



Minireview

Finding Needles in a Haystack with Light: Resolving the Microcircuitry of the Brain with Fluorescence Microscopy

Jong-Cheol Rah^{1,2,*} and Joon Ho Choi^{1,*}

¹Laboratory of Neurophysiology, Korea Brain Research Institute, Daegu 41062, Korea, ²Department of Brain & Cognitive Sciences, Daegu Gyeongbuk Institute of Science & Technology, Daegu 42988, Korea

*Correspondence: jcrah@kbri.re.kr (JCR); JoonChoi@kbri.re.kr (JHC)

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To understand the microcircuitry of the brain, the anatomical and functional connectivity among neurons must be resolved. One of the technical hurdles to achieving this goal is that the anatomical connections, or synapses, are often smaller than the diffraction limit of light and thus are difficult to resolve by conventional microscopy, while the microcircuitry of the brain is on the scale of 1 mm or larger. To date, the gold standard method for microcircuit reconstruction has been electron microscopy (EM). However, despite its rapid development, EM has clear shortcomings as a method for microcircuit reconstruction. The greatest weakness of this method is arguably its incompatibility with functional and molecular analysis. Fluorescence microscopy, on the other hand, is readily compatible with numerous physiological and molecular analyses. We believe that recent advances in various fluorescence microscopy techniques offer a new possibility for reliable synapse detection in large volumes of neural circuits. In this minireview, we summarize recent advances in fluorescence-based microcircuit reconstruction. In the same vein as these studies, we introduce our recent efforts to analyze the long-range connectivity among brain areas and the subcellular distribution of synapses of interest in relatively large volumes of cortical tissue with array tomography and superresolution microscopy.

Keywords: activity markers, circuit reconstruction, connectome,

electron microscopy, microcircuitry, superresolution microscopy

INTRODUCTION

To understand an electrical circuit, one needs to know which circuit elements are present and how they are organized. Because circuit function is critically dependent upon the arrangement of the elements, it is impossible to understand the function of a circuit without knowing what elements are connected and how those elements are wired. Likewise, to understand the neural circuits of the brain, one must know the activity patterns of a population of neurons as well as the connectivity among those neurons. If the identities of individual neurons can be clarified reproducibly in different experiments, information derived from various studies assessing functions and connectivity can be combined. However, despite recent progress in the systematic profiling of the morphological, physiological and molecular characteristics of neurons, reproducible identification across individual mammalian brains is far from being achieved in full (Peng et al., 2021; Zeng & Sanes, 2017). Therefore, to gain a comprehensive understanding of the neural circuit, an appropriate set of information must be accessed within a subject's brain. In this short review, we will discuss recent advances in anatomical

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methods that can be used to aggregate physiological information and present future perspectives on related work.

SHORTCOMINGS OF NEURAL CIRCUIT MAPPING WITH ELECTRON MICROSCOPY AND PUTATIVE SOLUTIONS

Electron microscopy (EM) provides sufficient resolution to resolve individual synapses and trace thin neurites. With the aid of rapid developments in artificial-intelligence-based morphological analysis (Beier et al., 2017; Januszewski et al., 2018) and various ingenious high-throughput EM image acquisition technologies, it has become possible to probe individual synaptic connections in cubic-millimeter-scale neural circuits (Table 1). Insightful review articles addressing the detailed advantages and limitations of these high-throughput EM technologies are available elsewhere (Briggman and Bock, 2012; Briggman and Denk, 2006; Helmstaedter et al., 2008; Kubota et al., 2018). Thus, in this minireview, we wish to highlight the technical hurdles facing EM-based circuit reconstruction in regard to retaining physiological information.

