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Fluorescence microendoscopy for *in vivo* deep-brain imaging of neuronal circuits

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Abstract

Imaging neuronal activity in awake, behaving animals has become a groundbreaking method in neuroscience that has rapidly enhanced our understanding of how the brain works. *In vivo* microendoscopic imaging has enabled researchers to see inside the brains of experimental animals and thus has emerged as a technology fit to answer many experimental questions. By combining microendoscopy with cutting edge targeting strategies and sophisticated analysis tools, neuronal activity patterns that underlie changes in behavior and physiology can be identified. However, new users may find it challenging to understand the techniques and to leverage this technology to best suit their needs. Here we present a background and overview of the necessary components for performing *in vivo* optical calcium imaging and offer some detailed guidance for current recommended approaches.

Keywords

functional imaging; microendoscopy; neuronal circuits; GRIN lens; GCaMP; GECI

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CRediT Authorship Contribution Statement

Brenton T. Laing: Conceptualization, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization. **Justin N. Siemian:** Conceptualization, Formal analysis, Writing – Original Draft, Writing – Review & Editing, Visualization. **Sarah Sarsfield:** Resources, Writing – Review & Editing, Visualization. **Yeka Aponte:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – Review & Editing.

Ethics Statement

All experimental protocols were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the approval of the National Institute on Drug Abuse Animal Care and Use Committee.

Declaration of Competing Interest

The authors declare no conflicts of interest.

1. Introduction

Optical monitoring of neuronal activity in freely moving animals has begun to crack the circuitry underlying interaction with the environment, physiology, and behavior. Recent functional imaging studies have significantly advanced our understanding of how specific neuronal circuits deep in the brain encode processes essential to survival such as feeding (Betley et al., 2015; Jennings et al., 2015; Douglass et al., 2017; Rossi et al., 2019), sleep (Cox et al., 2016; Weber and Dan, 2016), and nociception (Corder et al., 2019). Moreover, the use of these techniques has shed light on neuronal mechanisms involved in complex processes like place coding (Ziv et al., 2013; Kinsky et al., 2018), substance abuse (Siciliano et al., 2019), and learning and memory (Grewe et al., 2017; Kamigaki and Dan, 2017; Kitamura et al., 2017). The shift from electrical to optical recording methods has brought several key improvements including (a) the ability to target specific cell subpopulations with fluorescent, genetically-encoded activity sensors (e.g., calcium sensors), (b) the ability to record from the same exact population of cells over multiple sessions, (c) a vast increase in the numbers of simultaneously recorded cells, (d) the ability to map the relative spatial locations of groups of cells, and (e) increased accessibility to deep structures in the brain. Additionally, miniaturization of the microendoscope has allowed for interrogation of the neuronal circuits involved in the complex, waking behavioral states listed above, freeing researchers from the constraints of anesthetized or head-fixed configurations (Table 1). The decreasing costs of microendoscopy systems combined with increasingly user-friendly and elegant data analysis pipelines are also increasing the appeal of these approaches.

However, despite these advantages, the multi-faceted nature of *in vivo* functional imaging approaches can be overwhelming to inexperienced users. Obtaining satisfactory end results heavily depends on many factors including viral strategy, surgical technique, microscopy system, experimental design, programming experience, data preprocessing, and postprocessing analysis (Kamigaki and Dan, 2017). While excellent detailed methodological protocols have been described (Resendez et al., 2016; Yang and Yuste, 2017; Martianova et al., 2019; Lee and Han, 2020), the field is progressing rapidly, and additional recent advances have been made. Thus, here we present a background and overview of the key components for performing *in vivo* optical calcium imaging and offer some detailed guidance for current recommended approaches in microendoscopy.

2. Genetically encoded calcium indicators

The premise of *in vivo* functional imaging is to use changes in fluorescence of a genetically encoded calcium indicator (GECI) as a proxy for neuronal activity. Although indicators for other molecules such as GABA (Marvin et al., 2019), glutamate (Marvin et al., 2013), and dopamine (Patriarchi et al., 2018) are now available, this review will focus on GECIs as they are the most widespread in imaging studies. During an action potential, intracellular calcium levels increase, leading to increased fluorescence in neurons expressing GECIs, and this can be interpreted as an increase in cellular activity. By far the most widely used GECIs in optical imaging are the green fluorescent protein (GFP)-based calcium sensors of the GCaMP family. Initially developed in 2001 (Nakai et al., 2001), GCaMP was formulated as a fusion protein of GFP, the calcium-binding protein calmodulin (CaM), and the CaM-

interacting M13 peptide. A conformational change in the structure of GCaMP when calcium is bound allows GFP to fluoresce, and hence indicates the presence of the cation. Since then, seven main iterations of GCaMPs have occurred, with the vast majority of literature over the past decade coming from the use of GCaMP6 (Chen et al., 2013), which was designed in three kinetic variants: fast (GCaMP6f), medium (GCaMP6m), and slow (GCaMP6s). The latest round of structure-guided design has yielded the family of jGCaMP7 sensors, which offer improved sensitivity and signal-to-noise ratios (SNR) for the jGCaMP7s and jGCaMP7f variants, as well as optimization for neuropil imaging with jGCaMP7b ("high baseline"), and improved contrast with jGCaMP7c ("high contrast") (Dana et al., 2019).

