free in an open arena of 10’×10’ in size, and three 2000-frame (666 s) imaging sessions were performed. The OEC tracking was enabled in each session initially, and then disabled at half time. Between each imaging sessions, the mice were allowed to have resting breaks for ~5 minutes with tracking turned back on.
Figure 33. Neuron populational imaging with type II fiberscope. (A)-(C) Representative time-series images showing different neurons firing at different times. (D) Segmentation masks of the 60 neurons identified in this field of view.

Figure 33A-C show representative time-series images where different neurons were activated at different times. With a total of 6,000 imaging frames, our post-processing pipeline identified and segmented 60 neurons in the field of view (Figure 33D). The raw ΔF/F traces of each neuron over the three experiment sessions were calculated and plotted in Figure 34 (next page).
Figure 34. Raw ΔF/F of the 60 neurons along with the mouse velocity trace. Here data from three 2000-frames (666 s) imaging sessions are shown. Each imaging session started with tacking turned enabled, then disabled at half time. The solid dividing lines indicate the 5-min inter-session resting breaks for the mouse.
Figure 35. Behavior and fluorescence statistics over the three imaging sessions for mouse #1. (A) Photo of the arena with the mouse wearing the type II fiberscope. (B) Mouse movement trajectories over the 3 sessions, with blue/red color marking trajectories of the mouse when tracking was enabled/disabled. (C) Total distance traversed by the mouse in respective experiment sessions when tracking was turned on/off. The mouse consistently travelled more distance when tracking was enabled. (D) Total angular movement of the mouse measured in radians in respective experiment sessions when tracking was turned on/off. (E) Average fluorescence rates (area under the ΔF/F trace divided by time) of the 60 imaged neurons in respective experiment sessions when tracking was turned on and off. The neurons ensemble showed consistently increased fluorescence rates then tracking was turn on.

The behavior of the mouse was also recorded by a video camera (Figure 35A). The movement trajectories of the mouse were derived from the video footage and plotted in Figure 35B. The total distance traversed and the total turning angle of the mouse when tracking was turned on
and off were calculated for each experiment session, then plotted in Figure 35C-D. The results showed that the mouse travelled/turned more distance consistently in each session when the tracking was turned on, with a total increase of 125% in turning movements compared to the off case. The average fluorescence rates (defined as the area under the ΔF/F trace divided by time) of the 60 imaged neurons when tracking was enabled/disabled in each session were also derived and plotted in Figure 35E. When tracking was enabled, the neuron ensemble showed again consistently increased fluorescence rates of 82% on average across 3 sessions.

![Graphs showing distance travelled and angular movement](image)

**Figure 36. Impact of OEC tracking function on behavior and neural activities of 3 mice.** (A) Total distance traversed of the 3 mice when OEC tracking turned on and off. Results showed the 3 mice traveled more distance when OEC tracking turned on consistently. (B) Total angular movements of the 3 mice when OEC tracking turned on and off. The 3 mice also performed more turning movements when tracking was enabled.
enabled. (C) Fluorescence rates of 60 neurons of mouse #1 when OEC tracking was turned on and off, with their mean fluorescence rate shown in black. (D) Fluorescence rates of 54 neurons of mouse #2 when OEC tracking was turned on and off. (E) Fluorescence rates of 59 neurons of mouse #3 when OEC tracking was turned on and off. All 3 mice showed consistent increase in mean fluorescence rates when tracking was on.

The same 3-sessioned experiment was repeated on two other mice (#2 and #3) and their total distance traveled as well as fluorescence rates of the imaged neuron ensemble were derived from the imaging data and plotted. Figure 36A-B shows the total distance and angular movements of the 3 mice, and all the mice showed increased distance travelled/angle turned when tracking was on (with an average increase of 53% in distance, and an average increase of 64% in turning). 60, 54, and 59 neurons were identified respectively from 3 mice, and all three neuron ensembles showed increased mean fluorescence rates when tracking was enabled (with an average increasement of 64% out of N=173 neurons) in Figure 36C-E. With the OEC tracking functionality enabled, the mice tend to explore more and behave more freely, with its motor neurons firing more actively.
3.7.2 Angular motion correlation and neuron population statistics

Figure 37. Angular motion correlations and neuron population statistics of 3 mice. (A) Segmentation masks of the 60 identified neurons of mouse #1, with neuron #51 highlighted in red. (B) Pearson correlation (p<0.05) of the ΔF/F traces of the 60 neurons against angular speed, with the highlighted neuron #51 showing the highest correlation. (C) Representative calcium transient-triggered angular speed average of neuron #51 (calculated from N=84 epochs). (D) ΔF/F traces of neuron #51 (black) along with the angular speed trace (blue). (E) Fluorescence rates of the 60 neurons of mouse #1 during turning and non-turning, with their mean fluorescence rate shown in black. (F) Fluorescence rates of the 54 neurons of mouse #2 during turning and non-turning. (G) Fluorescence rates of the 59 neurons of mouse #3 during turning and non-turning. (H) Neuron populational statistics of the 3 mice, with 58/60, 27/54, and 55/59 neurons of the 3 mice respectively, all showing an increased fluorescence rate during turning motions.

Enabled by our unique twist-free imaging system, the connection between the motor cortex neuron activities and turning motions was further investigated by looked at the neuroimaging data collected with OEC tracking enabled. The ΔF/F traces of the 60 neurons of mouse #1 when OEC was enabled
were correlated against its corresponding angular speed trace, and their Pearson correlations (p<0.05) were plotted in Figure 37B. Among the 60 neurons, neuron #51 (Figure 37A) showed the highest correlation to locomotion speed. The calcium transients were identified from the ΔF/F trace of neuron #51, then the angular speed 3 seconds precede/succeed each calcium transient (total of N=84 transients) were averaged (in a way similar to spike-triggered average) and plotted in Figure 37C. The average angular speed curve showed a well-defined peak at the onset of calcium transients, indicating that neuron #51 is highly related to angular motion.

The time periods of turning and non-turning of mouse #1 were identified by thresholding the angular speed trace. Then, the fluorescence rates of the 60 neurons during turning and non-turning were calculated individually and plotted in Figure 37E, with 58 out of 60 neurons showing increased fluorescence rates during turning motions. The same analysis was repeated on mouse #2 and mouse #3, with 27 out of 54 neurons of mouse #2 showing an increased fluorescence rate during turning (Figure 37F), and 55 out of 59 neurons of mouse #3 showing an increased fluorescence rate during turning (Figure 37G). The neuron populational statistics of the 3 mice were combined and plotted in Figure 37H, with blue (orange) portions of the bar representing the total number of neurons with an increased (decreased) fluorescence rate during turning motions.
3.8 Imaging social neurons deep in the medical prefrontal cortex (mPFC) of freely behaving mice using type II fiberscope

Figure 38. Imaging mPFC neurons on a freely behaving mouse. (A)-(C) Representative time-series images showing different mPFC neurons firing at different times. (D) Segmentation masks of the 21 mPFC neurons identified in this FOV.

The medical prefrontal cortex (mPFC) of mice is known to be related to social anxieties. And the deep brain imaging capability of our type II fiberscope was validated by imaging the mPFC neurons on freely behaving mice. As described previously in section 3.1.2, the mouse model was prepared by AAV injection in mPFC then followed by implanting the 1-mm GRIN lens (~4 mm length, Inscopix) for imaging access. With the fiberscope focused to the mPFC neurons and fixed to the head, the mouse was set free in an open arena of 10’×10’. Its neural activities were captured by the
fiberscope at 3 frames/sec (Figure 38A-C) and its behavior was captured by a video camera (Figure 39A-B).

Figure 39. Mouse behavior tracking and ΔF/F of the mPFC neurons. (A) Photo of the mouse wearing the fiberscope behaving freely in the arena with added social stimulus. (B) Movement trajectories of the mouse before (blue) and after (red) introduction of the social stimulus. (C) Raw ΔF/F of the 21 neurons over the 2000-frame (666 s) imaging session. The red dashed line indicates the introduction of the social stimulus. The ΔF/F of neuron #6 is highlighted in the red box.
During the 2000-frame (666 s) imaging session, a pencil cup containing another mouse was introduced as a social stimulus at half time. Figure 39C shows the extracted ΔF/F of the 21 neurons based on the segmentation map in Figure 38D, where neuron #6 (highlighted in the red box) showed particularly strong reactions to the social stimulus.

**Figure 40. Response of neuron #6 in 3 trials.** (A) ΔF/F of neuron #6 over three trials, with red dashed line showing the introduction of the social stimulus in each trial. (B) Fluoresce rate of neuron #6 in each trial before (blue) and after (orange) introduction of the social stimulus.

The same imaging experiment with the same stimulus was repeated another 2 times and neuron #6 showed consistent increase in fluorescence activities after introduction of the stimulus (Figure 40A). The fluorescence rates of neuron #6 over the 3 trials before and after the social stimulus were calculated and plotted in Figure 40B, where its consistent increasement in fluorescence rate after stimulus was observed.
3.9 Chapter summary

This chapter described enabling method for fiberscope neuroimaging on freely behaving mice and as well as the fiberscope neuroimaging results. The chapter started with Section 3.1 delineating the animal preparation protocols, then moved on to Section 3.2 for a detailed description of the fiberscope head mounting method. Section 3.3 covered data processing method, and Section 3.4 through 3.6 reported the imaging results using type I fiberscopes. And finally, Section 3.7 and 3.8 documented the type II fiberscope imaging results for superficial and deep brain imaging, respectively.

