CODED COMPUTATIONAL ILLUMINATION AND DETECTION FOR THREE-DIMENSIONAL FLUORESCENCE MICROSCOPY

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Samuel J. Yang
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I certify that I have read this dissertation and that, in my opinion, it is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy.

Karl Deisseroth, Primary Adviser

Mark Horowitz

Gordon Wetzstein

Approved for the Stanford University Committee on Graduate Studies.

Patricia J. Gumport, Vice Provost for Graduate Education

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Abstract

In vivo calcium imaging enables the optical monitoring of neural activity at the level of individual neurons in real time, necessitating the development of high speed, three-dimensional (3D) fluorescence microscopy techniques with at least single-neuron spatial resolution. Because a typical widefield microscope intrinsically produces only two-dimensional images, various illumination and detection coding strategies have been implemented to address the challenge of 3D fluorescence microscopy, utilizing either precisely structured and temporally scanned illumination patterns, such as in two-photon laser scanning microscopy or coding of the emission, as in light field microscopy, respectively. However, many single-focal illumination coding strategies have limited acquisition speeds, while detection-coding-only strategies requiring computational reconstruction of the 3D volume are limited by optical aberrations of the tissue.

We present a 3D calcium imaging approach utilizing both multifocal scanned two-photon laser excitation for illumination coding and detection coding with the light field microscopy approach suitable for in vivo mammalian calcium imaging. A holographic 3D multifocal illumination pattern is targeted only towards pre-localized neurons avoiding the unnecessary illumination of other regions. The resulting fluorescence emission is coded and detected on an image sensor and deconvolution is used to recover the neural activity at each site. We present the design and optimization of such an imaging strategy, and validate the approach with experimental measurements. Finally, we demonstrate the application of this approach to in vivo mouse calcium imaging.
The design and implementation of another technique, frame-projected independent fiber photometry, enabling the optical recording and control of neural activity in freely moving mammals with region-level spatial resolution, is presented in a dedicated chapter as well, including simultaneous recording from multiple brain regions in a mouse during social behavior, two-color activity recording, and optical optogenetic stimulation eliciting dynamics matching naturally observed patterns.
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Chapter 1

Introduction

1.1 Scope and thesis organization

This dissertation describes two projects, first a frame-projected independent fiber photometry method, enabling region-level recording and optical manipulation of neural activity for freely moving animals, described exclusively in Chapter 3, and second, the development of a high speed, three-dimensional (3D) optical neuroimaging technique suitable for \textit{in vivo} imaging applications, described in all other chapters.

Most fluorescence microscopy techniques have historically been focused on two-dimensional (2D) imaging applications \cite{18}, but recent developments in optical neuroimaging applications, such as calcium imaging \cite{10}, have created the need for high speed, three-dimensional volume fluorescence microscopy methods for the optical recording of neural activity with single-neuron spatial resolution. As fluorescence imaging is a process which requires optical excitation at a particular range of wavelengths to produce fluorescence emission, typically at another range of wavelengths, most fluorescence microscopy techniques can be categorized into either illumination-coded or detection-coded sampling strategies, with various trade-offs and limitations in spatial and temporal resolution, field of view, depth of field and penetration depth in scattering tissue.

Recently, hybrid strategies involving both illumination and detection coding have been explored, but with several limitations. For example, in \cite{69, 68}, the particular
detection coding strategy requires imaging a 2D projection of a 3D volume, and is only suitable for sparse volumes where no two sources share the same \((x,y)\)-coordinate. Furthermore, limitations in the illumination strategies for fluorescence excitation included a limited field of view and requirement for much higher laser power, making the technique less scalable for recording from much larger neural populations. Finally, such joint illumination and detection coding techniques have not previously been adapted for \textit{in vivo} mammalian imaging, which poses its own unique set of challenges related to optical inhomogeneities.

We propose a hybrid computational illumination and detection coding strategy that overcomes these limitations and demonstrate its application for \textit{in vivo} mouse calcium imaging. Our illumination coding strategy utilizes a modified variant of the computer generated holography approach from [69], enabling both a larger field of view as well as the use of lower laser excitation powers. For the detection coding, we adapt the light field microscopy approach from [8] for \textit{in vivo} mouse imaging, enabling the resolution of more densely distributed sources, including those differing only by their \(z\)-coordinate. Finally, we demonstrate the application of the entire system for \textit{in vivo} mouse imaging.

This dissertation is organized as follows:

- Chapter 3 describes the design, implementation and characterization of frame-projected independent fiber photometry method, a method for region-level resolution optical recording and manipulation (via calcium imaging and optogenetics) of neural activity in freely moving mammals. The remaining chapters concern a different project and application, high speed 3D optical microscopy targeting single-neuron resolution optical recording in head-fixed but awake mammals.

- In Section 1.2 we review calcium imaging, the particular application motivating the need for 3D high speed optical microscopy techniques, and in Chapter 2 we review existing strategies for 3D high speed optical neuroimaging, categorized into illumination or detection coded methods.
• In Chapter 4 we describe the theory behind computer generated holography approaches for 3D illumination, including its use with two-photon excitation, and in Chapter 5 we describe our optimizations for increasing the field of view, reducing amount of laser power required and maximizing two-photon fluorescence excitation.

• Chapter 6 describes our detection coding strategy, including the adaptation of light field microscopy for this particular \textit{in vivo} imaging application.

• Chapter 7 describes our application of this system for \textit{in vivo} imaging and Chapter 8 concludes with a discussion on limitations and outlook to the future.

1.2 Calcium imaging

High speed, 3D fluorescence microscopy is an essential tool in cellular biology [9], developmental biology [42] and recently for neuroscience calcium imaging applications [29]. Calcium imaging of neuronal activity for \textit{in vivo} applications is a particularly challenging emerging application, requiring both biochemical tools for the calcium indicator, a suitable \textit{in vivo} imaging preparation and optical imaging tools compatible for \textit{in vivo} imaging.

In calcium imaging, optical imaging techniques are used to measure the change in concentration of calcium ions in neurons due to the firing of action potentials, fundamental electrical signals used to propagate information across neural circuits [29]. There are two classes of approaches which enable using optical fluorescence to measure the concentration of calcium ions: dye-based and protein-based approaches. These calcium imaging approaches may be applied for imaging neural activity in several different (non-human) brain tissue samples, ranging from \textit{in vitro} imaging applications, where neurons are cultured (grown) in a mostly 2D lab environment (e.g. a Petri dish), to slice preparations, where a thin 2D slice of brain tissue is removed from a recently-alive animal for acute imaging (short-term, several hours), to \textit{in vivo} applications, where neural activity in the brain of a live animal (either anesthetized, awake and head-fixed, or in the most challenging case, awake and freely moving) can
be monitored chronically (long-term, over months or years).

In a dye-based calcium imaging approach [49], a fluorescent dye with an absorbance spectrum that depends on the amount of bound calcium is distributed in bulk across a volume of neurons. The fluorescence of the dye will then be modulated by the local concentration of bound calcium. However, one limitation of this approach is that chronic imaging is difficult, requiring repeated access to the neurons for application of the dye solution. Another more fundamental limitation is that it is not possible to localize the dye to specific cell types or regions, a limitation similar to that for electrophysiological recordings of neural activity.

The second preferred approach, protein-based calcium indicators, involves expressing calcium-dependent fluorescent proteins in the neurons of interest. These genetically encoded calcium indicators (GECIs) [57] can be implemented using a viral injection to a particular brain region, or through the use of transgenic animals. Unlike with dye-based calcium imaging approaches, GECIs can be localized to specific cell types or regions through genetic targeting tools, and are suitable for chronic imaging. With transgenic animal approaches, there is no need to even utilize a physical injection to localize the virus or calcium indicator itself, as the calcium indicator is genetically defined.

With either the dye-based or protein-based approach for targeting the calcium indicator to the sample of interest, calcium imaging requires high speed fluorescence microscopy techniques for exciting fluorescence in the neurons, and subsequently reading out the fluorescence, a process repeated many times during a recording procedure lasting several minutes or longer. While the underlying electrical activity of the neural circuits occurs at the millisecond time-scale, the measurement of calcium activity is limited to a slower time scale. For example, the particular GECI used for the work in this dissertation, GCaMP6m, has a rise time of 0.2s and a decay time of 0.6s, necessitating a sample rate of approximately 10Hz [10].

While achieving such sample rates for 2D imaging is possible with a variety of fluorescence microscopy techniques, the extension to 3D imaging poses a challenge. Unfortunately, while most in vitro or in slice calcium imaging applications involve a mostly 2D sample, the neural circuits imaged in in vivo applications are fundamentally
Finally, one of the major applications of calcium imaging tools is enabling the observation of neural activity in various animal models of human-relevant phenomena such as learning, memory or socializing behaviors. Though tools exist for high speed 3D imaging of in vivo neural activity in simpler model organisms such as the C. elegans or larval zebrafish [2], to be able to correlate neural activity with a complex animal behavior, more advanced mammalian animal models, such as the mouse [81], need be used. In addition, while studying correlations in local neural circuits may require sampling of only a modest number of neurons, it is likely that complex behavioral phenomena will require optical microscopy tools for in vivo recording of large volumes of neurons. Furthermore, these imaging technologies need to be compatible with the use of optogenetics as well [7, 27, 99], to enable further investigation of the function of neural circuits beyond merely observing correlations.

This dissertation focuses on identifying and overcoming limitations of high speed 3D calcium imaging microscopy techniques, particularly for the application of in vivo mouse calcium imaging.
Chapter 2

Related Work

Various 2D and 3D fluorescence microscopy techniques have been adapted or developed particularly for high speed calcium imaging applications, both in vitro and in vivo. As discussed previously in Section 1.2, fluorescence imaging requires both excitation of fluorescence using an illumination light source of some kind, as well as detection of the resulting fluorescence emission.

In this chapter, these various microscopy techniques will be reviewed and categorized based on whether they employ illumination or detection coding strategies – that is, whether they rely on using precise spatially or temporally defined illumination patterns for imaging (e.g. point scanning approaches) or on spatially resolved detection (e.g. camera imaging approaches).

2.1 Uncoded methods

As a point of comparison for illumination and detection coded imaging methods discussed later, we consider here the uncoded imaging case.

2.1.1 Widefield microscopy

We first consider widefield microscopy and its application for 3D calcium imaging [70]. Widefield fluorescence microscopy utilizes an epifluorescence illumination system to
excite fluorescence across the entire 3D field of view, and images the resulting fluorescence emission across the 3D sample into a single camera exposure. The recorded light in this camera exposure consists of two components, an in focus image of the sample at the focal plane of the microscope, along with the rest of the light from out of focus objects. The depth range over which objects are in focus, termed depth of field, can be extended by reducing the numerical aperture of the imaging system, but only at the expense of total light collection efficiency, and as such, widefield microscopy is primarily a 2D imaging system, as illustrated in Figure 2.3. In order to record a 3D volume, one must translate either the sample or the imaging system across the \( z \)-dimension and acquire multiple 2D images.

This strategy has two major limitations. First, inertial scanning to achieve 3D imaging with a 2D imaging system limits the speed at which entire volumes can be imaged to typically a rate of \( 1 - 2 \text{Hz} \). Second, to image a single 2D plane, one must excite fluorescence across the entire 3D volume, the majority of which is imaged as out of focus light on the image sensor. Though 3D widefield deconvolution techniques [58] may be applied to remove out-of-focus light from each image, there still may exist substantial photon shot noise from the background and the sample is also subjected to repeated unnecessary light exposure, which may result in photo damage or photobleaching [80].

## 2.2 Illumination coded methods

The fluorescence microscopy techniques described in this section all utilize illumination coding strategies involving shaping a laser wavefront to produce precise spatially localized fluorescence excitation, with the goal of overcoming the previously described limitations of widefield microscopy.

### 2.2.1 Confocal microscopy

The fundamental idea behind confocal microscopy is the use of two optically conjugated pinholes to spatially confine both the excitation and emission light, enabling the
2D raster scanning acquisition of the same 2D image as in widefield microscopy but with very little light contribution from out-of-focus objects, a phenomena described as “optical sectioning” [94]. In the simplest implementation, a single laser is focused to a diffraction-limited spot through a pinhole to excite fluorescence, and the emission is also spatially filtered through a pinhole before being detected. Confocal microscopy is perhaps the first microscopy method practically applicable to 3D imaging given its optical sectioning capabilities, though its imaging rate is even slower than that of widefield microscopy given the need to raster scan a single excitation focal point.