Connectomic information on invertebrates such as *Caenorhabditis elegans* and fruit flies can be understood through comparisons of various functional studies performed in different individuals because the neurons of these organisms are relatively well classified morphologically and genetically (Hobert et al., 2016; Jenett et al., 2012; Yemini et al., 2021). On the other hand, in mammalian brains, neither location nor neurite morphology nor genetic profiling can be used to predict the identity of neurons. Therefore, to understand the circuit elements and connectivity of the mammalian brain, EM images must be correlated with images that show the physiological identity of neurons from the same brain tissue. These physiological images can vary depending on the biological questions to be addressed; the images may depict the firing properties of the neurons during a particular behavior as acquired from 2-photon Ca^{2+} images (Table 1), the long-range connectivity of the neurons from neurotracers, or cell types identified based on the expression of marker proteins (Adams et al., 2016). In most cases, identifying neurons in terms of their functional or molecular properties is a task best suited for fluorescence light microscopy (FM). Indeed, while large-scale EM studies on invertebrates provide rich functional and structural information, those concerning mammalian brains tend to focus on either analyzing structure and connectivity rules *per se* or adding functional information by means of correlative FM images (Table 1).

Correlative light and electron microscopy (CLEM) is technically challenging for many reasons. In addition to the difficulty of simultaneously preserving ultrastructure and fluorescence, the imaging axes of functional 2-photon microscopy and EM are usually perpendicular to each other (however, see [Andermann et al., 2013]). Thus, the number of neurons whose functions have been measured in the reconstructed volume is inevitably small. Furthermore, because the accuracy of the alignment between EM and FM images is at the level of somata, not the level of neurites, all neurites of the neuron of interest must be reconstructed to examine their connectiv-

ity. The results of axon tracing based on anisotropic EM images are often ambiguous because axons are thinner and ~7 times more numerous in a given volume than dendrites (Fiala and Harris, 1999; Kasthuri et al., 2015; Shepherd and Harris, 1998). Even artificial-intelligence-aided dense reconstruction of axons yields what are known as “edge” cases and requires extensive human correction of the segmentation results (Dorkenwald et al., 2019; Kasthuri et al., 2015; Motta et al., 2019).

We believe that the emergence of genetically encoded and EM-compatible tracers has opened a new path for efficiently studying long-range connectivity, molecular identity, and even firing properties with a limited temporal resolution (Atasoy et al., 2014; Han et al., 2012; Li et al., 2010; Lin et al., 2016; Schikorski et al., 2007; Zhang et al., 2019). In particular, genetically encoded dimerized enhanced soybean ascorbate peroxidase-2 (dAPEX2) directed to various subcellular organelles enriched in synapses immediately permits the anterograde labeling of multiple synaptic inputs. Ginty and colleagues showed that transduced dAPEX2 in the mitochondrial matrix of layer (L) 5 neurons leads to distinguishable labeling of the mitochondria throughout cell bodies and neurites without compromising ultrastructural preservation (Zhang et al., 2019). These authors also provided dAPEX2 constructs designed to target various subcellular compartments, including synaptic vesicles (SVs), plasma membranes (PMs), and the endoplasmic reticulum (ER). When the relative subcellular distribution of synapses from multiple brain regions is of interest, as in (Kim et al., 2021b), postsynaptically targeted neurons may be labeled with PM-targeted dAPEX2, while presynaptic groups of neurons are labeled with SV- and mitochondrial-matrix-targeted dAPEX2. Because the synaptic boutons are filled with SV and enriched with mitochondria, the origins of the boutons can be visualized without long and laborious axon tracing. The cell types of neurons, as defined based on the expression of marker proteins, can be visualized in EM by fusing the marker with dAPEX2, as demonstrated in (Martell et al., 2017; Rae et al., 2021). Finally, we suggest that photoactivable calcium-sensitive expression of dAPEX2 can enable selective EM-compatible labeling of neurons that are involved in a specific behavior (Lee et al., 2017). Let us assume that the question to be addressed is the local connectivity among neurons sharing orientation selectivity in the primary visual cortex (V1), as in (Ko et al., 2011). Neuronal mitochondria with specific orientation selectivity can be labeled with dAPEX2 by means of selective visual stimuli coupled with *in vivo* photoactivation. Because mitochondria are densely present in both axonal varicosities and dendrites, reconstructions around synapses with and without dAPEX2 can provide a statistical conclusion to this question (Fig. 1B, insets). Various light-dependent transcriptional regulation tools (de Mena et al., 2018) with different wavelengths allow orthogonal labeling of multiple activities (e.g., vertical vs horizontal orientation selectivity, see Fig. 1A).