3.10 Contributions

All the data processing, animal imaging experiments described in this chapter were performed by the author. The development of animal surgical protocol and head mounting procedure was led by the author, with contribution from fellow graduate student Honghua Guan. The head mounting adapter was designed by the author and fabricated by Honghua Guan. The IV injection of FITC-dextran described in Section 3.4 was performed by group member Dr. Defu Chen. The motor/mPFC mice involved in the imaging studies were courtesy of Dr. Hui Lu’s lab at the George Washington University.
Chapter 4  Miniature z-scanning stage for fiberscope neuroimaging

4.1  Introduction

Two-photon fiberscope allows several in vivo applications of TPM technologies, such as assessing the cervical consistency during pregnancy\(^9\) and neuroimaging on freely behaving rodents. In a fiberscope imaging setting, two-dimensional lateral beam scanning has been accomplished by various methods, including the use of a piezo-based scanner to perform spiral scan\(^92\), Lissajous scan\(^93\) or raster scan\(^94\), and the use of a miniature MEMS mirror\(^95,96\). To fully utilize the three-dimensional imaging capability of nonlinear microscopy, a depth or focus scan mechanism is needed. Conventionally, depth scanning has been achieved by mounting the fiberscope on a translational stage, or using a pull-back mechanism at the proximal end\(^97\). However, these methods have distinct limitations, as they increase the size of the instrument or have low accuracy, making them unsuitable for in vivo applications. In addition, the size and weight limit posed by mounting devices on the heads of small mammals such as mice for brain imaging, requires a mechanism that allows for accurate and reliable depth scanning, while maintaining a small form factor.

One way to achieve depth scanning is to vary the focal length of a lens directly with tunable lens technology\(^98,99\). However, for a two-photon fiberscope, a high numerical aperture (NA) lens with superior achromaticity is required\(^9,36,100,101\), and this is particularly challenging for a tunable lens\(^102\). Another way to achieve depth scanning is to directly translate the fiberscope optics, using technologies such as shape memory alloys (SMA)\(^103\), voice coil motors (VCM)\(^104\), or pneumatic actuation\(^105\). Among the various technologies, SMA wires have shown promise in extending 2D endoscopic imaging to 3D; by Joule heating, the SMA wire contracts and moves the fiberscope optics to change the beam focus\(^103\). However, this method suffers from hysteresis, and is susceptible
to changes in ambient temperature, making it difficult to achieve accurate and reliable depth scanning.

In this section, we describe a feedback-controlled SMA depth scanner which addresses these challenges. The core of the feedback loop was a Hall effect sensor. By measuring the magnetic flux density from a tiny magnet attached to the SMA wire, the contraction distance of the SMA wire could be accurately tracked in real time. The measured displacement was then fed to the PID algorithm running on a microprocessor, which computed the error between the command position and the current position of the depth scanner. The electrical current running through the SMA wire was then adjusted accordingly. This feedback-controlled SMA depth scanner had a tube shape with an outer diameter of 6.5 millimeters, and was designed to house a 2.1 mm fiberscope inside. The SMA depth scanner can travel up to 490 µm, and with an open-loop operation, it can move more than 350 µm within a second. With the feedback loop engaged, submicron positioning accuracy was achieved along with superior long term positioning stability. The high-precision positioning capability of the SMA depth scanner was further verified by depth-resolved fiberscope imaging of biological samples.

4.2 Methods

4.2.1 SMA and its hysteresis

SMA is a special kind of alloy that has memory of its original shape. When heated above the transition temperature, SMA will restore to its original shape if deformed\textsuperscript{106}. In this work, we employed the commercially available FLEXINOL\textsuperscript{®} wire, which was made from nickel-titanium shape memory alloy. SMA wire is compact, lightweight, and easy to use. By passing electrical current through the wire, Joule heating can cause the SMA wire to contract 3-5\% of its total length, and to generate sufficient pull force. Table 9 shows the key parameters of the SMA wire used in this work.
Table 9. Key Parameters of the SMA Wire

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter</td>
<td>125 μm</td>
</tr>
<tr>
<td>Activation temperature</td>
<td>90 °C</td>
</tr>
<tr>
<td>Resistance</td>
<td>75 Ω/m</td>
</tr>
<tr>
<td>Recommended current</td>
<td>320 mA</td>
</tr>
<tr>
<td>Pull force</td>
<td>223 g</td>
</tr>
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</table>

Figure 41. SMA wire characteristics. (A) Hysteresis response of the SMA wire. (B) Susceptibility of the SMA wire to ambient temperature change.

It is well known that shape memory alloy suffers from hysteresis; when heated or cooled, the SMA wire does not have the same response upon temperature change\(^{107}\). As shown in Figure 41A, when an SMA wire is heated, it follows the upper red contraction curve, but when it is cooling down, the wire follows the bottom blue relaxation curve. In addition to hysteresis, the SMA wire is susceptible to changes in ambient temperature. As shown in Figure 41B, a constant current was applied to an SMA wire, and it contracted in response to the rise in temperature. The contraction
could be easily perturbed by slight cooling (Air Blow), as depicted as ‘dips’ in the curve. The hysteresis response of the SMA wire, as well as its susceptibility to temperature perturbations posed major challenges in precise and robust control of its contraction and relaxation.

4.2.2 Feedback loop

To address these challenges, a feedback loop was introduced to actively stabilize the contraction or relaxation of the SMA wire. The feedback loop consisted of (1) a position sensor, (2) a microcontroller unit (MCU), and (3) the corresponding driving and amplification circuitry. The position sensor measured the SMA wire displacement. The measured displacement was then amplified and fed into the MCU. The MCU compared the measured position with the desired position set by the user (denoted as command position) and adjusted the electrical current in the SMA wire accordingly to deform the SMA further and bring the scanner to the command position.

The block diagram of the feedback loop is shown in Figure 42A.

![Feedback loop electronics](image)

**Figure 42. Feedback loop electronics.** (A) Block diagram of the feedback loop. (B) Photo of a Hall effect sensor. (C) Photo of the MCU and circuitry.

The position sensor consists of a Hall effect sensor and a neodymium magnet. The Hall effect sensor was fixed in position with the magnet attached to the moving part of the SMA depth
The displacement of SMA wire leads to a change in magnetic flux density around the Hall effect sensor. This change in magnetic field was further translated into voltages per the Hall effect. The small Hall voltage output from the sensor was then amplified and passed on to the digitizer of the MCU. In this way, contraction of the SMA wire could be accurately measured by the MCU. A photo of the Hall effect sensor is shown in Figure 42B.

The driving and amplification circuitry allows the MCU to track the displacement and control the SMA wire in real time. Figure 42C shows a photo of the MCU, and its driving and amplification circuitry. The driving circuitry was a current source that can be turned on and off at a high frequency via the pulse width modulation (PWM) functionality of the MCU. The PWM cycle period was set to 64 µs, orders of magnitude below the heating response time of the SMA wire, so that the wire was heated as if a constant current was applied. In addition, by changing the duty cycle of the on-off current, the heating power in the SMA wire could be accurately controlled. The amplification circuitry increased the small Hall sensor output. To ensure maximum sensitivity, this circuitry enhanced the Hall sensor output by subtracting the output voltage from a preset zero which was then amplified by 12 times to match the 5V input range of the MCU digitizer. The preset zero-point voltage was to ensure a zero output from the amplifier when the SMA wire was relaxed. The MCU digitizer was running at a sampling rate of 100 Hz, and every 100 milliseconds, an average of 10 latest samples was used as the input to the control algorithm.

The final link of the feedback loop was the PID algorithm running on the MCU. The MCU (Arduino Mega 2560) received the user-defined desired voltage from a computer and stored it in its memory. Then it compared the desired voltage with the current averaged voltage readout from its digitizer, computed the error between the two, and adjusted the heating power in the SMA wire based on PID algorithm\textsuperscript{108}. 
4.2.3 Construction of the SMA depth scanner

**Figure 43. Depth scanner schematics.** (A) Functional illustration of the depth scanner. (B) Perspective view of the depth scanner without the outer housing. (C) Picture of the SMA depth scanner with the encased fiberscope.

To adapt the scanner for use with a fiberscope, we modified the components to achieve a compact design with reasonable positioning precision. Figure 43A is the functional illustration of the depth scanner: an SMA wire and a rubber band are installed in an antiparallel fashion, pulling the fiberscope to opposite directions. They both have one end attached to the fiberscope, and the other end fixed in space. When a current is applied, the SMA wire heats up and contracts, pulling the fiberscope to the left in Figure 43 (towards the imaged sample). Conversely, when the current is
turned off, the SMA wire cools and relaxes; the rubber band then pulls the fiberscope back to the right (away from the imaged sample), as indicated by the two red arrows. The magnet is attached to the moving fiberscope, and the Hall effect sensor is fixed. The Hall effect sensor and the magnet are kept as close as possible to each other, ensuring maximum sensitivity.

Since only a single SMA wire was used in the design, the pull force was not balanced and the fiberscope had the tendency to twist itself during translation. To address this problem and minimize friction, a miniature guide rail with ball bearing was introduced, as shown in Figure 43B. The fiberscope was mounted on the moving carriage of the guide rail. The SMA wire and the rubber band were also attached to the moving carriage, pulling the fiberscope to opposite directions. The whole assembly was housed inside a gauge-3 hypodermic tube, with an outer diameter of 6.5 mm (Figure 43C). In this proof-of-concept design, the outer diameter of the depth scanner was primarily determined by the guide rail, which was purchased from available materials rather than constructed in house. We foresee that with proper design and customization, the outer diameter of the depth scanner can be potentially reduced to half of the current size.

