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Scientists compare a virus-based method with well-established approaches for functional neuroimaging in mice

Functional neuroimaging has become a powerful tool to better understand the functions and connectivity of different brain regions and how they relate to external stimuli and behavior. Many popular approaches to mesoscale, or intermediate, functional neuroimaging involve inferring neuronal activity indirectly by measuring optical signals from the brain, such as variations in reflected light resulting from real-time changes in blood flow around active neurons.

When performing functional neuroimaging in animal models, it’s common to use genetically engineered fluorescent indicators to greatly increase the sensitivity of the imaging method. For example, GCaMP6 is an artificial protein that acts as a calcium sensor; that is, when bound to calcium ions it absorbs blue light and then emits green light. Because calcium ions are transported into neurons when they fire, the fluorescence of GCaMP6 can be used as a proxy for neuronal activity. Scientists frequently use this calcium sensor to perform functional neuroimaging in live mice, whose skull is semi-transparent and allows for relatively easy optical imaging of the brain.

However, there are multiple ways in which one can get mouse neurons to express the GCaMP6 protein. A common approach is using mice with the GCaMP6 gene strategically inserted in the genome so that it is expressed in certain types of neurons. The main disadvantage of transgenesis (gene insertion) is that it is costly and time consuming to maintain the necessary mouse colonies and perform the required mouse crossings. Another viable strategy is using viral vectors to transfer the GCaMP6 gene directly into the brains of adult mice. Although more practical than creating transgenic mice, injecting viruses multiple times into the brain of a mouse is a very invasive procedure, which could negatively affect the experimental results.

Recently, researchers engineered a new variant of a virus (AAV.PHP.eB) that can cross the blood-brain barrier (BBB), which means that the GCaMP6 gene can be transferred to neurons by injecting the mouse in the veins in its tail rather than directly into its brain. Despite the vast potential of this approach to simplify mesoscale functional neuroimaging, there have been no reported comparisons with classical methods of inducing GCaMP6 expression. That’s why a trio of Canadian scientists conducted a comparative study between the BBB-crossing viral vector strategy and three well-established GCaMP6 transgenic lines in live mice.

The researchers first installed large transcranial windows in transgenic and brain-virus-injected mice. Then, they put the head-fixed animals in a dark chamber and periodically flashed a white light to evoke visual responses in the brain. They used a blue LED to trigger the fluorescence of GCaMP6 and a green LED to measure the base—or background—green reflectance of the brain to then compensate for it.

Overall, the results indicate that all three transgenic lines tested and the BBB-crossing viral vector are similarly useful for accurate mesoscale functional neuroimaging. One of the main strengths of the study was the multiple considerations that the scientists had to make to ensure a fair comparison of the approaches. These include the use of nonanesthetized mice, the selection of a visually evoked response paradigm, and the quantification of several measures. “We employed methodological strategies to avoid confounding factors that might limit the optical characterization of the different mouse lines,” highlights principal investigator Timothy Murphy of the University of British Columbia.

By proving the strengths of the BBB-crossing viral vector strategy against the also effective—but less convenient—transgenic approaches, these scientists are paving the way to simpler experimental pipelines for mesoscale functional neuroimaging. “Given the strong functional signals obtained using intravenous injection of AAV.PHP.eB and the ability to express genetic material without transgenesis or intracerebral injection, this viral vector will likely become an important experimental tool for the testing of novel fluorescent indicators of neuronal or glial activity in vivo,” concludes Murphy.

Summary

Scientists from the University of British Columbia have compared the effectiveness of different strategies to express a fluorescent indicator of neuronal activity in the brains of live mice. Their results show that a novel viral vector that can cross the blood-brain barrier is as good as many well-established techniques for functional neuroimaging, demonstrating the potential of this time-saving and less-invasive approach to make neurons express this specific protein.