While calcium imaging with single-wavelength GCaMPs has yielded many novel insights toward understanding the activity of neuronal circuits underlying behavior and pathology, there also was a need for calcium sensors that fluoresce at longer wavelengths. Longer wavelength light penetrates tissue more effectively (Nikolaos et al., 2008), as green light emission is absorbed by blood (Svoboda and Block, 1994), and thus red-wavelength imaging results in comparable image quality at lower illumination levels to reduce photobleaching and to enhance the quality of imaging in deep brain structures. Longer-wavelength light emission also facilitates "all-optical" approaches of circuit characterization, permitting the use of optogenetics combined with calcium imaging through independent spectral channels for photostimulation and recording (Akerboom et al., 2013). With these aims in mind, multiple red-shifted GECIs were developed from circularly permutated mApple and mRuby using structure-guided mutagenesis by testing variants with automated neuronal assays. These GECIs, including R-GECO and RCaMPs, are similar to GCaMP in that they incorporate calmodulin to bind calcium, as well as a binding peptide such as M13 or ckkap (Akerboom et al., 2013; Wu et al., 2014; Dana et al., 2016). These red-shifted GECIs have different advantages depending on the experimental application. For example, mApple-based GECIs are sensitive to calcium transients but exhibit photoswitching when illuminated with blue light, preventing simultaneous use with optogenetic photostimulation of the light-activated cation channel channelrhodopsin (Akerboom et al., 2013; Dana et al., 2016). On the other hand, RCaMP1, developed from mRuby, has lower sensitivity to calcium transients but is compatible with the blue light used for stimulation of channelrhodopsin, as it is not stimulated by blue light itself (Akerboom et al., 2013; Dana et al., 2016). The recent development of red-shifted opsins (e.g., Chrimson, bReaChES) allows latest-generation GCaMP indicators to be used in all-optical configurations (Klapoetke et al., 2014; Jennings et al., 2019). Another powerful approach offered by indicators accessible in different spectral channels is the simultaneous, parallel imaging of different genetically defined populations (Inoue et al., 2015; Dana et al., 2016). Continuing advances in cell targeting strategies will allow increasing specificity and control over opsin and calcium indicator targeting to facilitate our understanding of how the activity of neuronal circuits relates to behavior.

Importantly, there has been extensive use of ratio-metric (i.e, Fura-2 and Indo-1) and single-wavelength (i.e., Oregon Green BAPTA-1 and Fluo-4) dyes to study intracellular calcium dynamics in cultured cells and brain slices (Aponte et al., 2008; Paredes et al., 2008; Barreto-Chang and Dolmetsch, 2009). To use these dyes *in vivo*, multicell bolus loading (MCBL) was designed to microinject calcium sensitive acetoxymethyl (AM) ester

dyes into brain regions of interest (Stosiek et al., 2003). However, similar to the challenges encountered when using most dyes, this approach does not achieve high enough signal-to-noise ratio for *in vivo* imaging of subcellular structures (Garaschuk et al., 2006). The dye Cal-520 has been used due to its improved sensitivity over other dyes, but it is still only suitable *in vivo* for very signal-rich regions such as neocortical neurons or cerebellar Purkinje cells (Tada et al., 2014). Though other dyes such as Fluo-4 are preferred for detecting action potential events, they fade rather quickly and are not conducive for staining large populations (Golshani et al., 2009).

3. Cell targeting strategies

The molecular markers present in individual cells are indicators of the presence of structural machinery that could be related to function. Distinct brain regions are comprised of a diverse array of cells including neurons, oligodendrocytes, microglia, and astrocytes that can be categorized by their molecular markers, physical boundaries, or functional importance. Due to such diversity, the dissection of cellular populations into individual groups of neurons has been very challenging. For example, a given marker can indicate neurons that have a broad array of functions across brain regions and thus genetic strategies to specifically manipulate such neurons could not be achieved simply through cross-breeding of mouse strains. Initially, there was hope that microinjection of promoter-driven viruses would be sufficient to target molecularly defined populations in a region-specific manner (Klein et al., 2002). While these strategies can be effective at transducing a target population under highly transcribed promoters (Saito et al., 2013), it leaves the chance of ectopic expression in cells also exposed to the virus (one form of “leaky” expression).