4.2.4 Calibration of the Hall effect sensor

![Figure 44. Hall effect sensor calibration curve](image)

With the feedback loop, movement of the SMA wire could be stabilized with a user-defined digitized voltage. Calibration was performed to establish the relationship between the digitized
voltage and the actual displacement. We first calculated the exact size of individual pixels of a given camera. Then we captured on the same camera the displacement of the SMA scanner once it was steady in position at each command voltage. The photographed SMA travel was then translated into actual distance in microns. In this way, the relationship between the digitized voltage and the actual displacement was established, and the data points were fit to a 2\textsuperscript{nd} order polynomial (Figure 44). Submicron positioning accuracy could be achieved with the feedback loop engaged. As shown in Figure 44, the 400 µm travel range corresponds to 900 digitized voltage readout levels of the Hall effect sensor by the analog-to-digital converter (ADC) on the MCU of a 10-bit resolution, which gives ~0.44 µm step size on average. A better step size resolution can be achieved by using an MCU featuring a finer resolution ADC.
4.2.5 3D Fiberscope imaging system

Figure 45. Schematic of the 3D fiberscope imaging system. GP: Grating Pair; DM: Dichroic Mirror; BP: Band-pass Filter; SP: Short-pass Filter; PMT: Photomultiplier Tube; CL: Coupling Lens; DCF: Double-clad Fiber; FRS: Fiber-optic Resonant Scanner.

Figure 45 shows the schematic of the 3D fiberscope imaging system used in this study. The details of the two-photon fiberscope were described elsewhere\textsuperscript{33,92}. A Ti:Sapphire laser pre-chirped by a grating pair (GP) was coupled into the fiberscope through the core of a double-clad fiber (DCF) for excitation. The nonlinear optical signals from the samples were collected both by the core and inner clad of the DCF, and directed to a photomultiplier tube (PMT). The fiber-optic resonant scanner (FRS) created a spiral scanning pattern of ~200 µm in diameter. Depth-resolved images were taken as the SMA depth scanner accurately moved the fiberscope up and down.
4.3 Results and discussions

4.3.1 Positioning performance

Figure 46. Positioning performance characterizations. (A) Open-loop rapid operation of the SMA depth scanner. (B) Close-loop operation of the SMA depth scanner. (C) Stability of the SMA scanner in open/close-loop operations. (D) Perturbation test for close-loop operation of the SMA scanner.

To test the range of the depth scanner, the SMA wire was driven to its limit with the maximum heating current. The maximum heating current was set to 320 mA to prevent the SMA wire from burning, and a 490 μm scanning range was achieved. This corresponded to 3% of the total lengths of the 1.6 cm long SMA wire used in our system, which agreed well with the 3% to 5% contraction range specified by the manufacturer. Even with maximum current applied to the SMA wire and the
SMA wire reaching the actuation temperature of 90 degree Celsius, no obvious rise of temperature could be detected on the outer surface of the depth scanner housing. The reason was that there was little voltage drop across the SMA wire, yielding a heating power of merely 122 mW.

The actuation speed was tested under open-loop conditions where the SMA wire was given an instant jump (step drive function) of drive current to its maximum value (320 mA), and the movement was tracked in real time with the calibrated Hall sensor. As shown in Figure 46A, it took the SMA depth scanner 0.7 seconds to move about 369.6 µm in contraction, and 1.5 seconds to move 366.9 µm in relaxation. This open-loop movement was rapid and continuous, and the position of the scanner could be tracked in real time by the Hall effect sensor. This open-loop operation mode can be used in depth-priority scanning for imaging modalities with sufficiently high frame rate.

With the feedback loop engaged, the arbitrary positioning capability of the SMA depth scanner was tested by setting different command positions in the MCU, while tracking the change of the position over time. Figure 46B shows the change of position over time when given a series of different command positions at different times, and the actual positions closely followed the command positions. The positioning accuracy was defined as the root-mean-square (RMS) of the deviation between the actual position and the command position at a given steady state, and it was calculated to be 0.45 µm.

The positioning stability is crucial for imaging modalities with a relatively low frame rate, such as our two-photon fiberscope. Sharp images can only be acquired when the fiberscope position is stabilized. Figure 46C shows the stability of the depth scanner in close-loop operation as opposed to the open-loop constant current operation. In the close-loop case, the feedback loop locked the position of the depth scanner for a long time with negligible fluctuations. However, in the open-loop case, the depth scanner drifted dramatically and constantly, and it was unable to stabilize itself even after 100 seconds. Note that after a command was given to the feedback-controlled depth scanner, it took about 10 seconds for the SMA wire to stabilize at a target position, this was due to

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the conservative PID parameters chosen for better stability and accuracy. And this conservative PID tuning lead to an underdamped motion with a slow oscillation of about half a micron in amplitude. Since the response of the SMA wire is highly nonlinear, it is rather challenging to find a set of PID parameters that allowed for high speed actuation and superior accuracy at the same time. And this problem can be potentially addressed with more advanced nonlinear control algorithms\textsuperscript{109}.

Another unique feature of the feedback-controlled SMA depth scanner is that it can easily recover from perturbations. As demonstrated in Figure 46D, when the SMA wire was in a steady state for a given command position, a strong blow of cold air from a compressed-gas duster was applied. This caused a sudden drop in the SMA temperature, and hence the elongation of its length. Shortly afterwards, the SMA depth scanner managed to recover itself to the previous steady state by the feedback loop.
4.3.2 Depth-resolved fiberscope imaging

Figure 47. Fiberscope 3D depth-resolved imaging powered by the SMA depth scanner. (A) Depth-resolved blood vessel in the CUBIC mouse brain. (B) Depth-resolved somata and processes of OPCs in the Pdgfra-creER; RCE:loxP brain. (C) Depth-resolved somata and processes (myelin sheath internodes of oligodendrocytes) in the MOBP-EGFP brain. OPC: oligodendrocyte precursor cell.

The precision positioning capability of the SMA depth scanner was further verified by depth-resolved two-photon imaging of mouse brain samples with the 3D fiberscope imaging system shown in Figure 45. The fiberscope was first manually focused onto the surface of the sample to locate a region of interest, and then the SMA depth scanner was activated to move the fiberscope.
up and down to different focal depths. Imaging was performed using brain samples from three different lines of transgenic mice. The first was a mouse brain that underwent CUBIC optical clearing\textsuperscript{10}. Figure 47A shows the two-photon fluorescence images of a region in the brain in which a large branching blood vessel with autofluorescence is visible. The second brain sample was from a mouse that was bred by crossing *Pdgfra*-cre\textit{ER} mice (JAX 018280)\textsuperscript{111} with RCE:loxP mice (MMRRC 32037-JAX), where EGFP is expressed in oligodendrocyte precursor cells (OPCs). Our fiberscope imaging setup was able to resolve individual somata as well as the fine processes of OPCs at different depths (Figure 47B). The third brain sample was from an *MOBP*-EGFP mouse (MGI 4847238), in which EGFP is expressed in mature oligodendrocytes. As shown in Figure 47C, the somata of the oligodendrocytes and the individual myelin sheaths (internodes) formed by their processes are clearly resolved at different depths.

4.4 Chapter summary

In summary, we developed a feed-back controlled SMA depth scanner. The depth scanner had a small form factor with an outer diameter of 6.5 mm. It could travel up to 490 µm in distance. With open-loop operation, it could move more than 350 µm within one second. With the feedback loop engaged, submicron positioning accuracy was achieved along with superior positioning stability. It is expected that the size of the depth scanner can be further reduced by special customization, and the close-loop actuation speed can be improved by using more advanced nonlinear control algorithms. This design greatly extends the capabilities of fiberscopes by allowing stable, depth resolved imaging in biological tissues.

4.5 Contributions

The design, prototyping and non-imaging testing of the SMA device was performed solely by the author. The imaging test with the fiberscope described in Section 4.3.2 was led by the author, with
the contribution from senior graduate student (at the time) Wenxuan Liang. The brain samples were courtesy of Bergles lab in the neuroscience department of Johns Hopkins Medicine.
Chapter 5  Hand-held two-photon rigid probe for optical biopsy

5.1 Introduction

Optical biopsy refers to methods of using optical imagining or spectroscopy to evaluate tissue histopathology *in vivo* and *in situ* without tissue excision. Among various potential technologies for optical biopsy such as confocal laser scanning microscopy (CLSM), optical coherence tomography (OCT), and two-photon microscopy (TPM), TPM is of particular interest because of its capability to perform 3D imaging of unstained tissue at subcellular resolution. The near-IR excitation wavelength of TPM allows for deeper tissue penetration, and its confined nonlinear interaction helps reduce out of focus photodamage. Moreover, both two-photon fluorescence (TPF) from endogenous fluorophores (such as reduced nicotinamide adenine dinucleotide (NADH) and oxidized flavin adenine dinucleotide (FAD)) and second-harmonic generation (SHG) signals from collagen fibers can be acquired simultaneously with single-wavelength excitation, enabling label-free and simultaneous multi-contrast imaging. TPM has been approved for skin imaging in European Union, and it provides considerable label-free contrast for skin cancer detection. However, conventional bench-top multiphoton microscopes are bulky and not suitable for internal organ imaging. Recently, the development of two-photon fiberscopes enables label-free, *in vivo*, high-resolution and functional histological assessment of internal organs that was previously impossible. An alternative approach for TPM to reach internal organs was to use a rigid probe made of a gradient index (GRIN) relay lens to deliver the excitation light to and collect the fluorescence (or SHG) from tissue. Compared with the fiber-optic flexible endomicroscope, the rigid probe is more desirable in laparoscopic applications or in
interfacing with a biopsy probe (i.e., by going through the cannula of a biopsy needle). In addition, the rigid probe can be made smaller since the beam scanning mechanism can be packed outside the probe at its proximal end, offering more flexibility in design and additional functionalities. Nonetheless, all prior efforts enabling TPM imaging of internal organs involved surgical elevation of target organs to reduce motion artifact and most lacked a built-in mechanism for depth scanning\(^{70,72,74,75}\), which fell short of the \textit{in situ} requirement for optical biopsy.