Nonetheless, this method, like others, comes with limitations. Full structural reconstruction may be necessary because most neurons could have an intermediate receptive field and thus express both markers at a certain ratio. Many of the critical determinants of this method, such as sensitivity, half-

Table 1. Representative studies using large-scale EM

Species	Analyzed area	Function in question and correlated functional information	Method	Reconstructed volume (μm^3)	Pixel size (nm), section thickness (nm)	Reference
<i>C. elegans</i> Male	Complete Posterior	Connectome Sexually dimorphic circuits	ssTEM		N/A, 50	White et al., 1986 Jarrell et al., 2012
<i>C. elegans</i>	nervous system					
<i>Drosophila</i>	Mushroom body	Connectome	TEM-CA	750 × 370 × 250	3.6, 50	Scheffer et al., 2020; Zheng et al., 2018
<i>Drosophila</i>	Complete	Connectome	FIB-SEM	250 × 250 × 250	8, 2-4	Li et al., 2020; Scheffer et al., 2020
<i>Drosophila</i>	Medulla	Connectomic variations between columns	FIB-SEM	40 × 40 × 80	10, 10	Takemura et al., 2015
<i>Drosophila</i>	Olfactory glomerulus	Synaptic variations	TEM-CA	400 × 750 × 60	4, 40	Tobin et al., 2017
<i>Drosophila</i> larva	Mushroom body	Connectome	ssTEM		3.8, 50	Eichler et al., 2017; Ohyama et al., 2015
Zebrafish	Hindbrain	Eye movement Integration neurons by 2-photon microscopy	ATUM-SEM	220 × 110 × 60	5, 45	Vishwanathan et al., 2017
Zebrafish	Complete myelinated axons	Prey capture Avoidance	ATUM-SEM		56 (4 nm in ROI), 60	Hildebrand et al., 2017
Mouse	Retina	Visual responses by 2-photon microscopy	SBF-SEM	350 × 300 × 60	16.5, 23	Bae et al., 2018; Briggman et al., 2011; Kim et al., 2014
Mouse	V1 L2/3	Visual orientation preference by 2-photon microscopy	TEM-CA	450 × 350 × 52	4, 50	Bock et al., 2011
Mouse	V1 L2/3	Synapse size distribution	TEM; high-resolution, large-format camera	250 × 140 × 90	3.58, 50	Dorkenwald et al., 2019
Mouse	S1	Saturated reconstruction	ATUM-SEM	40 × 40 × 50	3, 30	Kasthuri et al., 2015
Mouse	S1BF L4	Saturated reconstruction	SBF-SEM	62 × 95 × 93	11, 28	Motta et al., 2019
Human	Temporal lobe fragment	Ultrastructural anatomy of the human cortex	ATUM-mSEM	3 mm × 2 mm × 180 μm	4, 33	Shapson-Coe et al., 2021

ssTEM, serial section transmission electron microscopy; N/A, not available; TEM-CA, transmission electron microscope camera array; FIB-SEM, focused ion beam scanning electron microscopy; ATUM-SEM, automated tape-collecting ultramicrotome scanning electron microscopy; ROI, region-of-interest; SBF-SEM, serial block-face scanning electron microscopy; ATUM-mSEM, ATUM multibeam SEM.

life, and physiological interference, remain unclear and are difficult to quantify. The lack of fine temporal resolution can be problematic for many experiments because it can obscure essential functional information, such as the pattern of activity and epoch sensitivity of the neurons.

