Review article

Breaking trade-offs: Development of fast, high-resolution, wide-field two-photon microscopes to reveal the computational principles of the brain

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\textbf{ARTICLE INFO}

\textbf{Keywords:}
- Two-photon microscope
- In vivo calcium imaging
- Large field-of-view
- Large objective lens
- Laser scanning microscope
- Cortical
- Optical invariant
- Large-scale recording

\textbf{ABSTRACT}

Information in the brain is represented by the collective and coordinated activity of single neurons. Activity is determined by a large amount of dynamic synaptic inputs from neurons in the same and/or distant brain regions. Therefore, the simultaneous recording of single neurons across several brain regions is critical for revealing the interactions among neurons that reflect the computational principles of the brain. Recently, several wide-field two-photon (2P) microscopes equipped with sizeable objective lenses have been reported. These microscopes enable large-scale in vivo calcium imaging and have the potential to make a significant contribution to the elucidation of information-processing mechanisms in the cerebral cortex. This review discusses recent reports on wide-field 2P microscopes and describes the trade-offs encountered in developing wide-field 2P microscopes. Large-scale imaging of neural activity allows us to test hypotheses proposed in theoretical neuroscience, and to identify rare but influential neurons that have potentially significant impacts on the whole-brain system.

\section{1. Introduction}

One of the most important missions of neuroscience is to reveal the computational principles underlying the cerebral cortex. To comprehensively understand how neurons in the cerebral cortex represent information, it is essential to record the activity of single neurons from living animals (Ohki et al., 2005; Pluta et al., 2017; Hafting et al., 2005; Harvey et al., 2012; Poulet and Petersen, 2008) because neurons are fundamental elements for the representation of information in the brain. Their activity (i.e., action potentials) is generated by the integration of synaptic inputs from other neurons (Jouhanneau et al., 2015; Froemke et al., 2007; Shu et al., 2003; Crochet et al., 2011). Synaptic inputs are transmitted from neurons in the same brain region, as well as distant brain regions (Wickersham et al., 2007; Petreanu et al., 2009; Zhang et al., 2014; Schwarz et al., 2015; Zingg et al., 2017). Therefore, to understand cortical computational principles, it is essential to simultaneously record the activity of single neurons from multiple brain regions and investigate dynamic interactions among neurons that constitute widely distributed cortical networks.

There are two major methods for recording the activity of multiple single neurons in vivo: electrophysiological techniques using extracellular electrodes and calcium imaging using a multiphoton microscope.

One of the main advantages of electrophysiological techniques is the direct recording of action potentials, which occur in the order of milliseconds. In recent years, high-density electrode Neuropixels with 960 recording sites on a single shank and Neuropixels2 with 5120 recording sites over four shanks have been developed (Jun et al., 2017; Steinmetz et al., 2021). Eight neuropixel probes are used to simultaneously record the activity of more than 2000 neurons from cortical and subcortical areas (Stringer et al., 2019b).

In contrast, calcium imaging allows us to capture calcium fluctuations derived from action potentials instead of sodium ion dynamics. One advantage of calcium imaging over electrophysiological techniques is the visualization of recorded neurons, which has the following further advantages: (1) The neural activity of small structures such as dendrites and axons can be recorded (Svoboda et al., 1997; Petreanu et al., 2009; Jia et al., 2010; Manita et al., 2015; Takahashi et al., 2016; Tanaka et al., 2018). (2) The same targets can easily be recorded across days (Holtmaat et al., 2009; Sadakane et al., 2015; Li et al., 2017). (3) The neural activity in minority groups of neurons (e.g., vasoactive intestinal peptide (VIP)-positive neurons in the cerebral cortex) can be efficiently recorded (Attinger et al., 2017; Garcia-Junco-Clemente et al., 2017). (4) The spatial location of the recorded neurons can be determined at the sub-micron level (Maruoka et al., 2017; Chettih and Harvey, 2019; Russell et al., 2018).

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https://doi.org/10.1016/j.neures.2022.03.010
Received 30 December 2021; Received in revised form 26 February 2022; Accepted 7 March 2022
Available online 4 April 2022
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Similar to electrophysiological techniques, in vivo calcium imaging has developed rapidly: genetically encoded calcium indicators (GECIs) (Chen et al., 2013; Inoue et al., 2019; Dana et al., 2019), miniature two-photon (2P) microscopy (Zong et al., 2017), and three-photon microscopes (Ouzounov et al., 2017; Yıldırım et al., 2019). One of the most notable developments in recent years has been wide-field 2P microscopes, which were developed by various research groups (Stiriman et al., 2016; Yu et al., 2021; Sofroniew et al., 2016; Rumyantsev et al., 2020; Ota et al., 2021). These microscopes enable us to record neural activity with single-cell resolution from a wide FOV across multiple brain regions.

In this paper, we describe the trade-offs encountered when expanding the field-of-view (FOV) of 2P microscopes and then describe the features of representative wide-field 2P microscopes that overcome the trade-offs. Finally, we discuss what is expected from the recording of neural activity using large-scale calcium imaging.

2. From conventional 2P microscope to wide-field 2P microscope

The wide-field 2P microscopes developed in recent years are scaled-up versions of conventional 2P microscopes. In this section, we first describe conventional 2P microscopes and their configurations. Since excellent review papers on conventional 2P microscopes have already been published (Denk and Svoboda, 1997; So et al., 2000; Zipfel et al., 2003; Helmcchen and Denk, 2005; Benninger and Piston, 2013; Ji et al., 2016; Yang and Yuste, 2017; Lecoq et al., 2019), we briefly introduce the fundamental points. Then, we discuss the trade-offs that arise from expanding the FOV of the microscope.

2.1. Two-photon excitation process

The main feature of a 2P microscope is the two-photon excitation (TPE) process. The TPE process is a nonlinear optical phenomenon in which a fluorophore simultaneously absorbs two photons and is excited to a higher energy level (excited state). After the transition to the excited state, it takes approximately $10^{-9}$ to $10^{-8}$ s to return to the ground state. The fluorophore emits a single photon during the return process. Calcium indicators emit fluorescence and their fluctuations reflect neural activity in calcium imaging. Importantly, the probability of TPE is proportional to the square of the light intensity such that the probability drops sharply away from the focal plane. This nonlinear property allows the 2P microscope to achieve a high optical resolution not only in the focal plane direction but also in the optical axis direction. In addition, this nonlinear property results in the use of near-infrared excitation light, which has a wavelength approximately twice that of one-photon (1P) excitation. Long-wavelength excitation light makes it possible to suppress light scattering and absorption in the brain, which is critical for observing deep regions of the brain.

2.2. Configuration of a 2P microscope

A 2P microscope is a type of laser scanning microscope. The specimen (sample) is scanned at high speed with the laser focused by the objective lens. Fig. 1A shows the excitation light path in the conventional 2P microscope. A laser beam emitted from a light source is expanded by a beam expander and reflected by the scanning mirrors. After passing through a scanning lens, tube lens, and objective lens, the laser is focused on the focal plane of the objective lens in the sample. The scan lens focuses the laser beam reflected by the mirror onto the focal plane with a fixed spot size independent of the mirror angle. The combination of the objective lens and tube lens achieves an infinity-corrected optical system, allowing the insertion of a dichroic mirror.