Though confocal microscopy achieves optical sectioning, the 2D acquisition rate is limited by the scanning time. Even multifocal confocal microscopy approaches typically implemented with a spinning disk pinhole array with higher 2D image acquisition rates have volume acquisition rates limited by the inertial scanning used to implement 3D imaging [56].

Another advantage of confocal microscopy over widefield microscopy that is not necessarily useful for in vivo calcium but quite useful for live cell imaging is that structured-illumination approaches for doubling the lateral spatial resolution are possible [77, 4]. These approaches take advantage of the fact that in a linear imaging system, as with fluorescence microscopy, the total point spread function of the imaging system is a product of the detection and illumination point spread functions (corresponding to a convolution in Fourier space). In the case where the two point spread functions are identical, the Fourier space convolution doubles the frequency support compared with a widefield microscope. Another equivalent way of viewing this phenomena is that illuminating the sample with a sinuisoidal spatial pattern at the spatial frequency limit of the main lens aliases high frequency information down to the lower frequency optical pass band, often denoted as the Moiré effect. By employing the use of multiple known illumination patterns and capturing and processing an image sequence, these approaches enable a doubling of the spatial resolution.

Confocal microscopy has successfully been applied for in vivo calcium imaging, but in drosophila [12]. This is primarily related to the fact that confocal microscopy still utilizes one-photon excitation and is subject to its limitations, as will be discussed next.
2.2.2 Two-photon laser scanning microscopy

Two-photon laser scanning microscopy involves raster scanning a single laser focus, but with two-photon fluorescence excitation in place of the one-photon excitation used in confocal microscopy, as shown in Figure 2.1. Pioneered by [18], the technique relies on the precise spatial confinement of light from the smaller two-photon excitation focal volume, so the technique does not require an emission pinhole as in confocal microscopy, and the total emission is instead directly detected, typically by a photomultiplier tube (fast single-pixel detector).

The principle of two-photon excitation is described in more detail in Section 4.2, but briefly, fluorescence excitation is no longer proportional to laser intensity as it is in one-photon excitation, but it is instead proportional to the square of the laser intensity, which results in a much more spatially confined excitation volume. In order to achieve the photon flux necessary for two-photon excitation, most approaches employ pulsed laser excitation with ~100 fs duration pulses with ~100MHz repetition rates in place of the continuous wave lasers used in confocal and light sheet microscopy.

Another important advantage for two-photon microscopy is that for most in vivo imaging applications, the penetration depth of the longer wavelength infrared sources enables light to be focused deeper in tissue without tissue absorption or scattering [35]. This unique and fortunate property of most biological tissue samples, in combination with the plethora of high quality visible-spectra fluorescent proteins, has enabled the use of two-photon microscopy in many in vivo deep tissue imaging applications.

Despite the deep penetration depth in tissue and precise spatial confinement of the illumination, two-photon laser scanning microscopy also suffers from slow 3D volume acquisition speeds due to the requirement for inertial scanning in the z-dimension. Recently, new approaches for parallel multifocal two-photon laser scanning microscopy have been proposed but have yet to be demonstrated for larger 3D volumes [11, 97, 44].

2.2.3 Random access two-photon scanning microscopy

Random access two-photon scanning microscopy is an adaptation of two-photon laser scanning microscopy for faster sampling of sparse 3D volumes [41]. Rather than raster
Figure 2.1: In two-photon laser scanning microscopy, where the 3D cubic volume consisting of neurons (gray ellipses) is being imaged, a precise single two-photon laser focus (red dot) is raster scanned (red arrows) across 2D or 3D one voxel at a time, where the emission is recorded with a photomultiplier tube (effectively a fast single-pixel camera), illustrated as the green square. Fluorescence emission only originates from neurons when they are excited by the laser, as illustrated by the green ellipse. At no point in time during the 3D scan is fluorescence excited in any of the deeper neurons, the primary advantage of two-photon microscopy.
scan an entire 3D volume using a single laser focus, here, a single laser focus is used to access a random, pre-defined subset of the entire 3D volume.

In order to achieve faster scan rates, the traditional galvanometer mirrors used for lateral scanning in two-photon laser scanning microscopy are replaced with even faster acousto-optic deflectors [74]. Importantly, the primary limit for 3D imaging in two-photon laser scanning microscopy, the scanning of the detection objective, is overcome with the use of a pair of acousto-optic deflectors instead [71].

While this technique has seen success in 3D in vivo calcium imaging applications, sampling 532 neurons at 56 Hz [41] in the mouse brain, some challenges remain for scaling the technique. First, the number of points that can be sampled in a given time period will be limited by a typical 80 MHz laser to 80 million points per second. After that limit, one will be limited by fluorophore saturation, photo damage, and the lifetime of the fluorescent protein, typically several nanoseconds [11]. Hence, as we will see, it is advantageous to consider multifocal illumination approaches with parallel detection.

### 2.2.4 Light sheet microscopy

Light sheet microscopy is unique among all of the methods discussed here in that it utilizes two orthogonal microscope objectives, rather than just one, one to deliver a thin sheet of excitation light, and the other to focus the excited emission onto a camera [38, 42]. The advantage of this approach, compared to widefield microscopy, is that to image any given 2D plane, fluorescence only within that plane need be excited, eliminating excess photobleaching and intrinsically enabling optical sectioning.

To enable 3D imaging, as with widefield, two-photon laser scanning, and confocal microscopy, the detection objective needs to be scanned in the z-dimension, and in addition, the light sheet needs to be scanned by a galvanometer mirror (but the illumination objective itself can remain stationary). The need for detection scanning is eliminated in a technique named SPED light sheet microscopy, discussed in Section 2.4.1.

As with confocal microscopy, it is possible to trade some acquisition speed for a
factor of two better spatial resolution compared with widefield microscopy by using structured illumination microscopy approaches [9]. In addition, while the light sheet is typically made by scanning a Gaussian beam or using a low numerical aperture cylindrical lens, Bessel beam approaches for structured illumination have been used to improve the trade-off between sheet thickness and field of view inherent in using Gaussian beams [65].

Overall, light sheet microscopy appears to be the tool of choice for both cellular and developmental biologists looking to do fast, high spatial resolution 3D volume imaging [86, 9]. For calcium imaging applications in neuroscience, light sheet microscopy is also well suited for imaging the larval zebrafish brain, where there exists convenient optical access from the side and the entire brain conveniently fits within the field of view of the microscope [2, 89, 87].

However, the traditional dual-objective light sheet microscope format does not work for mammalian brain imaging, where access only from the top is available. Recent work with oblique light sheet [20, 6] attempts to overcome this by both illuminating a sheet and imaging the orthogonal plane through the same high numerical aperture microscope objective. However, for in vivo mammalian imaging applications, scattering of the fluorescence emission in the tissue degrades resolution and limits penetration depth.

### 2.3 Detection coded methods

The methods in this section rely on widefield fluorescence excitation instead of structured laser illumination, utilizing various detection coding strategies to enable high speed 3D imaging instead.

One limitation inherent to these detection-coding only methods is that of the missing cone problem [33], where there exist certain 3D spatial frequencies that are unresolvable without the use of a structured illumination pattern.
2.3.1 Light field microscopy

Light field microscopy is a single snapshot 3D microscopy technique pioneered in [52], developed further in [53, 8] and applied toward 3D in vivo calcium imaging in [67, 13, 87]. This method utilizes a microlens array in front of the camera to code the 3D fluorescence emission onto the camera sensor in a single exposure. Utilizing an optical model consisting of the point spread function of each voxel in 3D, deconvolution methods may be applied to reconstruct a 3D volume from each camera image.

While the approach enables single snapshot 3D imaging, as illustrated in Figure 2.2, the extra speed is obtained at the expense of spatial resolution. In addition, the quality of the deconvolution results depends on the accuracy of the optical model, particularly for deep tissue imaging applications. More details on light field microscopy are given in Chapter 6.

Recently, a compressive light field microscopy approach has been proposed which may overcome many of these limitations [64].

2.3.2 Multi-focus microscopy

Multi-focus microscopy is a single snapshot 3D microscopy technique utilizing a custom optical element that enables images focused at different z-depths to be recorded at different spatial locations on the same image sensor [1]. This technique is similar to light field microscopy in that this detection coding strategy records an entire 3D volume in a single camera image. Also, there is a trade-off between the 2D field of view and spatial resolution, given the image sensor now needs to sample a 3D volume. However, unlike in light field microscopy, deconvolution isn’t required as each z-depth is focused to a different part of the image sensor.

Similar to 3D widefield deconvolution microscopy, out-of-focus light creates a background in each image though. In addition, the use of widefield detection will limit the penetration depth of the imaging method as well.
Figure 2.2: In light field microscopy, widefield fluorescence excitation (blue hourglass shape) is used to excite an entire 3D volume, from which the emission is coded and detected within a single camera exposure. Neurons of with varying levels of fluorescence emission are denoted in ellipses of various shades of green, and the total emission detected by the image sensor is a linear sum.
Figure 2.3: Widefield microscopy (no detection coding) versus light field microscopy (detection coding with a microlens array). In a widefield microscope, moving a resolution target away from the microscope leads to defocus, whereas the light field microscope enables imaging with reduced lateral spatial resolution across a range of z-depths.
2.4 Illumination and detection coded methods

The methods in this section utilize a combination of the illumination and detection coding strategies described previously.

2.4.1 SPED light sheet microscopy

As discussed in Section 2.2.4, one of the main limitations of the speed of volume acquisition in light sheet microscopy techniques is the inertial scanning of the detection objective. Recent work in applying extended depth of field microscopy approaches may remove the need to scan the detective objective though, enabling faster 3D volume imaging rates, in a technique named SPED light sheet microscopy [87]. In this approach, the light sheet serves as the coded illumination, exciting fluorescence within a selected 2D plane. While in a conventional light sheet microscope, the fluorescence emission is directly imaged by an orthogonal objective optomechanically focused to the illuminated plane, with SPED, the fluorescence emission is instead coded through a high refractive index block of media which induces spherical aberration and elongates the detection point spread function.

As a result, in SPED, the application of a detection coding scheme to extend the image depth of field eliminates the need to scan the detective objective to achieve 3D imaging. Given that the light sheet itself is easily scanned at much higher rates than the camera acquisition, SPED microscopy’s volume acquisition rate now becomes camera-limited, rather than inertial-scanning-limited. Hence, for whole zebrafish brain calcium imaging applications, volume acquisition rates of up to $12Hz$ are possible, up from $1 - 3Hz$ previously for inertial-scanning-limited light sheet microscopy approaches [87].

One limitation though is that computational deconvolution techniques must now be applied in order to produce the final image. Also, unfortunately, the other limitations of light sheet microscopy still persist, namely, the requirement for sample access on two sides for two orthogonal microscope objectives.
2.4.2 Multi-site two-photon microscopy

Multi-site two-photon microscopy utilizes simultaneous two-photon multifocal illumination of a predefined pattern of locations, where the emission is detected on a fast camera without any scanning required [60]. This technique has been extended to 3D for calcium imaging [69, 68], leveraging the use of an extended depth of field detection coding strategy to enable the simultaneous capture of a 2D projection across the 3D illuminated volume, as illustrated in Figure 2.4.

The multifocal illumination pattern targeting multiple sites in 3D simultaneously is achieved through the use of computer generated holography, where a phase spatial light modulator implements a phase hologram which directs the excitation light to the desired multiple focal volumes. Discussed further in Chapter 4, one of the major limitations of these computer generated holography illumination approaches is the trade-off between field of view and spatial resolution given by the limited pixel count of commercially available phase spatial light modulators.

Another important limitation of this two-photon computer generated holographic illumination technique is that it is often laser power limited since the two-photon-excited signal at each simultaneous site decreases quadratically instead of linearly with the total number of sites, as in one-photon illumination.

Finally, the particular detection coding strategy used in [69, 68] employs a cubic phase mask at the pupil plane of the imaging system, enabling the camera to acquire data to reconstruct a 2D projection across the 3D volume, rather than an image at the in-focus plane. While enabling scanless acquisition across the 3D volume, one limitation of this detection coding technique is that two locations differing only in their z-coordinate but sharing the same (x,y)-coordinates will necessarily be projected to the same measurement on the camera sensor, and has such, the success of this technique depends on sparsity of the 3D volume.
Figure 2.4: Multi-site two-photon microscopy uses a multifocal two-photon excitation pattern (red dots) to excite fluorescence in a subset of all the voxels in the 3D field of view, and detection coding is used to record the emission within a single camera exposure. The green ellipses indicated fluorescence excited in targeted neurons while no fluorescence is excited in the deeper neurons (gray ellipses).
2.5 Comparison of methods

The various 3D fluorescence microscopy methods discussed previously are compared in Table 2.1. The various criteria include:

- **inertial z-scanning** - Methods that do not require internal z-scanning to achieve 3D imaging are able to achieve higher volume acquisition rates.