Because of these challenges, a new standard was adopted for the induction of expression of a desired transgene in a cellular population of interest. By combining breeding and viral microinjection strategies, regionally restricted groups of neurons can be further subdivided by molecularly identified populations. The implementation of the Cre-Lox Flip Excision (FLEX) switch (Schnutgen et al., 2003), also known as DIO (Double-floxed Inverse Open reading frame), enabled a level of specificity that was previously unattainable by ensuring that expression occurs only in cells that express Cre recombinase. The FLEX switch strategy resolves ectopic expression by incorporating the key genetic construct for the transgene of interest in a non-transcriptionally functional, reverse orientation (Schnutgen et al., 2003; Atasoy et al., 2008; Sohal et al., 2009). By flanking the transgene of interest with a pair of heterotypic lox sites, such as loxP and lox2272, in opposing orientations, the inversion of the transgene proceeds in a Cre recombinase-dependent manner. Thus, cells expressing Cre recombinase will express functional transgenes, whereas cells lacking Cre recombinase will not. Alternatively, the system can be used for Cre-OFF transgene control by packaging the key construct in the forward direction whereby the FLEX switch reverses the transgene to the non-functional orientation. Variations of this system have been designed to allow simultaneous expression of Cre-ON and Cre-OFF constructs (Saunders et al., 2012). Recombinant viruses (typically adeno-associated viruses) expressing GECIs can be injected into transgenic Cre mouse lines or Cre recombinase can be introduced in a region-specific manner by viral injection (Gong et al., 2007). Constraining GECI expression to relevant cell types is critical to limit functional heterogeneity and to improve the ability

to detect a relationship between neuronal activity of a specific population of neurons and behavior.

4. Surgical techniques for deep-brain imaging

Although cranial window preparations may be sufficient for shallow (1 mm) depth imaging of calcium sensors using optics with appropriate working distances (Svoboda et al., 1997), deeper structures (> 1 mm) can only be accessed by relay lenses that extend the length of the imaging system to permit imaging in deep brain regions. Gradient-index (GRIN) lenses are used for this purpose. GRIN lenses are rod-shaped and are available in small diameters (< 1 mm) and multiple lengths. Their miniature profile makes them ideal for implantation into rodent brains and their optical properties make them ideal for use as relay lenses within microendoscopy systems (Jung and Schnitzer, 2003; Jung et al., 2004; Levene et al., 2004; Bocarsly et al., 2015).

First, a viral-GECI construct is injected, and in the same surgery, a GRIN lens can be implanted (Fig. 1A). Alternatively, the experimenter can perform two surgeries, the first for viral construct injection and the second for lens implantation. Whether one surgery or two, it is advisable to offset the injection (medial, lateral, anterior, or posterior) by at least 100 μm from the center of the desired imaging target to prevent the injection syringe from damaging tissue within the lens field of view (FOV). For GRIN lens implantation a path through the brain tissue must first be created, because implantation of a GRIN lens or GRIN lens cannula displaces tissue along the path (Bocarsly et al., 2015), resulting in tissue compression between the working distance of the lens and the focal plane. To avoid obscuring visualization with this compressed tissue, a number of strategies have emerged. One strategy is to slowly lower into the brain a sterile, beveled-tip syringe with the same diameter as the lens to clear a path for the implant. The sterile syringe should not be lowered all the way to the target site to prevent tissue damage in the FOV (Resendez et al., 2016). Extremely lateral regions may require microprisms, which are right-angled prisms attached to relay lenses allowing placement of the lens lateral to, instead of superior to, the region of interest. For implantation, a straight edge dissecting knife can be used to make a vertical and a lateral incision within the brain to clear the path (Andermann et al., 2013). Alternatively, for some deep brain implants the tissue 1 – 3 mm above the target site can be aspirated with a blunt-tip syringe manually or robotically (Liang et al., 2019). If using aspiration, continue until bleeding has ceased, because blood on the bottom of the surface of the implant will increase the inflammatory response and can obscure the lens (Liang et al., 2019). Finally, the GRIN lens or the GRIN lens cannula can be positioned in the brain at the appropriate working distance from the target. The clearing of excess tissue and blood during lens or cannula insertion is a critical step towards acquisition of high-quality *in vivo* calcium images, especially for deep brain implants. It is worth noting that after implantation of a GRIN lens, reactive glia form sheaths around the implant. This inflammatory reaction persists for at least two weeks after implantation, but largely subsides by week four (Bocarsly et al., 2015). For this reason, virally induced cellular expression of calcium indicators tends to precede clearing of the inflammatory response—highlighting that clearing of this response is a key contributor to improved visualization of cellular boundaries in the weeks that follow.

