Chapter 7

Visualizing and Measuring Neural Dynamics

After reading this chapter, you should be able to:

- Describe techniques for visualizing activity and cell proliferation in fixed tissue
- Describe classical techniques for measuring neurochemistry in vivo
- Describe biosensors useful for visualizing neural activity
- Describe biosensors useful for visualizing neurotransmission
- Describe strategies for visualizing and measuring activity from fluorescent biosensors
- Describe methods of image processing in visualization experiments

Techniques covered:

- **Static visualization of neural activity and function**: immediate early genes (IEGs), measuring cell proliferation with thymidine analogs (BrdU)
- **Measuring neurochemistry in vivo:** microdialysis, voltammetry, amperometry
- **Biosensors to visualize neural activity**: calcium sensors (calcium-sensitive dyes, genetically encoded calcium indicators), voltage sensors (voltage-sensitive dyes, genetically encoded voltage indicators)
- **Biosensors to visualize synaptic vesicle release:** FM dyes, genetically encoded pH sensors
- **Biosensors to visualize neurotransmitter/neuromodulator release**: genetically encoded neurotransmitter sensors, genetically encoded neuromodulator sensors
- **Strategies for visualizing and measuring activity from fluorescent biosensors**: fiber photometry, microendoscopy, cranial windows

Photographs in research articles, textbooks, and other publications can create the impression that an individual cell is static and unchanging—a collection of organelles in a cytoplasmic soup that sits silently within a tissue. These individual snapshots are extremely misleading. The living cell is a dynamic wonder—an exquisitely complicated machine full of activity that a scientist cannot see with the naked eye, especially not in a single photograph. In every

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cell, DNA is actively transcribed into mRNA, mRNA is shuttled out of the nucleus and translated into proteins, and proteins dynamically carry out the functions of the cell. These proteins change their activity depending on the other proteins they encounter and the cellular signals transduced from the plasma membrane. In the nervous system, neurotransmitters are released onto postsynaptic cells, altering intracellular signaling and electrical activity within these cells. Neurons fire electrical impulses, sometimes in a steady tonic rhythm and sometimes in phasic bursts. Cells are anything but static—we just cannot see their dynamic activity without using sophisticated techniques.

Methods to visualize the structure and localization of proteins and cells, as described in the last chapter, offer a good introduction to studying the activity that takes place in a neuron. However, these methods do not fully reveal the dynamic activity found within the cell. Methods of directly measuring the electrophysiological properties of cells were described in Chapter 4. The purpose of this chapter is to survey nonelectrophysiological methods used to visualize neural activity and neurotransmission. We begin with a description of how some functional processes can be measured in nonliving, fixed tissue. Next, we describe some classical methods of measuring real-time neurochemistry in neural tissue. Most of the chapter surveys methods of visualizing neural activity and neurotransmission using optical methods. We survey a variety of fluorescent biosensors useful for reporting neural activity, and describe various strategies for visualizing fluorescence in imaging experiments, especially in vivo.

STATIC MARKERS OF ACTIVITY

Although it is ideal to measure neural activity as it occurs, it is not always possible or practical. Some cellular functions can be studied by examining markers of activity in fixed, histological brain sections. There are two fundamental approaches to examining neural activity after it has already occurred: (1) measuring activity indirectly by measuring byproducts that accumulate during specific processes in active neurons; and (2) incorporating a marker into cells that can indicate the presence of activity during subsequent histological examination. In both methods, a scientist examines a snapshot of activity, a fixed representation of one moment in a dynamic process.

Assaying Neural Activity in Fixed Tissue

Neural activity leads to the transient and rapid (within minutes) transcription of a group of genes known as **immediate early genes (IEGs)**. These genes encode a diverse range of proteins, including transcription factors (**Fos**), cytoskeletal-interacting proteins (Arc), and phosphorylated ribosomal subunits (pS6). By using histological methods to stain for these markers, a scientist can indirectly determine which regions were active just prior to the time the brain

was fixed (Fig. 7.1). A scientist can use in situ hybridization to show expression of the IEGs or immunohistochemistry (IHC) to show expression of their protein products (Chapter 6). In the literature, staining for the IEG Fos is a very common proxy for measuring neural activity.

To perform an IEG experiment, it is necessary to compare IEG expression between a group of experimental animals and a control group—for example, a comparison between animals that received a stimulus compared to control animals that did not receive the stimulus. IEGs can also be used to screen the brain for populations of neurons that show increased activity during specific behaviors or physiological states. For example, some brain regions thought to promote sleep states in the brain were discovered by examining IEGs in animals that were asleep prior to fixation. In these types of studies, IEGs identify candidate populations that may participate in the regulation of a behavior or phenotype. Alternatively, using genetically modified organisms, it is possible to use the promoters for IEGs to drive expression of reporter genes to mark transiently active neurons. For example, the TRAP system uses the promoter for Fos to drive expression of fluorescent proteins to mark active neurons (Chapter 12).

Assaying Cellular Function in Fixed Tissue

To measure some functional processes in fixed tissue, a scientist can introduce a marker while the process occurs in live tissue, and then detect this marker in subsequent histological experiments. Two biological functions commonly assayed using these methods include cell proliferation and protein trafficking.

Assaying Cell Proliferation with Thymidine Analogs

As a cell divides, it progresses through different stages of the cell cycle. Cells in the DNA synthesis phase of mitosis can be marked by exposing them to

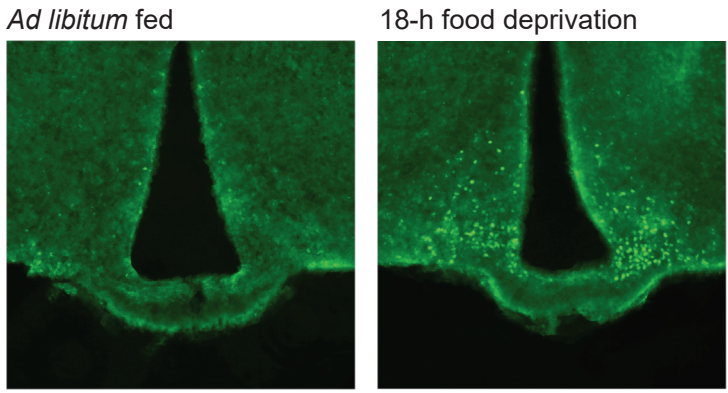

FIGURE 7.1 Fos staining as an indirect measure of neural activity. Representative brain images taken from the hypothalamic arcuate nucleus from (A) a mouse fed ad libitum (constant access to food), and (B) a mouse food deprived for 18 h. The brain sections were stained using immunohistochemistry for the immediate early gene Fos. Because Fos is located in the nucleus, Fos expressing cells resemble small ovals. The food-deprived mouse shows substantially more Fos-expressing cells, indicating that these cells were active in a food deprived state.

tagged DNA nucleoside analogs. For example, **bromodeoxyuridine (BrdU)**, a synthetic analog of the DNA nucleoside thymidine, or radioactive tritiated thymidine (3H-thymidine), can be injected into an animal or introduced into cells or tissue in culture media. Cells actively synthesizing DNA will incorporate BrdU or 3H-thymidine into their newly made DNA in place of endogenous thymidine molecules. In subsequent histology experiments, BrdU can be detected using IHC, and 3H-thymidine can be detected using autoradiography. The presence of BrdU or 3H-thymidine indicates that a cell was dividing around the time of injection (Fig. 7.2).