- **sparse 3D volume sampling** - While most methods densely sample the 3D volume, some methods are capable of sparse, random access sampling of a predefined set of locations within the 3D volume. Such 3D random access approaches are well suited for calcium imaging where the locations of neurons are fixed and can be predetermined.

- **three-dimensional (3D) resolvability** - All techniques with the exception of the multi-site two-photon imaging approach [69, 68], which relies on capturing a 2D projection of a 3D volume, can resolve points arbitrarily distributed in 3D up to their respective resolution limits.

- **two-photon (2P) penetration depth** - As discussed previously, the near-infrared wavelengths of two-photon excitation penetrate tissue with less scattering and absorption, enabling deeper imaging in tissue.

- **top access** - With the exception of the light sheet microscopy approaches which require both top and side access for two orthogonal microscope objectives, all other techniques compared here require top access only. Side access is possible for *in vivo* zebrafish whole brain calcium imaging, but not for *in vivo* mouse imaging.

- **application to *in vivo* imaging** - While many technologies could in principle be applied for *in vivo* calcium imaging in mouse, only a fraction have been, likely due to the unique set of challenges involved for *in vivo* mouse calcium imaging.

- **parallel readout** - Many techniques require sequential readout of signal using a single scanned laser focus, which will be fundamentally limited by laser
repetition rates or fluorophore lifetimes as discussed in Section 2.2.3. As such, techniques which are compatible with parallel excitation or readout are more scalable.

From Table 2.1, it is clear that the illumination coding strategies utilizing two-photon excitation have been the preferred method for \textit{in vivo} mouse imaging due to the better penetration depth of two-photon illumination. However, the lack of parallel readout opportunities will constrain the ultimate scalability of these sequential scanning techniques.

On the other hand, most detection-coding techniques with fast parallel camera readout have not been successfully applied for \textit{in vivo} mouse imaging, likely due to the lack of penetration depth.

One notable candidate technology that attempts to combine the best of both two-photon scanning illumination approaches with camera-based detection-coding techniques is the multi-site two-photon approach from \cite{69, 68}. However, as discussed in Section 2.4.2, this approach is unable to resolve two sources differing only in their $z$-position, and furthermore, has not yet been successfully applied for \textit{in vivo} mouse calcium imaging.

This dissertation extends the work in \cite{69, 68}, overcoming several limitations of the technology to make it suitable for \textit{in vivo} mouse calcium imaging.

2.6 Contributions

This dissertation makes the following contributions:

1. Design of a time-multiplexed scanning strategy for holographic two-photon illumination systems that overcomes two important limitations, field of view and laser power. In our system, the field of view is no longer limited by the pixel count of the phase spatial light modulator used to implement the computer generated holograms. In addition, by replacing simultaneous illumination with time-sequential multi-site illumination, we facilitate the use of lower laser powers for two-photon excitation.
Table 2.1: Comparison of various 3D fluorescence microscopy methods for calcium imaging

<table>
<thead>
<tr>
<th>method</th>
<th>no z-scanning</th>
<th>sparse sampling</th>
<th>3D resolvability</th>
<th>2P penetration depth</th>
<th>top access</th>
<th>in vivo</th>
<th>parallel readout</th>
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<td>widefield</td>
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<td>multi-site two-photon</td>
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<td>scanned multi-site two-photon (proposed)</td>
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For details on comparison criteria, see Section 2.5.
2. Implementation of a joint coded illumination and coded detection 3D calcium imaging method, using the multi-site time-division multiplexed illumination strategy in conjunction with a light field microscope. We discuss several additional design considerations related to implementation and present experimental results validating the coded illumination strategy described previously.

3. Application of this joint coding strategy toward \textit{in vivo} calcium imaging. Our approach leverages the extended field of view and lower laser illumination power requirements described previously, and represents the first application of a joint illumination and detection coding approach for \textit{in vivo} mouse imaging.

4. A second project distinct from the main focus of the dissertation, the design and implementation of a frame-projected independent fiber photometry combined recording and stimulation method.

The detection coding strategy using the light field microscope was first analyzed and applied toward \textit{in vivo} calcium imaging in a zebrafish in two papers published in Optics Express in 2013 and 2014 [8, 13]. The optimized illumination coding strategy was implemented in a paper published in Optics Express in 2015 [96], which included an initial application of 3D \textit{in vivo} mouse calcium imaging using the joint illumination and detection coding strategy. This dissertation expands on those works, describing additional considerations necessary and results for combining illumination and detection coding for 3D \textit{in vivo} mouse calcium imaging. Finally, the second project described in Chapter 3 was published in Nature Methods in 2016 [43].
Chapter 3

Frame-projected fiber photometry

This chapter describes a method of recording neural signals which is compatible with freely moving mammals and combines optogenetic stimulation and measurement. Supporting mobility does constrain resolution, so this system provides much lower resolution than the method in the following chapters. The work presented here is from [43], and this chapter stands independent of all of the other chapters in this dissertation.

3.1 Fiber photometry

The use of multi-unit electrical recordings enables the recording of electrical activity from neurons in various brain regions, but activity is sampled non-specifically from any active adjacent cells. Optical recording techniques leveraging calcium imaging, and in particular, genetically encoded calcium indicators, enable the cellular-level optical readout of activity from cell-type or projection specific neurons, enabling recording from a subset of a total set of neurons in a given recording volume.

However, many of these optical calcium imaging techniques require the animal be head-fixed, and of those that don’t and are compatible with freely-moving preparations, the designs, including head-mounted microscopes [24] and fiber bundles [21, 82], are often difficult to scale for recording from multiple regions due to physical constraints.
Fiber photometry is a technique that trades single-cell spatial resolution for a lightweight and flexible setup enabling region-level spatial resolution for completely freely moving imaging preparations [55, 76, 15, 31, 14, 51, 51, 98]. Here, a single optical fiber is implanted in a single brain region, and fluorescence is excited in the entire region through a tethered optical fiber, and the emission is detected on a single photodetector, enabling the fast, real-time readout of neural activity in a single brain region.

### 3.1.1 Contributions

This chapter of this dissertation describes a new more scalable approach for extending fiber photometry to recording from multiple brain regions, with the following contributions, described in [43]:

1. A new easy-to-use and scalable approach for simultaneously sampling activity from multiple implanted optical fibers using a fast sCMOS camera is designed and implemented.

2. A time-division multiplexing approach for multi-color and simultaneous optogenetic stimulation enables the combination of optical recording with optogenetic stimulation.

3. A method for quantifying signal crosstalk is presented and applied in the context of eliciting a naturally observed pattern of activity using artificial optical neural stimulation.

### 3.2 Multi-fiber recording

The work presented in this chapter builds primarily on the work from [31], where the goal is to scale the technique to record from more than one brain region simultaneously, in the same animal.

There existed several limitations of the previous approach:
Previously, a lock-in amplifier system was required to achieve sufficient detection sensitivity.

Optical alignment required focusing light from a several hundred micron diameter fiber onto a photodetector with area less than 1mm, a difficult task to achieve, resulting in reduced sensitivity or reliability in practice.

Simply scaling this approach would require as many lock-in amplifiers and precise photodetector alignments as the product of the number of regions times the number of color channels, making it costly and time-consuming to scale.

To overcome these limitations, we propose a new strategy whereby a fast sCMOS camera is used to image the bundle of fibers, in a strategy termed frame-projected independent-fiber photometry (FIP). This strategy has the following advantages:

- Optical alignment is now straightforward, as the camera image gives a clear indication of how to align the bundle of fibers, and there is no ambiguity about whether all of the fiber emission is being detected.

- The equipment and time cost for having many regions and channels is now similar to that of having a single channel, providing for a highly scalable approach.

As shown in Figure 3.1 from [43], we can record from up to at least seven brain regions using this approach, with the data shown in Figure 3.2 from [43]. In our implementation, we can also use an image splitter to enable dual color imaging, on the same camera, shown in Figure 3.3 from [43].

The final question, though, was whether the sCMOS camera was as sensitive as the previous photodetector with lock-in amplifier. To test this, we implemented a second setup, shown in Figure 3.4 from [43], where we could directly compare the sensitivity of these two approaches by recording neural activity and using a 50 : 50 beamsplitter to direct an equal amount of light to each half of the setup to directly compare these approaches. To accommodate the lock-in amplifier's requirement for time-modulated excitation light, we modulated the light source, though we note the FIP approach does not require, nor does it benefit from, having time-modulated...
Figure 3.1: Optical layout for multi-fiber frame-projected independent-fiber photometry (FIP) setup. A fast sCMOS camera is used to image a bundle of fibers, each implanted in a different brain region.

Figure 3.2: FIP calcium recordings from 7 brain regions simultaneously in a single awake, freely moving mouse.
excitation light. As illustrated in Figure 3.4, the signal to noise ratio (SNR) of the FIP based approach is actually higher, and the magnitude of the recorded neural activity is similar between the two approaches.

3.3 Time-division multiplexing for multi-channel illumination and acquisition

In order to enable multi-channel illumination and acquisition, we used a time-division multiplexing strategy, where we alternated various illumination sources. Compared with modulating excitation at different frequencies using a lock-in approach [31], the advantage of this approach is that there is absolutely no cross-contamination of detected signal, as each channel is sampled in an entirely different time bin, as illustrated in Figure 3.5 from [43]. However, crosstalk from the excitation still poses a challenge, particularly for combined optogenetic stimulation and recording, which will be considered next.
Figure 3.4: Head to head comparison of FIP with the previous lock-in detection approach from [31]. (a) The optical setup uses a 50 : 50 beamsplitter to simultaneously direct light to each detection method. (b) Raw signals detected simultaneously with each approach suggest the FIP camera approach (c) has higher signal to noise ratio (SNR) and (d) both approaches detect a signal of the same magnitude.
3.4 Characterizing crosstalk in optogenetic stimulation and recording

The combination of calcium imaging with optogenetic stimulation enables the joint observation and control of neural activity, but often with several challenges related to excitation crosstalk [30]. Here, the problem is that the excitation spectra of the calcium indicator and the opsin (protein for optogenetic stimulation) are always substantially nonzero across the same range of wavelengths, making it difficult to select an excitation wavelength that selectively targets one but not the other.

This is particularly a problem when the excitation light source meant for simply observing neural activity is strong enough to actually excite the opsin. Unfortunately, the severity of this effect will depend on a lot of factors, such as the particular calcium indicator and opsin used, the corresponding expression levels, and the choice of excitation wavelengths. Here, we propose a strategy for quantifying the magnitude of this excitation crosstalk.
We again use the time-division multiplexing strategy, but for the excitation illumination channel, we use another light source identical to the imaging excitation light source, shown at the bottom of Figure 3.5, except we can modulate the power and observe the magnitude of the neural response to increasingly bright exposures. As expected, we see that with sufficient power, we do see a sizable response, despite the illumination being at a sub-optimal part of the excitation spectra of the particular opsin used in this experiment.

From this experiment, a low enough imaging power can be selected that minimizes crosstalk. We can then employ the setup described in Figure 3.6 from [43] in order to combine both optical recording and optogenetic stimulation. The results are presented in Figure 3.7 from [43], where we are able to adjust the duration and intensity of the artificial optogenetic stimulation to elicit a neural response of similar magnitude to that observed in response to natural stimuli, a water reward.

3.5 Discussion

One limitation of the FIP approach presented here is that it is not immediately possible to have independent control of the illumination light sources for each fiber. This functionality would be important if the density of neurons or expression level varies
CHAPTER 3. FRAME-PROJECTED FIBER PHOTOMETRY

Figure 3.7: FIP-recorded, naturally observed response to a water reward and artificially elicited neural responses to orange and blue light with simultaneous calcium recording and optogenetic stimulation. The low power blue imaging light contributes little detectable excitation crosstalk, while the power of the orange excitation light can be adjusted to elicit a natural-looking neural response.

from region to region and the imaging excitation power needed to be adjusted accordingly, or for enabling independent optogenetic stimulation in all fibers. Some solutions may include optically positioning an independent excitation light source for each channel, or to perhaps use a digital-micromirror device (DMD) spatial light modulator as a fast shutter for modulating the illumination of each fiber independently.

In addition, the use of faster and more sensitive cameras in the future combined with new probes such as genetically encoded voltage indicators will enable the sampling of neural activity at faster rates.

