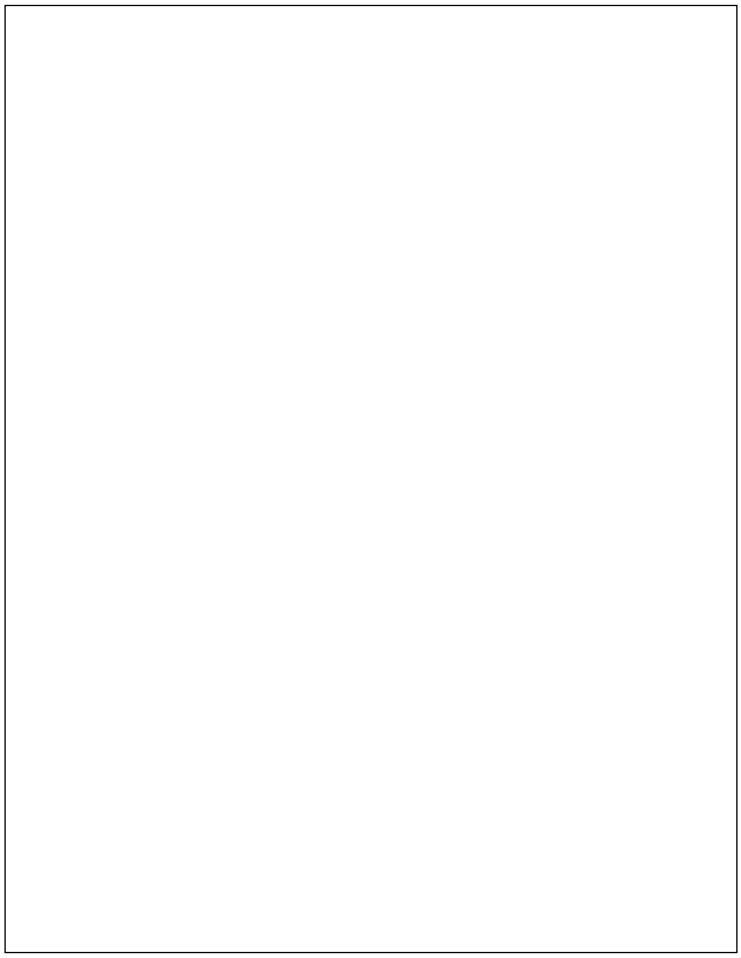

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Cellular resolution imaging of neuronal activity across space and time in the mammalian brain

Mitchell Clough[1] and Jerry L. Chen[1,2,3]

Abstract

The action potential has long been understood to be the fundamental bit of information in brain, but how these spikes encode representations of stimuli and drive behavior remains unclear. Large-scale neuronal recordings with cellular and spike-time resolution spanning multiple brain regions are needed to capture relevant network dynamics that can be sparse and distributed across the population. This review focuses on recent advancements in optical methods that have pushed the boundaries for simultaneous population recordings at increasing volumes, distances, depths, and speeds. The integration of these technologies will be critical for overcoming fundamental limits in the pursuit of whole brain imaging in mammalian species.

Addresses

[1] Department of Biomedical Engineering, Boston University, Boston, USA

[2] Department of Biology, Boston University, Boston, USA

[3] Center for Neurophotonics, Boston University, Boston, USA

Corresponding author: Chen, Jerry L (jerry@chen-lab.org)

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Introduction

To understand how the brain is capable of carrying out a certain behavior, it is necessary to determine the underlying neural code that can generate complex cognitive thought. To characterize the network activity patterns that form the basis of such computations, the ability to measure information processing at its native speed and fidelity in a comprehensive manner across all participating neurons has become both a scientific and engineering goal in neuroscience. Technologies for large-scale simultaneous recordings of neuronal populations spanning an entire brain will inform us how our nervous system represents the external environment, forms and recalls memories, makes decisions, and carries out actions.

Compared with electrophysiological methods, optical methods to noninvasively measure population activity provide distinct advantages in the ability to obtain precise spatial information, enabling the multimodal mapping of gene expression [1] and connectivity [2] that is critical for the dissection of circuit function. Continuing advancements in genetically encoded fluorescent activity sensors have driven the development of both one-photon and multiphoton microscopy for simultaneous population recordings at increasing volumes, distances, depths, and speeds. This review will focus on recent advancements in microscopy techniques that have pushed the boundaries across these different dimensions and discuss the prospects for potentially imaging activity across the entire mammalian brain.