These markers cannot indicate whether labeled cells continued to proliferate or stopped dividing to become a functional, differentiated cell. Therefore, a scientist can perform additional IHC experiments for proteins that are present only during specific stages of the cell cycle or in certain types of cells. For example, Ki67 and PCNA are proteins that are present during the active, proliferating stages of the cell cycle but not during the resting stage. Therefore, investigators can compare the number of proliferating cells that differentiated into postmitotic cells to the number of proliferating cells that continued to divide by combining BrdU/3H-thymidine labeling with IHC for Ki67.

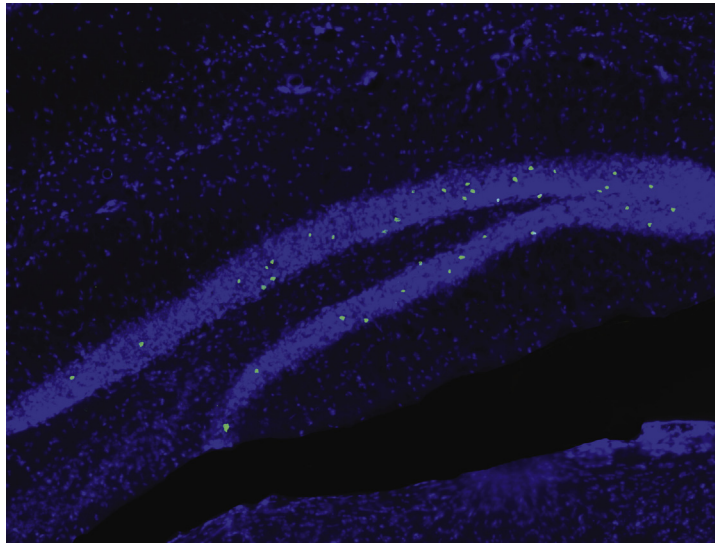

FIGURE 7.2 **BrdU staining.** In this experiment, a mouse was administered BrdU for 3 weeks before perfusion. A brain section containing the hippocampus was stained for the presence of BrdU using immunohistochemistry with a green fluorophore. All cells were visualized in blue using the DAPI nuclear stain (Chapter 6). Green fluorescence marks cells that were actively dividing during the administration of BrdU.

CLASSICAL METHODS OF MEASURING EXTRACELLULAR NEUROCHEMISTRY IN VIVO

Two classical techniques, still widely used today, measure neurochemicals (neurotransmitters, hormones, metabolites) in the extracellular environment over time: microdialysis and voltammetry/amperometry.

Microdialysis

Microdialysis is a technique used to sample chemical substances from the extracellular fluid in a specific location in the brain. Based on the principles of diffusion, a microdialysis probe creates a concentration gradient between the extracellular fluid in the probed brain region and a continuously perfused physiological solution (Fig. 7.3). The probe is made of a semipermeable membrane connected to two thin cannulae, with perfusion solution flowing into and out of the probed brain region. Because of the concentration gradient, substances present in the extracellular fluid will passively diffuse through the probe's membrane into the collected solution, the dialysate. This dialysate is collected continuously into a vial for an increment of 5–10 min for subsequent analysis using sensitive chemical methods, such as high-performance liquid chromatography, radioimmunoassay, or mass spectrometry.

Numerous factors affect the molecules that can be collected into the dialysis solution: the characteristics of the neurochemicals (size, charge, solubility), properties of the dialysis membrane itself (material, pore diameter), flow speed of perfusion, composition of perfusion solution, and the density of the brain tissue surrounding the microdialysis probe. These factors affect both the identity and the amount of substance that can be collected. Thus, the total amount of substance collected in the dialysate over a set amount of time,

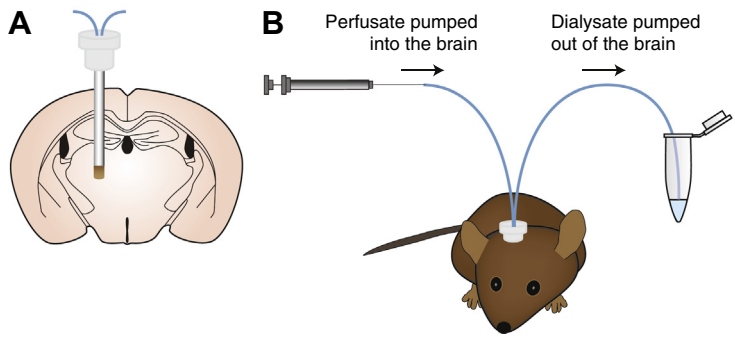

FIGURE 7.3 **In vivo microdialysis.** (A) A microdialysis probe is placed in a specific brain region of interest. The tip of the probe is composed of a semipermeable membrane allowing diffusion of substances in the extracellular fluid. (B) During a microdialysis experiment, a perfusate (usually artificial cerebrospinal fluid) is slowly pumped into the microdialysis implant. The dialysate solution that emerges out of the brain is collected over discrete time periods (usually 5–10 min increments).

known as **absolute recovery**, is not the true extracellular concentration. Instead, scientists present the concentration as **relative recovery**, the relative concentration of the substance in the dialysate from the probed brain region compared to the concentration in the perfusion solution.

It is possible to determine the actual concentration of a substance using at least two different methods: (1) measuring the relative recovery for different flow rates and extrapolating the information to a zero-flow rate, where recovery should theoretically be 100%; and (2) perfusing with known concentrations of the substance to determine the equilibrium point at which the collected concentration does not change, due to the lack of a concentration gradient. Some investigators calculate extracellular concentrations by calibrating probes in vitro, immersing the probes in beakers with known concentrations of the substance. However, because of the differences in the diffusion properties in a simple solution compared to the complex environment of the brain, it is not possible to equate in vitro with in vivo recovery to determine the true extracellular concentration of substances.