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Nicholas J. Michelson, Matthieu F. Vanni, and Timothy H. Murphy,
“Comparison between transgenic and AAV-PHP.eB-mediated expression of GCaMP6s using in vivo wide-field functional imaging of brain activity,” Neurophotonics 6(2), 025014 (2019). doi:10.1117/1.NPh.6.2.025014
Tiny implantable tool for light-sheet imaging of brain activity

Tools that allow neuroscientists to record and quantify functional activity within the living brain are in great demand. Traditionally, researchers have used techniques such as functional magnetic resonance imaging, but this method cannot record neural activity with high spatial resolution or in moving subjects. In recent years, a technology called optogenetics has shown considerable success in recording neural activity from animals in real time with single neuron resolution. Optogenetic tools use light to control neurons and record signals in tissues that are genetically modified to express light-sensitive and fluorescent proteins. However, existing technologies for imaging light signals from the brain have drawbacks in their size, imaging speed, or contrast that limit their applications in experimental neuroscience.

A technology called light-sheet fluorescence imaging shows promise for imaging brain activity in 3D with high speed and contrast (overcoming multiple limitations of other imaging technologies). In this technique, a thin sheet of laser light (light-sheet) is directed through a brain tissue region of interest, and fluorescent activity reporters within the brain tissues respond by emitting fluorescence signals that microscopes can detect. Scanning a light sheet in the tissue enables high-speed, high-contrast, volumetric imaging of the brain activity. Traditionally, using light-sheet fluorescence brain imaging with nontransparent organisms (like a mouse) is difficult because of the size of the necessary apparatus. To make experiments with nontransparent animals and, in the future, freely moving animals feasible, researchers will need to miniaturize these components.

A key component for the miniaturization is the light-sheet generator itself, which needs to be inserted into the brain and thus must be as small as possible to avoid displacing too much brain tissue. In a new study, an international team of researchers from the California Institute of Microstructure Physics (Germany), and Advanced Micro Foundry (Singapore) developed a miniature light-sheet generator, or a photonic neural probe, that can be inserted into the brain. The researchers used nanophotonic technology to create ultrathin silicon-based photonic neural probes that emit multiple addressable thin sheets of light with thicknesses <16 micrometers over propagation distances of 300 micrometers in free space. When tested in brain tissues from mice that were genetically engineered to express fluorescent proteins in their brains, the probes permitted the researchers to image areas as large as 240 μm × 490 μm. Moreover, the level of image contrast was superior to that with an alternative imaging method called epifluorescence microscopy.

Describing the significance of his team’s work, the study’s lead author, Wesley Sacher, says: “This new implantable photonic neural probe technology for generating light sheets within the brain circumvents many of the constraints that have limited the use of light-sheet fluorescence imaging in experimental neuroscience. We predict that this technology will lead to new variants of light-sheet microscopy for deep brain imaging and behavior experiments with freely moving animals.”

Such variants would be a boon to neuroscientists seeking to understand the workings of the brain.

Researchers used nanophotonic technology to develop a brain-implantable tool that can aid in the optical imaging of brain activity

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SUMMARY

Tools for optical imaging of brain activity in freely moving animals have considerable potential for expanding the scientific understanding of the brain. However, existing technologies for imaging brain activity with light have challenges. An international team of scientists has now developed an implantable probe for light-sheet imaging of the brain.
Novel calibration procedure for super-resolution brain imaging

A simple and robust procedure corrects a systematic error in microscopy, enabling precise imaging of biological tissue at greater depth.

Over more than a century, microscopists were hamstrung by this classic barrier until the invention of super-resolution fluorescence microscopy. One particularly powerful approach was developed in the late 1990s and coined stimulated-emission depletion (STED) microscopy. This technique requires the target sample to contain fluorophores, which are compounds that absorb light at one wavelength and then emit it at a longer one. In the simplest version of STED microscopy, fluorophores are excited in a circular spot by irradiation with a diffraction-limited focused laser. Then, a donut-shaped portion around the spot is irradiated with less-energetic light—the depletion beam—which switches off the fluorescence by the process of stimulated emission. Thus, the net effect is that only the fluorophores in the center of the donut re-emit photons, and because that area can be made arbitrarily small, this allows for super-resolution microscopy.