As it stands, the proposed approach enables the simultaneous parallel measurement of calcium activity across multiple distant brain regions, multicolor recording, and simultaneous recording and optogenetic stimulation. Despite only having region-level spatial resolution, the ease of use of the technique and compatibility with freely moving animal preparations makes it a powerful tool, particularly when combined with the high-speed 3D microscopy tools discussed in the remainder of this dissertation.
Chapter 4

Computer generated holography

Of the many possible illumination strategies, we consider in this work the use of computer generated holography. Compared to the use of amplitude-based illumination modulation [53, 82] implemented with digital micromirror devices, the advantages of computer generated holographic approaches include light transmission efficiency and straightforward compatibility for 3D illumination. Holography is the use of a phase element to focus collimated light from a laser to a defined 2D or 3D intensity distribution [26]. Recent computer generated holography techniques employ a programmable pixelated phase modulator to achieve the phase modulation, enabling these phase holograms to be computed and updated in real time [54]. This chapter explores the optical image formation model for computer generated holography, and considers the special case where two-photon excitation is also used.

4.1 Optical model

In a coherent illumination system, the complex wavefront at any plane perpendicular to the optical axis defines the resulting illumination at any other point later in the optical path. It is useful to characterize and modulate the complex electric field at one location in particular along the optical axis, the pupil or aperture plane of the final lens of the system, where there exists a simple Fourier relationship between the field at this plane, $H(u, v)$ and the field at the object plane after the lens, $U(x, y)$,
where \( U(x, y) = \mathcal{F}\{H(u, v)\} \) [26].

For 3D holographic illumination applications, we are interested in controlling \( H(u, v) \) to get the desired resulting object space intensity, \( I(x, y, z) = |U(x, y, z)|^2 \), typically a multifocal pattern, where the \( z \)-dependence has been introduced. The complex valued \( H(u, v) \) can be written as the product of an amplitude and phase term:

\[
H(u, v) = A(u, v) \exp(i\phi(u, v))
\]

In holographic illumination systems with phase-only modulation capabilities, \( A(u, v) \) is constrained by the fixed laser amplitude, typically approximated to be uniform or Gaussian, and the goal is to find a suitable phase profile, \( \phi(u, v) \).

If the phase-only modulator is a liquid crystal SLM with a full \( 0 - 2\pi \) modulation range at each of the \( N \times N \) pixels, then for a given 2D or 3D intensity pattern, algorithms exist to determine an appropriate \( \phi(u, v) = \phi_{\text{SLM}}(u, v) \) for a given \( I(x, y) \) [79, 54, 50, 78, 45], including the Gerchberg-Saxton algorithm presented in Appendix A. Figure 4.1 illustrates some examples of SLM phase values and various illumination patterns.

### 4.2 Two-photon excitation

In two-photon illumination strategies, the simultaneous absorption of two longer wavelength photons is used for excitation in place of a single shorter wavelength photon. As such, the signal no longer scales linearly with illumination power, but instead depends quadratically on the power. For pulsed lasers, the time-averaged two-photon signal rate is given by [95, 37] as

\[
R \propto \frac{P^2}{\tau f}.
\]  

(4.1)

Here, \( P \) is the average power, \( \tau \) is the pulse duration and \( f \) is the repetition rate. Hence, the fluorescence excitation now scales as \( I(x, y)^2 \).
Figure 4.1: Schematic of examples of computer generated holographic illumination patterns. The upper left panel illustrates a laser focusing to a single focal point at the focal plane when the SLM simply has a flat phase (equivalent to not having an SLM), where $\phi(u, v) = c$, where $c$ is a constant. In a beam steering example, a linear phase implemented on the SLM, $\phi(u, v) = c_1u + c_2v$ produces a lateral shift in the focal plane. In the beam focusing example, a quadratic phase, $\phi(u, v) = c(u^2 + v^2)$, produces an axial shift. Nearly arbitrary multifocal 3D illumination patterns can be produced using $\phi(u, v)$ computed from the phase retrieval algorithms described in Section 4.1. For the SLM phases, white-to-black represents $0 - 2\pi$. 
Figure 4.2: Images captured via macro photography illustrating fluorescence excited in a fluorescent dye solution in a cuvette using one photon widefield and two-photon multifocal SLM illumination. Though the apparent size of the two-photon illumination (two focal spots produced by the SLM) is larger than the actual size given the resolution limit of the macro photography lens, one can still appreciate from this demonstration the optical sectioning capabilities afforded by two-photon illumination approaches.

In particular, SLMs are increasingly used with infrared femtosecond pulsed lasers to implement patterned two-photon excitation for either multisite neuronal imaging [60, 69, 68] or multisite photoactivation [61, 63, 62], to either record or manipulate neural activity, using calcium indicators or light sensitive opsins, respectively. These two-photon approaches afford deeper tissue penetration, crucial for in vivo applications, but are often laser power limited since the signal at each simultaneous site decreases quadratically instead of linearly with the total number of sites.

Figure 4.2 illustrates the greater spatial confinement of light possible with two-photon excitation compared to one-photon widefield illumination. In the following chapter, we will consider strategies for optimizing two-photon holographic illumination to overcome these limitations.
Chapter 5

Optimizing holographic illumination

5.1 Extending field of view

While computer generated holography enables generating multifocal illumination patterns, two constraints limit the practical applicability of this technique. First, the accessible field of view is directly limited by the SLM hardware, and second, in two-photon applications, the total two-photon signal decreases linearly with the number of simultaneously illuminated sites. This chapter discusses optimizations made to extend the field of view and increase two-photon signal in practical computer generated holographic two-photon illumination implementations.

5.1.1 Limitations of spatial light modulators

As described in Chapter 4, typically phase spatial light modulators (SLMs) are used to implement computer generated holographic illumination strategies. However, the area over which light can be directed, termed field of view by [25], is fundamentally constrained by the resolution of the SLM. When the SLM image is optically magnified (e.g. by a 4f telescope) to exactly fill the back aperture of the main lens (to simultaneously maximize spatial resolution and light transmission), the number of
pixels, \( N \), then critically determines the maximum slope of the linear phase that can be represented without aliasing, which limits the maximal lateral shift accordingly to [25]:

\[
x_{\text{max}} = y_{\text{max}} = \frac{\lambda}{4NA} N.
\]

Here \( \lambda \) is wavelength, \( NA \) is the numerical aperture and \( N = L/d \), where \( L \) is the lateral dimension of the SLM and \( d \) is the SLM pixel pitch. The field of view (FOV) is then the lateral area spanning \( 2x_{\text{max}} \times 2y_{\text{max}} \) that the SLM can focus light to. Note that the intensity of the steered light decreases away from the center of the field of view as well within that region. Typical of values of \( N \) include \( N = 256 \) and \( N = 512 \), corresponding to the \( 256 \times 256 \) and \( 512 \times 512 \) phase SLMs used in the work in this dissertation. These values lead to a field of view of \( 140 \mu m \) and \( 280 \mu m \) wide, which is small compared with typical camera or laser scanning based fields of views at \( 500 + \mu m \).

### 5.1.2 Adding galvanometer mirrors

In many situations, the field of view accessible from the resolution of existing SLMs is not sufficient to cover the entire field of view of the main lens, as illustrated in Figure 5.1(a).

To address this, we propose using a pair of galvanometer scanning mirrors, imaged at a conjugate plane, to superimpose an additional linear phase term:

\[
\phi_{\text{galvos}}(u, v : \Delta x \Delta y) = c(u\Delta x + v\Delta y).
\]

Here \( c \) is a constant that depends on the wavelength and lens parameters. This linear phase term laterally shifts the SLM field of view by \( (\Delta x, \Delta y) \); by successively applying various lateral shifts over time, one can direct the smaller SLM field of view over a much larger region, as shown in Figure 5.1(b). The concept of using scanning mirrors to implement a linear phase function, which results in a lateral shift
Figure 5.1: Illustration of time-division multiplexing strategy for laterally extending the SLM field of view (FOV) from a conventional holographic illumination system (a) to our proposed approach (b), where a pair of galvanometer mirrors at a conjugate pupil plane enable lateral time-sequential scanning of one of 9 different holograms to each of 9 regions. Drawings are to scale for the Nikon 16× 0.8 NA objective lens (assuming 20mm field number) and 256 × 256 SLM used in our experiments.

in image space, was first proposed in [48] as a simple application of the shift theorem of Fourier transforms, but is the basis of laser scanning microscopy [3, 18]. In our proposed approach, the total phase modulation at any given point in time is simply the sum of the two:

$$\phi(u, v) = \phi_{SLM}(u, v) + \phi_{galvos}(u, v; \Delta x, \Delta y).$$

Figure 5.2 presents a schematic illustration of this approach for extending the field of view.

Though the multifocal illumination over the entire field of view is now achieved with smaller subsets illuminated sequentially instead of simultaneously, for many applications this sequential scanning is fast enough to be approximately simultaneous compared to the other relevant timescales. For example, in calcium imaging applications using the GCaMP6m calcium sensor, the 0.2s rise time and 0.6s decay time of the sensor indicate that sampling at a rate beyond 10Hz may not yield any more
Figure 5.2: Schematic illustrating the use of galvanometer scanning mirrors to superimpose an additional linear phase for scanning and extending the SLM field of view.

useful signal [10]. Indeed, many calcium imaging methods even employ single point-scanning techniques [41] where a single point is rapidly scanned across all locations sequentially.

The time it takes to scan all of the subsets in our approach is limited by the longer of the galvo positioning and SLM transition times, as illustrated in Figure 5.6. Fortunately, in a typical multiphoton microscope, the galvo positioning time is quite short, at less than 1 ms [32], so our approach enables scanning of the hologram at a rate limited primarily by the time it takes for the SLM to change the hologram. [47] explores in detail the positioning times and characteristics of galvanometer-based scanning systems.

Though other 3D single point-scanning approaches have achieved 3D scanning with much shorter 16.8 μs transition times using acousto-optic deflectors [41] leading to the ability to sequentially sample 53,400 voxels per second, slower multifocal simultaneous or scanned strategies may still be advantageous if there exist physical limitations to how much signal can be produced from excitation with a single femtosecond laser pulse (with a 80 MHz repetition rate laser, there are only 1300 laser
pulses within 16.8µs), such as fluorophore saturation or photodamage [40]. Hence our ability to generate multifocal illumination patterns over a larger field of view by using galvanometer mirrors to sequentially scan the smaller SLM field of view over a larger area may be particularly useful in these fluorophore-signal limited regimes.

5.1.3 Experimental setup

We implemented our time-division multiplexing illumination approach on a microscope equipped with a SLM-based illumination system and galvanometer scanning mirrors, as illustrated in Figure 5.3. We additionally incorporated a single-snapshot 3D imaging system (light field microscope) and a single-beam laser scanning two-photon imaging system to enable quantification of our two-photon excitation efficiency and 3D in vivo calcium imaging. See Appendix B for more information.

5.1.4 Experimental results

We analyzed 3D-reconstructed volumes [8] of a fluorescent dye solution illuminated with and without an extended field of view. In each case, the same 324 focal points in a $420\mu m \times 420\mu m \times 200\mu m$ volume were illuminated within a single camera exposure of $T = 100ms$ using $36mW$ (at the sample) to avoid saturation of the dye and camera. In Figure 5.4(a), we illuminated all focal points simultaneously with the galvanometer mirrors in a fixed position. The lateral extent of the $xy$-maximum projection after applying a 5% intensity threshold was $140\mu m$, matching the theoretical value of $140\mu m$ from Equation 5.1.

In Figure 5.4(b) we used time-division multiplexing of 9 SLM fields of view with 9 galvanometer positions to extend the field of view to $380\mu m$, an increase in area of 7.4 times. In addition, it should be possible to address the spatially varying diffraction efficiency [73] visible in Figure 5.4(a) by choosing the time-sequentially scanned fields of view to overlap further.