To perform optical biopsy, several issues and challenges need to be addressed for the rigid probe. Firstly, the probe should be compatible with existing biopsy protocols, i.e., the probe needs to be protected and durable, but at the same time it should have an appropriate length and small enough diameter to fit inside the cannula of a biopsy needle. This requires the objective lens and the beam delivery optics to be small in diameter. Secondly, the probe should be compact and flexible for clinical use, which calls for fiber-optical delivery of the excitation beam and signal photons, and a mechanism to manage/minimize temporal broadening of the femtosecond excitation pulses in optical fibers. Performance-wise, the probe should offer a histopathological resolution with an adequate imaging signal-to-noise ratio (SNR). For TPM, it requires a tight spatiotemporal confinement of the excitation beam at the tip of the probe\(^{120}\) and a superior collection efficiency. Thus, a miniature high-NA objective is needed. Function-wise, a built-in focus (or depth) scanning mechanism is desired to perform 3D imaging. Physically moving the probe for depth scanning is sub-optimal for \textit{in vivo} applications, since it requires an additional precision actuation and translation mechanism\(^{66,69,121}\), and the longitudinal probe translation would demand extra caution in avoidance of potential damages to the target tissue (e.g., tear or perforation) and even the probe itself. To realize focus scanning for TPM, a reasonably high NA for the excitation beam over the entire focus tuning range should be maintained, which poses an additional challenge in optics design for the rigid probe. Finally, the probe should provide a sufficient imaging speed in order to minimize motion artifacts introduced by physiological movements (breathing and heartbeat), and thus a fast beam scanner is highly desirable.
In this chapter, we developed a two-photon handheld rigid probe for optical biopsy that addressed these challenges. The handheld rigid probe consisted of two functional parts: a handheld compact scanning box (3D) and a compound GRIN objective. The compound GRIN objective was 15 cm long (including a micro-objective) with an outer diameter of 1.75 mm (including the housing hypodermic tube), and could fit within a 14-gauge biopsy needle. The scanning box included a MEMS mirror for 2D raster beam scanning up to 10 frames per second and a piezoelectric stage for focus scanning. Our optics design ensured a continuously tunable working distance from 100 μm to 300 μm in tissue and yielded a measured 120 μm field of view with a 0.855 μm lateral resolution. A single-mode fiber was used in our rigid probe imaging system for delivery of femtosecond pulses, whereas a multi-mode fiber with a large core diameter was used at the proximal end of the rigid probe to deliver the nonlinear optical signal (collected by the compound GRIN objective) to a separate detection module. The use of optical fibers for both excitation pulse delivery and nonlinear signal collection offers the flexibility for positioning the rigid handheld probe during imaging, which is particularly desirable for in vivo imaging. Depth-resolved two-photon autofluorescence (TPAF) imaging of internal organs and subcutaneous tumor on mouse models in vivo were performed with the biopsy needle compatible rigid probe. In addition, enabled by its high frame rate and 3D imaging capability, a first-of-its-kind depth-resolved two-photon optical biopsy of internal organ was successfully demonstrated. The optical biopsy was performed on mouse kidney in vivo and in situ without surgical elevation of the organ, and with the rigid probe going through a biopsy needle.
5.2 Miniature handheld rigid probe

Figure 48. Handheld rigid probe. (A) Handheld probe design schematic. μObj: Micro Objective; EFC: Excitation Fiber Connector and Collimator; CFC: Collection Fiber Connector; DM: Dichroic Mirror; L1-L4: Lens. (B) Photo of the handheld rigid probe. (C) Photo of the rigid probe inside a gauge-14 biopsy needle

As mentioned in the previous section, the design of our handheld rigid probe takes into account multiple target operational and performance features, including (1) compatibility with a biopsy needle, (2) an excellent SNR and resolution for label-free imaging, (3) capability of 3D focus scanning, (4) a high imaging framerate, and (5) a compact and flexible system for in vivo or clinical use. Figure 48 shows the detailed schematic of the handheld probe which consists of a scanning box and a compound GRIN objective. Lateral beam scanning was performed by a MEMS scanner, which was capable of raster scanning at 10 frames per second with a frame size of 234*234 pixels. Depth scanning is generally more challenging and several elegant methods have been demonstrated, including the use of an electrically tunable lens (ETL) in front of the objective lens\textsuperscript{122}. However, a typical ETL can only provide 10 to 25 diopters of focusing power tuning range,
which is not suitable for this application. In our design, depth scanning was realized by a piezoelectric stage (Figure 48A). By mechanically translating the input focus at the proximal end of the compound GRIN objective, the distal focus on the sample could be varied with minimal influence on the excitation NA.

5.2.1 Scanning box

The scanning box (Figure 48A) featured a MEMS scanner (A1S1.2, Mirrorcle Technologies, Inc.) for 2D lateral (X-Y) scanning, a piezoelectric micro translation stage (PP-18, Micronix) for focus (Z) scanning, a dichroic mirror for separation of signal beam from excitation beam and their corresponding relay optics. The excitation beam (denoted by red) from the PM-SMF was first collimated by a collimator (F230FC-780, Thorlabs), and then directed to the MEMS scanner for lateral 2D scanning. The MEMS scanner had a 1.7 mm aperture diameter, a resonance frequency of 1.3 kHz for both axes, and a maximum beam steering angle of 5 degrees. After the MEMS scanner, the beam was expanded by a pair of achromatic lenses (6.25 mm diameter, Edmund), as denoted by L1 (f=7.5 mm) and L2 (f=10 mm), and then refocused into the proximal (input) end of the compound GRIN objective by another achromatic lens L3 (f=10 mm). Depth scanning was realized by moving L3 along the optical axis with the piezoelectric translational stage. The nonlinear optical signal from the tissue was collected by the compound GRIN lens, collimated by L3, then deflected by a dichroic mirror. The signal from the dichroic mirror was re-focused by a singlet lens L4 (6 mm diameter, f=6 mm, Edmund) to the multi-mode fiber for detection. Given the fact that all the components in the compound GRIN lens were not optimized for achromatic operation, the detection optics were expected to suffer from severe chromatic focal shift, thus a multi-mode fiber with a large core (800 μm in diameter) was adopted to minimize the impact of chromatic focal shift on fluorescence collection efficiency.

The chassis of the scanning box was designed in house and CNC machined from aluminum. All the lenses and optical-mechanical components were either screwed or glued in their dedicated
mounting space machined on the chassis. The MEMS scanner was held in place with a 3D printed mount with two screws for minor adjustments. Apart from the MEMS scanner, all the alignments of optics were passive owing to the high precision CNC machining process.

5.2.2 Compound GRIN objective

Figure 49. Ray tracing of the compound GRIN objective. (A) Compound GRIN objective ray tracing model. (B) Distal focus (or imaging depth D1) within the sample vs. input (proximal) beam focus (D2). The compound objective had an ~7X magnification, corresponding to an ~50X axial magnification. (C) Excitation NA vs. distal focus (D1). The excitation NA dropped 10% over the entire depth scanning range. (D) RMS spot size/airy radius vs. distal focus (D1). A diffraction limited focused spot size can be achieved when the imaging depth is scanned between 97 μm and 270 μm.

Compatibility with a needle biopsy procedure is a major consideration in designing the compound GRIN objective. The objective should have an appropriate size and diameter to fit inside the cannula of a biopsy needle, and at the same time provide a superior resolution and sufficient field of view. We chose the dimension of the objective to be 15 cm long and 1.75 mm in diameter to fit inside the cannula of a gauge-14 biopsy needle (BARD® MAGNUM® MN1416). To ensure a good imaging quality, we have designed the objective as a compound lens consisting of a long doublet relay rod lens and a short micro-objective (Figure 49A). The micro-objective (GT-MO-080-018-810, GRINTech) has a high NA (0.8) at the sample and a 120 μm field of view. The
doublet relay rod lens serves to deliver the excitation beam and the fluorescence photons over a long distance (>10 cm) between the scanning box and the micro-objective. Due to the size constraint, we have chosen GRIN rod lenses of a 1 mm diameter to construct the doublet relay rod lens. In order to reduce the chance of beam clipping when the excitation beam is scanned off axis, a GRIN rod lens of 0.1NA was chosen for the proximal end of the doublet relay lens. To match the input NA of the micro-objective (0.19), a second GRIN rod lens of a 0.2NA was added between the first rod lens and the micro objective. As a result, the doublet relay lens consisted of a long 0.1NA (2.75 pitch, 122 mm) GRIN rod lens at the proximal end, and a short 0.2NA (0.75 pitch, 18 mm) GRIN rod lens at the distal end. This design also came with the advantage of reducing the total GRIN lens pitch number (as compared with directly using a single 0.2NA GRIN rod lens), and thus reduced optical aberration in the excitation beam path. The GRIN relay doublet and the micro-objective were housed inside a customized hypodermic tube of a 1.75 mm outer diameter, with a ~0.5 mm air gap in between the doublet and the micro objective.