Whole mammalian brain imaging: specifications and challenges

What would it take to image the activity of every single neuron in a mammalian brain? The engineering challenges are best understood by considering the physical requirements and the biological constraints. Development of optical systems has been focused on imaging the mouse brain which is composed of $\sim$75 million neurons that occupy a volume of $\sim$500 mm^3. To resolve the cell body of each neuron, an optical resolution of $\leq$5 μm is needed. One fundamental challenge is developing optical systems that are capable of imaging large volumes of tissue at high spatial resolution. The second fundamental challenge is the imaging speed required to capture population dynamics. The necessary speed depends on the signals being measured and the indicators of activity being used. The membrane action potential of a neuron is the direct measure of a neuron's activity. Detecting an action potential requires $\sim$1 ms temporal resolution. Combining the temporal and spatial resolutions required along with the volume of the brain means imaging 500,000 mm^3/s at 5 μm resolution to capture all the action potentials of the mouse brain. More modest speeds are sufficient when using calcium signals as a proxy for action potential firing activity. Calcium influx into the neuron occurs on the order of $\sim$100 ms, which translates to volume imaging rates of 5,000 mm^3/s.

Beyond the spatial and temporal requirements, a major consideration is the photon budget involved in exciting the fluorescent calcium or voltage indicators. Detection of action potential–related events is determined by the overall brightness, activity-related change in

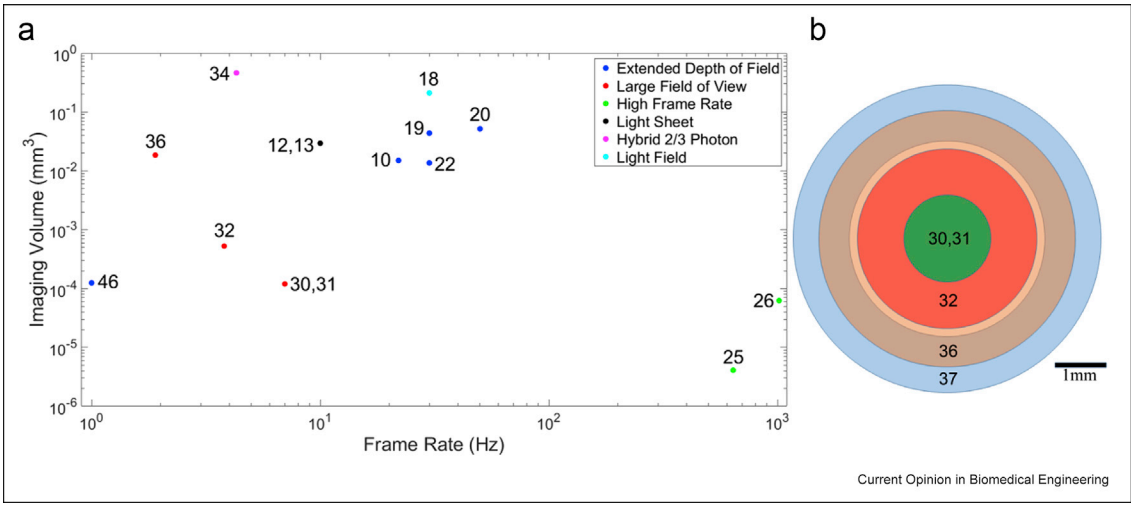
fluorescence, kinetics, and availability of the indicator [3–5]. Current calcium indicators are expressed in the cytosol, filling the volume of the neuron, with transients on the order of 100 milliseconds and signal changes up to 10-fold above baseline. Depending on how well the timing of spike-related events needs to be resolved, relevant calcium signals can be recorded at a rate in the range of ~1–30 Hz and at an average power of less than 30 mW per voxel. In contrast, voltage indicators need to be localized to the cell membrane and possess millisecond response times [6,7]. The differences in localization and kinetics severely limit the number of available excitable molecules during voltage imaging, requiring greater photo-energy to achieve comparable signal-to-noise as during calcium imaging. However, the light energy needed for excitation is ultimately limited by the amount of light the brain can safely accept without producing thermal damage and phototoxicity. Up to 400 mW at a 33% duty cycle across 1 h can be delivered before tissue damage is observed [8]. This indicates that an efficient photon budget is needed to achieve high-speed, large-scale excitation. The final major factor is the high degree of light scattering that occurs in mammalian brain tissue which limits the depth of imaging possible by reducing excitation efficiency as well as the ability to spatially resolve emitted fluorescence during detection [9].