These experimental results suggest that our time-division multiplexing approach is suitable for extending the field of view in holographic illumination applications beyond the limit imposed by the pixel count of the phase SLM used to realize the
Figure 5.3: Optical layout. The SLM, illuminated by a 920 nm femtosecond laser through a beam expander (BE), is imaged through lenses (L1, L2) and zero-order beam block (ZB) to the midpoint between two galvanometer scanning mirrors, and then to the objective pupil plane through a scan lens (SL), tube lens (TL) and short-pass dichroic mirror (D). Excited fluorescence filtered by an emission filter (F) is captured by either a photomultiplier tube (PMT) or light field microscope (LFM) with sCMOS camera, enabling single-snapshot 3D visualization of the excited fluorescence.
Figure 5.4: Experimentally measured increase in SLM field of view during a $T = 100\, ms$ exposure. Measured 3D illumination patterns in a fluorescent dye solution (maximum intensity projections shown) illustrate the field of view increase, denoted by dashed lines, from (a) $140\, \mu m \times 140\, \mu m$ without, to (b) $380\, \mu m \times 380\, \mu m$ with, time-sequential galvanometer scanning.

illumination. One difference with our strategy, compared to one involving a higher pixel count SLM, is that instead of illuminating all sites simultaneously, only the sites within a small field of view are illuminated at any given point in time. Though this is a disadvantage when truly simultaneous illumination is required, it is actually advantageous for two-photon excitation, as we will discuss next.

### 5.2 Increasing two-photon signal

This section discusses optimizations made to increase two-photon signal in practical computer generated holographic two-photon illumination approaches.
5.2.1 Time-division multiplexing

In two-photon excitation, signal rate depends quadratically on the average laser power $P$ \[95\] so the time-averaged signal integrated over a total period of exposure $T$ is

$$S \propto P^2T. \quad (5.2)$$

For an illumination pattern that divides the average laser power equally to $n$ excitation sites with equal focal volumes, the total time-averaged signal from the $n$ volumes is

$$S_{M=1} \propto n \left( \frac{P}{n} \right)^2 T, \quad (5.3)$$

which is a factor $n$ times smaller than Equation 5.2.

However, if we relax the requirement for simultaneity of the illumination, and allow for $M$ sets of $\frac{n}{M}$ sites to be sequentially excited over the duration of the exposure time $T$, the total time-averaged signal is

$$S(M) \propto n \left( \frac{PM}{N} \right)^2 \left( \frac{T}{M} - t_{SLM} \right), \quad (5.4)$$

where $t_{SLM}$ is the time it takes the SLM to change the illumination pattern between each set of spots. This is greater than Equation 5.3 by a factor of

$$Z(M : t_{SLM}) = M \left( 1 - M \frac{t_{SLM}}{T} \right). \quad (5.5)$$

For a given $t_{SLM}$, Equation 5.5 takes a maximum value of $\frac{M}{2}$ when $M = \frac{T}{2t_{SLM}}$. Hence if the laser power is held fixed and the SLM transition time $t_{SLM}$ is known, we can determine the optimum number of subsets to scan in order to maximize the total two-photon excited signal for a given total experimental exposure time.
5.2.2 Experimental results

We used the previously described experimental setup (see Appendix B for more information) to experimentally validate our expected signal increase from time-division multiplexing.

We first determined our SLM transition time to be $t_{SLM} = 7.5\text{ms}$ by steering a single focal spot between two photodiodes placed just after ZB in Figure 5.3 and measuring power as a function of time, plotted as the solid line in Figure 5.5, where the surrounding shaded region illustrates one standard deviation from three measurements.

To quantify the two-photon signal gain from time-division multiplexing empirically, we restricted the illumination in a fluorescent dye solution to the same 33 focal points within the same single SLM field of view (as in Figure 5.1(a)), to control for excitation and emission path vignetting effects, and measured the total detected counts on the image sensor as a function of the multiplexing factor, $M$, again with fixed laser power and camera exposure time of $T = 100\text{ms}$. A pockels cell shuttered the laser during the SLM transition periods in Figure 5.6. Measurements for each $M$ were normalized with $M = 1$, and compared with the expected results from Equation 5.5.

For $t_{SLM} = 7.5\text{ms}$ and $t_{SLM} = 10\text{ms}$, the maximum signal occurs at $M = 7$ and $M = 5$, respectively, compared with $M = 6.7$ and $M = 5$ predicted by Equation 5.5. We approximated the slower $t_{SLM} = 10\text{ms}$ SLM, by keeping the pockels cell
shuttered for this longer duration. The fact that the experimentally measured traces are slightly lower than the theoretically predicted curves might be explained by the limited precision with which we can synchronize our hardware, including the SLM, pockels cell and camera.

Finally, Equation 5.5 plotted in Figure 5.7 suggests that recent SLMs [83] with even shorter transition times may approach the limit where $t_{\text{SLM}}$ tends toward $0\text{ms}$, where the increase in signal should scale exactly linearly with the number of multiplexed subsets.

### 5.3 Additional considerations

As discussed in previous chapters, two-photon signal rate depends crucially on having a particular photon flux density in a given time. Typical commercially available femtosecond near-infrared lasers provide suitable power for single focal scanning methods such as two-photon laser scanning microscopy and random access two-photon microscopy. However, for computer generated holographic illumination applications
Figure 5.7: Theoretically computed and experimentally measured signal increase with SLM time-division multiplexing, for fixed average laser power, total number of illuminated sites and exposure time ($T = 100\text{ms}$ was used). SLMs with faster transition times will enable larger increases in total time-averaged two-photon signal with a greater number of subsets of focal sites illuminated sequentially. Error bars indicate one standard deviation.
utilizing multifocal illumination patterns, the available total laser power limits achievable signal, particularly for \textit{in vivo} imaging applications.

In addition to the time-division multiplexing approach proposed earlier, several more basic considerations from the two-photon laser scanning microscopy body of literature can be optimized for multifocal illumination with computer generated holography.

5.3.1 Laser repetition rate and pulse duration

The two-photon signal generation equation, given as Equation 4.1, highlights the important dependence on two parameters, $\tau$, the pulse duration and $f$, the repetition rate.

The pulse duration can be kept low by minimizing optical dispersion or using an adjustable pre-chirp to compensate for dispersion along the remainder of the optical path, and as a result, $\tau$ typically takes on a value of $100-200\text{fs}$.

However, the repetition rate, $f$, is a laser parameter which we have much more control over. In conventional two-photon laser scanning microscopy, lasers with $f = 80\text{MHz}$ [18] are typically used. However, lasers with repetition rates down to $f = 250\text{kHz}$, typically regenerative amplifiers, have been used to achieve more signal, particularly for deep tissue imaging applications down to depths of $1000\mu\text{m}$ and greater [84, 59].

Several multifocal two-photon imaging approaches in the past include widefield camera-based two-photon imaging approaches, where these regenerative amplifiers have been used as well [22, 16].

There are somewhat conflicting sources about whether the use of these low repetition rate laser systems induce more tissue photo damage or not. In [37], it is argued that photo damage scales nonlinearly with an even stronger power than the two-photon excitation effect, and as a result, [40] proposes a strategy for increasing the repetition rate to reduce the peak power even further to reduce photo damage. Similarly, [9] concludes that lower peak powers are advantageous for reducing photo damage, though in the context of one-photon fluorescence imaging of live cells.
However, [19] suggests the opposite, that a lower repetition rate will prevent photo damage. It may be possible to reconcile these seemingly contradictory results in the following way. It is likely that different mechanisms for photo damage may exist, and depending on the repetition rate and other parameters, possibly sample-dependent, different mechanisms of photo damage dominate. Hence, it could be that a repetition rate of $f = 80\, MHz$ is perhaps the worst repetition rate to use, and using either a higher or lower repetition rate could reduce photo damage.

Another potential concern is that peak powers that are too high (10$nJ/\mu m^2$) actually run into the realm of powers used in tissue ablation applications [91]. Importantly, one must consider the power at the focal volume, and not simply the power incident on the sample. For example, in [84], 200$nJ$ per pulse is incident at the surface of the tissue, but only 2$nJ$ per pulse remains deep down at a depth of 850$\mu m$ where the light is actually focused, as most of the power has been attenuated through the tissue.

As long as these limitations are taken into consideration when choosing to lower the repetition rate to increase the two-photon excitation efficiency, large signal gains may be realized in applications that are currently starved of laser power.

For example, in two-photon optical stimulation approaches utilizing optogenetics, where light is used to activate a light-sensitive protein rather than a fluorescent protein, many applications have barely enough laser power to demonstrate a proof of concept, let alone a scalable photo-activation technique. In [5], the limited output of Ti:Sapphire lasers operating near 80$MHz$ made it possible to stimulate at most 4 neurons (though the stimulation of only one neuron was demonstrated). The most neurons stimulated so far with single-cell precision has been ten [62]. Even in [63], where a regenerative amplifier was used, it was difficult to get enough power at the desired wavelength, and the technique was limited by laser power.

In principle, these two-photon optical neural stimulation techniques should benefit from the application of low repetition rate lasers as well, albeit with similar limitations. For example, consider the effect of laser saturation. From [66] Supplementary Figure 3, we know that for $\tau = 237\, fs$, $P = 20mW$, $f = 80\, MHz$, we appear to saturate the opsin’s photocurrent response with a dwell time of $t_{dwell} = 4\mu s$ corresponding
to 320 pulses. Let’s now consider a laser system with a repetition rate $f' = f/x$ which is a factor of $x$ times lower. Assuming the optical point spread function and other laser parameters (e.g. $\tau, P$) remain unchanged, then saturation would be achieved with only $320/x^2$ pulses. If we let $x = \sqrt{(320)}$, then we could saturate the opsin with a single laser pulse, with an effective dwell time of $t'_{dwell} = 1/f' = 0.22\mu s$, a factor of $17.9 \times$ faster than before. Assuming laser repositioning time is negligible, this suggests that using a repetition rate of $80/\sqrt{(320)} MHz$ will just barely saturate the opsin with each laser pulse and enable the scanning of $17.9 \times$ sites (e.g. neurons) within the same fixed time period.

Overall, the use of lower repetition rate pulsed lasers for optimizing multifocal two-photon excitation efficiency will become increasingly important given the move toward in vivo mammalian and to deeper imaging, both of which will require even more laser power. The success of this approach will require an awareness of various limitations, including saturation and photo damage.

### 5.3.2 Numerical aperture

In two-photon laser scanning microscopy, at the focal volume, the number of photons absorbed per fluorophore per laser pulse is given by [18]

$$n_a \approx \frac{P^2 \delta}{\tau f^2 \left( \frac{NA^2}{2hc\lambda} \right)^2}, \quad (5.6)$$

where $P$ is average laser power, $\delta$ is two-photon absorption cross section $\tau$ is the pulse duration, $f$ is the repetition rate, $NA$ is numerical aperture, $h$ is Planck’s constant, $c$ is the speed of light, $\lambda$ is wavelength.

From Equation 5.6, it is clear that the two-photon signal rate is proportional to $NA^4$, and that utilizing a high numerical aperture is a simple way to increase the signal rate. This relationship is plotted in Figure 5.8.

Increasing the numerical aperture of the system also results in an increase in detection side efficiency as well. Using $NA = n \sin(\theta)$, where $n$ is the refractive index
Figure 5.8: Normalized two-photon excitation efficiency as a function of numerical aperture.
of the immersion media, we can determine the solid angle of light captured by the lens from

$$\Omega = 2 \pi (1 - \cos(\theta)).$$  \hfill (5.7)

Figure 5.9 compares the total collected emission light as a function of numerical aperture. From this plot, it is clear that even small increases in $NA$ can yield large returns for two-photon imaging applications.

However, recent work suggests that multi-photon signal generation begins to decrease with too high of a NA when imaging through scattering tissue [93]. Hence, while having a higher collection NA will always improve efficiency, there may exist an optimal illumination NA which can be implemented by underfilling the back aperture of the microscope objective.
Chapter 6

Coded detection

While Chapters 4 and 5 concern a particular illumination coding strategy, computer generated holography, we now consider a particular detection coding strategy, light field microscopy. Though a typical widefield microscope configuration produces in focus images of a single 2D plane, and previous work described in Section 2.4.2 enabled the capture of 2D projections of a 3D volume using an extended depth of field detection coding strategy, the goal here is to identify a suitable 3D detection coding strategy for resolving arbitrary 3D points (within the spatial resolution limits of the imaging system).

We first review light field microscopy and its application in Section 6.3 for single snapshot volumetric imaging, without any illumination coding. The treatment here is from [8], a manuscript describing a wave optics model and volumetric deconvolution for the light field microscope, but with an emphasis on analysis of the 3D point spread functions suitable for 3D detection coding. Sections 6.4.1 and 6.4.2 discuss limitations of [8], limitations we sought to overcome by leveraging joint illumination and detection coding strategies.

6.1 Light field microscopy

First described in [52], light field microscopy utilizes the addition of a microlens array in between the camera and a standard widefield fluorescence microscope to enable
the coded capture and subsequent reconstruction of a 3D distribution of fluorescence.