Ray tracing simulation (Figure 49) was performed with ZEMAX to evaluate and fine-tune the performance of the compound GRIN objective. As mentioned before, focus scanning (D1) of the imaging beam within the sample was realized by translating the input beam focus (D2) to the compound objective. At a given D2, the corresponding D1 was numerically determined and plotted in Figure 49B. At the designed two-photon excitation wavelength (750 nm), moving D2 from -5 mm to 5 mm led to a change of D1 (depth scanning) from 302 μm to 97 μm. The compound GRIN objective had a transverse magnification of ~7X, which translated to an ~50X axial magnification, matching nicely the ~20 μm/mm slope in Figure 49B. The excitation NA was also plotted against depth scanning of D1 in Figure 49C, where the NA dropped about only 10 % (i.e., from 0.66 to 0.59) over the entire depth scanning range. The root-mean-square (RMS) spot radius on the focal plane determined by ray tracing was plotted against D1 as well and was compared with the airy radius (Figure 49D). The results showed that the diffraction-limited beam focusing (with the RMS spot radius smaller than the airy radius) could be achieved when the beam focus (D1) was tuned
between 97 μm and 270 μm.

### 5.3 Rigid probe imaging system

![Diagram of Rigid probe imaging system](image)

**Figure 50. Rigid probe imaging system.** M: Mirror; CL: Coupling Lens; PM-SMF: Polarization Maintaining Single-mode Fiber; PMT: Photomultiplier Tube; EF: Emission Filter; L: Lens; FC: Fiber Connector.

To make the handheld rigid probe flexible for *in vivo* and potential future clinical use, we have adopted fiber-optic delivery and collection to separate the compact 3D scanning handheld probe from the benchtop optics system (Figure 50). A single-mode fiber (SMF) was used to deliver the excitation beam to the handheld probe, and a multi-mode fiber was used to transmit fluorescence or SHG signals collected by the probe from the sample to the photon detection unit within the benchtop optics system.
Fiber delivery of femtosecond laser pulses from a Ti:sapphire laser has always been a challenge. Material dispersion and nonlinear effects in optical fibers require careful management, which would otherwise lead to severe temporal broadening of the laser pulses and thus dramatically reduce the fluorescence yield for TPM. Here we briefly describe the implementation of a dual-fiber based femtosecond pulse delivery scheme. As shown in Figure 50, a Ti:sapphire laser (120 fs, Chameleon Ultra II, Coherent, with a built-in pre-chirping unit) was first coupled into a 20 cm long polarization maintaining single-mode fiber (PM-SMF, PM780-HP, Thorlabs) for spectral broadening. The beam from the PM-SMF was then collimated and chirped by a grating pair (1200 lines/mm, Wasatch Photonics) to introduce anomalous dispersion. The negatively chirped pulse was re-coupled into another 1-meter-long PM-SMF of the same type to deliver the femtosecond pulse to the probe. With the anomalous dispersion provided by the grating pair balanced by the sum of the normal dispersion from the system (both fiber and probe), the pulse delivery system could deliver ~100 fs pulses up to 2nJ of pulse energy.

The nonlinear signal from the rigid endoscopic probe was collected by a multi-mode fiber with an 800 μm core diameter (FT800EMT, Thorlabs), and then delivered to the signal detection module (Figure 50). In the detection module, the signal from the multi-mode fiber was first collimated, then fed through an emission filter (FF02-694/SP-25, Semrock), and finally directed to a photomultiplier tube (H10771-40P, Hamamatsu) for detection. The fiber launch, the grating pair and the detection unit were mounted on an 18’×12’ breadboard with all-fiber-optic connections for easy integration with a portable femtosecond laser in the future.

5.4 Animal preparation for in vivo imaging

5.4.1 In vivo imaging protocol

For kidney and small intestinal imaging, we adopted the same imaging protocol as previously published. For kidney imaging, a small dorsal incision was created on an isoflurane sedated mouse, through which the left kidney was exteriorized and lifted for imaging. As for the case of
small intestine imaging, a small abdominal incision was created on another isoflurane sedated mouse. From the incision, a loop of small intestine was lifted and cut open to expose the mucosa for imaging. For kidney optical biopsy in a laparoscopic fashion, similarly, an isoflurane sedated mouse was fitted with a nose cone on a feedback-controlled heating pad. With the mouse lying sideways, the position of its kidney was identified, and a tiny dorsal incision (~2 mm) was created on top of the kidney. The rigid probe was first mounted sideways on a translational stage, with the probe going through the cannula of a gauge-14 biopsy needle. The cannula was then fixed, acting as a guide allowing the probe to slide inside. Aimed at the incision, the tip of the probe was adjusted carefully to go through the incision, until reaching the surface of the kidney. In vivo subcutaneous tumor imaging was performed with the tumor secured by a spring-loaded clamp. Images were first acquired with the rigid probe held carefully against the intact skin atop the tumor, then the tumor was cut open for further imaging. In cases of severe hemorrhage, a small piece of No. 0 cover glass was fitted on top of the tissue to control the amount of bleed. For all the in vivo imaging experiments, 30-50 mW of optical power at 750 nm was used.

5.4.2 Subcutaneous tumor model preparation

The A431 (human epidermoid carcinoma) cells were first cultured in Dulbecco’s modified eagle medium (DMEM, Gibco) supplemented with 10% (v/v) of fetal bovine serum (Sigma-Aldrich) at 37°C with 5% CO₂ and a humidified atmosphere. Five NCr nude mice (male, 6-8 weeks, Taconic biosciences) were injected subcutaneously with ~3 × 10⁶ A431 cells in the right flank. 7-10 days after tumor induction (tumor reaching 5-6 mm in diameter), the mice were isoflurane-sedated and prepared for imaging.

All animal housing and experimentation procedures were performed in accordance with the standards of humane animal care described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Protocols were approved by the institutional animal care and use committee of the Johns Hopkins University.
5.5 System performance characterization

**Figure 51. System characteristics.** (A) Autocorrelation trace of the excitation beam. The FWHM of the autocorrelation trace was ~100 fs, corresponding to an ~65 fs temporal pulse width (assuming a sech^2 pulse shape). (B) Wide-field reflectance image of a USAF resolution target. (C) The point-spread-function (PSF) of the two-photon fluorescence signal measured across a 200 nm yellow-green fluorescent bead. Experimental data were fit with a Gaussian profile and the FWHM of the PSF was found to be 0.855 μm.

Several key parameters such as the temporal pulse width, optical throughput, field of view (FOV), two-photon imaging resolution and maximum frame rate were experimentally investigated to assure the handheld rigid probe was performing as designed. We first tested our fiber-based pulse delivery scheme. The excitation beam from the tip of the probe was collimated by an aspherical lens with a short focal length, and then directed to a home-built interferometric autocorrelator for pulse width measurement. With the grating pair separation carefully tuned in the dual-fiber based grating pair dispersion management setup, the shortest achievable temporal pulse width of the excitation beam was ~65 fs at 750 nm, assuming a squared hyperbolic secant pulse shape (Figure 51A). Note that the resultant pulse width was shorter than the input pulse width (120 fs, FWHM) from the Ti:sapphire laser. This was due to the nonlinear effects in the fibers, and a resultant overall spectral broadening present in the pulse delivery system\(^{123}\). At the design wavelength of 750 nm, the optical throughput of the pulse delivery system (from the laser output to the rigid probe input) was measured to be 28%, and the optical throughput of the handheld rigid probe was 45%, yielding
an overall system optical throughput of 12.6%.

The FOV of the rigid probe was validated by imaging a USAF 1951 resolution target with the imaging system configured in a wide-field reflection imaging mode (excitation filter removed and PMT replaced by a photo diode). Figure 51B shows a representative wide-field microscopy image of the group 6 element 1 (64 line pairs/mm) of the resolution target. The FOV was estimated to be ~120 μm in diameter which agreed well with the specs of the micro-objective.

The two-photon imaging resolution was measured with 200 nm sub-resolution yellow-green fluorescence beads. Figure 51C shows the point spread function (PSF) measured by the fluorescent beads and its Gaussian fitting. The FWHM of the Gaussian fitted curve was calculated to be 0.855 μm.

The maximum frame rate that yielded a reasonable image size supported by our MEMS scanner was also investigated. For high frame rate scanning applications, the MEMS should be driven in a hybrid raster scanning mode, where one axis is given a sinusoidal voltage at a frequency close to its resonance for line scan, and the other axis is given a ramp signal for it to work in a quasi-static mode for frame scan. The resonance frequency of our MEMS scanner was 1.3 kHz for both axes, and when the X-axis was driven at 1.424 kHz, imaging at 10 frames per second could be achieved with each frame consisting of 234*234 pixels.
5.6 *In vivo* depth-resolved two-photon imaging of mouse internal organs

![Image](image_url)

**Figure 52.** Two-photon autofluorescence (TPAF) images of internal organs *in vivo*. (A) Representative *in vivo* depth-resolved TPAF images of mouse kidney cortex. RT: Renal Tubules; N: Nucleus; RCI: Renal Cortical Interstitium; L: Lumen. (500*500 pixels, 5-frames-averaged with 10 μs effective pixel dwell time, ImageJ ‘green’ color map, scale bar: 20 μm) (B) Representative *in vivo* depth-resolved TPAF images of mouse small intestinal mucosa. C: Crypt; G: Goblet Cell; CE: Columnar Epithelial Cell. (500*500 pixels, 10-frames-averaged with 20 μs effective pixel dwell time, ImageJ ‘green’ color map, scale bar: 20 μm)