LIMITATIONS OF NEURAL CIRCUIT MAPPING WITH LIGHT MICROSCOPY

Abbe's rule and conventional microscopy

The theory of image formation was proposed by Ernst Abbe. The theory suggests that an image arises from a diffraction process, specifically when incoming light waves from an object are diffracted by an objective lens (Hecht, 2016). Abbe

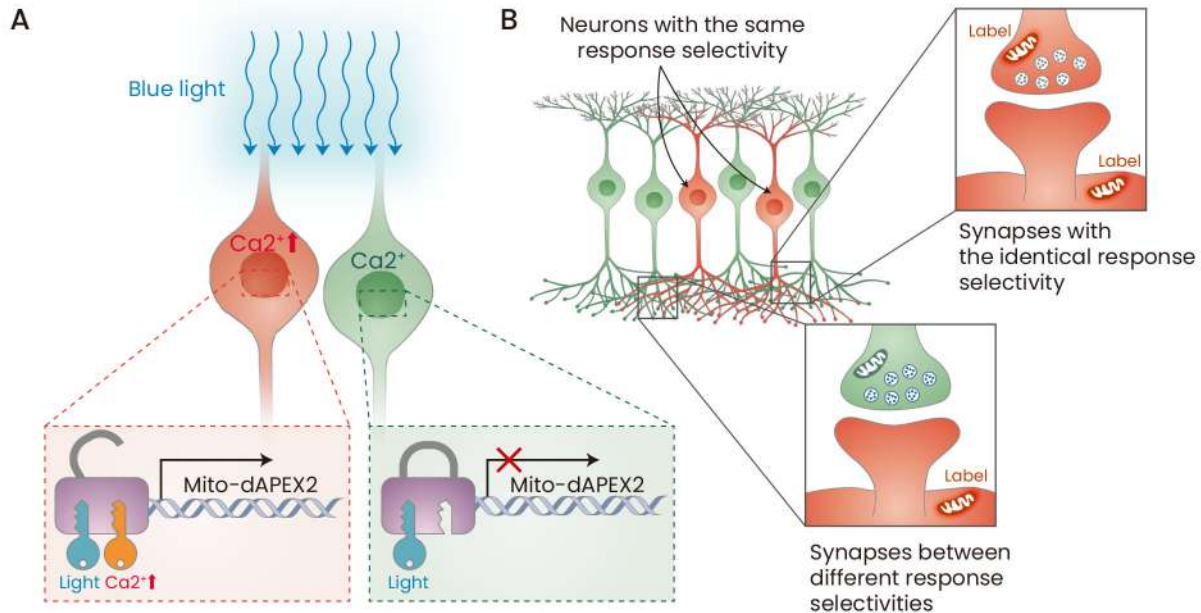


Fig. 1. A connectivity mapping strategy with functional information using photoactivable calcium-sensitive expression of dAPEX2.

(A) Light- and activity-dependent expression of dAPEX2, a genetically encoded and EM-compatible tracer, can be useful to examine one of the classical questions in neuroscience: whether neurons that fire together wire together. Because the Cal-Light system allows gene expression only when the Ca^{2+} concentration is elevated in the presence of blue light (Lee et al., 2017), repetitive vertically oriented visual stimuli paired with blue light will lead to expression of the target gene in a selective group of neurons whose receptive field matches the visual stimulation (red neuron in A). (B) If the target gene is dAPEX2 targeted to the mitochondrial matrix (Mito-dAPEX2; Zhang et al., 2019), the axons from the neurons with the vertical receptive field (red neurons in B) can be distinguished from the inputs from the neurons with no response (blue neurons in B) in EM by labeling of the mitochondrial matrix (insets). Thus, without reconstructing the entire volume, the relative ratio of the inputs can be calculated by a stereological approach. Figure was created with BioRender.com.