The objective lens collects the fluorescence emitted from the sample (Fig. 1B). The fluorescence wavelength is shorter than that of the 2P excitation light, so a dichroic mirror reflects the fluorescence and guides it to a photomultiplier (PMT). The fluorescence reaches the photocathode of the PMT and is converted into photocurrent. The photocurrent is finally converted into a voltage value (1D analog data) through current-voltage conversion. These 1D data are constructed into a 2D image of the specimen, referring to the scan angle of the mirrors.

2P microscopes commonly employ non-descanned detection (NDD) systems, in which the fluorescence signal leads to the PMT without passing through the scanning mirrors (Benninger and Piston, 2013). The position of the PMT can be determined relatively freely in the NDD system. Placing the detector closer to the objective lens shortens the fluorescence optical path and reduces the number of optical components through which the fluorescence passes to the detector, making it possible to collect the fluorescence more efficiently. It is important to note that 2P microscopes employing the NDD system do not need to form an image in the fluorescence optical path. The optical resolution is determined only in the excitation optical path.

2.3. Trade-off between wide FOV and high optical resolution

Microscope users will empirically notice a trade-off between the numerical aperture (NA) and the magnification of the objective lenses (Fig. 2). Here, we discuss the inevitability of this trade-off in terms of the geometrical optical properties of the light passing through the lens.

The NA of the objective lens is the main factor that determines the optical resolution of a microscope. In general, an objective lens with a larger NA provides an image with higher optical resolution. The NA is defined by

$$\text{NA} = n \sin \theta_e,$$

where $n$ is the refractive index of the medium in which the space between the specimen and the objective lens is filled, and $\theta_e$ is the angle between the marginal rays and the optical axis (Fig. 3A). The optical resolution of a microscope is typically represented by a point spread function (PSF), which is a response to a point source, that is, an impulse response in the imaging system. The optical resolution of a 2P microscope in an aberration-free and vignetting-less optical system is expressed in terms of the NA as follows (Zipfel et al., 2003):
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FWHM_{xy} = \begin{cases} 
0.3768 \lambda \text{NA} & (\text{NA} \leq 0.7), \\
0.3827 \lambda \text{NA} & (\text{NA} > 0.7), 
\end{cases} 

FWHM_z = \frac{0.6264 \lambda}{n - n^2 - \text{NA}^2}.

Here, FWHM_{xy} and FWHM_z are the full widths at half maximum (FWHM) of the PSF fitted as a Gaussian function in the lateral and axial directions, respectively. Eqs. (2) and (3) show that the larger the NA, the higher the optical resolution in all xyz directions.

To observe a wide FOV, the lens must have a low magnification. The diameter of the FOV (FOV_{dia}) of the 2P microscope is given by the following equation:

\text{FOV}_{dia} = 2r_f = 2f_{obj} \sin \theta_p,

where \( r_f \) is the radius of the FOV, \( f_{obj} \) is the focal length of the objective lens, and \( \theta_p \) is the maximum incident angle of the collimated light at the pupil of the objective lens (Fig. 1A). In Eq. (4), the FOV_{dia} is described using \( \sin \theta_p \) instead of \( \tan \theta_p \), assuming that the objective lens satisfies Abbe’s sine condition; indeed, commercially available microscope

Fig. 2. Magnification and NA of commercially available objective lenses. It can be seen that the lower the magnification of the lens, the lower the NA, and vice versa. Lenses with low magnification and high NA required for wide-field 2P microscopes are not available. Plotted for lenses described in Table 2 of Bumstead et al. (2019).

Fig. 3. (A) Definition of NA. (B) Trade-off between low magnification and high NA of the objective lens. If the focal length \( f_{obj} \) of the objective lens is increased to lower the magnification, \( \theta_p \) decreases. Consequently, NA decreases. (C) Solution to the trade-off shown in (B). Increasing the pupil diameter of the objective lens makes it possible to increase \( \theta_p \) while maintaining a large \( f_{obj} \). (D) Relationship between FOV_{dia} and pupil diameter of the objective lens. Inspired by Ji et al. (2016). Note that this relationship also depends on the maximum angle \( \theta_p \) of the collimated light entering the objective lens. Here \( \theta_p = 2.4563^\circ \).

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Objectives are designed to satisfy Abbe’s sine condition. No coma aberration occurs at the image points close to the optical axis for an objective lens that satisfies Abbe’s sine condition. To increase FOV$_{\text{def}}$, $\theta_p$ and/or $f_{\text{obj}}$ must be increased. Here, we consider increasing $f_{\text{obj}}$ because the angle $\theta_p$ is typically limited to ~1–5 degrees to suppress aberrations (Ji et al., 2016). However, if $f_{\text{obj}}$ is increased, $\theta_p$ decreases, as shown in Fig. 3B, resulting in a lower NA. Thus, there is a trade-off between low magnification and high NA (i.e., a trade-off between a wide FOV and high optical resolution).

### 2.4. Objective lens with large pupil diameter to achieve low magnification and high NA

The trade-off between wide FOV and high optical resolution is overcome by an objective lens with a large pupil diameter (Fig. 3C). The pupil diameter of the objective lens ($d_{\text{pupil}}$) is expressed using the focal length of the objective lens ($f_{\text{obj}}$):

$$d_{\text{pupil}} = 2r_p = 2f_{\text{obj}}NA,$$

where $r_p$ is the laser radius of collimated light at the pupil of the objective lens (Fig. 1A). From Eqs. (4) and (5), we obtain the relational equation between the pupil diameter of the objective lens and the diameter of the FOV. Fig. 3D indicates that a wide FOV (low magnification) and single-neuron resolution (high NA) can be achieved simultaneously using an objective lens with a larger pupil diameter.

It should be kept in mind that the NA of an objective lens is one of the main factors determining the optical resolution of a microscope. It is also essential to minimize optical aberrations in the entire optical system, including the tube lens, scan lens, and scanning mirrors. In 2P imaging, aberrations reduce not only the optical resolution, but also the excitation efficiency. Low excitation efficiency reduces the quality of images, resulting in neural activity data with a low signal-to-noise ratio (SNR).

Furthermore, a large $\theta_p$, which is a parameter that determines the NA of the objective lens, does not necessarily result in a brighter and clearer image for in vivo 2P imaging. The large $\theta_p$ results in a long light-path length, which causes scattering of the excitation light in the brain. Consequently, the excitation efficiency decreases. Underfilling the back aperture of the objective lens has been proposed to solve this problem (Tung et al., 2004; Helmchen and Denk, 2005; Kondo et al., 2017). The excitation NA is determined independently of the collection NA in a 2P microscope employing NDD. By decreasing the excitation laser radius at the pupil of the objective lens ($r_p$), the effective excitation NA can be reduced while maintaining a high collection NA. This technique has also been implemented in wide-field 2P microscopes to collect fluorescence signals efficiently (Sofroniew et al., 2016; Ota et al., 2021).