Although STED microscopy was a true breakthrough for observing the morphology of live neurons at higher resolution, there is still room for improvement. In a recent study, a team of scientists led by Dr. U. Valentin Nägerl from Université de Bordeaux developed a simple yet effective calibration method that allows for more precise STED imaging at higher tissue depths. Their approach is based on analyzing and correcting for one of the main sources of systematic error in STED microscopy for biological samples: spherical aberration of the depletion beam. When imaging a tissue sample at depths higher than 40 μm, the depletion beam suffers various types of defocusing and degradation (aberration) and loses its carefully crafted shape, which is essential to the STED method. Spherical aberration is the biggest offender and was the one the researchers targeted. Their strategy was to first prepare a brain tissue phantom sample, a gel-based proxy with a refractive index similar to that of the actual brain. This phantom sample contained homogenously dispersed fluorophores and gold nanoparticles, which allowed the team to clearly visualize and quantify how the shape of the depletion beam got distorted as it penetrated deeper. Then, they calculated the necessary pre-adjustments that should be made to the depletion beam according to tissue depth so that its final shape more closely matches the ideal one. The adjustments were made using adaptive optics, which is a technology originally developed by astronomers to improve telescopic images that suffer from aberrations caused by the earth's atmosphere. By the process of stimulated emission, the net effect is that only the fluorophores in the center of the donut re-emit photons, and because that area can be made arbitrarily small, this allows for super-resolution microscopy—a technique that is specifically adjusted for deep tissue imaging. The results were quite convincing: corrected STED images captured the fine details of deeper neuronal dendrites much better than standard STED images. "Using our calibration strategy, we could measure neuronal structures as small as 80 nm at a depth of 90 μm inside biological tissue and obtain a 60 percent signal increase after correction for spherical aberration," says Nägerl. "Our approach is not limited to brain samples; it could be adapted to other tissues with known and relatively homogeneous refractive indices, as well as other types of preparations, such as single layer cells, where light scattering is negligible."

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Scientists proceeded to image live neural tissue. They compared the results of regular STED microscopy, corrected STED microscopy, and two-photon stimulated emission depletion microscopy (2P-STED), and achieved 80 nm resolution of imaging neuron dendritic spines through 90 micron brain tissue. This is noteworthy because super-resolution is hard to achieve in thicker tissue—particularly the highly scattering quality of brain tissue." Yi explains that the advance will facilitate study of neural activities and interactions. Considering this novel calibration process is robust, straightforward to implement, and relatively inexpensive, it could be easily incorporated into standard laboratory practices to obtain better results with STED microscopes, so long as the prepared phantom sample matches the optical properties of the biological specimen. In this regard, Nägerl states: "Our approach is not limited to brain samples; it could be adapted to other tissues with known and relatively homogeneous refractive indices, as well as other types of preparations, even potentially in the intact, live mouse brain."
Scientists Use Neuroimaging to Study Slow Oscillation Activity Pattern in the Brain During Sleep

Neuroimaging, coupled with innovative statistical analysis methodologies, has enabled researchers to observe patterns in brain activity in greater detail than ever before. Functional connectivity analysis, one such approach, employs the sequential correlation of spontaneous activity in different brain regions to create a “map” of areas sharing functional properties, called a functional connectivity pattern. This clarifies the organization of the brain and is useful in the diagnosis of various mental health disorders.

With recent progress in optical imaging and in transgenic mice studies, scientists can now extract detailed functional connectivity patterns from the whole brain at higher frequencies, reaching from 0.4 to 4 Hz. This frequency range corresponds to the “delta band,” in which a peculiar phenomenon called the “slow oscillation” appears. The slow oscillation occurs exclusively during sleep or under anesthesia and causes large, nearly periodic fluctuations in neuronal activity throughout the entire brain.

Despite being a hallmark of the sleeping state, much about the slow oscillation and the delta band remains unknown. For example, researchers don’t know if the slow oscillation affects the functional connectivity patterns differently for distinct brain states, such as natural sleep or anesthesia-induced sleep. Moreover, it remains unclear if the slow oscillation replaces the usual delta band activity in the wakeful state or if it superimposes it.