found that a larger aperture—and thus, a higher numerical aperture (NA)—results in a higher resolution, as described in the following simple equation:

$$d = \frac{0.61\lambda}{NA} \quad (\text{Thorn, 2016})$$

The NA also affects the resolution in the z (axial) direction:

$$d_z = \frac{2\lambda}{NA^2} \quad (\text{Thorn, 2016})$$

Another way of describing the resolution of a microscope is the point spread function (PSF), which corresponds to an image of an infinitesimal object blurred by diffraction. The size of the PSF reflects the resolution of the imaging system in terms of both the theoretical and experimental limits. Usually, the full width at half maximum (FWHM) of the PSF matches Abbe's rule. In an invariant space system (i.e., an aberration-free system such that the PSF is the same everywhere), one can think of an image as a convolution of the imaged object and the PSF (Hecht, 2016).

Effectively, in the field of neuroscience, two enhanced green fluorescent protein (EGFP)-expressing dendrites separated by a lateral distance of less than approximately 205 nm will appear as a continuous structure. Worse, neuronal struc-

tures separated by less than approximately 465 nm of axial space will appear to be in physical contact even under ideal conditions when the objects exist on a single imaging plane.

In practice, however, because most neurons have radially oriented neurites, the objects on the focal plane are often obscured by out-of-focus structures. Therefore, an imaging method that can selectively produce clear images of the focal plane within thick brain tissue is required. This is one of the critical reasons why confocal laser scanning microscopy is such a widely adopted imaging tool in neuroscience. In confocal microscopy, optical sectioning is achieved by illuminating an object with focused laser light and detecting the light through a pinhole that eliminates light originating from out-of-focus points. Despite this advantage, confocal microscopy is not appropriate for studying connectivity due to its insufficient resolution, especially along the axial dimension. Many imaging methods have been developed and adopted for neural circuit reconstruction to circumvent this insufficient resolution while preserving the advantages of FM.

Physical methods to circumvent the resolution problem

To circumvent the limited resolution of FM imposed by the properties of light, some physical solutions have been suggested, namely, expansion microscopy and array tomography

(Micheva and Smith, 2007; Wassie et al., 2019). A method of isotropic expansion of biological specimens was initially suggested by Boyden's research team (Chen et al., 2015; Wassie et al., 2019). They took advantage of the expandability of a polymer network. By cross-linking the network of swellable polyelectrolyte hydrogel in brain tissue, they achieved magnification by a factor of 4.5 or more without noticeable distortion of the tissue while preserving the antigenicity of the tissue (Chen et al., 2015; Truckenbrodt et al., 2018). This method effectively allows an optical resolution comparable to that of superresolution microscopy to be achieved using conventional confocal microscopy. However, the typical challenges of confocal microscopy still hinder large-scale circuit mapping. Compared to other FM imaging methods, confocal microscopy requires a long imaging time due to the scanning of the excitation laser, suffers from increased photobleaching because of the prolonged exposure of the fluorophore to the laser, and has relatively low sensitivity because the pinhole tends to block a substantial fraction of the in-focus signal. Because expanded tissues have a lower fluorophore density per unit volume and thus require slower scanning, the problems of confocal microscopy described above will be more severe with expansion microscopy. Combination with selective plane illumination microscopy (SPIM), in which the optical resolution is provided by an orthogonally applied excitation beam in the form of a thin sheet of light (Huisken et al., 2004), could potentially solve some of these problems (Bürgers et al., 2019).