6.1.1 Optical layout

As seen in Figure 6.1 from [8], a light field microscope consists of a microlens array placed where the image sensor would go in a typical widefield fluorescence microscope. Instead, the image sensor images the plane exactly one microlens focal length behind the microlens array. In practice, it is difficult to position the image sensor this close to the microlens array, so a 4f relay lens system can be used to image the sensor to this plane.

While the pitch of the microlens array determines the trade-off between peak lateral spatial resolution and z-range over which reasonable lateral resolution is achieved (see [8] for details), to ensure the microlenses don’t limit spatial resolution and the entire image sensor is utilized without any overlap of any two individual microlens images, the f-number of the microlens should be

\[ N = \frac{M}{2NA}. \]  

(6.1)

Here, \( M \) and \( NA \) are the magnification and numerical aperture of the microscope objective.

6.1.2 Point spread function

Compared to a widefield microscope, the point spread function of a light field microscope has several notable properties which make it a suitable 3D detection coding strategy. First, objects below or above the native focal plane of a widefield microscope will have a very diffuse and large point spread function, growing in area at a rate proportional to \( z^2 \), whereas in the light field microscope, as illustrated in Figure 6.2 from [8], while the total support of the point spread function does grow larger, the number of nonzero pixels in the point spread function appears to grow very slowly.
Figure 6.1: Light field microscopy implementation on a conventional widefield fluorescence microscope. A camera images through a 4\textit{f} relay a plane one microlens focal length behind a microlens array, which is placed at the camera port.
Figure 6.2: Light field microscopy optical setup, illustrating a point spread function for an in-focus (left) and out-of-focus (right) point source.
In the widefield microscope case, there are several problems with such point spread functions which limit 3D resolvability. First, if an in-focus point produces a point spread function at a single pixel which is nearly saturated on the image sensor, then the limited dynamic range of the camera will constrain the depth range over which it is possible to image, let alone resolve, point objects. For example, with a 30000 : 1 dynamic range of a typical sCMOS camera sensor, a point source with a point spread function exceeding $\sqrt{30000} \approx 173$ pixels wide will be undetectable on the image sensor, assuming the camera exposure is configured such that another in-focus point source of identical brightness nearly saturates the image sensor.

Another challenge with point spread functions that are distributed over more image sensor pixels is that Poisson-distributed photon shot noise will exist at every detector pixel in conjunction with the detector pixel’s read noise. Indeed, in super-resolution localization microscopy techniques, there is a well known trade-off with pixel size and various noise sources [85]. The light field microscope detection coding strategy is not immune to this problem, and though a thorough analysis has yet to be done, the roughly constant number of nonzero pixels in the PSF suggest noise should have an equal impact on objects at varying depths.

A second property of the light field microscope point spread function is that, for a well-corrected doubly telecentric imaging system, which most widefield microscopes are, the point spread function is shift-invariant for shifts equal to the pitch of the microlens array. This important property makes it feasible to determine the point spread function for any location in the volume, given only knowledge of the point spread functions for locations residing directly in front of a single microlens array. In practice, this reduces the computation and storage required for computing and evaluating the optical model by 4 orders of magnitude. In the next section, we will consider how these point spread functions maybe used to represent the image formation model.
6.2 Image formation model

This section describes the image formation model suitable for light field microscopy in fluorescence imaging applications.

6.2.1 A linear system

Implicit in the discussion about point spread functions in Section 6.1.2 is the assumption that these point spread functions accurately describe the image formation model. More precisely, the image formation model is a linear system, where the system behavior is completely described by the point spread functions, if the following assumptions hold:

- Fluorescence emission is isotropic, radiating uniformly in all directions. Note that this is in contrast to light field photography applications where the ability to capture specular highlights in the scene is an important advantage of light field approaches.

- The sample is transparent. All fluorescence emission is neither attenuated, refracted nor otherwise modulated by the sample before it is captured by the microscope.

- The fluorescence emission is incoherent. The detected image of any two point sources existing together must be the linear sum of their individual point spread functions.

Given the above assumptions, we can then explicitly model the linear image formation as

\[ f = Hg, \quad (6.2) \]

where \( g \) is the vectorized volume, with dimension \( n_{\text{voxels}} \times 1 \), \( f \) is the vectorized camera image, with dimension \( n_{\text{pixels}} \times 1 \), and \( H \) is a \( n_{\text{pixels}} \times n_{\text{voxels}} \) matrix representing the
Figure 6.3: The image formation model in light field microscopy can be represented by a linear system.

Forward optical model. This is represented in Figure 6.3 from [8]. Here, $n_{\text{voxels}}$ is the number of voxels in the volume being reconstructed, typically $n_{\text{voxels}} \approx 10^6$, and $n_{\text{pixels}}$ is the pixel count of the camera, typically $n_{\text{pixels}} \approx 10^6$ for a sCMOS camera. The $i^{th}$ column of $H$ represents the (vectorized) point spread function for the $i^{th}$ voxel. In contrast, the $j^{th}$ row of $H$ represents the 3D volume a the $j^{th}$ image sensor pixel samples, and is an informative notion about the 3D spatial frequencies being sampled, a superset of the 3D spatial frequencies that can be reconstructed.

As discussed in Section 6.1.2, the fact that the point spread function is shift-invariant for shifts equal to the microlens array pitch makes it such that we need only determine the columns of $H$ corresponding to the voxels directly below or above the image of a single microlens array, as we can simply laterally translate that set of point spread functions to get the point spread function for any other voxel. We next consider how to determine these point spread functions.
6.2.2 A wave optics model

The forward optical model of the light field microscope can be computed numerically using scalar wave optics theory. For reasonable numerical apertures, such as the 0.8NA used in the work presented in this dissertation, a scalar model is sufficient, but for higher numerical aperture objective lenses, it may be necessary to use a vectorial wave optics model to correctly account for polarization-state effects [88].

More detail is given in [8], but briefly, as Figure 6.4 from [8] illustrates, we can simulate the point spread function of a given voxel by first treating the source as a spherical wave, using Fourier analysis to propagate the electric field from the image plane to the plane of the microlens array, followed by Fresnel propagation through the remainder of the optical path, before taking the magnitude of the electric field to get the intensity point spread function detected by the image sensor.

As Figure 6.5 from [8] illustrates, the simulated point spread functions from using this approach resemble the experimentally measured point spread functions with reasonable accuracy compared to the previously used geometric ray optics model of light
CHAPTER 6. CODED DETECTION

Figure 6.5: The experimentally measured (with a sub-diffraction-limit-size fluorescent bead) and numerically simulated (with a scalar wave optics model) point spread function.

propagation [52, 53]. However, there exists a dark cross-shaped feature at the center of the center-most microlens image which is more prevalent in the experimentally measured point spread function, which suggests that future work may yet improve the accuracy of this optical model.

6.3 3D imaging without illumination coding

Given the forward optical model of image formation, \( H \), we can proceed to conduct 3D volumetric imaging without any illumination coding, by simply using widefield fluorescence excitation, and recording the resulting camera image, \( f \), and then applying deconvolution to recover our best estimate of \( \hat{g} \), work described in [8].

Because \( H \) has dimension \( \sim 10^6 \times 10^6 \) and is too large to store densely in computer memory, let alone compute the pseudo inverse for, we instead implement operators corresponding to multiplication with \( H \) and \( H^T \), and then use iterative deconvolution methods to recover \( \hat{g} \). One particular iterative deconvolution algorithm which accurately models the Poisson noise statistics of \( f \) is Richardson-Lucy deconvolution [39], described in Appendix A.
6.4 Limitations of light field microscopy

We now consider two limitations of this light field microscope imaging approach.

6.4.1 Long object problem

One limitation of the optical modeling and inverse problem solving of the previous sections is that there is an implicit assumption that all of the detected light on the image sensor originates from the 3D volumetric field of view being reconstructed.

When this does not hold, the reconstruction will often fail to identify the correct solution. This happens, for example, when there is fluorescence coming from adjacent regions or regions well below or above the depths being reconstructed.

In the context of computed tomographic imaging research, this is analogous to the so called long object problem, where one attempts to reconstruct only a portion of an object given a limited set of measurements [17, 46, 75, 92].

In the widefield fluorescence excitation case, where no illumination coding is used, for most calcium imaging applications in mouse, there is almost certainly going to be fluorescence excited in deeper regions outside of the 3D field of view as illustrated in Figure 2.2, and hence, the reconstruction quality will be degraded by this long object problem. As will be discussed, this issue can be overcome with more spatially precise illumination coding strategies.

6.4.2 Tissue scattering

Another limitation of the optical modeling is that the model assumes there is no tissue scattering or attenuation of any fluorescence emission. While the assumption holds reasonably well for in vivo imaging of the zebrafish, it begins to break for in vivo mouse imaging.

Figure 6.6 shows several empirically measured point spread functions, measured in either the mouse brain or in a container of (transparent) fluorescent dye. These point spread functions were measured by focusing the laser to a single location and recording the excited fluorescence. While the measurements in the dye solution match
the simulated point spread functions produced from our numerical optical modeling (see Figure 6.5), the measurements from the mouse brain look quite different.

A careful analysis of the differences reveals two different phenomena. First, the images of the point in the individual microlens images are blurred, as the light is distributed over more pixels. This effect is likely the result of nearly-isotropic scattering of the emission. Second, in some cases, the entire point spread function is shifted in one direction by several pixels. A possible explanation for this is if there is a refractive index change between two tissue interfaces not orthogonal to the optical imaging axis, where the light should refract from the optical axis according to Snell’s law.

While it would be in theory possible to empirically measure the point spread function for all of the voxels being reconstructed in the light field microscope approach without any illumination coding, in practice, this would be challenging since the optical aberrations are likely spatially varying and one would have to make many measurements (about $10^6$).

However, if we introduce the idea of illumination coding to greatly restrict the number of voxels that need to be recorded from, it then becomes feasible to empirically measure the point spread function for this more limited set of voxels. This is precisely the approach that we will use to overcome the limitation of tissue scattering, as we will discuss further in the next chapter.
Figure 6.6: Point spread functions measured empirically in a transparent fluorescent dye solution (green) and *in vivo* in a mouse brain (magenta). Scale bar indicates the microlens pitch (125µm).
Chapter 7

Experiments

The focus of the work presented in this dissertation is the development and application of a 3D calcium imaging technique suitable for \textit{in vivo} mouse calcium imaging, and while Chapters 5 and 6 discuss the illumination and detection coding strategies, respectively, this chapter discusses their integration together. The application of the method for \textit{in vivo} calcium imaging is discussed, along with some of the challenges unique to imaging awake mammals.

7.1 Microscope implementation: illumination and detection coding

Using the optimizations from the previous chapters, we can now implement a computational 3D microscopy approach using both illumination and detection coding. The method is similar to that of [68], except with the microlens array in our light field microscope [8] in place of the cubic phase mask [68]. We used the previously described optical setup, equipped with both a coded illumination and coded detection path (see Appendix B for more information). Figure 7.1 describes the \textit{in vivo} imaging setup where we can image a mouse as it is awake and running on a linear track.

The method works in the following way.

1. Identify the locations of the neurons in 3D by using conventional two-photon
Figure 7.1: The head-fixed mouse is able to run freely under the microscope objective on a linear track affixed to an XYZ-stage mounted rigidly to an optical table.
laser scanning microscopy to scan every voxel in the 3D field of view in a process lasting several minutes.

2. Once a set of neurons is selected for recording, scan a single laser focus onto each site to excite fluorescence, to determine its unique point spread function (coded diffraction pattern) on the image sensor.

3. Use a combination of coded illumination and coded detection to record fluorescence representing neural activity only from pre-determined sites onto a sequence of camera frames with 100 ms exposure times.

4. Process each camera frame with the pre-measured point spread functions using Richardson-Lucy deconvolution (see Appendix A) to recover the neural activity at each site for each camera frame.