### 2.5. Design of a wide-FOV 2P microscope based on optical invariant

The trade-off between the wide FOV and high NA can be reconsidered from the viewpoint of the optical invariant (Beiser, 1986, 1995; Bumstead et al., 2019). Here, we extend the trade-off discussed in Section 2.4 and show that similar trade-offs also occur in the excitation optical path through scanning mirrors, a scan lens, and a tube lens.

The optical invariant is an essential variable in the design of a microscope. It is conceptually interchangeable with the etendue, which means that the luminance of a surface light source is conserved in an optical system if there is no loss or vignetting of light. A large optical invariant indicates that a large amount of light is transmitted through the optical components of a microscope.

First, we discuss the optical invariant for excitation (Fig. 4). For two pupils (two laser irradiation surfaces) formed on the scanning mirror and objective lens, the following optical invariant is valid:

$$r_m \sin \theta_m = r_p \sin \theta_p,$$

where $r_m$ and $\theta_m$ represent the laser beam radius and the scan angle of the scanning mirror, respectively. Similarly, for the two images (the intermediate image and the image on the focal plane) formed by the scanning and objective lenses, the following optical invariant is valid:

$$r_s \sin \theta_s = n r_p \sin \theta_e,$$

where $r_s$ and $\theta_s$ represent the height and focal angle of the image formed by the scanning lens, respectively. The other symbols are as previously described.

Noting that $r_m \sin \theta_m = n r_p \sin \theta_e$ is derived from Eqs. (1), (4), and (5), we can obtain a relational expression for the optical invariant of the excitation optical path ($I_e$), as follows:

$$I_e = r_m \sin \theta_m = r_p \sin \theta_e = n r_p \sin \theta_s.$$

These equations show that the excitation light propagates through the optical components, while the products of $r$ and $\sin \theta$ in pupil 1, image 1, pupil 2, and image 2 are preserved as constants. This optical invariant is also known as the $\theta D$ product (Beiser, 1995). The final equation in Eq. (8) is the product of NA and FOV radius. Since $I_e$ is a conserved quantity (constant), FOV and NA are mutually exclusive. Eq. (8) represents the trade-off between FOV and NA.

The optical invariants of optical components can be calculated in isolation by considering the light path through each of them. Importantly, the optical invariant of the entire system ($I_e$) is limited to the lowest value of the optical invariants calculated for each optical component. For example, even if a large optical invariant of the scanning mirror ($r_m \sin \theta_m$) is achieved by increasing the diameter of the laser projected onto the mirror and/or increasing the scan angle of the mirror,
| Name of 2P microscope | (Reference) | Objective lens | PSF | Representative recording condition1 | Representative recording condition2 |
|-----------------------|-------------|----------------|-----|-------------------------------------|-------------------------------------|
| **TRAPPs**            | (Stirman et al., 2016) | Custom-made objective lens | Immersion: Air NA: 0.43 | Lateral FWHM: 1.2 μm, Axial FWHM: 1.25 μm (Center, 275 μm depth) | Pathway1 POV: 0.75 mm × 0.75 mm, Sampling rate: 0.05 Hz, Number of pixels: 512 × 512, Imaging depth: 256 μm |
|                       |             |                 |      | Radial FWHM: 1.2 μm, Axial FWHM: 1.25 μm (Edge, 275 μm depth) | Pathway2 Same as parameters at pathway1 (Two sub-regions in the 47 mm POV are imaged independently) |
| **Divisep**           | (Yu et al., 2022) | Custom-made objective lens | Immersion: Air NA: 0.48 | Lateral FWHM: ~1 μm, Axial FWHM: ~8 μm (Across a 5-mm POV and up to 500-μm imaging depth) | Pathway1 POV: 0.75 mm × 0.75 mm, Sampling rate: 0.05 Hz, Number of pixels: 256 × 256, Imaging depth: 256 μm |
|                       |             |                 |      | POV: 0.75 mm × 0.75 mm, Imaging depth: 262 μm | Pathway2 Same as parameters at the pathway1 (Two sub-regions in the 47 mm POV are imaged independently) |
| **2p-ARAM**           | (Sofoanis et al., 2018) | Custom-made objective lens | Immersion: Water Excitation NA: 0.6 | Lateral FWHM: 0.86 μm, Axial FWHM: 4.09 μm (Center, 450 μm depth) | Pathway1 POV: 0.6 mm × 0.6 mm × 4 regions, Sampling rate: 9.6 Hz, Pixel size: 1.2 μm × 1.2 μm, Imaging depth: Layer 2/3 (Four sub-regions are imaged in sequence) |
|                       |             |                 |      | Radial FWHM: 1.7 μm, Axial FWHM: ~8 μm (Polar plane: unit: on-axis) | Pathway2 POV: 0.6 mm × 0.6 mm × 4 regions, Sampling rate: 9.6 Hz, Pixel size: 1.2 μm × 1.2 μm, Imaging depth: Layer 2/3 (Four sub-regions are imaged in sequence) |
|                       |             |                 |      | Lateral FWHM: < 1 μm, Axial FWHM: ~8 μm (Polar plane: unit: off-axis) | Pathway1 POV: 0.6 mm × 0.6 mm × 4 regions, Sampling rate: 9.6 Hz, Pixel size: 1.2 μm × 1.2 μm, Imaging depth: Layer 2/3 (Four sub-regions are imaged in sequence) |
| **Multi-area two-photon microscope** | (Chen et al., 2016) | 16X OPTIC LWD (Nikon) Water-immersion objective NA: 0.6 | (Effective excitation NA: 0.53) | Lateral FWHM: 0.36 μm, Axial FWHM: 5.2 μm (Polar plane: unit: on-axis) | Pathway1 POV: 0.6 mm × 0.6 mm × 4 regions, Sampling rate: 9.6 Hz, Pixel size: 1.2 μm × 1.2 μm, Imaging depth: Layer 2/3 (Four sub-regions are imaged in sequence) |
|                       |             |                 |      | Lateral FWHM: < 1 μm, Axial FWHM: ~8 μm (Polar plane: unit: off-axis) | Pathway2 POV: 0.6 mm × 0.6 mm × 4 regions, Sampling rate: 9.6 Hz, Pixel size: 1.2 μm × 1.2 μm, Imaging depth: Layer 2/3 (Four sub-regions are imaged in sequence) |
| **XLPLUDX41 (Olympus)** | (Bumstead et al., 2016) | XPLN SERIES (Olympus) Water-immersion objective NA: 0.6 | (Effective excitation NA: 0.22) | Lateral FWHM: 0.36 μm, Axial FWHM: 6.4 μm (ACROSS a 7-mm-diameter FOV) | Pathway1 POV: 0.6 mm × 0.6 mm × 4 regions, Sampling rate: 9.6 Hz, Pixel size: 1.2 μm × 1.2 μm, Imaging depth: Layer 2/3 (Four sub-regions are imaged in sequence) |
|                       |             |                 |      | Lateral FWHM: 1.2 μm, Axial FWHM: 8 μm | Pathway2 POV: 0.6 mm × 0.6 mm × 4 regions, Sampling rate: 9.6 Hz, Pixel size: 1.2 μm × 1.2 μm, Imaging depth: Layer 2/3 (Four sub-regions are imaged in sequence) |
| **Super-widefield TPLSM** | (Terada et al., 2018) | XPLN SERIES (Olympus) Water-immersion objective NA: 0.6 | (Effective excitation NA: 0.22) | Lateral FWHM: 0.36 μm, Axial FWHM: 6.4 μm (ACROSS a 7-mm-diameter FOV) | Pathway1 POV: 0.6 mm × 0.6 mm × 4 regions, Sampling rate: 9.6 Hz, Pixel size: 1.2 μm × 1.2 μm, Imaging depth: Layer 2/3 (Four sub-regions are imaged in sequence) |
|                       |             |                 |      | Lateral FWHM: 0.36 μm, Axial FWHM: 6.4 μm (ACROSS a 7-mm-diameter FOV) | Pathway2 POV: 0.6 mm × 0.6 mm × 4 regions, Sampling rate: 9.6 Hz, Pixel size: 1.2 μm × 1.2 μm, Imaging depth: Layer 2/3 (Four sub-regions are imaged in sequence) |
| **Multi-beam two-photon microscope** | (Ramyantsev et al., 2020) | 5.0× Planap (Leica) | Immersion: Air NA: 0.5 | Radial FWHM: 2.0 μm, Axial FWHM: 1.8 μm | Pathway1 POV: 0.6 mm × 0.6 mm × 4 regions, Sampling rate: 9.6 Hz, Pixel size: 1.2 μm × 1.2 μm, Imaging depth: Layer 2/3 (Four sub-regions are imaged in sequence) |
|                       |             | Working distance: 5 mm |      | Radial FWHM: 2.0 μm, Axial FWHM: 1.8 μm | Pathway2 POV: 0.6 mm × 0.6 mm × 4 regions, Sampling rate: 9.6 Hz, Pixel size: 1.2 μm × 1.2 μm, Imaging depth: Layer 2/3 (Four sub-regions are imaged in sequence) |
| **FASIO-3PM**         | (Ota et al., 2021) | Custom-made objective lens | Immersion: Air Excitation NA: 0.4 | Lateral FWHM: 1.61 μm, Axial FWHM: 7.07 μm (Center, 190 μm depth) | Pathway1 POV: 0.6 mm × 0.6 mm × 4 regions, Sampling rate: 9.6 Hz, Pixel size: 1.2 μm × 1.2 μm, Imaging depth: Layer 2/3 (Four sub-regions are imaged in sequence) |
|                       |             | Working distance: 5 mm |      | Lateral FWHM: 1.58 μm, Axial FWHM: 6.91 μm (Edge, 190 μm depth) | Pathway2 POV: 0.6 mm × 0.6 mm × 4 regions, Sampling rate: 9.6 Hz, Pixel size: 1.2 μm × 1.2 μm, Imaging depth: Layer 2/3 (Four sub-regions are imaged in sequence) |
|                       |             | Working distance: 4.5 mm |      | Lateral FWHM: 1.58 μm, Axial FWHM: 6.91 μm (Edge, 190 μm depth) | Pathway1 POV: 0.6 mm × 0.6 mm × 4 regions, Sampling rate: 9.6 Hz, Pixel size: 1.2 μm × 1.2 μm, Imaging depth: Layer 2/3 (Four sub-regions are imaged in sequence) |