Array tomography (AT) is another physical solution to overcome Abbe's rule. Because AT relies on iterative wide-field FM imaging of ultrathin serial sections of brain tissue, the axial resolution of this imaging technique depends solely on the thickness of the tissue, not on Abbe's rule (Micheva and Smith, 2007). Furthermore, because all the pixels have the same chance of antigen-antibody reactions, this method enables quantitative immunohistochemical analysis. A reasonably high (~80%) synapse detection accuracy has been predicted and demonstrated at an isotropic optical resolution of 200 nm (Rah et al., 2013). Furthermore, the minimal thickness of the sections allows repetitive antibody elution and staining (Micheva and Smith, 2007; Micheva et al., 2010b). In practice, various studies have taken advantage of this technique to successfully unveil the subcellular distribution or molecular diversity of synapses (Bloss et al., 2016; Kim et al., 2021b; Micheva et al., 2010a; 2021; Rah et al., 2013; 2015). Nevertheless, despite the advantages described above, AT also has inherent limitations. Because AT is based on wide-field FM, dense reconstruction of local connectivity is difficult to resolve. Furthermore, the wave nature of light imposes a lateral resolution limit on this technique, and thus, the detection of small synapses can be ambiguous. We believe that local circuits could be resolved via AT in combination with Brainbow (expression of fluorescent proteins) (Cai et al., 2013; Livet et al., 2007; Loulier et al., 2014), although this possibility currently remains unexamined experimentally. Superresolution microscopy providing a resolution as high as a few nm on the matching thickness of the serial sections could overcome the resolution limit of this imaging technique. However, because of the long imaging time and fast

bleaching of superresolution microscopy imaging, this task is not as easy as it sounds. Below, we introduce a brief conceptual introduction to several widely used superresolution microscopy techniques, such as photoactivated localization microscopy (PALM), structured illumination microscopy (SIM), and stimulated emission depletion (STED), to discuss the advantages and disadvantages of microscopic techniques as a possible solution for FM-based microcircuit reconstruction.

Recent progress in superresolution imaging (or nanoscopy)

A wide variety of FM techniques have emerged in an attempt to overcome the diffraction limit (Fig. 2; Sahl et al., 2017). Among these superresolution techniques, scanning-based methods can acquire images directly, similar to a confocal microscope. The methods of STED and reversible saturable/switchable optical linear fluorescence transitions (RESOLFT) directly silence diffraction-limited areas such that the effective PSF is reduced to 40 nm (Vidomini et al., 2018). PALM and stochastic optical reconstruction microscopy (STORM) use the flickering kinetics of a single molecule, known as stochastic kinetics, to computationally detect the molecule's position. These methods have the highest resolution of all techniques (Betzig et al., 2006; Sahl et al., 2017). To collect a sufficient number of flickering events, special fluorophores and a large amount of imaging (>10,000 frames per image) are required (Khater et al., 2020). In SIM, the sample is illuminated with several rotated and phase-shifted periodic patterns, and the obtained images contain high-resolution information in a resolvable regime, similar to moiré fringe patterns (Gustafsson, 2000; 2005). Through linear image postprocessing of SIM in Fourier space and the reverse Fourier transformation, it is possible to recover an image with a twofold increase in resolution (~100 nm). This process provides sufficient resolution for synapse imaging while requiring only relatively simple and fast computational processes (Khater et al., 2020). On the sample preparation side, SIM is not restricted by labeling and requires a relatively low dose of light, making live cell imaging easier (Sahl et al., 2017). Finally, by utilizing higher harmonic orders with brighter illumination (saturated SIM), the resolution can be increased even further with a nonlinear reconstruction method, which is computationally complex and heavy (Gustafsson, 2005).

Future perspectives

The abovementioned superresolution techniques provide sufficient optical resolution while maintaining the advantages of FM, such as compatibility with functional imaging, genetic or immunochemistry-based molecular labeling, and relatively straightforward image segmentation due to the high contrast of FM. However, in terms of imaging speed, superresolution microscopy imaging is not significantly advantageous over EM. Although the field of view of superresolution microscopy tends to be wider than that of EM, it is still typically smaller than 50 μm . Furthermore, it takes 3-10 min to acquire a one-tile superresolution image (Huszka and Gijs, 2019; Kim et al., 2021a). Therefore, the long imaging time required to obtain images over a sufficiently large volume to address biological questions is a critical limiting factor. In fact, whereas various high-throughput EM imaging methods have been developed