There are multiple advantages to using microdialysis to measure neurochemicals in the brain. Microdialysis collection of extracellular fluid can be followed by sensitive chemical assays capable of identifying most types of small molecules. Therefore, unlike voltammetry or most of the optical imaging approaches described later in the chapter, microdialysis can be used to measure virtually any molecule within the extracelluar fluid. Furthermore, microdialysis can be combined with other methods including extracellular electrophysiological recordings, optical imaging of neural activity or neurotransmission, and neuromodulation techniques. Finally, microdialysis can be used to deliver compounds through the perfusion fluid in a procedure called **reverse microdialysis**. This method of drug delivery allows for greater control over the flow of the pharmacological agent compared with pressure injection through a cannula.

The greatest disadvantage of microdialysis is low temporal resolution. The concentration of neurochemicals that can be collected in the dialysate is typically very low, requiring a high volume of fluid to be collected for analysis. This low yield can require long sampling periods (5−10 min), which is an adequate timescale for measuring long-term changes in neurochemical concentrations but inadequate for detection of neurotransmitter release at a physiological time scale.

Voltammetry and Amperometry

Voltammetry is a technique used to detect neurochemicals capable of undergoing oxidation reactions. These neurochemicals include neurotransmitters such as serotonin and the catecholamines (e.g., epinephrine, norepinephrine, and dopamine). A scientist inserts a carbon fiber microelectrode into the brain and applies a specific voltage. When the chemicals encounter the surface of the electrode, they undergo an oxidation reaction, releasing electrons that

produce a measurable change in current. The magnitude of the current is proportional to the number of molecules oxidized. Therefore, it is possible to detect the presence and relative concentration of these neurochemicals at a physiological timescale.

A commonly used form of voltammetry is **fast-scan cyclic voltammetry (FCV)**, which has a high temporal resolution (fractions of a second). In FCV, the voltage of the electrode is shifted back and forth from a nonoxidizing potential to an oxidizing potential within milliseconds. The scientist can then plot the amount of measured current versus the applied voltage, producing a **cyclic voltammogram**. Because electroactive neurochemicals are oxidized and reduced at different potentials, a cyclic voltammogram can be used as a fingerprint to identify the specific neurotransmitter detected at the electrode. Another subtype of voltammetry is **amperometry**. Unlike in FCV, amperometry holds the electrode at a specific, constant voltage. All molecules that can be oxidized at that potential will be detected. Thus, one limitation of amperometry compared with FCV is that it is difficult to identify the specific compound detected by the electrode solely based on the shape of the current. However, in amperometry, measured currents can be averaged over longer time periods, allowing more precise measurements of the relative concentrations of neurochemicals.

Taken together, voltammetry/amperometry techniques have a high temporal resolution and can readily identify electroactive neurochemicals including a variety of neuromodulators. However, these techniques are relatively difficult to learn to perform and analyze. The ease and efficiency of newer methods of visualizing neurotransmission using fluorescent biosensors and dyes, described below, are gradually phasing out the use voltammetry/amperometry as a widely used research tool.

BIOSENSORS FOR VISUALIZING NEURAL ACTIVITY

Chapter 1 discussed methods of noninvasively visualizing activity in an entire brain, such as fMRI or PET, with a spatial resolution of millimeters (about 100,000 neurons) and a temporal resolution of several seconds. Chapter 4 discussed electrophysiological methods of recording neural activity with a spatial resolution of a single neuron (depending on the type of electrode and location in neural tissue) and a temporal resolution of milliseconds. Optical methods of visualizing neural activity, described here, combine advantages of both noninvasive brain imaging and electrophysiology techniques: the ability to measure activity in single neurons or thousands of neurons at once with the temporal resolution of milliseconds to seconds, depending on the specific technique. These methods can be used to visualize activity in dissociated cells in culture, tissue slices in bath media, and even intact brains in living organisms.

Visualizing neural activity depends on specialized fluorescent molecules that report changes in calcium concentration or membrane potential. For example, scientists can visualize neural activity using modified fluorescent

proteins engineered to increase fluorescence depending on the intracellular calcium concentration or membrane voltage. Alternatively, there are a number of organic dyes that also fluoresce depending on calcium levels or membrane potential. Dyes tend to exhibit better temporal properties and signal-to-noise characteristics than proteins. However, genetically encoded proteins can be targeted to specific cell types in the brain, allowing a scientist to observe activity in molecularly defined circuits (Chapter 12). Unlike electrophysiology techniques, scientists can measure neural activity from specific populations of neurons, even within a heterogeneous population of cell types. Table 7.1

TABLE 7.1 Commonly Used Neural Activity Sensors.

Category	Examples	Advantages	Limitations
Genetically encoded calcium indicators	GCaMP	Can be genetically targeted to specific populations of neurons	Relatively long signal decay time (milliseconds). Photobleaching can decrease the signal strength
Ratiometric calcium indicator dyes	Fura-2, Indo-1	High signal-to-noise ratio. Ratiometric properties allow for precise calibration	More complicated data acquisition and measurement than nonratiometric dyes. Buffering of intracellular Ca^{2+} can alter signaling pathways if present at high concentrations
Nonratiometric calcium indicator dyes	Fluo-3, Fluo-4, calcium Green-2	Direct fluorescence intensity changes correlated with Ca^{2+}	Cannot be calibrated, therefore prone to artifacts due to photobleaching, concentration, and other experiment-specific conditions. Buffering of intracellular Ca^{2+} can alter signaling pathways if present at high concentrations
Genetically encoded voltage indicators	Archon	Can be genetically targeted to specific populations of neurons. High temporal resolution (microseconds)	Lower signal-to-noise ratio than calcium indicators
Voltage sensitive dyes	Di-8-ANEPPS, RH 414	High temporal resolution (microseconds)	Lower signal-to-noise ratio than calcium indicators

compares various biosensors used to visualize neural activity. Methods of measuring and imaging emitted light from biosensors and dyes are described later in the chapter.

Biosensors for Imaging Calcium Dynamics

Intracellular calcium is central to many physiological processes, including ion channel gating, second messenger pathways, and neurotransmitter release. In neurons, increases in membrane potential cause increases in intracellular Ca^{2+}; thus, changes in calcium concentration can indirectly indicate changes in neural activity. In a calcium imaging experiment, scientists either express a **genetically encoded calcium indicator (GECI)** in populations of neurons or administer a calcium indicator dye. Then, using a light meter or fluorescent microscope, it is possible to correlate the amount of light emitted by molecules with the relative activity levels of the neurons.