Imaging neuronal activity of increasing volumes

Developing technologies to match the physical and biological requirements for whole brain imaging is a formidable challenge. Individual efforts, thus far, have been focused on pushing the capabilities along a subset of dimensions, which can still be used for specific experiments (Figure 1). Volumetric imaging has applications for comprehensively measuring local circuit dynamics and understanding functional relationships in the architectural organization of brain areas. Several methods using temporally or spatially structured illumination have been applied to increase imaging volume while maintaining sufficient temporal resolution. Widefield one-photon fluorescence microscopy has traditionally provided large field of view imaging without scanning. Because of the lack of spatial confinement, axial information about the sample is normally not present. To address this, modulated-illumination extended-depth-of-field imaging (MI-EDOF) can be used to gain axial resolution [10]. This technique makes use of a deformable mirror (DM) to increase the depth of field of a standard widefield imaging setup [11]. The DM allows for rapidly sweeping the focus of the microscope axially which allows in-focus imaging of a whole volume at frame rates limited by the camera. By controlling the intensity of the illumination light as a function of the focal depth and then using the optical transfer function to perform deconvolution, the axial position of each pixel in the image can be estimated.

Light-sheet microscopy makes use of laser light focused along one axis and detection optics arranged perpendicularly to the excitation light to optically section a volume at high speeds. This is typically accomplished through the use of two separate objectives, one for fluorescence excitation and one for fluorescence detection. Although this configuration is amenable to imaging the brains of small organisms such as zebrafish, it presents steric challenges for imaging larger mammalian brains. One method to address this issue, called swept confocally-aligned planar excitation (SCAPE) [12,13], makes use of oblique plane microscopy (OPM) [14]. SCAPE allows for 3D volumetric

Figure 1



(a) Survey of current imaging systems (numbered references) developed for cellular resolution brain imaging plotted along imaging volume and temporal resolution. **(b)** Total field of views of current imaging systems.

imaging at volume rates of 10 Hz in mice. A downside to the original implementation of SCAPE [12] was that the excitation light sheet tilted as a volumetric scan was performed. This creates a point-spread-function (PSF) which is variable across a scan and adds computational complexity to the exact 3D reconstruction of the scanned volume. More recently, SCAPE 2.0 [13] and another implementation of OPM called scanned oblique plane illumination microscopy (SOPi) [15,16] tackle this issue so that the excitation light sheet maintains a constant angle relative to the sample across a volumetric scan.

Light field microscopy (LFM) is another technique which enables volumetric imaging using single photon excitation. LFM involves inserting a microlens array at the image plane of a widefield fluorescence microscope and then placing a camera at the focal plane of the microlens array. This enables capturing the 2D location and 2D angle of the emitted light and by postprocessing, the full 3D light field that produced the detected light can be recovered [17]. LFM therefore allows for volumetric imaging without the need for scanning. Recently, a new computational technique called seeded iterative demultiplexing (SID) has been developed to allow for use of LFM in mammalian cortex [18]. Making use of SID allowed for imaging of a 900 x 900 $\times$ 260 μm volume at 30 Hz at depths up to 380 μm in mouse cortex.

Because widefield, light-sheet, and light field microscopy make use of one-photon excitation, depth penetration is typically limited to less than the first 150 μm of the brain surface. Two-photon microscopy improves on the depth penetration because of the longer excitation wavelength and its optical sectioning capabilities through multiphoton absorption. Raster scanning of a Gaussian PSF comes with a trade-off between imaging volume and speed. However, extending the functionality of two-photon imaging to 3D can be achieved by generating an axially elongated PSF. This has been demonstrated in vivo by use of a spatial light modulator [19] and an axicon [20]. By elongating the PSF, it is possible to generate an extended-depth-of-field image to achieve volume imaging at the same rate as one would normally image a 2D plane. In dense labeling conditions, the lack of depth information results in difficulty resolving activity from overlapping structures. This can be overcome post-hoc through computational analysis to demix signals based on their spatial and temporal structure [21].

The lack of depth information provided by a single Bessel beam can alternatively be overcome with volumetric two-photon imaging of neurons using stereoscopy (vTwINS) [22]. By generating a PSF with two axially elongated arms at an angle to each other, vTwINS makes use of stereoscopy to disambiguate signals coming from different depths. The v-shaped PSF of the microscope generates two images of each neuron, separated by a distance related to (1) the angle between the two arms of the PSF and (2) the depth of the neuron in the tissue. As a result, the 3D volume is compressed to a 2D image, but information about the axial position of each neuron is preserved.

Imaging neuronal activity at increasing speeds

The development of genetically encoded voltage indicators and novel indicators of neurotransmitters such as glutamate with millisecond kinetic properties necessitates the development of imaging systems at kilohertz temporal resolution. Random-access scanning technologies provide some increase in temporal resolution by restricting scan time to voxels of interest [23]. Detecting and correcting for brain motion is a challenge for random-access scanning, which can be addressed with scanning of multiple subvolumes [24] or through closed-loop online motion correction. Despite this, sampling time per voxel remains the same and thus imaging time scales with increasing voxels. To increase throughput, parallel scanning of multiple beams can be used. Multiple spatially arranged beams can be generated using diffractive optic elements or spatial light modulators [25]. Camera sensors or multianode photomultiplier tubes (PMTs) are required to spatially resolve the emitted fluorescence from each beamlet and thus can suffer from spatial crosstalk at increasing depths because of light scattering.