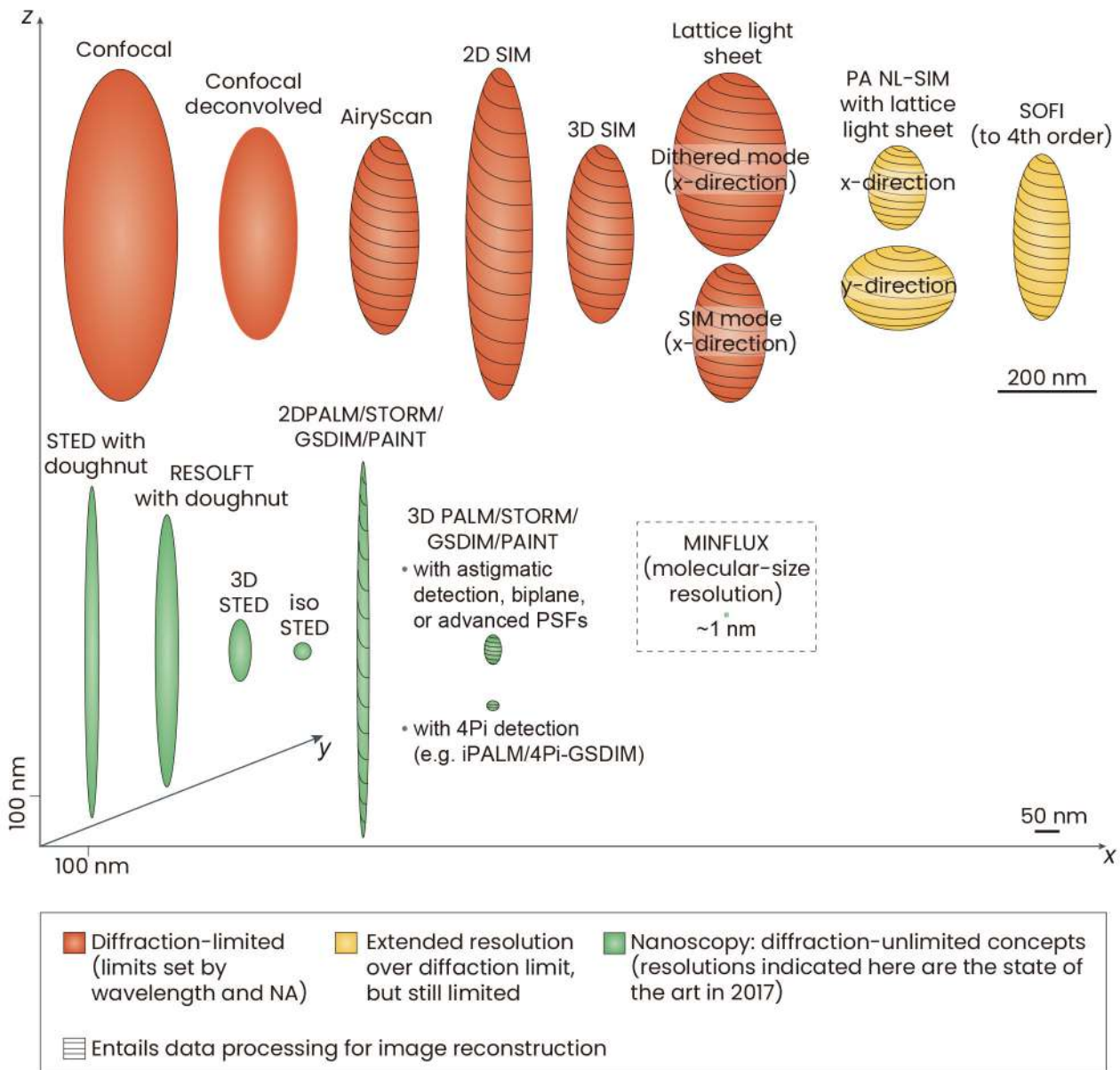


Fig. 2. Comparison of the imaging resolution of different microscopy techniques. The ellipsoids indicate the 3D resolution of the listed methods. Each can be interpreted as an uncertainty range from where detected photons originate or the PSF of that technique. PSFs of the diffraction-limited methods, such as confocal microscopy, SIM, and lattice light-sheet microscopy, are shown in orange (assumed emission wavelength: ~ 650 nm). PSFs of the further extended resolution techniques over the diffraction limits are shown in yellow. Finally, PSFs of the diffraction-unlimited techniques such as STED, PALM, RESOLFT, and minimal emission fluxes (MINFLUX) are shown in green. Adapted from the article of [Sahl et al. \(2017\)](#) (*Nat. Rev. Mol. Cell Biol.* 18, 685-701) with original copyright holder's permission.

and utilized in neural circuit analysis (Table 1), superresolution microscopy has been mainly developed for studying fine structure or dynamic molecular changes in neuroscience ([Ji et al., 2008](#); [Maglione and Sgrist, 2013](#); [Tao et al., 2012](#)). SIM seems to be the best fit for large-scale circuit reconstruction because of the following attributes. SIM provides the minimal necessary optical resolution for reliable identification

of synaptic connections, i.e., ~ 100 nm ([Gustafsson, 2000](#); [Kim et al., 2021a](#); [Rah et al., 2013](#); [Wu et al., 2013](#)). To take full advantage of FM, multiple fluorescent channels with conventional fluorophores can be utilized. Compared with other superresolution microscopy techniques, the image acquisition speed is relatively fast because of the relatively large field of view ([Fiolka, 2013](#); [Gustafsson, 2005](#)). The shortcomings

of SIM, preventing it from being an ideal technique, are excessive imaging time and the inability to image thick tissue sections effectively. Three-dimensional sectioning provides approximately twice the resolution of conventional microscopy, approximately 300 nm, and is applicable only to thin samples such as cell cultures, as opposed to thick and highly diffractive matter such as brain tissue (York et al., 2012). A long image acquisition time for a field is unavoidable because SIM uses a series of sinusoidal illumination patterns.