5. Fluorescence microendoscopy systems

To visualize calcium dynamics in subsurface regions, implants must be used to relay excitation light to—and emitted signal from—GECI-expressing neurons. The widely used technique of fiber photometry is perhaps the most basic method to do this. A single optical fiber is implanted above a brain region of interest, and the measurements collected represent the overall, population-wide fluorescence of GECI-expressing cells. This technique allows access to neuronal populations deep in the brain, and animals can move freely during recording sessions. Moreover, the small footprint of the implant allows users to monitor multiple sites across the brain simultaneously, for example different hemispheres or a pair of connected neuronal populations (Sych et al., 2019) which requires more complex systems for imaging (Lecoq et al., 2014) at multiple sites (Barretto et al., 2011). This approach can enable photostimulation in one region through one optical fiber while recording the population activity of another region through a second optical fiber (Nieh et al., 2016). Since fiber photometry lacks single-cell resolution, it is generally best-suited for monitoring neuronal populations with predicted response properties that are not widely heterogeneous, otherwise changes in fluorescence may be diluted or undetectable. However, this will often be unknown *a priori*.

Functional dissection of neuronal populations is critical to understanding how the brain drives behavior. Even within a given region, a molecularly defined set of neurons will likely be comprised of a diverse collection of cell types (Mickelsen et al., 2019). Thus, while fiber photometry is a useful tool to capture the net activity of a population, analysis of cells as single units to understand the complexity and range of activity patterns has emerged as the key strategy for identifying nodes within a neuronal circuit. Interfacing a microscope with an implanted GRIN lens allows the visualization of the FOV *in vivo* with cellular and subcellular resolution (Broussard et al., 2018). By pairing this technology with GECIs, the calcium dynamics of single cells deep in the brain can be monitored in real time via one- or two-photon microscopy.

Two-photon microscopy was originally proposed in the dissertation of Maria Göppert-Mayer (Göppert-Mayer, 1931). This approach uses a pair of low energy photons generated by near infra-red light for excitation of the fluorophore of interest. The photons arrive at a fluorophore molecule nearly simultaneously, within 0.5 femtoseconds of each other, and the fluorophore emits a single photon of the wavelength corresponding to its profile (Helmchen and Denk, 2005). This results in low axial spread of the point function and enables optical sectioning along the z-axis with substantially reduced out-of-focus multiphoton absorption (Rubart, 2004; Helmchen and Denk, 2005). The primary advantages of two-photon microscopy are the use of long-wavelength light to excite fluorophores, which decreases the damage that occurs from short wavelengths (Chen et al., 2002), and the resolution, which enables visualization of calcium concentration dynamics in subcellular compartments (Meng et al., 2019) as transients driven by cellular events including action potentials (Yasuda et al., 2004). For excitable cell types like neurons, calcium concentration can vary widely between the soma and spines. Change in calcium concentration is dependent on net influx, extrusion, and buffering of calcium. Because buffering of calcium occurs very rapidly in the spines of the dendritic compartment, this calcium flux can have a local influence without changing

the electrical potential of the entire dendrite. Therefore, high resolution imaging at a fast sampling rate is required to image these fast calcium events. Another major advantage of the two-photon system is the ability to simultaneously excite two fluorophores—one that is calcium-sensitive and one that is not. Use of a calcium-insensitive fluorophore that is visible at baseline can aid in identifying cellular structures and reduces sensitivity of the data to background fluctuations. In addition, the two-photon system reduces photobleaching and photodamage outside of the focal plane by focusing light onto the laser focal volume (Yang and Yuste, 2017). With proper habituation and careful task design, calcium dynamics can be monitored in awake head-fixed animals during behaviors of interest (Dombeck et al., 2010). For example, measurement of neuronal responses to environmental cues, rewards/punishments, or drugs of abuse lend themselves well to two-photon microscopy. However, two-photon microscopy has not yet been commercially optimized for use in freely moving animals because of challenges related to the beam scanning mechanism (Yang and Yuste, 2017).