Temporal resolution can also be increased by combining new imaging systems with post-hoc computational imaging. A two-photon scanned line angular projection (SLAP) microscope that uses tomography by scanning line foci across a two-dimensional sample plane at multiple different angles has recently been developed [26]. Although spatial information along one scanned line focus collected through a single PMT is compressed into a single data point, measurements across different angles overlap at only one point in space. Assuming that neuronal activity is sparse across time and using prior structural information obtained through a reference image, neuronal activity can be spatially localized from the acquired data through demixing. The SLAP system was able to achieve frame rates of 1 kHz.

Scanning two planes simultaneously can also increase the imaging speed of a microscope. This has been accomplished by using a spatial light modulator (SLM) to create multiple beamlets, which target different regions in the sample [27]. The result is an image which is a superposition of each of the individual planes being imaged. By taking images of each of these planes individually and using the spatial information from those images, the neuronal calcium signals can be demixed. SLM-based multiplane scanning along with random-access scanning and SLAP all require prior spatial information about the sample.

Spatial crosstalk can be avoided through spatiotemporal multiplexing [28–32], which involves splitting the laser beam into multiple different beam paths and then delaying the different beams so that each light pulse arrives at the sample at different times. The emitted fluorescence is detected by a single detector as in a standard 2P system, and the area of origin for the detected fluorescence is demultiplexed according to the timing of the laser pulses. Crosstalk is minimized by ensuring the interpulse interval is sufficiently longer than the fluorescence lifetime of the indicator being used [29,32]. Spatiotemporal multiplexing allows for similar multiplane imaging as previously described [27] but does not require postprocessing of the images acquired to demix the spatial location of the neuronal calcium signals.

A final approach to increasing imaging speeds is to treat each neuron as a single voxel and devise methods to more efficiently excite all indicators expressed in a single cell with a single laser pulse. Temporal focusing can be used to decouple the lateral and axial resolution of the microscope [33], creating PSFs on the order of 5 μm × 5 μm x 10 μm. PSFs of this size maintain the needed spatial resolution for neuronal imaging and enable scanning of one neuron per laser pulse per voxel at a time. Increased excitation efficiency enables increases in imaging speed while maintaining signal-to-noise and can be scaled up using spatiotemporal multiplexing [34].

Imaging neuronal activity at increasing distances

The need to investigate interactions in neuronal activity patterns between brain areas requires imaging at increasingly large fields of view (FOV) while maintaining cellular resolution. Surgical implants like "crystal skull" now provide optical access to the entire dorsal mouse cortex such that multiple cortical areas can be imaged simultaneously [35]. Most standard commercial off-the-shelf 2P microscopes can only record from an area of $\sim$1 mm^2 which limits the ability to record from multiple cortical regions. Increasing the FOV of a 2P microscope while maintaining a high numerical aperture (NA) has required larger custom optics that allow for a wider scan angle and a larger beam diameter at the back pupil of the objective.

The development of the two-photon random-access microscope (2P-RAM) involved designing custom optics that allowed for imaging across a 5 mm diameter at an NA of 0.6 [36]. With this system, scanning of multiple areas must be done sequentially which decreases the temporal resolution proportionally to the number of regions being scanned. To dissociate the frame rate with the number of areas being simultaneously imaged, other designs have been created which combine a large FOV with spatiotemporal multiplexing [30–32] (Figure 2). In one implementation, beamlets are targeted onto different regions of interest through lateral x/y-movement of independent "focal plane units" which introduce an offset of the respective beamlet from the optical axis of the first scan lens. This offset is converted into a pivoting angle of the beamlet onto scan mirrors that synchronously scan the beamlets at the sample [30,31]. Independent z-positioning is further achieved using electrically tunable lenses. In the twin-region, panoramic 2P microscope, independent steering mirrors are used to deflect each beamlet path along the optical axis and then the beamlets are recombined onto the same scan engine [32].

Postobjective scanning can also increase the FOV of a 2P microscope. This has been enabled by the development of high-NA long working distance objectives that provide space to introduce small mirrors on a rotation mount in the space between the objective and the brain, providing a donut-shaped FOV [37]. Using postobjective scanning, an FOV of 6 mm in diameter was achieved without an increase in size of the PSF across the FOV.