Data for calcium imaging experiments typically show changes in fluorescence intensity over time, normalized to initial levels of fluorescence (F_0). The degree of change of fluorescence over time ($\Delta F/F_0$) is dependent on the change of activity within the neuron, the concentration of the calcium indicator, and the sensitivity of the light detector measuring fluorescence. Typical calcium imaging experiments report changes of $\Delta F/F = 1\%-20\%$ with a temporal resolution of milliseconds.

Genetically Encoded Calcium Indicators

GECIs are fusions of fluorescent proteins (especially GFP) and endogenous calcium-binding proteins. For example, **GCaMP** is an engineered GECI that reports changes in calcium based on a fusion between green fluorescent protein (GFP) and the calcium binding protein calmodulin (Fig. 7.4). When calmodulin binds to Ca^{2+}, a conformational change causes an increase in GFP fluorescence. These proteins can also reveal activity in neural processes including axons and synaptic terminals. Several variants of GCaMP have been engineered that have different molecular properties. GCaMP8s, for example, has a high signal-to-noise ratio, but is slow to decay and therefore cannot accurately report activity on a millisecond timescale. GCaMP8f has a faster decay time, but a smaller signal-to-noise ratio.

Genetically encoded calcium indicators have proven extremely useful for studying the activity patterns of specific populations of neurons in vivo. By expressing these indicators in discrete cell types, especially in heterogeneous populations of neurons, it is possible to correlate neural activity with specific behaviors or phenotypes. Different strategies of recording neural activity in vivo are discussed at the end of this chapter.

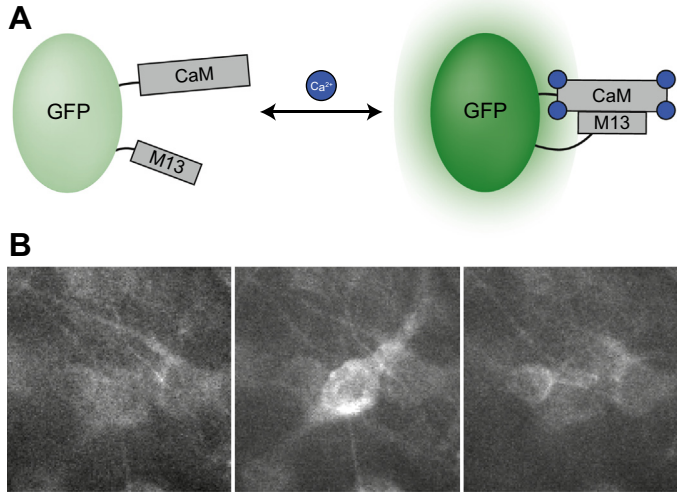

FIGURE 7.4 **Calcium imaging using GCaMP.** (A) GCaMP is composed of green fluorescent protein (GFP) fused to a calmodulin (CaM) protein and a peptide sequence called M13. In the presences of Ca^{2+}, a conformational change causes an increase in GFP fluorescence intensity. (B) Example of GCaMP fluorescence in vivo. These three images show the same specimen containing neurons expressing GCaMP. Neurons exhibit different levels of fluorescence over time that correlate with their neural activity.

Calcium Indicator Dyes

Calcium indicator dyes, organic molecules that change their spectral properties when bound to Ca^{2+}, can be categorized as ratiometric or nonratiometric. **Ratiometric dyes** are excited by or emit at slightly different wavelengths when they are free of Ca^{2+} compared to when they are bound to Ca^{2+}. Thus, they can report changes in Ca^{2+} through changes in the ratio of their fluorescence intensity at distinct wavelengths. A common ratiometric indicator is Fura-2, which emits at 510 nm, but the wavelength at which it is excited can range from 340 to 380 nm in response to calcium binding. Thus, monitoring the ratio of the fluorescence intensity emitted by Fura-2 at 340 nm to the fluorescence emitted at 380 nm can report changes in calcium concentration. Ratiometric dyes allow investigators to correct for background changes in fluorescence unrelated to calcium dynamics, such as artifacts related to photobleaching, variations in illumination intensity, or differences in dye concentration.

Nonratiometric dyes report changes in Ca^{2+} directly with changes in either excitation or emission fluorescence intensity. The common nonratiometric indicators Fluo-3 and Fluo-4 exhibit predictable increases in emission fluorescence intensity with increases in calcium concentration. While

the direct relationship between fluorescence intensity and calcium concentration is sensitive for detecting changes due to calcium binding, the measurement is also prone to detecting changes based on dye concentration and other experiment-specific conditions.

Biosensors for Imaging Membrane Voltage

Techniques that visualize changes in membrane potential are the closest analogs to electrophysiological recordings, as they directly report voltage changes in neuronal membranes with a high temporal resolution. Like calcium imaging, it is possible to image membrane voltage using **genetically encoded voltage indicators (GEVIs)** or using voltage-sensitive dyes.

Genetically Encoded Voltage Indicators

GEVIs are useful for examining molecularly defined subsets of neurons. GEVIs report changes in membrane potential with increases or decreases in fluorescence, allowing for the optical detection of action potentials or even subthreshold changes in membrane potential. Despite much effort, these tools have historically been inadequate for precise measurements of membrane potential. However, new, improved tools are making more widespread use of GEVIs a reality. For example, **Archon** is a relatively new GEVI that features improved signal size and signal-to-noise ratios over previous proteins.

Voltage-Sensitive Dyes

Voltage-sensitive dyes shift their absorption or emission fluorescence based on the membrane potential, allowing a scientist to gauge the global electrical state of a neuron. Unlike extracellular electrophysiology techniques, it is possible to detect subthreshold synaptic potentials in addition to spiking activity. These dyes allow activity measurements in large populations of neurons at once, a task that would otherwise require large electrode arrays. There are a variety of voltage-sensitive dyes available, each differing in signal duration, intensity, signal-to-noise ratio, and toxicity. Most dyes exhibit small signal changes of $\Delta F/F = 0.1{-}1\%$), depending on the concentration of the dye and the degree of change in membrane potential. Large changes in membrane potential (~ 100 mV) can produce changes of $\Delta F/F = 5{-}6\%$. Therefore, compared with calcium imaging, there is less signal-to-noise intensity. However, the temporal resolution is much better, with observable changes in microseconds in contrast to milliseconds.