Single-photon microscopy utilizes epifluorescence to illuminate a FOV under a miniaturized microendoscope (“miniscope”) in freely moving animals. The single-photon system is substantially lighter and smaller than commercial two-photon systems, enabling the head-mounted miniscope (Ferezou et al., 2006) to be tethered to an LED light source via a fiber optic cable (Fig. 1A). In addition, less motion artifact is observed in freely moving animals compared to head-fixed animals (Ghosh et al., 2011). The resolution is sufficient to visualize individual cells, and also dynamics in dense dendritic fields (Murayama et al., 2007). Acquisition can occur at rapid frame rates (>100 Hz) to allow for time-locking calcium events and behavioral events (Flusberg et al., 2008). Following habituation, neuronal activity during natural, learned, and complex behavioral paradigms in freely moving animals can be studied. Miniscopes are small enough for mice to conduct tasks such as the rotarod test, foot fault, and the Morris Water Maze (Lee et al., 2016). Single-photon microendoscopy combined with an elevated plus maze test identified vasoactive intestinal peptide neurons from the broader GABAergic circuitry in the prefrontal cortex by their ability to gate mouse activity on the open arms of the elevated plus maze (Lee et al., 2019), indicating their critical role in control of anxiety-like behavior. Taken together, these studies are examples of how *in vivo* calcium imaging can be used to enhance our understanding of neural mechanisms underlying behavior. Ready-to-use miniscope systems are commercially available through vendors such as Inscopix and Doric Lenses. They can also be assembled from an open-source parts list costing just over $1000 USD (http://miniscope.org).

Selection of an imaging system should be made when considering the experimental question to leverage the advantages of fiber photometry, single-photon microendoscopy, or two-photon microendoscopy. In addition, by understanding the disadvantages and challenges attributed to each method, experiments can be designed within the limitations of the selected imaging system. Careful consideration about the costs and skills required for the implementation of any *in vivo* imaging system can help users choose the setup that is most appropriate for their research (Table 1).

6. Data analysis

An important factor to consider when designing optical imaging experiments is the approach for data extraction and analysis following collection. Depending on acquisition parameters, functional imaging dataset file sizes approach or exceed 1 GB/min, which necessitates careful consideration of all subsequent data handling, processing, and storage. Moreover, these datasets require preprocessing before any downstream analysis can be performed. This can pose a significant barrier to new users without sophisticated programming skills. Here we summarize current approaches for analysis of optical imaging datasets.

Preprocessing pipelines are compatible with one or more time series file types, such as AVI or TIFF, which may differ from the file type collected by the recording software. Converting data formats with ImageJ/FIJI (Schindelin et al., 2012) is generally successful and recommended in such cases.

Typically, calcium imaging analysis begins with a motion correction step to remove artifacts evoked by natural brain movement, which can be exaggerated in freely moving animals (Fig. 1B). Subsequent neuronal activity extraction is critically dependent on a stable video, and thus removing movement artifacts is vitally important. For small FOVs, movement problems can be solved under assumptions of translational movement with standard rigid motion correction methods (Thevenaz et al., 1998), where for example all frames are matched to a standard template. However, as the size of the FOV grows, non-uniform motion artifacts arise across the FOV, requiring methods that correct for movement in a piecewise manner, which the non-rigid motion correction (NoRMCorre) approach sought to address (Pnevmatikakis and Giovannucci, 2017). However, by using block-based registration, assumptions and restrictions are made regarding the form and extent of movements that may not always be accurate to single-photon microendoscopic data. Moreover, since all frames from a given dataset are registered to one reference frame, movement correction errors can be prone to propagation. Recent pipelines for single-photon microendoscopic data typically employ NoRMCorre or combine rigid and non-rigid modules to increase processing efficiency and decrease registration time (Lu et al., 2018; Giovannucci et al., 2019).

Following motion correction, the main analysis task is “source extraction.” Calcium-imaging data are innately high-dimensional ($n_{\text{pixels}} \times n_{\text{frames}}$) and meaningful interpretation of the data requires the extraction of the spatial locations of putative neuronal components and their respective fluorescent signals across time. This was initially performed via region-of-interest (ROI) analysis, which involved manually circling clusters of pixels suggestive of GECI expression. While successful in some applications, ROI analysis is not well-suited to datasets containing a high density and large number of GCaMP-expressing cells, mainly due to labor intensity and difficulties associated with the manual separation of signal crosstalk (overlapping and/or out-of-focus regions). ROI analysis is also susceptible to background contamination. ROI analysis can be conducted with the ImageJ MosaicSuite plugins (Sbalzarini and Koumoutsakos, 2005). Python implementations of ROI analysis are also available in SIMA and SamuROI (Kaifosh et al., 2014; Rueckl et al., 2017).

Automated source extraction began with a combination of principal component analysis (PCA) to find and discard dimensions encoding noise, followed by independent component analysis (ICA) for extracting spatial footprints and fluorescent traces (Mukamel et al., 2009). While faster and less prone to crosstalk than ROI analysis, PCA/ICA has several pitfalls as well. First, the number of neuronal components must be manually entered following visual estimation. Second, as a stochastic algorithm, PCA/ICA results are likely to be different across repeated analyses of a dataset. Third, it is ill-suited to datasets from neuronal populations with sparse activity levels and low SNR. Finally, PCA/ICA is a linear demixing method and does not perform well when neural components substantially overlap (Pnevmatikakis et al., 2016; Lu et al., 2018).