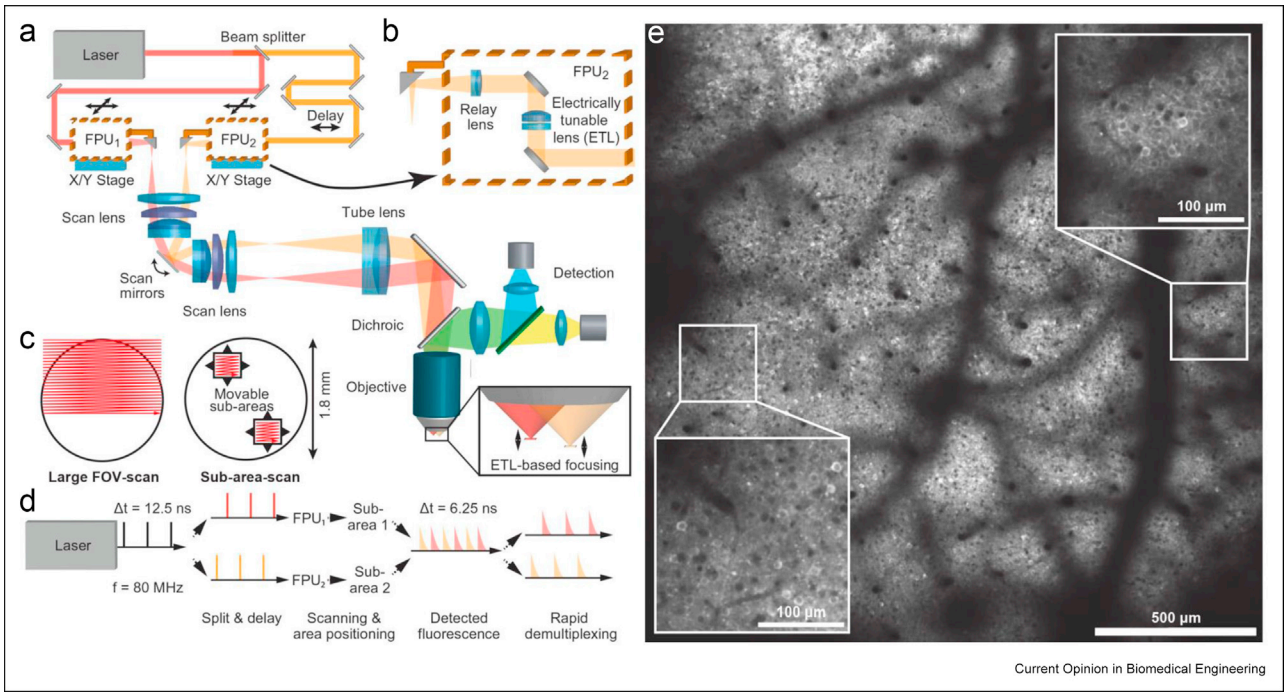
Beyond a 6 mm separation distance, geometric constraints become less restrictive for integrated imaging using multiple microscope systems. A dual-axis two-photon microscope uses two independent microscopes and microendoscopes that can be positioned at any spatially nonrestrictive locations across the brain [38]. This overcomes the limitations of single-objective techniques that require the imaged regions be located under a relatively flat field of view.

Imaging neuronal activity at increasing depths

Most of the advancements described earlier have been focused on relatively superficial imaging at the cortical level. While deeper, subcortical imaging can potentially be achieved surgically through the implantation of a prism [39,40] or gradient index lens [41,42], noninvasive imaging remains a challenge. Increasing depth penetration with 2P imaging can be achieved by underfilling a high-NA objective, thereby reducing the effective excitation NA [43]. Lower NA excitation avoids some inefficiencies related to refractive index mismatch and light scattering deep within the tissue that is more prominent with higher NA excitation, but this occurs at the expense of resolution. Both resolution and excitation efficiency can be maintained deeper into tissue through the use of direct wavefront sensing and adaptive optics which directly measure the tissue-induced aberrations and correct for such distortions in the excitation wavefront [44].

Alternative to 2P microscopy, three-photon (3P) microscopy offers an increase in signal-to-background ratio

Figure 2



Multiarea Two-Photon Microscope. (**a**) Diagram of system capable of simultaneous imaging across cortical areas. **(b, c)** Focal plane units enable independent positioning of subareas within large field of view. **(d)** Multibeam imaging is enabled by spatiotemporal multiplexing and demultiplexing. **(e)** *In vivo* images of mouse cortex taken from the multiarea two-photon microscope. (adapted from ref. 31).

and possible imaging depth [45]. Three-photon microscopy makes use of longer wavelength excitation light (1300 or 1700 nm) than 2P and thus suffers less from scattering on the excitation side and allows for deeper imaging. The high peak power needed to generate three-photon excitation involves constructing lasers with lower repetition rates (~1 MHz) than their 2P counterparts (~80 MHz). This lower repetition rate increases the dwell times necessary to ensure that sufficient numbers of indicator molecules are excited to generate an acceptable signal-to-noise ratio which effectively limits the speed of imaging.