A team of scientists from Washington University conducted an in-depth study comparing the correlation structures of multiple brain states in genetically engineered mice in both the delta and the infraslow band, the latter of which is the one conventionally used in functional connectivity analyses. Moreover, they assessed brain activity by imaging both calcium fluorophores and hemoglobin, as well as by acquiring electroencephalography and electromyography signals to accurately determine the arousal state of the animals.

To conduct the imaging experiments, the researchers installed a transparent plexiglass window onto the skull of mice. Then, they shone LEDs of appropriate frequencies onto the brain to excite the fluorescent molecules and to compensate for the base background noise. The arousal states studied were wake, non-rapid eye movement (NREM), and two sleep states induced by different types of anesthetic drugs. The team used principal component analysis, a statistical decomposition approach, to determine whether the slow oscillation can be separated from wake-like functional connectivity patterns.

One of the main findings of this study was that simply subtracting a few of the principal components of the delta band activity in sleeping mice was enough to produce correlation patterns similar to those in a wake state. “These results prove that wake-like cortical activity persists during brain states dominated by the slow oscillation—meaning NREM sleep and anesthesia—regardless of the mechanism of slow oscillation induction,” explains Lindsey M. Brier, the corresponding author of the paper. “Thus, during these brain states, the slow oscillation superimposes on, rather than replaces, persistent wake-like ongoing activity.”

This study was notably more comprehensive than previous ones because multiple sleep states were considered, as well as different fluorescent targets and supporting signals. These broad experimental conditions will provide a solid foundation for evaluating the relationship between the slow oscillation and functional connectivity, which in turn will help scientists better understand the differences between wakefulness and sleep. This may lead to the answers to important questions in medicine and biology.

As Brier highlights, “Our study provides opportunities for further research that could demonstrate the mechanisms of sleep and sleep disturbances related to disease. Our results can help the scientific community understand the association between sleep and Alzheimer’s disease, and the effect of anesthesia on postoperative delirium.”

Neuroimaging studies shed light on the nature of the “slow oscillation,” a brain activity pattern that characterizes sleep states
To understand how the mammalian brain processes information, makes decisions, and forms memories, we need to understand the mechanisms underlying the functions of neural circuits. This is best achieved through visual documentation, aided by microscopy. Beginning with simpler efforts involving light microscopes, scientists have used increasingly advanced techniques, including in vivo imaging, to study the brain. However, detailed observation of the neural circuitry at the synapse level, from a whole brain perspective, is still relatively elusive to modern microscopy, particularly because of the opaqueness of the brain tissue and the size of mammalian brains.

To enhance the resolution of images observed through light microscopy, a technique called expansion microscopy has been introduced, which relies on physical expansion of tissues through imbibition of water-adsorbent polymers. The expanded tissues show greater transparency, which is suitable for subsequent light microscopy. However, visualizing large biological specimens like neurons at sufficiently higher speed and resolution is still challenging.

Although light-sheet fluorescence microscopy (LSFM) has been applied in some studies for volumetric imaging of large brain cells like neurons, the technique achieves resolution of up to 250 nm only due to diffraction-related limitations. Although light-sheet fluorescence expansion microscopy (LFSFM) has been applied, scientists from Germany and the United Kingdom have successfully developed a light-sheet fluorescence expansion microscopy technique that unravels brain cell morphology with unprecedented finesse and speed, thereby putting neurobiology research on fast track.