Constrained nonnegative matrix factorization (CNMF) was introduced to simultaneously denoise, deconvolve, and demix calcium imaging data, accounting well for overlapping spatial footprints (Pnevmatikakis et al., 2016). Optimized for two-photon imaging data, CNMF works best in situations with high SNR, stable FOV, and simple spatiotemporal structure, similar to another two-photon data analysis package, Suite2p (Pachitariu et al., 2017), available in MATLAB and Python. However, CNMF does not perform well on single-photon microendoscopic datasets as the background is not modeled well. Thus, extended CNMF for microendoscopic data (CNMF-E) was developed to model a more accurate and flexible spatiotemporal background and incorporates better algorithms to initialize and fit neural components, producing better results in low SNR conditions and improving downstream analyses as compared to PCA/ICA extractions (Zhou et al., 2018) (Fig. 1C). As with PCA/ICA, CNMF-E can have problems with false-negative and false-positive ROI identification (although manual intervention steps have been successfully implemented), depends on parameter tuning, and will fail with a non-stable imaging field (Lu et al., 2018). Python and MATLAB each support CNMF in the Calcium Imaging Analysis (CAIMAN) pipeline (Giovannucci et al., 2019). CNMF-E is available in MATLAB, and core components of NoRMCorre and CNMF-E have been combined into a continuous analysis pipeline.

The EZCalcium pipeline was recently introduced as a graphical user interface (GUI)-based approach in MATLAB for users without prior programming experience (Cantu et al., 2020). This pipeline is currently optimized for two-photon imaging data, but its open source nature should allow a module such as CNMF-E to be easily integrated. Another source extraction module called A Technique for Extracting Neuronal Activity from Single Photon Neuronal Image Sequences (TENASPIS) is available in MATLAB (Kinsky et al., 2018; Mau et al., 2018), but has only been used for hippocampal populations and has not been benchmarked against other source extraction methods.

A recent end-to-end pipeline developed for microendoscopic data (MIN1PIPE) purported to solve both the movement correction problem (outperforming NoRMCorre) and source extraction problem (outperforming PCA/ICA and CNMF-E) (Lu et al., 2018). MIN1PIPE does not completely eliminate the need for some manual ROI pruning, but it is nearly automated, and the involved parameter-setting is straightforward and error-tolerant. MIN1PIPE’s precision may be improved with future advances, but it is likely the most easily

implemented and accurate pipeline currently available for single-photon microendoscopic data. MIN1PIPE code is available for MATLAB.

Finally, one of the most powerful capabilities enabled by optical imaging is the ability to revisit the same FOV and neuronal populations across multiple sessions, and thus longitudinally monitor within-neuron changes in activity, which is crucial to research areas such as learning and long-term memory. Automated implementations of inter-session cell registration are available in CAIMAN and CellReg (Sheintuch et al., 2017; Giovannucci et al., 2019).

File sizes, hardware capabilities, and preprocessing methods chosen will all determine the time cost associated with optical imaging datasets. Processing speeds are substantially increased by spatial and/or temporal downsampling, which are often required as many analysis methods hold the video in random access memory (RAM) for analysis. Thus, computers with 32 GB RAM or more are generally desirable for analysis on local machines, and computers with up to 128 GB RAM have been used in published benchmark comparisons between analysis methods (Zhou et al., 2018). Cloud computing services have been used to significantly speed up processing, offering a promising avenue for future optimization (Namboodiri et al., 2019).

After obtaining the extracted data, the experimenter must overcome the next obstacle of figuring out what to do with it. This “post-processing analysis” will be determined by the experimental question. Simple baseline-corrected, time-locked fluorescence changes can be used to report how neurons from a dataset respond acutely to behavioral events such as foot shocks, social interaction, or food consumption (Jennings et al., 2015; Zhou et al., 2018; Jennings et al., 2019; Rossi et al., 2019) (Fig. 1D). More sophisticated analyses may seek to determine how hippocampal place cells encode spatial location (Ziv et al., 2013; Kinsky et al., 2018), how neuronal responses change over time (Namboodiri et al., 2019; Otis et al., 2019; Rossi et al., 2019), or how activity data from large populations of neurons can be used to decode behavioral states (Gründemann et al., 2019; Rubin et al., 2019). While the possibilities for data analyses and presentation are boundless, some consideration of post-processing analysis is required prior to experimentation to ensure all relevant collected data may be a factor in subsequent steps. Combining functional imaging methods with recent advances in sophisticated behavioral scoring such as SimBA and DeepLabCut (Nath et al., 2019; Nilsson et al., 2020) is likely to yield detailed insights into the relationship between neuronal activity and behavior in the near future. See Table 2 for a list of selected methods for functional imaging analysis.