BIOSENSORS FOR VISUALIZING NEUROTRANSMISSION

A critical component of neural signaling is the exocytosis of neurotransmitters from vesicles into the synaptic junction and the binding of neurotransmitters to

receptors on the postsynaptic membrane. Neurotransmission can be visualized by imaging the docking and recycling of synaptic vesicles or by imaging the binding of neurotransmitters to binding proteins or receptors on the pre- or postsynaptic cell. Table 7.2 compares various methods of visualizing neurotransmission. Methods of visualizing emitted light are discussed at the end of this chapter.

Biosensors for Imaging Presynaptic Vesicle Release

FM Dyes

FM dyes are lipophilic styryl dyes that fluoresce when bound to membranes. Typically performed in cell culture preparations, a scientist adds dye to the medium to label the membrane surface. Neurons are stimulated so that all synaptic vesicles undergo exocytosis and endocytosis; the stained membranes are internalized during endocytosis. The rest of the dye is washed away, leaving behind clusters of stained recycled synaptic vesicles. During an experiment, the investigator images the fluorescence that occurs as the stained

TABLE 7.2 Commonly Used Neurotransmission Sensors.

Category	Examples	Advantages	Limitations
FM dyes	FM1-43, FM4-64	Can be used to visualize synaptic vesicle docking and recycling	Used only in cell culture models. Cannot be genetically targeted to neurons. Cannot identify contents of synaptic vesicles
Genetically encoded synaptic vesicle release biosensors	Synapto-pHluorin	Can be genetically targeted to specific neurons	Cannot identify contents of synaptic vesicles
Genetically encoded neurotransmitter biosensors	iGluSnFR iGABASnFR	Can be genetically targeted to specific neurons. Specific to glutamate or GABA release	Provides measure of relative release over time instead of absolute concentration
Genetically encoded neuromodulator biosensors	dLight GRAB-DA GRAB-ACh GRAB-NE GRAB-5HT	Can be genetically targeted to specific populations of neurons. Specific for various types of neuromodulators	Provides measure of relative release over time instead of absolute concentration

synaptic vesicles release dye during further exocytosis. FM dyes have been used to characterize neurotransmitter release mechanisms, measure the kinetics of vesicle recycling, and observe vesicle movements. The major disadvantages of using FM dyes are that they are most optimally used in cell culture preparations and that they cannot identify or discriminate between specific neurotransmitters/neuromodulators.

pH-Sensitive Fluorescent Proteins

Neurotransmitter release can also be examined using the genetically encoded neurotransmission reporter **synapto-pHluorin**, a pH-sensitive mutant of GFP fused to a synaptic vesicle protein. Synapto-pHluorin GFP molecules decrease fluorescence in relatively acidic environments. Because the interior of synaptic vesicles is acidic (pH $\sim$ 5.7) relative to the cytoplasm of the cell, synapto-pHluorin proteins inside of vesicles are in an off state. When the vesicle fuses with the plasma membrane during exocytosis, the pH rises to extracellular levels, increasing the fluorescence. Therefore, an increase in fluorescence reports an increase in vesicle release of neurotransmiter. Like FM dyes, the major disadvantage of measuring neurotransmission using synapto-pHluorin is that it cannot identify or discriminate between specific neurotransmitters or neuromodulators.

Biosensors for Imaging Neurotransmitter/Neuromodulator Signaling

Relatively new, genetically encoded tools allow for the visualization of specific neurotransmitter and neuromodulator release into the synapse (Fig. 7.5). These biosensors are typically composed of a fusion between a protein that binds the neurotransmitter/neuromodulator and a fluorescent protein that increases signal when the neurotransmitter/neuromodulator is present.

Neurotransmitter Signaling

The canonical neurotransmitters glutamate and GABA can be visualized in the synapse using the genetically encoded biosensors **iGluSnFR** and **iGABASnFR**, respectively. These fusion proteins combine a modified GFP protein with a protein the normally binds to glutamate or GABA (Fig. 7.5A–D). When the neurotransmitter binds to the sensor, there is a transient increase in fluorescence. These biosensors can be expressed on the presynaptic or postsynaptic cell membranes to reliably measure glutamate or GABA dynamics in the synapse.

Neuromodulator Signaling

Neuromodulators (such as dopamine, norepinephrine, acetylcholine, and serotonin) are produced in discrete populations of neurons and released