To demonstrate the feasibility of the probe for optical biopsy *in vivo*, we performed depth-resolved two-photon auto-fluorescence (TPAF) imaging of internal organs on live mice. Here we chose kidney and small intestine as the organs of interest, and for initial performance testing, surgeries were performed to elevate these organs from the body to suppress motion during imaging (laparoscopic imaging was also performed later without surgeries for organ elevation). A single
PMT was used to detect all the autofluorescence signals ranging from 400 nm to 720 nm, including those from intrinsic fluorophores such as NAD(P)H and FAD. Figure 52A shows some representative images of mouse kidney cortex in vivo where the renal tubule (RT) and dark nucleus (N) are evident on the surface of the cortex. As the imaging depth was tuned 24 μm below the surface, the empty lumen (L) of the renal tubule shows up. Figure 52B shows representative images at subcellular level acquired from villi of mouse small intestinal mucosa. On the surface of the mucosa, one can appreciate the tiling pattern of the columnar epithelial (CE) cells, with occasional mucus-secreting goblet (G) cells identified as dark patches surrounded by epithelial cells. Crypts (C) of the small intestinal mucosa are also visible in the same field of view. As the depth is tuned 20 μm below the surface towards the basal side, columnar epithelial cells in another orientation (with elongated columnar shape) emerge near one of the crypts.
5.7 *In vivo* two-photon imaging of subcutaneous tumor

![Figure 53. TPAF images of normal mouse skin and A431 subcutaneous tumor.](image)

We further demonstrated the probe’s capability of label-free imaging of cancerous tissue by performing TPAF imaging of A431 (human epidermoid carcinoma) subcutaneous tumor *in vivo* on a mouse model by gently placing the probe in direct contact with the intact skin and the exposed tumor core. Figure 53 shows the representative images collected by the rigid probe of the subcutaneous tumor as well as normal skin, with Figure 53A showing the keratinocytes of normal intact skin\(^{125}\), Figure 53B showing the cells of intact skin directly on top of the subcutaneous tumor, and Figure 53C-D showing the cells of the exposed tumor core. In the tumor core, cellular features
such as large nuclei, pronounced variation in cell size and shape, and disorganized cellular arrangement can be directly visualized, which are hallmarks of cancer cells\textsuperscript{126,127}.

### 5.8 Optical biopsy of internal organ

![Figure 54. Optical biopsy of mouse kidney. (A) Photo of the rigid probe based optical biopsy setup. (B)-(D) Representative depth-resolved TPAF images of mouse kidney cortex acquired during optical biopsy. RCI: Renal Cortical Interstitium; RT: Renal Tubules; L: Lumen. (234*234 pixels, unaveraged raw frames with 2 μs pixel dwell time, ImageJ ‘green’ color map, scale bar: 20 μm)](image)

The \textit{in vivo} TPAF imaging of internal organs described previously involved surgical elevation of the organ from the body for minimizing motion artifacts, which did not satisfy the \textit{in situ} requirement of optical biopsy. Here we report TPAF optical biopsy of mouse kidney \textit{in vivo} and \textit{in situ} with the rigid probe, where no surgeries were performed on the organ. Figure 54A shows the experimental setup, where imaging was performed laparoscopically on the kidney of an anesthetized mouse with the rigid probe delivered through a biopsy needle cannula. Figure 54B-D show the representative TPAF images acquired at different depths of the kidney cortex.
Microscopic structures of the kidney cortex such as renal tubules (RT), lumen (L) of the renal tubule and renal cortical interstitium (RCI) could again be visualized by the rigid probe at an imaging speed of 10 FPS, but with a reduced image quality when compared with Figure 52A. This was due to a much shorter pixel dwell time and downsampling (~2X reduction of scan line density) in the 10-FPS scanning mode.

5.9 Chapter Summary

In summary, we developed a biopsy-needle compatible varifocal two-photon handheld rigid probe for depth-resolved optical biopsy of unlabeled biological tissues in vivo and in situ. The probe could perform TPAF imaging near histopathological resolution with a good SNR owing to careful temporal pulse management for the excitation pulses, the use of a high NA objective and the excellent nonlinear light collection efficiency. The probe was compatible with the needle biopsy protocol, and its small diameter (1.75 mm) and sufficient length (15 cm) fit inside the cannula of a 14-gauge biopsy needle. The probe was capable of 3D imaging, with a 120 μm field of view and a 200 μm focus scanning range. Imaging could be performed in real time at 10 FPS with an integrated MEMS scanner. Depth-resolved TPAF imaging of healthy mouse internal organs and subcutaneous tumor in vivo were demonstrated. With our rigid probe, microscopic structures of kidney cortex and small intestinal mucosa could be clearly visualized in a label-free fashion, and tumor cellular features such as large nuclei, pronounced variation in cell size and shape, as well as disorganized cellular arrangement could be clearly resolved. In addition, depth-resolved optical biopsy of internal organ was demonstrated on mouse kidney for the first time with the organ untouched, and the rigid probe going through a biopsy needle.

5.10 Contributions

The initial design and construction of the rigid probe device were performed by Dr. Gunnsteinn Hall. The author later significantly improved the devices in imaging SNR and frame rate and built
circuitries and software for high-speed *in vivo* imaging. All the *in vivo* imaging experiments were led by the author, with the assistance from Dr. Defu Chen and Wenxuan Liang.
Chapter 6  Conclusion and future perspectives

6.1  Conclusion

In this dissertation, we explored two types of miniaturized two-photon microscopy imaging systems (fiberscope and rigid probe) for their respective applications. Chapter 2-4 covered our efforts in enabling the two-photon fiberscope imaging system for imaging of both superficial and deep neurons on freely walking mice. And Chapter 5 covered our hand-held two-photon rigid probe for optical biopsy.

In Chapter 2, the design and construction of our fiberscope imaging system were described in detail. Our fiberscope imaging system included an optoelectrical commutator (OEC) with active rotational tracking and compensation to allow the animal to walk and rotate in arbitrary patterns during two-photon imaging of neural activities. Thus, for the first time, dynamic imaging of neural activities with subcellular resolution became possible on true freely rotating/walking rodents. Our fiberscope system was also equipped with a GRISM (grating and prism) per-chirper, which improved the quality of femtosecond pulses delivered through fiber and resulted in an ~2X increment in neuroimaging signal \textit{in vivo}. In addition, this chapter also described the design and construction of our new type II fiberscope based on the composite fiber cantilever, which enabled imaging deep neurons through GRIN lenses, as well as a much larger FOV with moderate loss of resolution compared to our legacy type I fiberscope.

The next chapter (Chapter 3) detailed the methods and apparatus that enabled our two-photon fiberscope system to perform neuroimaging reliably, including the animal surgery protocol, head mounting hardware and procedure, data processing methods, and our efforts in \textit{in vivo} cerebral vasculature imaging for system performance validation. In addition, neuroimaging results collected
by both our type I and type II fiberscopes were presented, with type I fiberscope focused on high resolution imaging of somatic and dendritic calcium dynamics, and type II fiberscope focused on neuron populational imaging in a larger FOV as well imaging mPFC neurons deep in the brain.

Chapter 4 described our efforts in developing a SMA-based feedback-controlled depth scanner for fiberscope imaging. The depth scanner had a small form factor with an outer diameter of 6.5 mm. It could travel up to 490 µm in distance and could provide submicron positioning accuracy along with superior positioning stability. The SMA depth scanner greatly extended the capabilities of fiberscopes by allowing stable, depth resolved imaging in biological tissues, and could potentially be used for head-mounted neuroimaging allowing real time focal plane tunability while the imaging subject (mouse) was running around freely.

In Chapter 5, we developed a biopsy-needle compatible varifocal multiphoton handheld rigid probe for depth-resolved optical biopsy of unlabeled biological tissues in vivo and in situ. The probe could perform TPAF imaging near histopathological resolution with a good SNR. It was compatible with the 14-gauge biopsy needle protocol, with a 1.75-mm outer diameter and 15-cm probe length. The probe was also capable of 3D imaging at a maximum speed of 10 FPS, with a 120 µm field of view and a 200 µm focus scanning range. With our rigid probe, depth-resolved optical biopsy of internal organ was demonstrated on mouse kidney for the first time through a biopsy needle.

In conclusion, with the engineering efforts described in Chapter 2-4, two-photon neuroimaging on freely behaving mice could be performed repeatedly and reliably with our fiberscope imaging system. This gives the research community a powerful imaging tool with unparalleled resolution and SNR (compared to miniature single photon fluorescence microscope) for fundamental neuroscience studies. The rigid probe described in Chapter 5 also extended application of two-photon microscopy to clinics, giving the clinicians an optical biopsy tool that do not require tissue excision, alternative to the conventional needle biopsy protocol.
6.2 Future perspectives: fiberscope

6.2.1 Imaging wider and faster

Imaging wider and faster has always been the pursuit of future developments in nonlinear microscopy for neuroimaging in vivo. However, for any point-scanning imaging modalities, there is tradeoff between FOV and imaging speed (frame rate), a bigger FOV would usually come at a loss of imaging frame rate. In addition, higher imaging speed would also come at the cost of imaging SNR, as the pixel dwell time shortens with increase of frame rate. So is true for our two-photon fiberscope technology. In Chapter 3, type II fiberscope imaging of motor cortical neurons was reported with 300 μm FOV and 3 FPS speed, which was indeed a compromise between FOV, frame rate, and imaging SNR.

Nevertheless, a few strategies are available to overcome the tradeoff. The decrease of SNR at high frame rate can be mitigated by using newer generations of GCaMPs such as jGCaMP7 series, where a ~3X improvement in signal strength was reported compared to the current GCaMP6 series\textsuperscript{128}. This would allow a ~3X improvement in scanning speed (resonance frequency) or FOV while maintaining the current signal strength. The tradeoff between FOV and frame rate can also be mitigated by radial down-sampling. Testing with a high-speed type I fiberscope had shown that 26 FPS speed could be realized at 150 μm at 8X radial down-sampling (64 circles/frame) when imaging GCaMP6 neurons\textsuperscript{129}.