Recent attempts have been made to overcome the limitations imposed by a slower frame rate using 3P microscopy. Namely, a Bessel beam has been used to perform EDOF imaging with a 3P microscope [46]. This technique was able to achieve PSFs which were axially elongated to 20–90 µm while maintaining a similar lateral resolution to normal 3P excitation. Recently, 2P and 3P excitation have been used simultaneously in a technique called Hybrid Multiplexed Sculpted Light Microscopy (HyMS) [34]. HyMS combines spatiotemporal multiplexing, temporal focusing, and one pulse per voxel excitation to achieve imaging of a 690 x 675 x 1,000 µm volume at a rate of 13.0 Hz for the 2P volume and 4.3 Hz for the 3P volume, sufficient for volume scanning across an entire cortical column.

Conclusion

In conclusion, active and diverse developments in novel imaging systems are pushing the capacity for population-level neuronal imaging across multiple spatial and temporal dimensions. Currently, no one-size-fits-all solution exists. The appropriate technology to be used depends on the specific biological question to be studied. Whether the current set of technologies are capable of achieving the goal of imaging an entire mammalian brain is unclear. Continuing efforts to integrate the technologies described can serve to push the boundaries to reach the necessary theoretical specifications. In parallel, ongoing improvements in the sensitivity and signal-to-noise of genetically encoded sensors will help to lower some of these theoretical barriers. It is clear that the limit has not been reached and there is ample room for further advances to potentially observe the entire brain in action.

Declaration of competing interest

Nothing declared.

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References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. Lovett-Barron M, Andalman AS, Allen WE, Vesuna S, Kauvar I, Burns VM, Deisseroth K: **Ancestral circuits for the coordinated modulation of brain state**. *Cell* 2017, **171**:1411–1423.e17.

2. Lee W-CA, Bonin V, Reed M, Graham BJ, Hood G, Glattfelder K, Reid RC: **Anatomy and function of an excitatory network in the visual cortex**. *Nature* 2016, **532**:370–374.

3. Weisenburger S, Prevedel R, Vaziri A: **Quantitative evaluation of two-photon calcium imaging modalities for high-speed volumetric calcium imaging in scattering brain tissue**. *bioRxiv* 2017. https://doi.org/10.1101/115659.

4. Sjulson L, Miesenböck G: **Optical recording of action potentials and other discrete physiological events: a perspective from signal detection theory**. *Physiology* 2007, **22**:47–55.

5. Wilt BA, Fitzgerald JE, Schnitzer MJ: **Photon shot noise limits on optical detection of neuronal spikes and estimation of spike timing**. *Biophys J* 2013, **104**:51–62.

6. St-Pierre F, Marshall JD, Yang Y, Gong Y, Schnitzer MJ, Lin MZ: **High-fidelity optical reporting of neuronal electrical activity with an ultrafast fluorescent voltage sensor**. *Nat Neurosci* 2014, **17**:884–889.

7. Kralj JM, Hochbaum DR, Douglass AD, Cohen AE: **Electrical spiking in Escherichia coli probed with a fluorescent voltage-indicating protein**. *Science* 2011, **333**:345–348.

8. Podgorski K, Ranganathan G: **Brain heating induced by near-infrared lasers during multiphoton microscopy**. *J Neurophysiol* 2016, **116**:1012–1023.

9. Helmchen F, Denk W: **Deep tissue two-photon microscopy**. *Nat Methods* 2005, **2**:932–940.

10. Shain WJ, Vickers NA, Li J, Han X, Bifano T, Mertz J: **Axial localization with modulated-illumination extended-depth-of-field microscopy**. *Biomed Opt Express* 2018, **9**:1771–1782.

11. Shain WJ, Vickers NA, Goldberg BB, Bifano T, Mertz J: **Extended depth-of-field microscopy with a high-speed deformable mirror**. *Opt Lett* 2017, **42**:995–998.

12. Bouchard MB, Voleti V, Mendes CS, Lacefield C, Grueber WB, Mann RS, Bruno RM, Hillman EMC: **Swept confocally-aligned planar excitation (SCAPE) microscopy for high-speed volumetric imaging of behaving organisms**. *Nat Photonics* 2015, **9**:113–119.