We have recently developed a new imaging technique called structured illumination microscopy of the putative region of interest with ultrathin sectioning (SIM-PRIUS), which combines SIM with AT (Kim et al., 2021a). To reduce the imaging time, SIM images are selectively acquired from regions with putative synapses of interest as determined based on a set of low-magnification images (Kim et al., 2021a). Furthermore, because SIM-PRIUS relies on ultrathin sectioning of the specimen, the axial resolution is determined by the physical thickness of the tissue. Indeed, when the target structure is as sparse as a particular set of synapses (e.g., synapses sourced from a specific brain region), synapse identification can be achieved with greater accuracy and a 95% reduction in imaging time (Kim et al., 2021a). With the rapid development of artificial-intelligence-dependent object recognition techniques, we believe that this concept can enable expeditious and accurate detection from a relatively large volume of brain tissue.

CONCLUSION

Despite the initial worries about connectomic research highlighted by Seung and Movshon's argument, many studies have successfully demonstrated the advances that connectomic studies can provide. It is now widely agreed that brains cannot be understood without knowing how neural circuits are organized. A proper understanding of neural circuits can finally be derived when a set of information about molecules defining the cell types and long-range connectivity, physiological characteristics, and local connectivity is collectively understood. In this respect, we believe that FM is the technique that is best positioned to provide the necessary elements if there is sufficient support from digital image processing, automatic hardware control and feedback systems, and molecular tool development.

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AUTHOR CONTRIBUTIONS

J.C.R. and J.H.C. designed the article and interpreted the relevant literature. J.C.R. and J.H.C. drafted the article.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

ORCID

Jong-Cheol Rah <https://orcid.org/0000-0003-3780-0522>

Joon Ho Choi <https://orcid.org/0000-0002-2090-8724>

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