By combing the above strategies, one can conclude that an ~500 μm FOV could be imaged at 26 FPS by the type I fiberscope (not considering the objective/scanning size limit). Given the type II fiberscope had reduced collection NA of 0.5, which would lead to a 63% loss in the solid angle of fluorescence detection (as compared to the 0.8NA micro-objective). To maintain the same signal strength, the type II fiberscope could operate at a reduced frame rate of 26*(1-0.63)=9.6 FPS with an ~500 μm FOV.
6.2.2 Chronic imaging and dual probe imaging

An important feature for chronic neuroscience study is the capability to image the same neurons over multiple days. This would require the fiberscope to have a repeatable and detachable head mounting mechanism. Such feature could be realized by modifying the head mounting adapter (HMA) and adding a detachable baseplate (DB). Alignment pins and locking screws should be added as guides for alignment and locking the DB with the HMA. After the 1st time fiberscope imaging, with the HMA + DB cemented to the head restraining bar (HRB) of the mouse, the fiberscope + HMA could be detached from the DB (which is cemented to the HRB), then re-attached to the DB hopefully with high precision and repeatability in the next imaging session. In this way, our fiberscope imaging system could be used for day-to-day imaging of the same neurons repeatedly.

Since our fiberscope had a small outer diameter of 3 mm, it occupies a very small footprint on the head of the mouse. Thus, mounting two fiberscopes on the same head of mouse became possible. This would allow simultaneous multi-regional brain imaging on freely behaving mice, and example applications would include (but not limited to): M1+M1, V1+V1, M1+V1 multi-regional imaging on freely behaving mice. In addition, since our type II fiberscope was capable of imaging deep neurons through GRIN lens implants, multi-regional imaging such as V1+CA1 imaging could be imagined as well. This would require a redesign of our HMA with reduced footprint, and also a modified animal surgeries protocol for multi-regional AAV injection and glass window/GRIN lens implantations. In addition, a two-channel OEC would be needed to actively unwind the two fiberscopes mounted on the head of a same mouse.

6.3 Future perspectives: rigid probe

The rigid probe reported in Chapter 5 only had one signal detection channel and imaged combined auto-fluorescence of the tissue. In the future, more detection channels could be easily added to the
rigid probe imaging system, potentially allowing applications such as high-resolution metabolic functional imaging by simultaneous NADH and FAD detection\textsuperscript{70}, as well as evaluation of breast cancer malignancy by SHG imaging during needle biopsy\textsuperscript{130}, and preterm birth risk assessment\textsuperscript{67}. In addition, nonlinear microscopy techniques such as coherent anti-stock Raman scattering (CARS)\textsuperscript{131}, three-photon excited fluorescence (3PEF)\textsuperscript{132}, and third harmonic generation (THG)\textsuperscript{133} could also be integrated in the rigid probe, which would introduce more imaging contrast, and potentially improve the diagnostic outcome of optical biopsy.
Appendix A: ZEMAX (ZPL) script for dispersion calculations

PI=4*ATAN(1)
C=3e8
WL=0.92
!WL=WAVL(1)
DWL=0.001
FOR J=1,4,1
    WAVL(J)=WL+(J-3)*DWL+0.0005
!WAVL(1)=WL+(J-3)*DWL+0.0005
RAYTRACE 0,0,0,0,J
!RAYTRACE 0,0,0,0,1
VEC1(J)=2*PI*1e3*OPTH(NSUR())/WAVL(J)
!VEC1(J)=2*PI*1e3*OPTH(NSUR())/WAVL(1)
VEC2(J)=2*PI*0.3/WAVL(J)
!VEC2(J)=2*PI*0.3/WAVL(1)
PRINT "Pathlength=", OPTH(NSUR())
PRINT "Phase=", VEC1(J)
NEXT
!WAVL(1)=WL
A$=5DATE()
PRINT "ZEMAX GVD","",A$
PRINT "Wavelength Frequency"
PRINT WL," ", (VEC2(2)+VEC2(3))/2
FOR J=1,3,1
    VEC3(J)=VEC2(J+1)-VEC2(J)
!PRINT "DOmega=", VEC3(J)
!PRINT "DPhi=",VEC1(J+1)-VEC1(J)
VEC4(J)=(VEC1(J+1)-VEC1(J))/VEC3(J)
!PRINT "Tau=",VEC4(J)
NEXT
!PRINT "Tau(fs)"
!PRINT VEC4(1)," ", VEC4(2)," ", VEC4(3)
FOR J=1,2,1
    VEC4(J+3)=2*(VEC4(J+1) - VEC4(J))/VEC3(J)
NEXT
!PRINT "GVD(fsec)^2 TOD(fsec)^3 TOD/GVD(fsec)"
GVD=(VEC4(5)+VEC4(4))/2
TOD=(VEC4(5) - VEC4(4))/VEC3(2)
OPTRETURN 0=GVD
OPTRETURN 1=TOD/GVD
Appendix B: OEC microcontroller firmware

(Arduino C)

```
#include <Encoder.h>
#include <EEPROM.h>
#define RESOLUTION 8192.0
#define CTRL_LOOP 10

enum MODE {FB,JOG};
MODE mode=JOG;
int pwmPin=2;
int adcPin=0;
int dirPin=23;
int enaPin=24;
int pulseRemain=0;
int current=0;
int target=0;
int limit=1760;
int dir=0;
int intCnt=0;
String inString = "";
String cmd="";
int phaseAPin=20;//green wire
int phaseBPin=21;//gray - wire
Encoder enc(phaseAPin,phaseBPin);
int encCnt=0;
float curAngle=0.0;
float angleRange=2;
int ledPin=53;
int ledSuppPin=52;
int monitorPin=40;
int loopCnt=0;

void pulse(int pin){
digitalWrite(pin,HIGH);
delayMicroseconds(50);
digitalWrite(pin,LOW);
}

void setup() {
    pinMode(pwmPin,OUTPUT);
    pinMode(enaPin,OUTPUT);
    pinMode(ledPin,OUTPUT);
    pinMode(ledSuppPin,INPUT);
    pinMode(monitorPin,OUTPUT);
    digitalWrite(dirPin,HIGH);
    digitalWrite(enaPin,LOW);
    Serial.begin(115200);
    while(!Serial){;}
    // initialize timer1
    noInterrupts();      // disable all interrupts
    TCCR1A = 0;
    TCCR1B = 0;
    TCNT1  = 0;
    OCR1A = 63;          // compare match register 16MHz/256/1000Hz
    TCCR1B |= (1 << WGM12);   // CTC mode
    TCCR1B |= (1 << CS12);    // 256 prescaler
    TIMSK1 |= (1 << OCIE1A); // enable timer compare interrupt
    interrupts();            // enable all interrupts
    current=(EEPROM.read(0)<<8)|EEPROM.read(1);
    target=current;
    enc.write((EEPROM.read(2)<<8)|EEPROM.read(3));
}
ISR(TIMER1_COMPA_vect)     // timer compare interrupt service routine
{
    digitalWrite(monitorPin,HIGH);
    switch (mode){
        case FB:
            digitalWrite(ledPin,HIGH);
            if (curAngle>angleRange)
            {
                digitalWrite(dirPin,HIGH);
                current--;
            }
```

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pulse(pwmPin);

if (curAngle<-angleRange)
{
    digitalWrite(dirPin,LOW);
    current++;
pulse(pwmPin);
}
break;
case JOG:
digitalWrite(ledPin,LOW);
if (current!=target)
{
    if (current<target){
        digitalWrite(dirPin,HIGH);
        current++;
    }
    if (current>target){
        digitalWrite(dirPin,LOW);
        current--;
    }
pulse(pwmPin);
    if (current==target)
    {
        Serial.print(current);Serial.println(" OK");
    }
    break;
}
if (loopCnt==CTRL_LOOP)
{
    encCnt=enc.read();
    curAngle=float(encCnt)/RESOLUTION*360.0;
    loopCnt=0;
    Serial.println(current);
    //Serial.print("n");
} else loopCnt++;
digitalWrite(monitorPin,LOW);

void loop() {
while (Serial.available() > 0) {
    int inChar = Serial.read();
    if (isAscii(inChar)) inString +=
(char)inChar;
if (inChar == "n") {
    cmd=inString+"0";
inString = "";
if (cmd[0] == 'Q')
    {
        Serial.print(current);
        Serial.print("t");
        Serial.println(encCnt);
        cmd="";
    }
if (cmd[0] == 'J')
    {
        String jogCount=cmd.substring(1);
        if (jogCount.length()>0)
        target=current+jogCount.toInt();
else target=current;
mode=JOG;
    cmd="";
    }
if (cmd[0] == 'F')
    {
        enc.write(0);
        mode=FB;
        cmd="";
    }
if (cmd[0] == 'S')
    {
        EEPROM.write(0,current>>8);
        EEPROM.write(1,current&0xFF);
        EEPROM.write(2,encCnt>>8);
        EEPROM.write(3,encCnt&0xFF);
        cmd="";
    }
if (cmd[0] == 'R')
    {
        EEPROM.write(0,0);
        EEPROM.write(1,0);
        EEPROM.write(2,0);
        EEPROM.write(3,0);
current=0;
target=0;
enc.write(0);
cmd="";
    }
}
}
Appendix C: Optical autocorrelation

Intensity autocorrelation

Figure 55. Intensity autocorrelation. (A) Schematic of the intensity autocorrelation. (B) Phase matching condition in the nonlinear crystal for intensity autocorrelation. \( k_n \) and \( n_n \) are wave vectors and refractive indices of the crystal each beam sees. (C) Photo of the output from an intensity autocorrelator. M: Mirror; BS: Beam Splitter; L: Lens; XTAL: Nonlinear Crystal; SHG: Second Harmonic Generation; SFG: Sum Frequency Generation.

\[
(k_1 n_1 + k_2 n_2) \cos \alpha = k_3 n_3
\]

\[
k_1 = k_2 = \frac{1}{2} k_3
\]
As shown in Figure 55A, an intensity autocorrelator is a modified Michaelson interferometer. In one of the arms, the beam is displaced with a pair of mirrors, resulting in two parallel beams at the output. The two parallel beams are focused by a lens (L) into a nonlinear crystal (XTAL). The nonlinear interaction of the two beams in the crystal generates a 3rd beam with doubled the wavelength via the sum frequency generation (SFG) process. The time-averaged intensity of the SFG beam as a function of the delay (\(\tau\)) is known as the intensity autocorrelation of the optical pulse.