**SUMMARY**

Addressing deeper questions in neurobiology requires advanced microscopy techniques that allow simultaneous, fast, and high-resolution mapping of neural circuits spread across large areas of the brain. However, existing microscopy techniques seldom meet all these requirements. A group of scientists from Germany and the United Kingdom has successfully developed a light-sheet fluorescence expansion microscopy technique that unravels brain cell morphology with unprecedented finesse and speed, thereby putting neurobiology research on fast track.
Focused Optogenetic Activation at the Subcellular Level

Optogenetics, a method used for controlling neuronal activity with light stimulation, has revolutionized our understanding of neuronal circuits. In optogenetics, light of specific wavelengths can be used to activate or inactivate genetically engineered neurons expressing specific light-sensitive proteins called channelrhodopsins. However, while current optogenetic techniques allow the activation of small groups of neurons, specific activation at a subcellular level has not been extensively explored, despite its potential in delineating synapse-level neuronal communication. Moreover, techniques that allow such restricted stimulation of channels at the micro- and nanoscale levels are currently unavailable.

To address this gap, a multinational team of researchers tested a new approach for focused subcellular stimulation. They used two light beams of different wavelengths—an activating beam and an overlapping donut-shaped inactivating beam—to activate channels at the subcellular level. Their findings revealed that the co-illumination approach could successfully create a central region of activation surrounded by a ring of inactivation. Moreover, light with a wavelength of 405 nm appeared to be ideal for channel activation in cases where strong photocurrents and efficient inactivation using 594-nm co-illumination were required.

Although the channel currents generated in the present study were not sufficient to allow the nanoscale activation of subcellular domains within neurons, the study represents an important step in the development of techniques that could allow such interventions in the future. The expected discovery of channel variants with stronger photocurrents, as well as new optogenetic tools with faster “on–off” switches, could bolster the development of strategies for focused nanoscale activation. “The present study provides the guiding framework for bioengineering to establish techniques that improve the current resolution of optogenetic activation,” says Dean.

Such techniques could drive the detailed investigation of subcellular biology in neurons and aid our understanding of the precise mechanisms that underlie brain function.

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Modified Two-Photon Microscopy Technique to Image Astrocytes

Astrocytes are a type of glial cell that supports non-neuronal cells in the brain. They are involved in cellular functions like injury response, transportation of blood-borne components to the neurons, energy regulation, and providing electrical insulation for unmyelinated axons. They are also important in regulating essential physiological functions like attention and sleep. Previous studies have shown that these functions of astrocytes involve signaling mechanisms mediated by calcium ions.

Microscopy techniques have been successful in the live imaging of calcium signaling in neurons. However, the same cannot be said of the larger astrocytes, which are relatively “silent”—electrically speaking. Scientists from the USA, led by Kira E. Poskanzer, assistant professor at the Department of Biochemistry and Biophysics, University of California, San Francisco, have now attempted to solve this unique problem. They successfully enhanced the existing imaging technique of two-photon microscopy to better live-image astrocytes “flagged” for calcium ion activity.

Poskanzer sees a need for improved imaging of astrocytes. “To better understand the role of astrocytes in neural circuits, optical tools tailored specifically to capture their complex spatiotemporal calcium ion dynamics, in multiple dimensions, are the need of the hour,” she says.

Two-photon microscopy is often used to image high-light-scattering biological samples, such as brain tissue. Moreover, this technique employs near-infrared light, which is low-scattering, and shows low absorption in biological tissues. Once the scientists determined the best imaging modality to enhance, their next step was to determine an active optical element that would allow physiological multiple-plane observation of calcium ion dynamics in the astrocytes. For this, they used the deformable mirror (DM).

The research team employed microelectromechanical systems (MEMS)-based DMs as the active optical element to axially scan the astrocyte of interest in vivo. MEMS incorporates electrical and mechanical components that allow the “deformation” of the mirror in an optimal manner. Specifically, the use of MEMS-based DMs allowed the scientists to view any astrocyte, and its calcium ion activity, in different z-planes, separated by distances as short as 53 μm, effectively achieving a deep image that was comparable to a three-dimensional image.

The significance of this advancement could be considerable. “The enhanced two-photon microscopy technique that we propose will allow researchers to noninvasively study any calcium ion-signaling brain cell,” says Poskanzer. “In addition, we have succeeded in stretching the limits of two-photon microscopy to include volumetric imaging of relatively undetectable astrocyte calcium signaling.”