The parameters are based on published information and are not filled in for unclear values.
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Table 2
Cambridge Technology’s Resonant Mirror (CRS Series).

| Specifications                  | CRS 4 kHz | CRS 8 kHz | CRS 12 kHz |
|--------------------------------|-----------|-----------|------------|
| Resonant frequency (Hz)        | 3938      | 7910      | 12,000     |
| Mirror size (mm)               | 12.7      | 7.8 × 5.5 | 7.8 × 5.5  |
| Clear aperture (mm)            | 12 × 9.25 | 7.2 × 5.0 | 7.2 × 5.0  |
| Maximum scan angle (degrees,  | 26°       | 26°       | 10°        |
| peak-to-peak)                  |           |           |            |
| Optical invariant              | 1.04      | 0.56      | 0.21       |

A smaller optical invariant at the rear aperture of the objective lens ($r_p \sin \theta_p$) does not transmit all the light from the scanning mirror to the sample. The optical invariant of this system is limited to $r_p \sin \theta_p$. Increasing the value of $r_p \sin \theta_p$ for all the optical components is necessary to increase the optical invariant for the entire microscope system, resulting in a contiguous-wide FOV (large $r_f$) and high-NA system simultaneously.

Next, we discuss the optical invariants in the optical fluorescence path (Fig. 1B). As fluorescence from the neurons spreads without directionality, it enters the full aperture of the objective lens. The optical invariant for collection ($I_c$) in a 2P microscope using the NDD system is determined independently of the excitation light path.

$$I_c = n r_d \sin \theta_c = r_d \sin \theta_d,$$  

where $\theta_c$ is the maximum angle of the collected fluorescence from the neurons to the objective lens with respect to the optical axis and $r_d$ and $\theta_d$ represent the radius and angle of the fluorescence projected to the photocathode of the PMT, respectively. An optical design with $\theta_d < \theta_c$ ($I_c < I_e$) allows us to collect more fluorescence from the neurons, resulting in an image with a high SNR. As mentioned above, 2P microscopes employing the NDD system do not need to form images in the fluorescence pathway. The optics of the excitation pathway determine the spatial resolution. The design concept of $\theta_d < \theta_c$ is derived from the advantage that a less aberration optical design is not required in the fluorescence pathway relative to the excitation pathway. This advantage is particularly useful in the design of large 2P microscope lenses.

2.6. Optical invariant of resonant mirrors

Now that optical design software (e.g., Zemax) has enabled end users of microscopes to design their own lenses (objective lens in Table 1), the optical component that limits the optical invariant for excitation ($I_e$) is

![Fig. 5. (A) Conventional 2P imaging. The FOV is ~ 500 μm × 500 μm, which is too small to observe multiple brain regions. (B) Sub-regions 2P imaging mode. Neural activity is recorded from two or more small FOVs (~ 500 μm × 500 μm), arbitrarily selected from a large region. (C) Full-region 2P imaging mode. The neural activity is recorded using a contiguous-wide FOV. The figure below shows the FOV observed in each imaging mode, with one block as the FOV of the conventional 2P microscope. In sub-regions 2P imaging, the target brain regions for observation need to be predetermined; therefore, this imaging mode promotes hypothesis-driven research. In contrast, the full-region imaging mode promotes data-driven research because neural activity is recorded in a non-biased manner.](attachment:fig5.png)
considered to be the scanner, not lenses. This has motivated us to discuss the optical invariants of the scanners. The scanners generally implemented in 2P microscopes are polygonal, galvanometer, and resonant scanners. Polygonal scanners are less suitable for calcium imaging because zoom scanning is not possible. When monitoring relatively fast dynamics, such as calcium fluorescence changes, a combination of galvanometer and resonant scanners to scan a continuous plane is widely used. The selection of the resonant mirror is of great importance for increasing $I_2$ because the resonant mirrors have a lower optical invariant than the galvanometer mirrors.