7. Conclusions

Over the past decade we have observed rapid development towards state-of-the-art techniques for imaging neuronal activity deep in the brain. Advancements in deep brain neuronal imaging have led to insights about how central inputs and outputs relate to hunger and thirst (Betley et al., 2015; Lutas et al., 2019), taste (Barretto et al., 2015; Patel et al., 2019), sleep (Weber et al., 2018; Blanco-Centurion et al., 2019), narcoleptic cataplexy (Sun et al., 2019), pain (Hua et al., 2020), anxiety (Shin et al., 2018), locomotion

(Karnani et al., 2020), social interaction (Francis et al., 2017; Lu et al., 2017), threat responses (Gore et al., 2014), defensive strategies (Lecca et al., 2020), and drug abuse (Cameron et al., 2019; Heinsbroek et al., 2020). The increasing popularity of population-level analyses will continue to elucidate neuronal linkages to behavior (Gründemann et al., 2019; Rubin et al., 2019; Xu et al., 2020). Still, opportunities for major technical advancements remain, including optimization of surgical procedures, improvement of the endoscope-to-GRIN lens interface using screw-on microscope bodies, and miniaturization of the implant footprint for simultaneous multi-site recordings (de Groot et al., 2020). In addition, technical improvements to manipulate focus along the z-axis in single-photon systems and biological improvements for multi-channel imaging of cellular nuclei markers in combination with cytosolic markers to help distinguish cells will be beneficial. Once these challenges are resolved, versatile and low-cost imaging systems, potentially including *in vivo* two-photon imaging in freely moving animals (Helmchen et al., 2001; Engelbrecht et al., 2008; Helmchen et al., 2013), will take aim at the growing array of deep brain cell types defined by molecular characteristics (Saunders and Sabatini, 2015), anatomical connections (Chen et al., 2018; Li et al., 2020), and activity patterns (Guenthner et al., 2013; Grewe et al., 2017; Gründemann et al., 2019; Rubin et al., 2019).

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Highlights

- Calcium imaging is a powerful tool for studying neuronal function
- The many options for this technique make it flexible yet challenging
- Deep brain imaging is possible with the use of GRIN lenses
- We explain basic concepts and outline approaches to various experimental scenarios