13. Voleti V, Patel KB, Li W, Campos CP, Bharadwaj S, Yu H, Ford C, Casper MJ, Yan RW, Liang W, *et al.*: **Real-time volumetric microscopy of in vivo dynamics and large-scale samples with SCAPE 2.0**. *Nat Methods* 2019, **16**:1054–1062.

14. Dunsby C: **Optically sectioned imaging by oblique plane microscopy**. *Opt Express* 2008, **16**:20306–20316.

15. Kumar M, Kishore S, Nasenbeny J, McLean DL, Kozorovitskiy Y: **Integrated one- and two-photon scanned oblique plane illumination (SOPi) microscopy for rapid volumetric imaging**. *Opt Express* 2018, **26**:13027–13041.

16. Kumar M, Kozorovitskiy Y: **Tilt-invariant scanned oblique plane illumination microscopy for large-scale volumetric imaging**. *Opt Lett* 2019, **44**:1706–1709.

17. Prevedel R, Yoon Y-G, Hoffmann M, Pak N, Wetzstein G, Kato S, Schrödel T, Raskar R, Zimmer M, Boyden ES, *et al.*: **Simultaneous whole-animal 3D imaging of neuronal activity sing light-field microscopy**. *Nat Methods* 2014, **11**:727–730.

18. Nöbauer T, Skocek O, Pernía-Andrade AJ, Weilguny L, Traub FM, Molodtsov MI, Vaziri A: **Video rate volumetric Ca^{2+} imaging across cortex using seeded iterative demixing (SID) microscopy**. *Nat Methods* 2017, **14**:811–818.

19. Lu R, Sun W, Liang Y, Kerlin A, Bierfeld J, Seelig JD, Wilson DE, Scholl B, Mohar B, Tanimoto M, *et al.*: **Video-rate volumetric functional imaging of the brain at synaptic resolution**. *Nat Neurosci* 2017, **20**:620–628.

20. Lu R, Tanimoto M, Koyama M, Ji N: **50 Hz volumetric functional imaging with continuously adjustable depth of focus**. *Biomed Opt Express* 2018, **9**:1964–1976.

21. Pnevmatikakis EA, Soudry D, Gao Y, Machado TA, Merel J, Pfau D, Reardon T, Mu Y, Lacefield C, Yang W, *et al.*: **Simultaneous denoising, deconvolution, and demixing of calcium imaging data**. *Neuron* 2016, **89**:285–299.

22. ** Song A, Charles AS, Koay SA, Gauthier JL, Thiberge SY, Pillow JW, Tank DW: **Volumetric two-photon imaging of neurons using stereoscopy (vTwINS)**. *Nat Methods* 2017, **14**:420–426.
Volume imaging is achieved using stereoscopic scanning of v-shaped axially elongated excitation beams.

23. Duemani Reddy G, Kelleher K, Fink R, Saggau P: **Three-dimensional random access multiphoton microscopy for functional imaging of neuronal activity**. *Nat Neurosci* 2008, **11**:713–720.

24. Nadella KMNS, Roš H, Baragli C, Griffiths VA, Konstantinou G, Koimtzis T, Evans GJ, Kirkby PA, Silver RA: **Random-access scanning microscopy for 3D imaging in awake behaving animals**. *Nat Methods* 2016, **13**:1001–1004.

25. Bahlmann K, So PTC, Kirber M, Reich R, Kosicki B, McGonagle W, Bellve K: **Multifocal multiphoton microscopy (MMM) at a frame rate beyond 600 Hz**. *Opt Express* 2007, **15**:10991–10998.

26. ** Kazemipour A, Novak O, Flickinger D, Marvin JS, Abdelfattah AS, King J, Borden PM, Kim JJ, Al-Abdullatif SH, Deal PE, *et al.*: **Kilohertz frame-rate two-photon tomography**. *Nat Methods* 2019, **16**:778–786.
Kilohertz frame rate imaging is achieved using tomographic approach to line scanning

27. Yang W, Miller JK, Carrillo-Reid L, Pnevmatikakis E, Paninski L, Yuste R, Peterka DS: **Simultaneous multi-plane imaging of neural circuits**. *Neuron* 2016, **89**:269–284.

28. Amir W, Carriles R, Hoover EE, Planchon TA, Durfee CG, Squier JA: **Simultaneous imaging of multiple focal planes using a two-photon scanning microscope**. *Opt Lett* 2007, **32**:1731–1733.

29. Cheng A, Gonçalves JT, Golshani P, Arisaka K, Portera-Cailliau C: **Simultaneous two-photon calcium imaging at different depths with spatiotemporal multiplexing**. *Nat Methods* 2011, **8**:139–142.