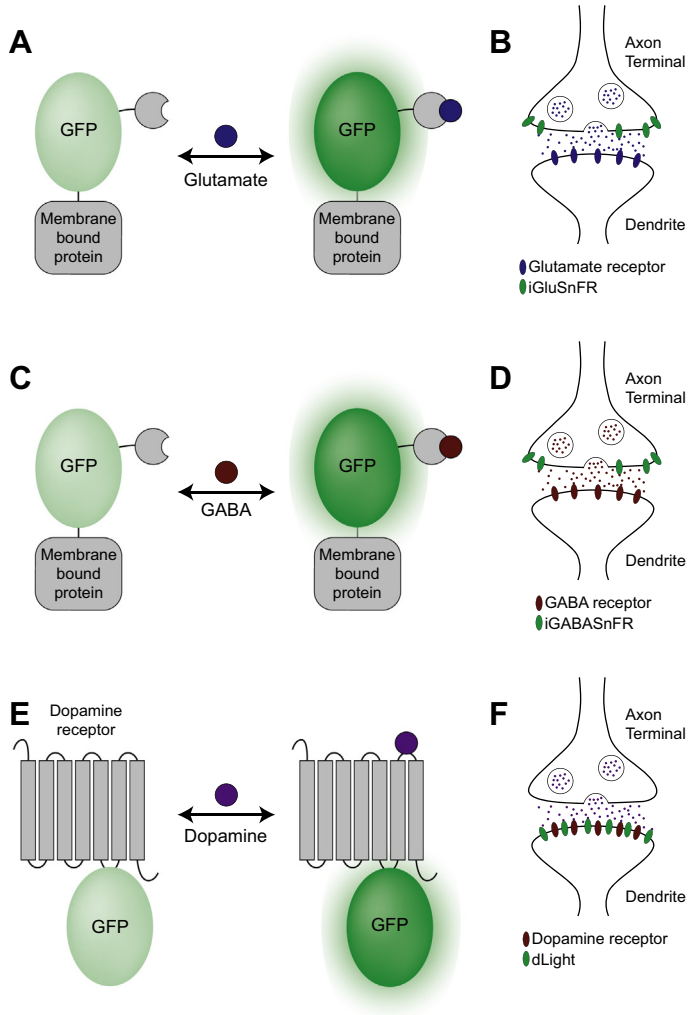

FIGURE 7.5 **Imaging neurotransmission.** (A) iGluSnFR is composed of green fluorescent protein (GFP) fused to a glutamate binding protein and a membrane-bound protein. In the presence of glutamate, a conformational change causes an increase in GFP fluorescence intensity. (B) iGluSnFR can be expressed on the pre- or postsynaptic membranes, but is commonly expressed on the presynaptic membrane to visualize glutamate transmission from a genetically determined population of interest. (C) iGABASnFR is composed of green fluorescent protein (GFP) fused to a GABA binding protein and a membrane bound protein. In the presence of GABA, a conformational change causes an increase in GFP fluorescence intensity. (D) iGABASnFR can be expressed on the pre- or postsynaptic membranes, but is commonly expressed on the presynaptic membrane to visualize GABA transmission from a genetically determined population of interest. (E) Neuromodulator sensors, such as dLight, are composed of green fluorescent protein (GFP) fused to an intracellular domain of neuromodulator receptor, such as the dopamine D1 receptor. In the presence of the neuromodulator in the synapse, a conformational change causes an increase in GFP fluorescence intensity. (F) Neuromodulator receptors can be expressed on the pre- or postsynaptic membranes, but are commonly expressed on the postsynaptic membranes of brain regions of interest.

throughout the brain to affect neuronal excitability and synaptic plasticity in postsynaptic cells. Neuromodulators play a major role in reward processing, sleep and wakefulness, emotional valence, learning and memory, and many other cognitive phenotypes. Therefore, there is great interest in reliably measuring neuromodulator release at specific sites within the brain.

Like the neurotransmitters described above, genetically encoded neuromodulator sensors consist of a modified GFP reporter and a protein that binds to the neuromodulator, usually a receptor (Fig. 7.5E–F). For example, fluorescent dopamine sensors are transmembrane fusion proteins between GFP and dopamine receptors such that the binding of dopamine to the biosensor increases GFP fluorescence. **dLight** consists of a modified GFP fused to the dopamine receptor D1 and **GRAB-DA** consists of a modified GFP protein fused to the dopamine receptor D2. Similar sensors can optically detect the release of a variety of neuromodulators including acetylcholine (**GRAB-ACh**), norepinephrine (**GRAB-NE**), and serotonin (**GRAB-5HT**). Importantly, scientists are also engineering these sensors to utilize different fluorescent proteins such that multiple neuromodulators can be detected simultaneously. For example, GCaMP can be used together with a dopamine sensor designed with a red fluorescent reporter to simultaneously image dopamine release and calcium transients on postsynaptic neurons.

STRATEGIES FOR VISUALIZING AND MEASURING ACTIVITY FROM FLUORESCENT BIOSENSORS

To visualize neural activity or neurotransmission in cultured cells (Chapter 13), a scientist can add a dye directly to the culture media or use a DNA delivery system (e.g., transfection or viral gene delivery) to express any of the genetically encoded biosensors described above. Alternatively, a scientist can express a genetically encoded biosensor in a specific population of neurons in vivo (Chapter 12), collect primary cultured neurons or brain slices, and then image activity in a cell culture or slice preparation. In these conditions, a fluorescent microscope (Chapter 5) can directly image cells in a secure and stable environment.

Visualizing neural activity and neurotransmission in vivo is inherently more difficult than cultured preparations because of the need to access the brain. A scientist must not only express a genetically encoded biosensor in a specific population of neurons, but also employ a strategy to excite the biosensors with light and measure the emitted light as a readout of neural activity. There are a variety of strategies to visualize fluorescence in vivo depending on the needs of the investigator (Fig. 7.6).

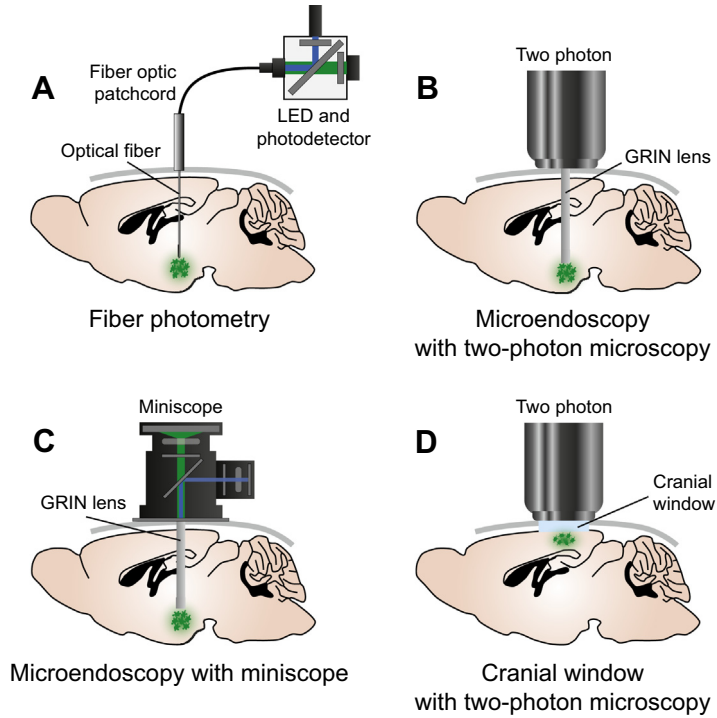

FIGURE 7.6 Methods of visualizing fluorescent biosensors in vivo in rodents. (A) Fiber photometry methods consist of implanting an optical fiber above a brain region of interest and connecting the fiber to a light source and photodetector via a longer (~2 m) fiber optic patchcord. The animal can freely move about its cage while the photometry rig delivers light at an appropriate excitation wavelength and measures the total amount of light at the emission wavelength. Thus, fiber photometry measures total population dynamics. (B) Microendoscopy methods require the use of specialized lenses, often GRIN lenses, to image fluorescent activity deep in the brain. The GRIN lens can interface with the objective of a two-photon microscope in head-fixed mice to produce high-frequency images of neural activity. Alternatively, (C) microendoscopy can be combined with lightweight head-mounted miniscopes, complete fluorescent microscopes capable of imaging activity in freely moving mice. (D) Cranial windows allow for imaging of fluorescence in superficial cortical cells. Typically, cells observed via cranial windows are exited and observed using two-photon microscopy in head-fixed mice; however, smaller two-photon microscopes can also be implanted on an animal's head to image activity in freely moving mice.