For this particular experiment, fluorescence representing calcium activity of the neurons [10] in an awake, head-fixed mouse, shown in Figure 7.2, is imaged through the cranial window using the following approach. In the first step, lasting several minutes, single-beam laser scanning two-photon imaging [3] yields high-spatial resolution images identifying the locations of neurons at each of 51 z-depths defined by an SLM-implementation of a quadratic phase. We then select for high-speed recording 104 neurons of interest spanning a volume of dimension 600 µm × 600 µm × 200 µm from this 3D image stack and grouped them into $M = 5$ subsets, color-coded in Figure 7.3. Next, we use our time-division multiplexing approach with 250 kHz laser with 71 mW (at the sample) to excite fluorescence at all sites, which is recorded through the microlens array and in a single $T = 100 ms$ camera exposure. From each camera image we extract the fluorescence contribution from each of the illuminated sites (more below), and by recording an image sequence, we can sample the underlying calcium activity at each site at a rate of 10 Hz, for a total duration of 50 seconds, as shown in Figure 7.4.
Figure 7.2: 3D two-photon \textit{in vivo} calcium imaging. (a) Neuronal activity in barrel cortex of an awake, head-fixed mouse is recorded. (b) 104 recording sites, centered on neurons, divided into $M = 5$ subsets (annotated by number and subset) and spanning $600 \mu m \times 600 \mu m \times 200 \mu m$, are selected from a single-beam raster-scanned two-photon image stack, and then illuminated using our time-division multiplexing approach, enabling the sampling of calcium signals across all sites.
7.2 **In vivo calcium recording results**

In total, the $M = 5$ illuminated sub-regions span an area about 4 times larger than the original SLM field of view of $300\mu m \times 300\mu m$, and Figure 7.4, which illustrates the 10 most active sites, demonstrates that we observe spontaneous neural activity in sites within every region. From previous experience, if there were any errors in the accuracy of the 3D geometric calibration, oftentimes we would fail to record any activity form a particular subset of neurons.

Additionally, the signal increase from multiplexing, in combination with other efficiency improvements in Chapter 5, enabled *in vivo* mouse cortex recording using only $71mW$. Taken together, these represent a significant improvement in field of view and requisite laser power over the work reported in Figures 3 and 4 of [68], where $1200mW$ was required to record from 107 sites in a mouse hippocampus slice *in vitro*, across a smaller $250\mu m \times 250\mu m \times 50\mu m$ volume. Importantly, the limited kinetics of the GCaMP6m calcium sensor [10] enabled us to time-division multiplex our sampling of the sites with $T = 100ms$ corresponding to a 3D volume sampling rate of $10Hz$ without sampling any less information than if we had sampled all sites simultaneously as in [68].

Another important advantage of this approach over the work in [68] is the ability to record from two sites differing only by their $z$-coordinate. Using the cubic phase mask detection coding approach, the emission from these two sites would be projected to the same image sensor pixels, and would not be distinguishable. However, by using the light field microscope for detection coding, although we do not demonstrate it explicitly here in this work, it should be possible to more easily resolve emission from the two sites in this case, at least within the resolution limits of the light field microscope [8].

For completeness, the following describes how the calcium measurements shown in Figure 7.4 were extracted from raw camera images of the illuminated sites. Our light field microscope projects fluorescence from each site in 3D to a unique image sensor diffraction pattern [8], which is either determined analytically [8] or, in this work, calibrated empirically by recording an image of each site illuminated in isolation. As
Figure 7.3: 3D two-photon in vivo calcium imaging. Calcium signals ($\Delta F/F$, a normalized measure of activity) across all sites at a rate of 10Hz ($T = 100\text{ms}$).
Figure 7.4: 3D two-photon in vivo calcium imaging. Top 10 largest magnitude responses.

discussed previously in Chapter 6, the analytically computed point spread functions require the assumption of a transparent non-scattering media, which begins to break for deeper imaging depths in the mouse brain.

Nevertheless, because each raw camera image represents a linear superposition of the known, possibly overlapping, diffraction patterns of each of the illuminated sites, we can as in [8], but for a much smaller inverse problem, use Richardson-Lucy deconvolution to solve for the fluorescence at each site. The $\Delta F/F$, a normalized measure of activity, of each site is plotted in Figure 7.4, and is computed in the following way. After the Richardson-Lucy deconvolution of all images, we are left with the fluorescence at each site across time. To normalize the time series from each site, we divide it by a baseline fluorescence computed for each individual site. Following [10], we choose the 25th percentile fluorescence value in the time series for a given site to be the baseline fluorescence for that site (this gives us a robust approximation of the minimum). The $\Delta F/F$ represents the neural activity of a particular site at a given point in time.

One difficulty in validating the neural recording results presented here is that we do not have a ground truth for comparison. We considered the possibility of using external stimuli to repeatedly evoke neural responses, such as responses to visual or somatosensory stimuli [41], but determined that we could not reliably produced such responses repeatedly across different imaging sessions. This could be due to intrinsic
variability in neural signaling, or to our inability to appropriately target the correct brain region for a given sensory stimulus.

A potential way to avoid this issue of having a repeatable neural signal is to simultaneously record neural activity using both our approach and an existing reference approach. However, one challenge is that the illumination coding strategies of different approaches would undoubtedly interfere with each others’ detection approaches. A solution to this would be to interleave two imaging methods, synchronizing them within tens of milliseconds precision. Interleaving our proposed approach with conventional two-photon laser scanning microscopy might be sufficient for validating 2D imaging, but for 3D imaging, a faster approach like that of [41] would need to be used as the reference.

Overall, we conclude from these initial results that the method works as a proof of concept, but future work will require a more rigorous validation of the spatial resolution of the technique as a function of imaging depth.

### 7.2.1 Motion-related challenges

One potential challenge for in vivo neural recording of awake, behaving animals is that locomotion of the animal may cause the brain to move relative to the microscope, introducing motion-related artifacts. This is particularly important because oftentimes there are real correlations between neural activity and locomotion that could be confounded by brain motion.

For single point scanning based imaging approaches, the effect of brain motion is to cause a shift in the location of a given neuron in the image. Fortunately, if the motion was in-plane only, or if a fast 3D scan was done, it may be possible to do post hoc motion correction by aligning subsequent video frames to a reference frame, much like video stabilization algorithms in computer vision [28].

Even for camera-based approaches, brain-motion will manifest itself as blur in the image, which is possibly invertible, or at the very least, detectable.

However, for random access imaging methods, it is not readily obvious if there are motion-related artifacts in the recorded data, since only a sparse subset of a 3D
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volume is sampled, and any potential motion-correction methods need to work in real time by dynamically shifting the illumination pattern in response to shifts in the brain position relative to the microscope, likely a difficult task.

To approach this problem, we first sought to minimize all external sources of brain motion, by ensuring the mouse head plate was head-fixed to the microscope via large 1” diameter stainless steel posts, and that the microscope was rigidly attached to the optical table (see Figure 7.1).

We then quantified the residual brain motion to see if it would significantly impact our ability to record neural activity with single-cell precision. To achieve this, we conducted two-photon laser scanning microscopy of a single 2D plane of neurons in a head-fixed mouse as it was free to run in place along a linear track under the microscope, shown in Figure 7.1, and recorded both its progression along the linear track and its neural activity with single-neuron spatial resolution.

We then identified two time periods lasting 10s each, where the mouse was at rest in one case, and running at full speed down the linear track in the other, and averaged the corresponding image frames of the neurons in the brain. The result, shown in Figure 7.5, highlights the slight misalignment in frames captured while the mouse was running, indicated by the blurred image at the bottom right of the figure. We can attempt to infer the magnitude of the brain motion by artificially blurring the left image to find the blur kernel size that produces the right image, and from this analysis we conclude the magnitude of the motion is on the order of several microns.

This promising result suggests that with a rigidly built head-fixation setup, the effects of brain-motion without any correction are limited in magnitude, and perhaps could be mitigated by some sort of averaging strategy whereby rather than recording from a single location on a neuron, we might integrate signal across the entire neuron, such that any motion-related shift is small relative to the recording area. This might be easily implemented in our microscope by using the galvanometers we used for extending the field of view, for also implementing a small cellular-sized 2D scan pattern which would be applied simultaneously to any illuminated focal sites.

In a final test, we applied our proposed approach to record from adjacent neurons
Figure 7.5: Residual brain motion is characterized by conducting two-photon laser scanning microscopy of the neurons in the same 2D plane while a mouse is either stationary or running at full speed along the linear track. An average of recorded frames across 10s are shown at the bottom (scale bar 15\(\mu m\)). These measurements suggest the magnitude of typical motion-related effects is at most 20 – 30% the size of a neuron.
in an attempt to quantify spatial resolution during locomotion. We again monitored the position along the linear track, and sought to identify any signs of signal cross-contamination specific to periods of locomotion. This time, we imaged a different region in a different mouse, ventral CA1, imaged through a 0.5mm × 1mm cannula, using AAVDJ-CaMKIIalpha-GCaMP6f, recorded at 10Hz using a 80MHz Ti:Sapphire laser (instead of the 250kHz used previously) with 25× 0.95NA Olympus objective.

The results are shown in Figure 7.6. As illustrated by the two arrows, we record the activity of two adjacent neurons with minimal cross-talk between the two even during periods of locomotion at the full speed.

Of course, this measurement of spatial resolution may depend on many other factors, including the precise surgery preparation, the imaging depth, and the precision of the geometric calibration, and the experiment proposed here is by no means a definitive measure of expected resolution, but more of a proof of concept experiment that brain-related motion need not be a significant detriment to the application of neural imaging techniques that sparsely sample 3D volumes and hence cannot implement post hoc motion correction.
Figure 7.6: Calcium imaging using our proposed approach during locomotion. (a) 10 cells are targeted for recording, including several adjacent pairs of cells. (b) The recorded neural activity is juxtaposed with (c) the locomotion of the mouse along the linear track. Shaded regions indicate time periods of locomotion.
Chapter 8

Discussion

This dissertation describes contributions to two different projects, frame-projected independent-fiber photometry and a joint detection and illumination coding strategy optimized for 3D in vivo mouse calcium imaging.

Optimizations to the coded illumination field of view, laser and optical parameters, along with adaptations to the light field microscopy detection coding strategy, enabled the successful demonstration of this approach for imaging neural activity in an awake mouse.

8.1 Limitations

Though the peak power at each focal point in our multifocal scanned approach is lower than that of single point-scanning approaches [41], it is still higher than that required for simultaneously illuminating all sites, which may lead to fluorophore saturation or photodamage [40, 19]. Although we showed signal gains for the two-photon excitation case, this approach should work for higher order multiphoton signal generation as well.

At present, our approach only extends the lateral, and not axial field of view, but new hardware developments, including other phase modulators such as tunable lenses and deformable mirrors could also be combined to extend the axial field of view as well.

The spatial resolution of our 3D neural activity recording technique will depend on
several factors, mostly related to the tissue depth. A bound on the spatial resolution is given by the size of the focused laser spots produced by computer generated holography, which is diffraction and scattering-limited at best. Hence, we should expect the imaging depth to be no better than with conventional two-photon laser scanning microscopy with adaptive optics. Another factor limiting spatial resolution is the ability to resolve the fluorescence from multiple sources, which likely will depend both on the number and position of the sources. However, there may be opportunities in optimizing the time-multiplexed illumination and detection coding approaches to maximize the spatial resolution given a particular imaging sample, as we discuss in Section 8.2.

### 8.2 Future research directions

Future work will involve scaling the technique to record from a much larger number of neurons. In the work presented in Chapter 7, a factor of 7 times more laser power was required to conduct two-photon laser scanning imaging to identify the location of the neurons than has been used in other reported work, suggesting the use of a better surgical preparation for *in vivo* imaging may enable the recording from 7 times as many neurons.

In addition, the recordings done in this work were with only 71mW of power, and with a more efficient optical path with 30% efficiency [69], about 500mW of power should be available at the sample, yielding another factor of $7 \times$.

The recent advent of significantly faster phase spatial light modulator hardware [83] will make it possible to extend the time-division multiplexing approach to get even more signal, as illustrated in Figure 8.1.

Though we quantified the effect of brain motion and found it to be minimal for our application, certain applications may require more precise targeting of light that adapts dynamically to slight shifts in brain motion. For this, it may be useful to construct a system which can enable holograms to be updated in real time to respond to shifts in the position of the brain relative to the microscope. This should in principle be possible as the galvanometer mirror response times are 1ms or less, which is much
shorter compared to our 100\(ms\) exposure times. However, SLMs with faster response times may be necessary to be able to compensate for motion in the \(z\)-direction.

We demonstrated here the switch from using a 80\(MHz\) repetition rate laser system to a 250\(kHz\) system, but it remains to be seen whether the repetition rate could be further optimized given knowledge of the dark state relaxation times of the particular fluorophore or opsin [19], to enable even higher two-photon excitation efficiency.

At that point, challenges may exist in developing both improved detection coding strategies as well as reconstruction algorithms for resolving the activity of thousands of sources recorded within a single camera exposure. In particular, characterizing the penetration depth and spatial resolution of the imaging system in \textit{in vivo} imaging applications will be an important but challenging task for future work.