The CRS series of resonant mirrors from Cambridge Technology (Table 2) are used for the 2P microscopes. It can be observed that a resonant mirror with a lower resonance frequency can achieve a larger optical invariant. Therefore, a 4 kHz resonant mirrors (CRS 4 kHz) is expected to provide the widest FOV and highest NA among the three types of resonant mirrors. However, it should be noted that the spatial resolution is limited not only by the optical resolution but also by the pixel size. The pixel size must be smaller than the cellular size. More pixels are required for wide-FOV scanning because the pixel size is smaller than the cellular size, which results in a lower frame rate. Furthermore, if a scanning mirror with a low resonant frequency (CRS 4 kHz) is used, the frame rate will be even slower than when using CRS 8 kHz or CRS 12 kHz. This is a trade-off between a wider FOV and faster frame rate (this trade-off is discussed in Section 3.3). The decision on which resonant mirror to use should be made according to the intended purpose, considering the spatial resolution and frame rate.

### 3. Recent efforts toward development of wide-field 2P microscopes

The FOV of conventional 2P microscopes is approximately 500 $\mu$m × 500 $\mu$m, which is not appropriate for the simultaneous observation of multiple brain regions, even in the small brain of the mouse (Fig. 5A). Recently, several groups have reported new 2P microscopes for recording neural activity in multiple brain regions (Table 1). Here, we introduce resonant-galvo-based 2P microscopes that can record neural activity with single-cell resolution from a wide FOV across multiple brain regions: a twin region, panoramic 2-photon (Trepan2p) microscope (Stirman et al., 2016), a dual independent enhanced scan engines for large field-of-view two-photon imaging (Diesel2p) (Yu et al., 2021), a 2-photon random access mesoscope (2p-RAM) (Sofroniew et al., 2016), a multi-beam 2P microscope (Rumyantsev et al., 2020), and a fast-scanning high optical invariant two-photon microscope (FASHIO-2PM) (Ota et al., 2021).

The imaging modes of these 2P microscopes for observing multiple brain regions are classified into two categories: the sub-regions imaging mode (Fig. 5B) and full-region imaging mode (Fig. 5C). The sub-regions imaging mode is an imaging mode in which neural activity is observed from two or more small FOVs (approximately 500 $\mu$m × 500 $\mu$m) arbitrarily selected from a large region. In this imaging mode, it is necessary to determine which brain region to focus on in advance. Therefore, it is suitable to conduct research using a hypothesis-driven approach. Full-region imaging, on the other hand, allows the observation of a continuous-wide FOV (several tens of times larger than that of conventional 2P microscopes). Neural activity can be recorded in a non-biased manner from a wide FOV; therefore, it would be suitable for conducting research with a data-driven approach. Note that each microscope does not necessarily implement one imaging mode or another, but some microscopes implement both imaging modes.

#### 3.1. Trepan2p and Diesel2p

Trepan2p can record neural activity from multiple distant brain regions through temporally multiplexed pathways (Stirman et al., 2016). An 80 MHz pulsed laser is split into two optical paths using a beam splitter. Each beam is independently positionable in X, Y, and Z by a steering mirror and electronically tunable lens introduced into each optical path. One path is 1.87 m longer than the other and is therefore delayed by 6.25 ns, which is half the pulse repetition frequency of the laser (80 MHz). This delay shifts the time at which the calcium sensor is

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**Fig. 6.** (A) Frame rate as a function of FOV for 2P microscopes discussed in Section 3. The solid circles denote the typical values shown in the original papers for each microscope. The relational equation between $F$ and FOV for each microscope is as follows: $f = d \times v$; FOV is valid for the 2p-RAM and FASHIO-2PM. $f = 2 \times d \times v$; FOV is valid for Diesel2p. $f = 4 \times d \times v$; FOV is valid for the multi-beam 2P. For Trepan2p, the FOV's and frame rates shown in Stirman et al., (2016) are plotted. All lines are calculated, not including the time taken for the scanning mirror to return to the starting point of the scan. Inspired by Rumyantsev et al. (2020). (B) Optical invariant at the front focal plane of the objective lens for 2P microscopes shown in (A). The y-axis represents the product of NA and $r_s$, which is equal to the last equation in (Eq. 8). The red and blue lines represent the maximum values of the optical invariant calculated based on the specifications of the CRS 8 kHz and CRS 12 kHz resonant mirrors, respectively. The maximum value of the optical invariant for the CRS 4 kHz resonant mirror is 1.04, which is greater than the range of the graph. The resonant mirrors installed in each microscope are shown in the bar graphs. In FASHIO-2PM, an optical invariant of 0.6. is achieved by selecting a high-precision CRS 8 kHz resonant mirror and using it beyond the specifications. The objective, scanning, and tube lenses are custom-made to preserve the optical invariant from the resonant mirror to the sample. (C) Scanning speed of resonant mirrors in the focal plane ($v$) for 2P microscopes shown in (A), $v$ is calculated based on $v = 2 \times 2\tau_f$ × Resonant frequency, assuming that round-trip scanning is possible for all microscopes. The $v$ values for each microscope are as follows: $v = 14$, 400 mm/s in 2p-RAM, $v = 15$, 620 mm/s in multi-beam 2P, $v = 47$, 460 mm/s in FASHIO-2PM, $v = 23$, 730 mm/s in Diesel2p, and $v = 3938$ mm/s in Trepan2p. The high 2P excitation efficiency and high sensitivity detection system of FASHIO-2PM achieves the fastest $v$. The $v$ of Diesel2p is half that of FASHIO-2PM, but $f$ is almost equivalent to that of FASHIO-2PM (2048 pixels) in (A) because the temporal multiplexed system allows two FOVs to be scanned simultaneously. The $v$ of Trepan2p is calculated using the parameters of scanning a 0.5 mm × 0.5 mm FOV shown in Stirman et al., (2016).
excited in each light path, so that the fluorescence in each light path can be detected by a single PMT without temporal overlap.

In Trepan2P, the relay, scanning, tube, and objective lenses are designed using OpticStudio (Zemax, LLC). The objective lens has a focal length of 27.5 mm and an NA of 0.43. The CRS 4 kHz resonant mirror is installed. The combination of temporal multiplexing and these optical components enables the simultaneous measurement of neural activity from two arbitrary sub-regions (500 μm × 500 μm) in a 3.5 mm diameter FOV. It is also possible to record neural activity from a continuous plane of 9.6 mm²; however, in that case, the frame rate is ~ 0.1 Hz. Therefore, Trepan2P is suitable for the simultaneous observation of two or more small sub-regions (Fig. 5B).