30. Voigt FF, Chen JL, Krueppel R, Helmchen F: **A modular two-photon microscope for simultaneous imaging of distant cortical areas in vivo**. In *Multiphoton microscopy in the biomedical sciences XV*. International Society for Optics and Photonics; 2015. 93292C.

31. Chen JL, Voigt FF, Javadzadeh M, Krueppel R, Helmchen F: **Long-range population dynamics of anatomically defined neocortical networks**. *eLife* 2016, **5**, e14679.

32. Stirman JN, Smith IT, Kudenov MW, Smith SL: **Wide field-of-view, multi-region, two-photon imaging of neuronal activity in the mammalian brain**. *Nat Biotechnol* 2016, **34**:857–862.

33. Prevedel R, Verhoef AJ, Pernía-Andrade AJ, Weisenburger S, Huang BS, Nöbauer T, Fernández A, Delcour JE, Golshani P, Baltuska A, *et al.*: **Fast volumetric calcium imaging across multiple cortical layers using sculpted light**. *Nat Methods* 2016, **13**:1021–1028.

34. ** Weisenburger S, Tejera F, Demas J, Chen B, Manley J, Sparks FT, Martínez Traub F, Daigle T, Zeng H, Losonczy A, *et al.*: **Volumetric Ca2+ imaging in the mouse brain using Hybrid multiplexed sculpted light microscopy**. *Cell* 2019, **177**:1050–1066.e14.

Temporal focusing, spatiotemporal multiplexing, two- and three-photon imaging are all combined to achieve simultaneous volume imaging across a cortical column and below to the hippocampus.

35. Kim TH, Zhang Y, Lecoq J, Jung JC, Li J, Zeng H, Niell CM, Schnitzer MJ: **Long-term optical access to an estimated one million neurons in the live mouse cortex**. *Cell Rep* 2016, **17**: 3385–3394.

36. Sofroniew NJ, Flickinger D, King J, Svoboda K: **A large field of view two-photon mesoscope with subcellular resolution for in vivo imaging**. *eLife* 2016, **5**, e14472.

37. * Terada S-I, Kobayashi K, Ohkura M, Nakai J, Matsuzaki M: **Super-wide-field two-photon imaging with a micro-optical device moving in post-objective space**. *Nat Commun* 2018, **9**: 3550.

This system demonstrates large field of view two-photon by performing raster scanning after the objective.

38. Lecoq J, Savall J, Vučinić D, Grewe BF, Kim H, Li JZ, Kitch LJ, Schnitzer MJ: **Visualizing mammalian brain area interactions by dual-axis two-photon calcium imaging**. *Nat Neurosci* 2014, **17**:1825–1829.

39. Andermann ML, Gilfoy NB, Goldey GJ, Sachdev RNS, Wölfel M, McCormick DA, Reid RC, Levene MJ: **Chronic cellular imaging of entire cortical columns in awake mice using microprisms**. *Neuron* 2013, **80**:900–913.

40. Chia TH, Levene MJ: **Microprisms for in vivo multilayer cortical imaging**. *J Neurophysiol* 2009, **102**:1310–1314.

41. Jung JC, Mehta AD, Aksay E, Stepnoski R, Schnitzer MJ: **In vivo mammalian brain imaging using one- and two-photon fluorescence microendoscopy**. *J Neurophysiol* 2004, **92**:3121–3133.

42. Levene MJ, Dombeck DA, Kasischke KA, Molloy RP, Webb WW: **In vivo multiphoton microscopy of deep brain tissue**. *J Neurophysiol* 2004, **91**:1908–1912.

43. Kondo M, Kobayashi K, Ohkura M, Nakai J, Matsuzaki M: **Two-photon calcium imaging of the medial prefrontal cortex and hippocampus without cortical invasion**. *eLife* 2017, **6**, e26839.

44. Liu R, Li Z, Marvin JS, Kleinfeld D: **Direct wavefront sensing enables functional imaging of infragranular axons and spines**. *Nat Methods* 2019, **16**:615–618.

45. ** Ouzounov DG, Wang T, Wang M, Feng DD, Horton NG, Cruz-Hernández JC, Cheng Y-T, Reimer J, Tolias AS, Nishimura N, *et al.*: ***In vivo* three-photon imaging of activity of GCaMP6-labeled neurons deep in intact mouse brain**. *Nat Methods* 2017, **14**:388–390.

This is the first demonstration of deep non-invasive functional calcium imaging into hippocampus using three photon microscopy.

46. Rodríguez C, Liang Y, Lu R, Ji N: **Three-photon fluorescence microscopy with an axially elongated Bessel focus**. *Opt Lett* 2018, **43**:1914–1917.