Fiber Photometry

Fiber photometry is an optical technique for measuring population-level changes in neural activity or neurotransmission in a freely moving animal (Fig. 7.6A). After transducing neurons of interest with a genetically encoded biosensor, a scientist implants an optical fiber into the brain just above an area of interest. The optical fiber consists of a very thin fiber optic wire connected to a shaft implanted on the skull. During an experiment, a scientist attaches the

exposed shaft of the implant to a fiber optic patchcord. The other end of the patchcord is connected to a fiber photometry control system that is capable of delivering light to excite the fluorescent proteins, as well as quantifying the emitted light with a photodetector.

Specialized optics and filters within the control system allow proper transmission of the correct excitation wavelength to the brain and transmission of the correct emission signal from the brain to the imaging device. For example, in GCaMP imaging, lasers or light emitting diodes in the control system deliver ~480 nm light through the patchcord into the brain, while a photoreceiver measures ~530 nm light. Many fiber photometry experiments also illuminate neurons with an isosbestic wavelength of light, such as 405 nm, that should not cause excitation of GCaMP; this signal controls for any movements or artifacts that may contribute to the fiber photometry signal. The light sources drive 480 and 405 nm wavelengths at alternating frequencies so it is possible to measure GCaMP emissions due to each signal.

Fiber photometry is useful for recording fluorescence intensity from a genetically defined population of neurons and can be used to image neural activity at the soma or axonal projections, or to image neurotransmission at the synapse. Although animals are tethered to patchcords, they can generally move freely about their cages. Many scientific manufacturers sell turnkey fiber photometry systems containing all of the necessary hardware and software to perform an experiment. Furthermore, fiber photometry can be combined with other experimental strategies, such as optogenetics, to measure neural activity with simultaneous neuromodulation of other brain areas. The main limitation to fiber photometry is that it measures the total fluorescent activity of a neural population as opposed to fluorescence in individual neurons. Therefore, fiber photometry cannot detect different activity patterns within a neural population.

Microendoscopy

Microendoscopy is the process of implanting a specialized lens into the brain that can relay light between the adjacent brain structures just beneath the lens and the objective of a fluorescent microscope for high-frequency image acquisition (Fig. 7.6B and C). The most common lenses in microendoscopy are gradient refractive index lenses, popularly referred to as **GRIN lenses**. These lenses are cylindrically shaped with a flat bottom surface. The design of the lens affects the optical path of light by varying the index of refraction within the lens itself. This property greatly reduces aberrations in the light signal and allows for measuring light at high resolution deep within the brain. As opposed to fiber photometry recordings, microendoscope recordings produce detailed images of the brain that can be used to identify fluorescence in individual neurons or neural processes. Additionally, high-frequency image acquisition allows a scientist to produce movies of fluorescence activity within a neural field over time.

A microendoscope lens can be attached to different kinds of fluorescent microscopes. Larger microscopes, especially two-photon microscopes, can be attached in experiments in which animals are headfixed so that they cannot move freely (Fig. 7.6B). Because two-photon microscopes restrict fluorescent excitation to a narrower plane of focus than traditional one-photon microscopes (Chapter 5), they can produce very clear, detailed images of fluorescent activity with high resolution. However, headfixed mice cannot engage in freely moving behaviors. Alternatively, microendoscope lenses can also be attached to miniaturized microscopes, popularly referred to as **miniscopes**, so lightweight that they are capable of being directly mounted on a rodent's head (Fig. 7.6C). During a surgery, a scientist typically implants the GRIN lens into the brain and a baseplate over the skull; during an experiment, the miniscope can be attached to the baseplate for the duration of the experiment. Miniscopes allow for fluorescent imaging of neural activity or neurotransmission in freely moving, behaving animals. Recent developments in miniscope technology have enabled researchers to perform dual-color imaging, wireless imaging, and even two-photon imaging.

Cranial Windows

Cranial windows are translucent interfaces between the brain and a microscope that allow for imaging of superficial structures, such as the cerebral cortex. To produce a cranial window, a scientist performs a craniotomy to remove part of the skull and replaces it with a glass coverslip. Alternatively, a scientist can thin and polish an area of the skull so that it becomes translucent. During an imaging experiment, animals are typically headfixed beneath the objective of a large two-photon microscope (Fig. 7.6D), although newer, lightweight two-photon miniscopes make it possible to image activity in freely moving animals.

IMAGE PROCESSING IN VISUALIZATION EXPERIMENTS

Just as scientists take care in processing static microscopic images of neurons (Chapter 5), there are necessary steps in acquiring and processing high-frequency images of the brain used to show changes in neural activity over time. In particular, in vivo imaging poses significant challenges that require sophisticated analytical techniques that are still being refined and improved. In general, image processing includes the following steps:

- **Registration** is the process of aligning images in the same coordinate system. Imaging brains in freely moving animals will inevitably result in motion artifacts such that the precise location of a neuron may be slightly shifted in one image compared to another. Using a variety of mathematical techniques, registration algorithms correct for these motion artifacts by

lining up all the images in a series based on specific visual features within most or all of the images.

- After registration is complete, the areas in the images where the signal will be measured are determined. These areas, referred to as **regions of interest (ROIs)**, can be selected manually depending on the research question of the investigator. Alternatively, algorithms have been created that automatically detect ROIs using statistical methods to determine which pixels likely comprise cell bodies or other neural features. These algorithms are particularly useful when imaging hundreds of neurons simultaneously. In many imaging experiments, researchers measure changes in fluorescence of ROIs over time, which can indicate changes in neural activity or other cellular processes. In these cases, once ROIs are determined, the analysis software then extracts a fluorescent trace by measuring the fluorescence of each ROI across the series of images.
- One of the goals of calcium imaging is to infer electrophysiological spiking activity from fluorescent calcium transients. This inference is made more difficult due to the fact that recording of fluorescent signals often contains background noise and artifacts unrelated to the actual fluorescent signal itself. To enhance the signal-to-noise ratio of recordings, a scientist can use mathematical **deconvolution** techniques to break down a complex trace into its component parts. The temporal dynamics of calcium indicators have been extensively characterized, allowing deconvolution algorithms to more accurately infer electrophysiological spike times from noisy calcium signals.

Using these computational imaging tools, an investigator generates recordings of deconvolved activity of individual neurons. These traces can be used to determine which neurons respond to certain kinds of stimuli, whether activity in specific neurons correlates with behavior, or how the activity patterns of individual neurons change over time.