There may be many interesting opportunities to maximize information content and source separability by leveraging multiple different time-multiplexed illumination patterns in combination with multiple detections within a single sampling period, which could be achieved by operating the camera at a higher frame rate (in our work, we only used 10\% of the camera’s maximum frame rate). In the most advanced case, this optimization would account for optical scattering effects and potential signal
cross-talk, and optimize the sampling strategy for all sites. Often in computer generated holography, the holograms include so-called ghost spots which are unwanted but predictable concentrations of illumination power. By using multiple illumination patterns and detections, it may be possible to correct for the impact of any of these ghost spots.

Another method for extending the imaging depth or number of imaged sources may be to utilize any possible temporal correlations from the signals from the recording sites, solving a global optimization problem across all frames rather than on a per-frame basis as done here.

Lastly, our work utilized computer generated holography with a SLM to generate multifocal illumination patterns in tissue, and for most our work, we worked at more superficial regions of the brain where it was reasonable to assume the illumination light was minimally scattered. Much recent work in adaptive optics using phase SLMs for focusing laser through scattering media should readily be applicable to increase the imaging depth of our approach.

Similarly, widefield adaptive optics approaches for modifying the emission before being detected by the camera might be useful in optically processing the data to minimize the impact of noise and to maximize separability of the signal.

\section{Closing remarks}

We have addressed the field of view and two-photon signal limitations of holographic SLM-based illumination approaches by using a time-division multiplexing strategy to time-sequentially tile a larger field of view. We demonstrate that for applications where strictly simultaneous illumination is not required, such as 3D \textit{in vivo} neuronal calcium imaging, a larger accessible field of view and additional signal can be achieved with little additional cost.
Appendix A

Algorithm details

A.1 Multi-plane Gerchberg-Saxton algorithm

Monochromatic propagating light can be represented at any plane as a complex electric field function, with an amplitude and phase component at each spatial location. The Gerchberg-Saxton algorithm [23] concerns the determination of the phase component at one plane given an amplitude constraint at that and another plane related by some physical propagation function (e.g. Fourier transform).

In the context of computer generated holographic illumination applications, this phase retrieval algorithm is used to determine the phase pattern to be implemented (e.g. on a phase spatial light modulator) to produce a desired intensity pattern (the modulus squared for the electric field). The input to Algorithm 1, then, is TargetAmplitude, the desired amplitude function (square root of the desired intensity distribution) at the sample plane, as well as SourceAmplitude, the fixed amplitude of the illuminating laser (typically a Gaussian function), and some physically-accurate wave propagation function connecting these two optical planes, ForwardPropagate and BackPropagate.

The multi-plane version of the Gerchberg-Saxton was first proposed in [34] and later applied toward optical tweezing in [79]. Here, described in Algorithm 2, rather
APPENDIX A. ALGORITHM DETAILS

Input: \textit{SourceAmplitude, TargetAmplitude}  
Output: RetrievedPhase  
\[ A = \text{BackPropagate}(\text{TargetAmplitude}) \]
\begin{algorithm}
while ErrorExceedsCriterion do  
\begin{align*}
B &= \text{Amplitude}(\text{SourceAmplitude}) \otimes \exp(i \cdot \text{Phase}(A)) \\
C &= \text{ForwardPropagate}(B) \\
D &= \text{Amplitude}(\text{TargetAmplitude}) \otimes \exp(i \cdot \text{Phase}(C)) \\
A &= \text{BackPropagate}(D)
\end{align*}
end while
\end{algorithm}
\[ \text{RetrievedPhase} = \text{Phase}(A) \]

\textbf{Algorithm 1: Single-plane Gerchberg-Saxton Algorithm}

than have a single laser amplitude constraint, \textit{SourceAmplitude}, and a single sample amplitude constraint at one z-plane, this modified algorithm allows for \( k \) amplitude constraints at \( k \) z-planes, \( \{\text{TargetAmplitude}(k)\} \) so long as a physically accurate propagation function for each z-plane is known, \( \{\text{ForwardPropagate}(k)\} \) and \( \{\text{BackPropagate}(k)\} \).

\begin{algorithm}
Input: \textit{SourceAmplitude, \{TargetAmplitude\}(k)}  
Output: RetrievedPhase  
\[ A = \sum_k \text{BackPropagate}(k)(\text{TargetAmplitude}(k)) \]
\begin{algorithm}
while ErrorExceedsCriterion do  
\begin{align*}
B &= \text{Amplitude}(\text{SourceAmplitude}) \otimes \exp(i \cdot \text{Phase}(A)) \\
& \text{for all } k \text{ do} \\
C(k) &= \text{ForwardPropagate}(k)(B) \\
D(k) &= \text{Amplitude}(\text{TargetAmplitude}(k)) \otimes \exp(i \cdot \text{Phase}(C(k))) \\
& \text{end for} \\
A &= \sum_k \text{BackPropagate}(k)(D(k))
\end{align*}
end while
\end{algorithm}
\[ \text{RetrievedPhase} = \text{Phase}(A) \]
\end{algorithm}

\textbf{Algorithm 2: Multi-plane Gerchberg-Saxton Algorithm}

In the single-plane Gerchberg-Saxton implementation for 2D computer generated holographic applications where the phase spatial light modulator is placed at the pupil plane of the imaging system, typically \textit{ForwardPropagate} is the band-limited Fourier transform operator and \textit{BackPropagate} is the inverse Fourier transform [26].
In the extension to 3D with the multi-plane Gerchberg-Saxton algorithm, the propagation functions \{ForwardPropagate_{(k)}\} and \{BackPropagate_{(k)}\} are implemented using a combination of Fourier transform operators as well as Fresnel diffraction, using the Fresnel transfer function propagator [90]

\[
U_2(x, y) = \mathcal{F}^{-1}\{\mathcal{F}\{U_1(x, y)\}H(f_X, f_Y)\}, \tag{A.1}
\]

where \(U_1(x, y)\) and \(U_2(x, y)\) are the source and target electric fields and the transfer function \(H\) is

\[
H(f_X, f_Y) = e^{i\nu z} \exp[-i\pi \lambda (f_X^2 + f_Y^2)], \tag{A.2}
\]

where \(\nu\) is the wave number and \(\lambda\) is the illumination wavelength.

### A.2 Richardson-Lucy deconvolution

Richardson-Lucy deconvolution, first proposed in [72], has been applied to a variety of image reconstruction problems in microscopy, where Poisson-distributed photon shot noise is the dominant noise source [39]. In this noise regime, the Richardson-Lucy deconvolution algorithm will accurately produce the maximum likelihood estimate, and hence is the algorithm of choice.

The algorithm attempts to solve the following linear system

\[
f = Hg, \tag{A.3}
\]

where \(f\) is the noisy vectorized measurement of the true vectorized signal \(g\) projected through a linear system matrix \(H\) with Poisson-distributed noise.

The likelihood function is
The following Richardson Lucy update rule can be iteratively applied to yield the maximum likelihood estimate $\hat{g}$:

$$g^{(k+1)} = H^T (f \circ (Hg^{(k)})^{-1}) \circ (H^T 1^{-1}) \circ g^{(k)}. \quad (A.5)$$

Here $k$ denotes the iteration number, $\circ$ is the Hadamard or element wise product, and $1$ is a vector of ones of same size as $g$.

As discussed previously, this algorithm is well suited for image reconstruction problems in microscopy, where Poisson photon shot noise is the dominant noise source. In addition, inverting or determining the pseudo inverse of $H$ is not required – only functions which implement $H$ and $H^T$ are required. Hence, this algorithm can be applied to invert problems with system matrices that are too large to invert or even represent densely.

Note: Equation A.5 is equivalent to Equation 9 of [8] and the equations in [39] with $b = 0$.

Algorithm 3 illustrates a practical implementation of this method, where $\epsilon$ is some user-defined stopping criteria, and $\cdot$ and $/$ represent element-wise multiplication and division, respectively, and $1$ is a vector of ones of same dimension as $f$. The algorithm requires only the noisy measurement image, $f$, and two functions implementing the system matrix $H$ and its transpose $H^T$. 

$$L(g) = \prod_i \text{Poisson}(f_i | Hg). \quad (A.4)$$
**Input:** $f$, $MatrixH$, $MatrixHTranspose$

**Output:** $\hat{g}$

$\hat{g} = MatrixHTranspose(f)$

$\hat{f} = MatrixH(\hat{g})$

while $|f - \hat{f}|^2 > \epsilon$ do

$r = f / \hat{f}$

$u = MatrixHTranspose(r) / MatrixHTranspose(1)$

$\hat{g} = u \cdot \hat{g}$

$\hat{f} = MatrixH(\hat{g})$

end while

**Algorithm 3:** Richardson-Lucy deconvolution
Appendix B

Optics hardware details

B.1 Optical setup

This section describes the individual components used to implement the optical system from Figure 5.3.

In the excitation path, 920 nm pulses from a 80 MHz Ti:Sapphire laser (Coherent Chameleon Ultra II) seed a regenerative amplifier (Coherent RegA 9000) producing 1.8 W at 250 kHz with 170 fs pulses. A Pockels cell (Conoptics 350-80-LA-02-RP KD*P) both reduces power and serves as a high-speed shutter. The beam passes through a half wave plate (Thorlabs AHWP10M-980) and is expanded using achromatic doublets (Thorlabs) to slightly overfill a 6.14 mm × 6.14 mm, 256 × 256 resolution, 0 − 2π phase SLM (Meadowlark HSP256-1064-P8) at a 15 degree angle from the normal. The SLM is imaged (f1 = 300 mm, f2 = 200 mm) onto the midpoint between two 5 − mm galvanometer mirrors (Cambridge Technology 6215H), spaced 1 cm apart. A beam block (2 mm diameter steel pin in a glass window, Thorlabs WG12012-B) blocks zero order undiffracted light. Finally, two lenses (Leica scan lens VIS-IR-TCS-SP2, f = 39 mm and Thorlabs tube lens ITL200, f = 200 mm) bring the conjugated SLM and scanning mirrors through a dichroic (Semrock FF670-SDi01-25x36) and onto the objective pupil (Nikon 16 × 0.8 NA), after which 20% of the total laser power remains.

The fluorescence emission path follows the light field microscope configuration in
APPENDIX B. OPTICS HARDWARE DETAILS

Emission passes through a filter (Semrock FF01-535/50), an identical tube lens, microlens array (RPC Photonics, 125 $\mu m$ pitch, f/10), two relay lenses (Nikon 35mm, Nikon 50mm), and an sCMOS camera (Hamamatsu Orca Flash4.0 V2). A MATLAB program (MathWorks) controls all hardware with a data acquisition card (National Instruments PCIe-6343).

We used 3D holograms computed with the multi-plane Gerchberg-Saxton method [79]. For calibration and characterization experiments, we used a green fluorescent dye (Sharpie highlighter) in a cuvette (Thorlabs CV10Q3500) capped with a 0.17mm coverslip. Our amplifier compressor stage was adjusted to compensate for pulse dispersion from the optics.

B.2 Geometric calibration

With the geometric calibration procedure in [69], we determined the 2D affine transforms between each z-depth of the light field microscope and SLM field of view, for each of 9 galvanometer mirror positions. For each mirror position at two z-depths, the 2D transform was measured by illuminating multiple focal spots in fluorescent dye and recording the resulting 3D light field coordinates; the transforms for the other z-depths were linearly interpolated.

B.3 Modifications for in vivo experiments

To enable 3D in vivo calcium imaging with a modified version of [68] the following changes were made. Two flip mirrors (Newport 8892-K) enabled switching between the 80MHz and 250kHz lasers, and between a photomultiplier tube (Hamamatsu H10770P A-40) and the sCMOS camera/microlens array, enabling single-beam 2D laser-scanning two-photon microscopy [15]. We extended the geometric calibration procedure from Appendix B.2 to include this third coordinate system by registering the corners of the 2D scanned field of view in dye with the light field microscope coordinates at each z-depth. We used a 512 $\times$ 512 resolution, but slower ($t_{SLM} = 10ms$), SLM (Meadowlark HVHSPDM512-532) and to match the larger 19.2mm SLM
size to the objective pupil, we adjusted the beam expansion (BE) accordingly and used $f_1 = 750mm$ and $f_2 = 150mm$.

We imaged 6 months after a chronic cranial window was implanted [36]. Briefly, mice were sedated with isoflurane and a 5\textit{mm} diameter, #1 thickness cover slip (Warner) was implanted over barrel cortex after injection of 1000\textit{$\mu$L} of AAVdj-Camk2a-GCaMP6m virus [10]. In addition, a plate for head-fixation during imaging was affixed to the skull using dental cement (Parkell). The Stanford University Institutional Animal Care and Use Committee approved all experimental protocols.
Bibliography