The FWHM of the PSF in the axial direction is 12.1 μm. As the diameter of the cell body of layer 2/3 cortical neurons is approximately 10 μm, the optical resolution is expected to be improved to accurately record the neural activity excluding the neuropile signal.

Recently, Smith et al. introduced a new wide-field 2P microscope called Diesel2p (Yu et al., 2021). In Diesel2p, adaptive optics is implemented, and a new objective lens with an NA of 0.54 is mounted to achieve a higher optical resolution. The lateral FWHM is ~ 1 μm and the axial FWHM is ~ 8 μm across a 5 mm × 5 mm FOV. As with Trepan2P, Diesel2p uses temporal multiplexing, while the implementation of an 8 kHz resonant mirror allows for a much higher frame rate than Trepan2P. They are able to record 5874 active neurons from two FOVs of 1.5 mm × 5 mm at 3.85 Hz. A particularly exciting feature is the ability to monitor two overlapping regions simultaneously using different parameters (frame rates, imaging fields, and pixel numbers). This feature allows for multiscale imaging. While recording the activity of more than 400 neurons in a 750 μm × 750 μm FOV at 15 Hz, the activity of the neurons in an arbitrary sub-region (250 μm × 250 μm) within that FOV can be recorded faster frame rate (60 Hz).

3.2. 2p-RAM

The 2p-RAM (Sofroniew et al., 2016) is equipped with a CRS 12 kHz resonant mirror. The scanning range of the mirror in the focal plane is as narrow as 0.6 mm. For wide-field imaging, three galvanometer mirrors (termed a virtually conjugated galvo pair, VCGP) are used to move the small 0.6 mm wide region over a wide area, enabling 4.4 mm × 5.4 mm imaging at 0.7 Hz. Although the sampling rate for this wide-field imaging is relatively slow for recording neural activity, the 2p-RAM is capable of simultaneously observing four different brain regions (four 0.6 mm × 0.6 mm FOVs) at 9.5 Hz.

The most important feature is the remote focus (RF) unit, which is located conjugate to the focal position in the sample. The RF unit consists of a custom RF objective and lightweight mirror mounted on a voice coil, which enables the observation depth to be adjusted up to 1 mm by moving the mirror position along the optical axis. The custom-made objective lens is a water immersion lens with an incident pupil diameter of 25.6 mm, focal length of 21 mm, NA of 0.6 for excitation, and NA of 1.0 for collection to collect as much fluorescence from the specimen as possible. In addition, a condenser lens is custom-made, and the space between the lens and the glass-covered PMT is filled with oil to increase the refractive index. This technique prevents light loss between the objective lens and the PMT. The field curvature is greater than 100 μm at the edge of the field of view. This optical aberration along the galvanometer scan is corrected by adjusting the focus position using the RF unit. However, the speed of the voice coil cannot keep up with that of the resonant scanner. As a result, the correction is not completed within a small region of 0.6 mm width, leaving a field curvature of several tens of μm.

Other researchers have extended the scope of the 2p-RAM. For example, Lu et al. combined the 2p-RAM with a volumetric imaging module based on Bessel focus scanning to measure the neural activity from a 3020 μm × 1500 μm × 60 μm volume (Lu et al., 2020). They succeeded in measuring the activity of 9247 GABAergic neurons at 1 Hz.

Microscopes incorporating temporal-division multiplexing/spatio-temporal multiplexing in a 2P-RAM have also been reported (Tsyboulski et al., 2018; Orlova et al., 2020).

3.3. Multi-beam 2P microscope

The 2P microscope is a scanning microscope, which results in a lower frame rate for wide-field imaging. Rumyantsev et al. discussed this point using the following equation (Rumyantsev et al., 2020):

\[
\text{FOV} = d \times v/f,
\]

where \(d\) (mm) is the pixel width along the slow-axis scanner (galvanometer mirror), \(v\) (mm · s⁻¹) is the speed at which the laser beam is swept across the specimen using the fast-axis scanner (resonant mirror), \(f\) (s⁻¹) is the frame rate, and \(\text{FOV}\) (mm²) is the observation area, not \(\text{FOV}_{\text{frame}}\) in Eq. (4). In Eq. (10), the FOV and \(f\) are inversely proportional to each other. The wider the FOV, the lower the frame rate, as discussed in Section 2.6. The frame rates of Trepan2P and 2p-RAM for wide-field imaging are approximately 1 Hz (Fig. 6A), which may fail to capture all calcium events. Therefore, as Rumyantsev et al. pointed out, Trepan2P and 2p-RAM are suitable microscopes for sub-regions imaging and not full-region imaging.

To resolve the trade-off between a wide FOV and a high frame rate, Rumyantsev et al. developed a multi-beam 2P microscope that realizes a new temporal multiplexed approach (Rumyantsev et al., 2020). Four different FOVs of 500 μm × 500 μm are simultaneously scanned by equally dividing the laser beam into four beams. In their system, the relational equation between the FOV and the frame rate is formulated as follows:

\[
\text{FOV} = 4 \times d \times v/f.
\]

This equation means that their multiplexed approach enables them to simultaneously monitor an FOV four times wider than conventional microscopes, while maintaining a frame rate that is as fast as that of conventional microscopes. The laser intensity is 1/4; however, commercially available 2P lasers are generally utilized with a considerably reduced intensity (less than 1/4 of maximum power) to avoid brain damage. Even with divided lasers, it is possible to achieve sufficient intensity to observe neural activity by increasing the laser power. They recorded the neural activity in the visual cortex in a 2 mm × 2 mm FOV at 17.5 Hz.

The multi-beam 2P microscope has 16 independent beam pathways. A 2 mm × 2 mm FOV is divided into 4 × 4 segments of 500 μm × 500 μm. Four sub-regions (parts of the FOV) 500 μm apart can be simultaneously scanned with four beams. This scanning phase is switched four times to observe a 2 mm × 2 mm FOV. The regions excited in each phase are switched every 400 ns using three pairs of electro-optic modulators (EOMs). In this multibeam scan, 16 sub-regions of 500 μm × 500 μm are positioned in a square shape on the FOV, so the relation \(r_f = \sqrt{\text{FOV}}/2\) holds. The frame rate \(f\) is inversely proportional to \(\sqrt{\text{FOV}}\) rather than to \(\text{FOV}\). Therefore, compared with other microscopes, the frame rate decreases more slowly as the FOV increases (Fig. 6A). Note that \(r_f\) is restricted by the optical invariant of the resonant mirror. The multi-beam 2P is equipped with a 8 kHz resonant mirror as a fast-axis scanner.