CONCLUSION

For several decades, measuring neural activity was synonymous with measuring electrical activity using electrophysiology techniques. In the modern era of neuroscience, investigators have several additional tools to measure neural dynamics in vitro and in vivo. In particular, the development of fluorescent biosensors to visualize neural activity and neurotransmission allows scientists to measure dynamic processes in specific populations of neurons. Combined with innovations in microscopy, especially two-photon microscopy and head-mounted miniscopes, scientists can literally see neural activity as it occurs in real time in awake, behaving animals.

SUGGESTED READING AND REFERENCES

Books

Yuste, R. (Ed.), 2010. Imaging: A Laboratory Manual. Cold Spring Harbor Laboratory Press.

Review Articles

Aharoni, D., Khakh, B.S., Silva, A.J., Golshani, P., 2018. All the light that we can see: a new era in miniaturized microscopy. Nat. Methods 16, 11–13.

Girven, K.S., Sparta, D.R., 2017. Probing deep brain circuitry: new advances in vivo calcium measurement strategies. ACS Chem. Neurosci. 2, 243–251.

Guzowski, J.F., Timlin, J.A., Roysam, B., McNaughton, B.L., Worley, P.F., Barnes, C.A., 2005. Mapping behaviorally relevant neural circuits with immediate-early gene expression. Curr. Opin. Neurobiol. 15, 599–606.

Jennings, J.H., Stuber, G.D., 2014. Tools for resolving functional activity and connectivity within intact neural circuits. Curr. Biol. 6, R41–R50.

Ji, N., Freeman, J., Smith, S.L., 2016. Technologies for imaging neural activity in large volumes. Nat. Neurosci. 19, 1154–1164.

Pnevmatikakis, E.A., 2019. Analysis pipelines for calcium imaging data. Curr. Opin. Neurobiol. 55, 15–21.

Tian, L., Akerboom, J., Schreiter, E.R., Looger, L.L., 2012. Neural activity imaging with genetically encoded calcium indicators. Prog. Brain Res. 196, 79–94.

Yang, W., Yuste, R., 2017. In vivo imaging of neural activity. Nat. Methods 14, 349–359.

Primary Research Articles—Interesting Examples from the Literature

Airan, R.D., Meltzer, L.A., Roy, M., Gong, Y., Chen, H., Deisseroth, K., 2007. High-speed imaging reveals neurophysiological links to behavior in an animal model of depression. Science 317, 819–823.

Betley, J.N., Xu, S., Cao, Z.F.H., Gong, R., Magnus, C.J., Yu, Y., Sternson, S.M., 2015. Neurons for hunger and thirst transmit a negative-valence teaching signal. Nature 521, 180–185.

Cao, G., Platisa, J., Pieribone, V.A., Raccuglia, D., Kunst, M., Nitabach, M.N., 2013. Genetically targeted optical electrophysiology in intact neural circuits. Cell 154, 904–913.

Goldsmith, C.J., Stadele, C., Stein, W., 2014. Optical imaging of neuronal activity and visualization of fine neural structures in non-desheathed nervous systems. PLoS One 9, e103459.

Gunaydin, L.A., Grosenick, L., Finkelstein, J.C., Kauvar, I.V., Fenno, L.E., Adhikari, A., Lammel, S., Mirzabekov, J.J., Airan, R.D., Zalocusky, K.A., Tye, K.M., Anikeeva, P., Malenka, R.C., Deisseroth, K., 2014. Natural neural projection dynamics underlying social behavior. Cell 157, 1535–1551.

Jennings, J.H., Ung, R.L., Resendez, S.L., Stamatakis, A.M., Taylor, J.G., Huang, J., et al., 2015. Visualizing hypothalamic network dynamics for appetitive and consummatory behaviors. Cell 160, 516–527.

Krasnow, A.M., Ford, M.C., Valdivia, L.E., Wilson, S.W., Attwell, D., 2018. Regulation of developing myelin sheath elongation by oligodendrocyte calcium transients in vivo. Nat. Neurosci. 21, 1.

Lutas, A., Kucukdereli, H., Alturkistani, O., Carty, C., Sugden, A.U., Fernando, K., Diaz, V., Flores-Maldonado, V., Andermann, M.L., 2019. State-specific gating of salient cues by midbrain dopaminergic input to basal amygdala. Nat. Neurosci. 22, 1820–1833.

Marvin, J.S., Borghuis, B.G., Tian, L., Cichon, J., Harnett, M.T., Akerboom, J., Gordus, A., Renninger, S.L., Chen, T.W., Bargmann, C.I., Orger, M.B., Schreiter, E.R., Demb, J.B., Gan, W.B., Hires, S.A., Looger, L.L., 2013. An optimized fluorescent probe for visualizing glutamate neurotransmission. Nat. Methods 10, 162–170.

Mu, Y., Bennett, D.V., Rubinov, M., Narayan, S., Yang, C., Tanimoto, M., Mensh, B.D., Looger, L.L., Ahrens, M.B., 2019. Glia accumulate evidence that actions are futile and suppress unsuccessful behavior. Cell 178, 27–43.

Scholl, B., Thomas, C.I., Ryan, M.A., Kamasawa, N., Fitzpatrick, D., 2021. Cortical response selectivity derives from strength in numbers of synapses. Nature 590, 111–114.

Vaidyanathan, T.V., Collard, M., Yokoyama, S., Reitman, M.E., Poskanzer, K.E., 2021. Cortical astrocytes independently regulate sleep depth and duration via separate GPCR pathways. eLife 17, 10.

Wang, J.W., Wong, A.M., Flores, J., Vosshall, L.B., Axel, R., 2003. Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. Cell 112, 271–282.

Protocols

Gaffield, M.A., Betz, W.J., 2007. Imaging synaptic vesicle exocytosis and endocytosis with FM dyes. Nat. Protoc. 1, 2916–2921.

Greenberg, D.S., Wallace, J.J., Kerr, J.N., 2014. Imaging neuronal population activity in awake and anesthetized rodents. Cold Spring Harb. Protoc. 2014.

Resendez, S.L., et al., 2016. Visualization of cortical, subcortical and deep brain neural circuit dynamics during naturalistic mammalian behavior with head-mounted microscopes and chronically implanted lenses. Nat. Protoc. 11, 566–597.

Royle, S.J., Granseth, B., Odermatt, B., Derevier, A., Lagnado, L., 2008. Imaging phluorin-based probes at hippocampal synapses. Methods Mol. Biol. 457, 293–303.