The complex electric field of the input beam \(E(t) = A(t)e^{j\omega t}\) has intensity \(I(t) = |E(t)|^2\), for beam 1 and 2, we have:

\[
I_1(t) = \frac{1}{4} I(t), \quad \text{Eq. 16}
\]

\[
I_2(t) = \frac{1}{4} I(t - \tau). \quad \text{Eq. 17}
\]

The intensity of the SFG beam \(I_{SFG}(t)\) is directly proportional to the intensities of the two input beams (since two beams are coming in at oblique angles, and the SFG beam is generated in another direction, E-fields do not add up directly for nonlinear interaction and phase matching):\(^{134}\)

\[
I_{SFG}(t) \propto I_1(t)I_2(t) \propto I(t)I(t - \tau). \quad \text{Eq. 18}
\]

The signal detected by a realistic detector \(I_M\) is the time-averaging of the \(I_{SFG}(t)\), as a function of the time delay \(\tau\):

\[
I_M(\tau) \propto \int_{-\infty}^{+\infty} I(t)I(t - \tau)dt. \quad \text{Eq. 19}
\]

The expression shown in Eq. 19 is the exact mathematical autocorrelation of input intensity \(I(t)\), and by varying the delay \(\tau\) in the autocorrelator and record the SFG signal intensity at each \(\tau\), one can measure the intensity autocorrelation of a pulse. The full width half maximum (FWHM)
of the autocorrelation function can be used to infer the actual pulse width. For the $sech^2$ pulse profile, its actual pulse width (FWHM) is $\sim 0.65$ times of the FWHM of its autocorrelation function.

The intensity autocorrelator can give direct measurement of the autocorrelation function of the pulse profile. However, it is rather difficult to align, as the SFG signal will only be generated when the crystal is at its appropriate phase matching angle, and the time delay $\tau$ is zero. Experimentally, this is a blind two-dimensional parametric search, which can take hours to complete. In addition, the misalignment of beam 1 and 2, as well as the aberrations in lens (L) will further decrease the signal strength, making the alignment process even harder.
Interferometric autocorrelation

An alternative approach is interferometric autocorrelation, with colinearly aligned beams.

![Interferometric autocorrelation diagram](image)

\[ k_1 n_1 + k_2 n_2 = k_3 n_3 \]
\[ k_1 = k_2 = \frac{1}{2} k_3 \]

**Figure 56. Interferometric autocorrelation.** (A) Schematic of interferometric autocorrelation. (B) Phase matching condition in the nonlinear crystal for interferometric autocorrelation. \( k_n \) and \( n_n \) are wave vectors and refractive indices of the crystal each beam sees. M: Mirror; BS: Beam Splitter; L: Lens; XTAL: Nonlinear Crystal; SHG: Second Harmonic Generation; CF: Color Filter.

**Figure 56A** shows the schematic of an interferometric autocorrelator, instead of using two parallel beams in the ‘cross’ configuration, the interferometric autocorrelator is a Michaelson interferometer plus a nonlinear crystal for second harmonic generation (SHG). The combined beams from the interferometer are focused by a lens (L) into the crystal, and then a SHG signal is
A color filter (CF) is used to filter out the longer wavelength, and the SHG signal is then picked up by a detector for measurement.

Here, at the crystal, the electrical fields of beam 1 and 2 are added up coherently, and the intensity of SHG beam $I_{SHG}(t)$ is\textsuperscript{135}:

$$I_{SHG}(t) \propto \left| (E_1(t) + E_2(t))^2 \right|^2 = \left| (E(t) + E(t - \tau))^2 \right|^2.$$  \hspace{1cm} \text{Eq. 20}

The signal detected by a realistic detector $I_M$ is the time-averaging of the $I_{SFG}(t)$, as a function of the time delay $\tau$:

$$I_M(\tau) \propto \int_{-\infty}^{+\infty} \left| (E(t) + E(t - \tau))^2 \right|^2 dt.$$ \hspace{1cm} \text{Eq. 21}

Plug in $E(t) = A(t)e^{j\omega t}$ we have:

$$I_M(\tau) \propto \int_{-\infty}^{+\infty} \left| (E(t) + E(t - \tau))^2 \right|^2 dt$$

$$= \int_{-\infty}^{+\infty} \left| (A(t)e^{j\omega t} + A(t - \tau)e^{j\omega(t-\tau)})^2 \right|^2 dt$$

$$= I_{bg} + I_{int}(\tau) + I_{\omega}(\tau) + I_{2\omega}(\tau),$$

where $I_{bg}$ is a constant background term, $I_{int}(\tau)$ is the intensity autocorrelation term, $I_{\omega}(\tau)$ is the oscillating term with frequency $\omega$, and $I_{2\omega}(\tau)$ is the oscillating term with frequency $2\omega$:

$$I_{bg} = \int_{-\infty}^{+\infty} |A(t - \tau)|^4 + |A(\tau)|^4 dt = 2 \int_{-\infty}^{+\infty} I(t)^2 dt,$$ 

\hspace{1cm} \text{Eq. 23}

$$I_{int}(\tau) = \int_{-\infty}^{+\infty} 4|A(t - \tau)|^2 |A(t)|^2 dt = 4 \int_{-\infty}^{+\infty} I(t)I(t - \tau) dt,$$ \hspace{1cm} \text{Eq. 24}

$$I_{\omega}(\tau) = 4 \int_{-\infty}^{+\infty} Re[(I(t) + I(t - \tau))A^*(t)A(t - \tau)e^{-j\omega \tau}]dt,$$ \hspace{1cm} \text{Eq. 25}

$$I_{2\omega}(\tau) = 2 \int_{-\infty}^{+\infty} Re[A^2(t)(A^*(t - \tau))^2 e^{-2j\omega \tau}]dt.$$ \hspace{1cm} \text{Eq. 26}
At $\tau = 0$, the function maximizes:

$$I_{bg} + I_{int}(\tau) + I_\omega(\tau) + I_{2\omega}(\tau) = (2 + 4 + 8 + 2) \int_{-\infty}^{+\infty} I^2(t) dt.$$  \hspace{1cm} \text{Eq. 27}

When $\tau \to \infty$, the function is at its background value:

$$I_{bg} + I_{int}(\tau) + I_\omega(\tau) + I_{2\omega}(\tau) = 2 \int_{-\infty}^{+\infty} I^2(t) dt.$$  \hspace{1cm} \text{Eq. 28}

The function also has a minimum value 0. The peak to background ratio of interferometric autocorrelation is $(2 + 4 + 8 + 2):2 = 8:1$ (a perfect 50:50 power splitting is assumed).

For a chirp-free pulse, the FWHM of the envelope of the interferometric autocorrelation trace can be used to derive the pulse width, in the same way as the intensity autocorrelation (where $sech^2$ pulse profile has a factor of 0.65). Compared to intensity autocorrelation, interferometric autocorrelation has a constant background term $I_{bg}$. This makes the alignment process much easier as the optimal crystal angle $\theta$ can be found by directly maximizing the background SHG signal, without the need to simultaneously match the time delay $\tau$. And once the optimal crystal angle $\theta$ is found, the delay $\tau$ can be found by scanning the interferometer arm and monitoring the detector output. Owing to its ease of alignment, interferometric autocorrelation remains a popular tool for femtosecond pulse characterization, although its measurement results can be confounded by pulse chirp.

Interestingly, the background term $I_{bg}$ of interferometric autocorrelation is exactly proportional to the two-photon fluorescence yield term, where the two-photon time-averaged fluorescence $F$ is:

$$F \propto \int_{-\infty}^{+\infty} I^2(t) dt \propto I_{bg}.$$  \hspace{1cm} \text{Eq. 29}

This made interferometric autocorrelator a useful tool for optimizing the grating/GRISM separation of our two-photon fiberscope imaging systems.
Home-built interferometric autocorrelator

Figure 57. Home-built interferometric autocorrelator. CC: Corner Cube; BS: Beam Splitter; LS: Loudspeaker; BBO: Beta Barium Borate Crystal; CF: Color Filter; CM: Curved Mirror.

Figure 57 shows the picture of our home-built interferometric autocorrelator. Here, two corner cubes (CC, also known as hollow retroreflectors) were used in place of mirrors to further ease the alignment processes. It also relaxed the stability requirement for the scanning motion, allowing a loudspeaker (LS) to be used for delay scanning. A curved mirror (CM) was utilized instead of a regular lens for focusing the beam into the crystal, which minimized the built-in dispersion of the autocorrelator, and made the focal position in the crystal wavelength independent. The nonlinear crystal was made of beta barium borate (BBO). For measuring laser input power of >100 mW, a Si photodetector could be used as the detector. And for low power applications, such as measuring the pulse widths from the fiberscope tip, PMTs were typically used. The SHG signal was sampled by NI DAQ cards using MATLAB, and FWHM of the signal envelope was derived to find the
pulse width. In this setup, due to the orientation of the BBO crystal, p-polarized beam (parallel to the table) was required as input to the autocorrelator.
Bibliography