Although no new objective lens was developed, Rumyantsev et al. designed a custom 4 × 4 lens array to collect the fluorescence emitted from 16 different sub-regions of the FOV. Each lenslet is connected to a plastic optical fiber, and the fluorescence collected in each lenslet is guided to 16 different PMTs. Since four sub-regions of the FOV are excited simultaneously in the microscope, there is crosstalk between the signals acquired in each PMT. They formulated the fluorescence contamination between simultaneously scanned sub-regions and established a method that allows signal unmixing even in optically uniform tissues, such as the brain, and stitched the images almost continuously. In this manner, the multi-beam 2P microscope realizes the full-region imaging.
3.4. **FASHIO-2PM**

The last microscope introduced is the FASHIO-2PM (Ota et al., 2021). The FASHIO-2PM is equipped with custom-designed large objective, scanning, and tube lenses. The pupil diameter of the objective lens is 56 mm, the focal length is 35 mm, and the NA is 0.8. The excitation light passes through the center of the 28 mm diameter objective lens, resulting in an effective excitation NA of 0.4, which is sufficient to observe single neurons in the brain (Zipfel et al., 2003). We selected a high-precision CRS 8 kHz resonant mirror and designed an optical path in which the expanded laser beam is projected to the short diameter of the mirror size instead of the clear aperture, and the mirror is scanned over the maximum scan angle of 26 degrees. Thus, FASHIO-2PM achieves an optical invariant of 0.6 by utilizing a CRS 8 kHz resonant mirror beyond its specification (Fig. 6B). This setting achieves more than 2.5 times optical invariant than the CRS 12 kHz, and 2 times faster scan speed than the CRS 4 kHz. The custom-made lenses preserve the optical invariant from the resonant mirror to the sample, resulting in both contiguous-wide FOV and high NA, while providing a fast frame rate to record neural activity.

Before describing a further feature of FASHIO-2PM, we would like to discuss another trade-off between a high SNR of the acquired image and the short pixel dwell time (the time for which the laser dwells on each pixel position) in 2P calcium imaging. Microscopes for calcium imaging are required to achieve a high frame rate to follow the calcium dynamics caused by action potentials. However, when wide-field imaging is performed with a high frame rate, the pixel dwell time is shortened and the probability of 2P excitation of the fluorophore decreases. Consequently, the SNR of the acquired images decreases. A high laser intensity allows brighter images but may cause a higher risk of brain damage (Podgorski and Ranganathan, 2016). It is essential to suppress aberrations in the optical system and efficiently induce 2P excitation.

In FASHIO-2PM, the trade-off between the high SNR and short dwell time is overcome by realizing a practically aberration-free optical system. In the excitation path, 80% of the light reflected by the scanning mirror is contained within a radius of 1.1 μm on the focal plane over the entire FOV. The profile of an encircled energy function is almost equal to one with the diffraction limit, and the Strehl ratio is ~ 0.99 over the FOV. In addition, we developed a large pre-chirper to further improve 2P excitation efficiency. Due to high 2P excitation efficiency over the entire FOV, we can record neural activity at the edge of the FOV with the same quality as at the center of the FOV. In the fluorescence path, the objective lens that achieves a fluorescence NA of 0.8, twice the excitation NA, collects more fluorescence from the neurons to improve the overall SNR. We also developed a large-aperture GaAsP PMT with a high current output (14 mm square aperture, 50 mA output current) to increase signal detection sensitivity. The high 2P excitation efficiency and highly sensitive detection system in FASHIO-2PM enables us to record neural activity with a high SNR regardless of the short pixel dwell time (~ 18–36 ns which is approximately four times shorter than that of conventional 2P microscopes). Therefore, FASHIO-2PM can achieve a full-region imaging mode without image stitching. Finally, we have been able to monitor neural activity from more than 16,000 neurons in a contiguous 3 mm × 3 mm FOV of cortical layer 2/3 at a 7.5 Hz sampling rate. We have also succeeded in recording the activity of 6235 neurons from layer 5 without causing laser-induced brain damage.

The high SNR recording with a short pixel dwell time provides the fastest v (47, 460 mm/s) in wide-field 2P microscopes (Fig. 6C). By increasing v in (Eq. 10), FASHIO-2PM achieves a frame rate that is as high as or higher than that of microscopes with a temporal multiplexed system while maintaining a wide FOV (Fig. 6A). This indicates that FASHIO-2PM has the potential to record neural activity from a wider FOV at a higher frame rate if the laser is split to scan multiple brain regions simultaneously. In FASHIO-2PM, the activity of cortical layer 2/3 neurons can be recorded with 20–30% of the maximum 2P laser power. The decrease in the excitation light intensity caused by the splitting of the laser and the consequent decrease in the SNR of the acquired image can be overcome by increasing the laser power (i.e., outputting a laser with 40–60% of the maximum power).

3.5. **Other 2P microscopes for wide FOV imaging**

Prior to the 2P microscopes described above, Tsai et al. built a large-FOV 2P microscope with an off-the-shelf objective lens in 2015 (Tsai et al., 2015). Similarly, Bumstead et al. optimally combined commercially available optical components (objectives, relay lenses, mirror

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**Fig. 7.** Hub-like neurons (n = 15) functionally connected with more than 120 neurons across brain regions. White lines indicate functional connections between neurons pairs. Hub-like neurons are located at the points where the white lines concentrate in a high density. Neurons located in different brain regions are colored differently.
scanners, and emission collection systems) to maximize the optical invariant and constructed a wide-FOV 2P microscope (Bumstead et al., 2019). The FOVs of these microscopes are 10 mm and 7 mm, respectively. However, the optical resolution of the axial axis (14 μm and 28 μm) does not provide single-neuron resolution. Their challenging research suggests that developing a lens that provides both low magnification and high NA is essential for wide-FOV imaging with single-cell resolution.

In addition to Trepan2p and 2p-RAM, other 2P microscopes that simultaneously record neural activity in multiple brain regions have been reported. Similar to Trepan2p, Chen et al. developed a 2P microscope in which a laser is split into two optical paths to simultaneously record neural activity from two different cortical regions (Chen et al., 2016). Lecoq et al. developed a dual-axis 2P microscope using microlenses (Lecoq et al., 2014). Terada et al. developed an interesting system to switch the FOV position by inserting a micro-opto-mechanical device into the post-objective space (Terada et al., 2018). Recently, Clough et al. constructed a new microscope, a quad-area large-FOV 2P microscope (Quadroscope) (Clough et al., 2021). In the Quadroscope, Ytterbium-fiber lasers with a 31.25 MHz repetition rate are separated into four optical paths to excite four brain regions at 8 ns intervals. A custom-designed objective lens of 0.52 NA is employed, and two ~3.0 mm FOVs are superimposed to provide a total FOV of ~4.8 mm FOV. They succeeded in measuring neural activity at 30 Hz from four sub-regions independently (0.75 mm × 0.75 mm each).

4. Future outcomes expected from large-scale imaging with single-cell resolution

The development of wide-field 2P microscopes has dramatically increased the number of neurons observed using calcium imaging. Recently, some studies have also recorded activity from a large number of neurons using volumetric calcium imaging (multiline imaging at different depths) from the same brain region (Weisenburger et al., 2019; Han et al., 2019; Onda et al., 2021). We briefly describe the expectations for large-scale imaging along with the research results.