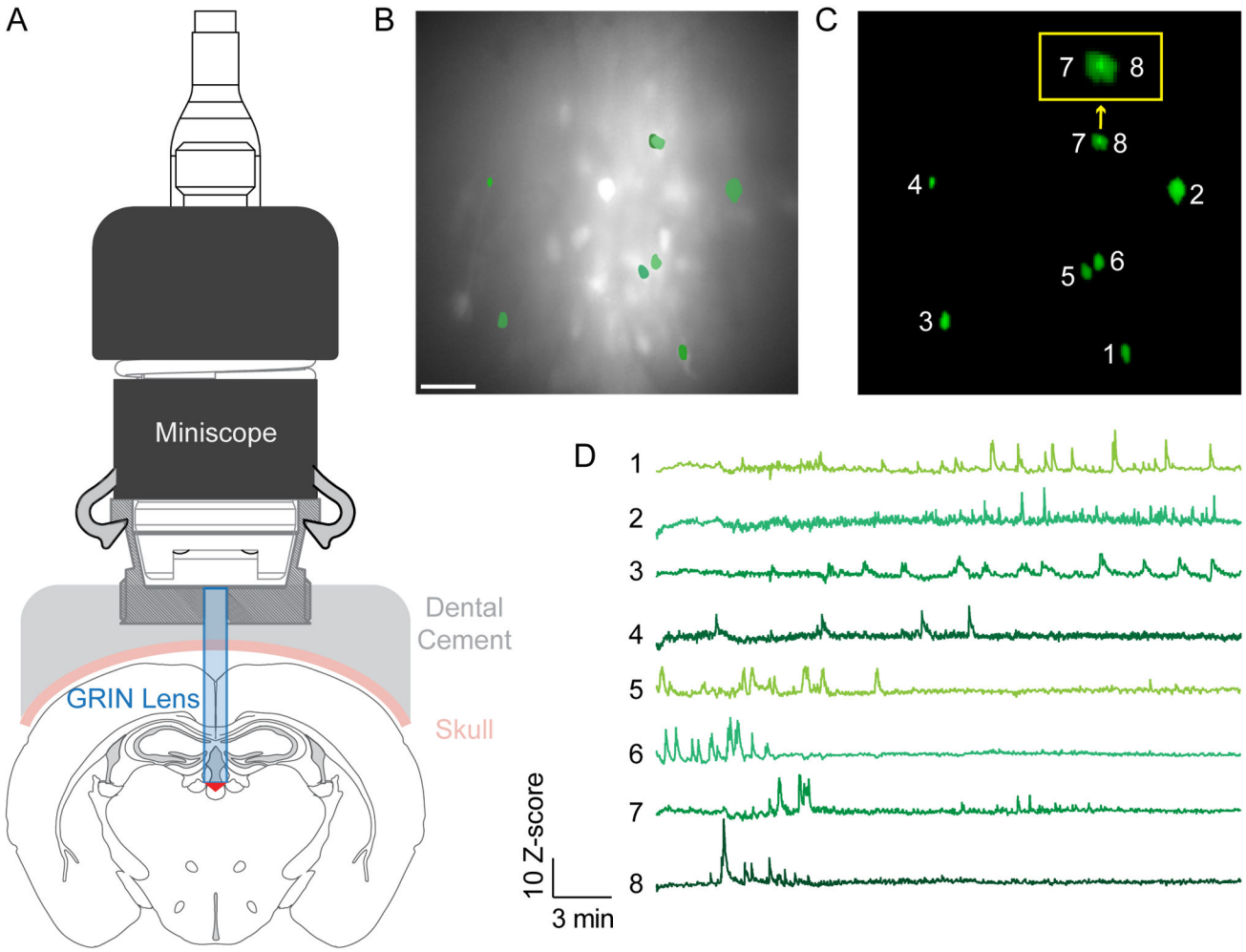

Figure 1.

Functional imaging of neuronal activity from a deep-brain region *in vivo* using a single-photon miniscope.

A) Schematic cross-sectional representation of a 500-μm GRIN lens inserted above the paraventricular thalamus (PVT) and miniscope attachment for imaging in freely moving mice. An adeno-associated virus with jGCaMP7f (AAV9-syn-FLEX-jGCaMP7f-WPRE; Addgene:104492-AAV9) was injected into the PVT of *Drd2-Cre* transgenic mice (MGI:3836635). **B)** Representative image depicting the maximum intensity per pixel from a motion-corrected video. Scale bar = 100 μm. **C)** Selected sample ROIs from (B) extracted by CNMF-E. (Inset outlined in yellow is a zoomed-in view of neurons 7 and 8, demonstrating the ability of CNMF-E to demix overlapping signals). **D)** Calcium traces extracted by CNMF-E corresponding to ROIs in (C). Permission to publish miniscope drawing granted by Doric Lenses Inc.

Table 1.

Microendoscopy system features

Feature	Fiber Photometry	Single-photon Imaging	Two-photon Imaging
Resolution	Population dynamics, no single-cell resolution	Cellular resolution, overlapping cells in Z-space	Subcellular resolution, optical sectioning
Restraint	Freely moving	Freely moving	Typically head-fixed; freely moving not widely available
Motion Correction	Second channel used to remove motion artifacts	Required	Required
Skill required	+	++	+++
Footprint	Small	Moderate, dual-site imaging possible	Large
Cost	$	$$ − $$$	$$$$

Table 2.

Directory of selected methods for functional imaging analysis.

Analysis	Link	Reference
ROI analysis	http://mosaic.mpi-cbg.de/?q=downloads/imageJ	(Sbalzarini and Koumoutsakos, 2005)
PCA/ICA	https://github.com/mukamel-lab/CellSort	(Mukamel et al., 2009)
SIMA	https://github.com/losonczylab/sima	(Kaifosh et al., 2014)
SamuROI	https://github.com/samuroi/SamuROI	(Rueckl et al., 2017)
CAIMAN/CNMF	https://github.com/flatironinstitute/CaImAn-MATLAB	(Pnevmatikakis et al., 2016; Giovannucci et al., 2019)
CNMF-E	https://github.com/zhoupc/CNMF_E https://github.com/etterguillaume/MiniscopeAnalysis (Includes NoRMCorre)	(Zhou et al., 2018)
MIN1PIPE	https://github.com/JinghaoLu/MIN1PIPE	(Lu et al., 2018)
Suite2p	https://github.com/MouseLand/suite2p	(Pachitariu et al., 2017)
EZCalcium	https://github.com/porteralab/EZcalcium	(Cantu et al., 2020)
TENASPIS	https://github.com/SharpWave/TENASPIS	(Kinsky et al., 2018; Mau et al., 2018)
CellReg	https://github.com/zivlab/CellReg https://github.com/JinghaoLu/CellReg	(Sheintuch et al., 2017)