Overall, the scientists were successful in the optical design and implementation of a two-photon microscope that incorporates a DM for axial scanning. This achievement could enable more in-depth exploration of relatively undetectable non-neuronal physiological components in the brain.

Further fine-tuning of the optical features of the enhanced two-photon microscopy presented in this study will surely add to the ever-growing knowledge of the inner workings of the human brain.

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SUMMARY

Astrocytes, well-known for their supportive roles in the brain, have important functions in regulating sleep and attention, mediated by complex cell signaling dynamics dependent on calcium ions. Until recently, owing to limitations in microscopy techniques, it was difficult to visually observe these dynamics. In a new study published in Neurophotonics, scientists from the USA successfully enhanced two-photon microscopy using a deformable mirror to observe astrocyte calcium signaling in detail.
High-Resolution Nanoimaging for Assessing Protein Interactions in Neurons

Scientists develop a new technique—fluorescence lifetime imaging nanoscopy—that allows the determination of cellular protein interactions at the nanoscale.

The researchers' findings effectively showcase the utility of FLIN in biological research. While traditional FRET can offer information on whether proteins interact, it cannot reveal the specific nanodomains where these interactions occur. FLIN is a valuable tool that overcomes these shortcomings and provides a comprehensive understanding of protein signaling, especially in cellular structures as small as dendritic spines.

With technological advancements, the focus of research has shifted towards delineating cell biology at the molecular scale. The development of FLIN is an important milestone in this journey. This approach breaks several barriers preventing the nanoscale-level examination of cell signaling and could have far-reaching applications across several fields of biology, biochemistry, and medicine.

Heat map showing that FLIN offers a much greater resolution at the nanoscale level than the currently used technique FLIM, allowing improved assessment of protein interactions even within cellular nanodomains, such as the dendritic spines of neuronal cells.

The ability to visualize protein interactions at nanoscale levels is important for understanding cell signaling—the processes by which cells communicate—and for subsequently delineating cell behavior. Several techniques are used to dissect these interactions. One such technique is fluorescence lifetime imaging microscopy (FLIM), in which proteins of interest are tagged with fluorescent molecules called fluorophores and monitored for gradual changes in fluorescence intensities. FLIM can be used to measure Förster resonance energy transfer (FRET), a process in which an excited donor fluorophore transfers energy to a nearby acceptor fluorophore. FRET can be measured to obtain a better understanding of the positions of adjacent molecules. However, FRET-FLIM is usually performed using traditional microscopy techniques and is thus limited by the diffraction barrier, which causes the signals of neighboring proteins to overlap and prevents their resolution.

To overcome this problem, researchers from Canada developed a new technique called fluorescence lifetime imaging nanoscopy (FLIN). Their new technique combines FLIM with stimulated emission depletion (STED), a method used to resolve diffraction-related limitations. They performed simulations to evaluate the benefits of FRET-FLIN over FRET-FLIM in spatially receiving protein interactions at the nanoscale level. For these experiments, they used a custom-built STED microscope and fixed hippocampal neurons, focusing on their dendritic spines—the 0.5 to 2-μm-long protein-packed neuronal projections that are crucial for signal transmission.

Their initial experiments demonstrated the superior resolution power of FLIN. While FLIM generated blurry protein heat maps during simulations, FLIN allowed the visualization of discrete protein nanoclusters. With the help of multiple proteins tagged with immunofluorescent probes, the researchers found that FLIN was useful for visualizing FRET within nanoclusters of immunolabelled proteins. They also demonstrated that the immuno-FRET-FLIN approach can discriminate between proteins present within short distances of each other. Finally, they confirmed that the FRET-FLIN method allows the evaluation of different degrees of interactions between proteins at the subspine level.

The researchers' findings effectively showcase the utility of FLIN in biological research. While traditional FRET can offer information on whether proteins interact, it cannot reveal the specific nanodomains where these interactions occur. FLIN is a valuable tool that overcomes these shortcomings and provides a comprehensive understanding of protein signaling, especially in cellular structures as small as dendritic spines.

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