4.1. Proposal for a new information-processing mechanism

Theoretical neuroscientists have proposed many hypotheses to understand how information is represented in the cerebral cortex (Dayan and Abbott, 2001; Trappenberg, 2009; Gerstner et al., 2014). These hypotheses assume much larger numbers of neurons than are monitored in experimental studies. However, recent reports of large-scale neural activity recording methods have been bridging this gap. Stringer et al. recorded the activity of over 10,000 neurons from the V1 region while presenting 2800 different natural image stimuli to mice (Stringer et al., 2019a). They found that the variance of the population activity of neurons represented in n-dimensional space decays following a power law of $n^{-d}$ ($a \approx 1 + 2/d$), where $d$ is the dimension of the visual stimulus. This result suggests that the information representation of neuronal populations in the sensory cortex does not follow efficient coding but lies on a smooth manifold. Rumyantsev et al., who developed the multi-beam 2P microscope, clarified the relationship between the information represented by a large number of neurons and noise (fluctuations in neural responses). Individual neurons are thought to show stochastic responses to repeated presentations of the same stimuli, and these fluctuations limit the coding accuracy of external stimuli. However, they presented mice with two types of visual stimuli and found that noise did not interfere with the discrimination between neuronal responses to the two stimuli. By recording the activity of thousands of neurons, researchers first demonstrated how the noise of individual neurons collectively affects neural population representations in a living animal brain.

As these studies have shown, one of the benefits of large-scale imaging is the discovery of a new aspect of information representation produced by collective neuronal activity (Stringer et al., 2019b). Similar to the study by Rumyantsev et al., large-scale recording and data analysis also provide new insights into the assessment of how interest statistics converge as the number of neurons increases (Stringer et al., 2021).

4.2. Identification of rare but highly influential neurons

Another attractive feature of large-scale imaging is the detection of rare samples in the brain. Recent studies have found various heavy-tailed distributions in the brain; firing rate, synaptic weight, the number of synaptic contacts, etc (Buzsáki and Mizuseki, 2014). Large-scale imaging allows the detection of rare samples located at the tails of such distributions. Note that just because a sample is rare, this does not mean that its activity has a small effect on the brain. For example, a functional network analysis based on partial correlation coefficients between calcium activity of more than 10,000 neurons in cortical layer 2/3 has revealed hub-like neurons that are functionally connected to more than 100 neurons. The hub-like neurons are located at the tail of the degree distribution and are extremely rare (0.1%–0.01% of neurons used for analysis). Intriguingly, the hub-like neurons form the functional connectivity with not only neurons in local brain regions but also ones in distant brain regions (Fig. 7). This connectivity property suggests a possibility that the hub-like neurons have a significant impact on the activity of the overall brain. In network science, hubs are known to play important roles in maintaining a network structure against attacks (Albert et al., 2000; Cohen et al., 2000), spreading phenomena (Pastor-Satorras and Vespignani, 2001), and synchronization (Nishikawa et al., 2003; Wang and Slotine, 2005). It would be interesting to investigate the functional significance of hub-like neurons in future studies.

Moreover, it has been reported that the activation of a small number of neurons by optogenetic techniques can change the activity of not only the targeted neurons but also other neurons, which influences learning-related behavior for external stimuli or the environment (Carrillo-Reid et al., 2019; Robinson et al., 2020). Although large-scale imaging was not performed in these studies, it is worth noting that the study of rare samples can be further developed by combining it with single-cell optogenetics. Loss-of-function and gain-of-function experiments can also be helpful for estimating the effect of rare samples on collective neuronal activity in the brain (Peron et al., 2020; Marschel et al., 2019).

Similar to wide-field 2P imaging, wide-field 1P imaging has revealed functional maps (Ferezou et al., 2007; Manita et al., 2015), functional connectivity (Mohajerani et al., 2010), information flow for spontaneous activity (Mohajerani et al., 2013), stimulus-evoked activity (Kuroki et al., 2018), and behavior-related activity (Allen et al., 2017; Makino et al., 2017; Gidat et al., 2018; Musali et al., 2019; Esmaeili et al., 2021) across brain regions. The neural activity extracted using wide-field 1P imaging is a population activity that involves the activity of multiple single neurons. Fast and contiguous-wide 2P imaging is expected to reveal the fundamental rules behind the population activity in terms of single-cell activity.

5. Conclusions

This review discusses three trade-offs in the development of a wide-field 2P microscope: (1) The trade-off between wide FOV and high NA,
(2) the trade-off between wide FOV and high frame rate, and (3) the trade-off between high SNR of the acquired image and short pixel dwell time. In practice, all these parameters are intertwined. Therefore, it is necessary to simultaneously improve all the parameters to develop a wide-field 2P microscope. We should keep in mind that the performance of the microscope should not be evaluated based on a single parameter but should be comprehensively evaluated based on all parameters (including the FOV, spatial resolution, frame rate, and aberrations), as well as physiological parameters (such as the number of neurons recorded, SNR of neural activity, and brain damage).

Now that lenses with large pupils for wide-field 2P imaging, such as those used in Trepan2p, Diesel2p, 2p-RAM, Quadroscope, and FASHIO-2Pm have been designed, the birth of a new scanning method is particularly desirable. Temporal multiplexing would be one method of satisfying this requirement (Stirnim et al., 2016; Chen et al., 2016; Clough et al., 2021). However, the inter-pulse intervals between the split lasers are limited by the fluorescence lifetime of the GECIs. If short lifetimes and bright GECIs are developed, it will be possible to shorten the intervals and increase the number of imaging planes.

The wide FOV microscopes introduced in this paper use 2D-raster scan methods with the galvo-galvo system or resonant-galvo system. The problem with raster scanning is that the scanning time increases in proportion to the FOV (more precisely, the number of pixels). To overcome this problem, arbitrary line scanning, which scans only the target of interest (e.g., neurons), has been proposed (Katona et al., 2012; Göbel et al., 2007; Nikolenko et al., 2007). Although it is challenging to correct movement artifacts after image acquisition, such scanning systems enable us to avoid scanning uninteresting structures, such as blood vessels. As new scanning methods, kilohertz 2P fluorescence microscopy using FACED (freepace angular-chirp-enhanced delay) (Wu et al., 2020) and optical bead microscopy using MaxiMuM (many-fold axial multiplexed module) (Demas et al., 2021) have also been reported.

New optical systems and optical designs are essential to overcome the limitations of the trade-offs in wide-field 2P microscopy. However, considering the tremendous progress made in the past decade, this may be solved sooner than possibly imagined. The number of neurons to be recorded will be much larger, and it will not be long before vast amounts of neural activity can be recorded, as much as 10¹⁰.

Acknowledgments

This research was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI (Nos. JP20H05775 (issued to M.M.), JP20K06934 (issued to K.O.), RIKEN Special Postdoctoral Researchers Program (H.U.), and AMED-Brain/Minds Project (grant number 21dm0207001h0008 to M.M.).

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