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REVIEW

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Calcium Imaging in Vivo: How to Correctly Select and Apply Fiber Optic Photometric Indicators

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ABSTRACT

Fiber-photometric is a novel optogenetic method for recording neural activity in vivo, which allows the use of calcium indicators to observe and study the relationship between neural activity and behavior in free-ranging animals. Calcium indicators also convert changes in calcium concentration in cells or tissues into recordable fluorescent signals, which can then be observed using the system of fiber-photometric. To date, there is a paucity of relevant literature on the proper selection and application of fiber-photometric indicators. Therefore, this paper will detail how to correctly select and apply fiber-photometer indicators in four sections: the basic principle of optical fiber photometry, the selection of calcium fluorescent probes and viral vector systems, and the measurement of specific expression of fluorescent proteins in specific tissues. Therefore, the correct use of suitable fiber optic recording indicators will greatly assist researchers in exploring the link between neuronal activity and neuropsychiatric disorders.

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Introduction

As an important second messenger, intracellular free calcium Ca^{2+} is involved in the regulation of growth, development of organisms[1–3]and enter neurons during action potential (AP) firing and synaptic inputs. AP firing and synaptic inputs can therefore be assessed, sometimes quantitatively, by measuring changes in intracellular $[Ca^{2+}]$.[4] Calcium imaging can be used to observe and record changes in calcium ion concentration, and the more commonly used calcium imaging techniques include single-photon calcium imaging, two-photon calcium imaging, fiber-optic recording, and deep-brain microcalcium imaging and the like. Two-photon in vivo calcium imaging offers deeper tissue penetration and high resolution but suffers from inevitable photobleaching and requires head fixation, which limits the animal's freedom of movement.[5] Single-photon in vivo calcium imaging is suitable for long-term observation of population neuronal activity with fewer restrictions on animal movement, but it has higher background fluorescence and is prone to errors due to light scattering.[6] Compared with other calcium imaging methods, fiber photometry can measures presynaptic calcium activity in the axon terminal population, is less destructive to neighboring structures than other techniques, does not require immobilization of the animal, and allows the animal to move freely.[7–9] With the implantation of optical fibers in the brain tissue, the fluorescent signals emitted by the deep brain will be relayed out on the surface of the fiber, detected by the optical system, which in turn allows the optical method to break through the traditional imaging depth limitation and enable optical detection of neuronal activity in the deep brain region of active animals. Its widespread application in neuroscience research extends not only to foundational studies in cognition, behavior, and psychology, where it serves as an independent technique to explore the interactions between specific brain regions and neurons, but also in the field of neurological disorders. Here, it can be combined with

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functional magnetic resonance imaging to mechanistically reflect blood oxygen level-dependent signals of individual neuron populations, or integrated with neurotransmitter probes to monitor changes in neurotransmitter levels.[10,11]

Even though GRIN lens-coupled miniscopes enable cellular-resolution imaging in freely moving animals,[12,13] fiber photometry provides a minimally invasive, high-sensitivity approach for monitoring population-level neural activity across multiple brain regions with cost-effectiveness and behavioral compatibility.[14,15] Thus, photometry serves as a complementary approach to cellular-resolution imaging, prioritizing circuit-wide activity readouts over single-cell specificity. Currently, calcium fiber photometry systems are primarily categorized into single-channel and multichannel systems. The single-channel fiber photometry system can only detect fluorescence signal changes from a single channel, which is highly limited in scope. In contrast, the multichannel fiber photometry system can simultaneously monitor multiple channels, enabling the detection of neuronal activity across different brain regions at the same time or cellular activity changes in multiple mice subjected to the same stimulus simultaneously.[16,17] Compared to single-channel systems, multichannel fiber photometry systems offer a broader range of applications and can support more diverse experimental designs.

Moreover, fiber photometry is increasingly seen as the technique of choice to measure neurotransmitter dynamics in vivo in rodents.[18] And with the development of genetically encoded calcium indicators and the widespread use of fiber-optic technology, this has provided important technical support for in-depth interpretation of the pathogenesis of neurological diseases,[19,20] while optical methods have been further developed.[16,21] Moreover, Several studies have found that transgenic mice with genetically encoded fluorescent indicators combined with the Cre-LoxP recombinase system are more likely to label specific types of neurons so that neuronal activity is translated into changes in fluorescence intensity.[22,23] In fact, Monitoring neurotransmission in the brain of freely moving animals is important to understand how real-time neuronal activity and specific behavioral events are correlated. For example, voltammetry-based methods combined with wireless telemetry (as cited in[24]) have successfully tracked dopamine dynamics during reward-related behaviors in rodents.Identifying this relationship can help elucidate the underlying circuitry of various brain regions, and how that circuitry is manipulated by various toxins, diseases.[9]

The above confirms the importance of fiber-photometry in the study of the pathogenesis of neurological diseases. The exploration and screening of appropriate fiber-photometry indicators has become an even more important part of the study of the biomolecular mechanisms of neurological diseases. Therefore, this paper will review the selection and application of fiber-photometry indicators in terms of the selection of calcium fluorescent probes and viral vector systems and the expression measures of fluorescent proteins in specific tissues. These will help to further explore the relationship between neuronal activity and animal behavior and associated disease phenotypes.

The basic principle of optical fiber photometry

Fiber optic photometry is an optical technique in which light is used to trigger and measure fluorescence fluctuations caused by conformational changes in an expressed biosensor (Figure 1). Excitation light of a specific wavelength is transmitted through an implanted optical fiber and the emitted fluorescence is returned to the photodetector through the same fiber. A digital light intensity signal is then generated, which is assumed to reflect the relative number of target-bound sensors at the fiber tip. Since the detected signal comes from the tissue surrounding the fiber tip, which may range from 50 to 400 μm, this reflects a regional or "global" reading. However, because biosensors are genetically encoded, their expression can be targeted to defined circuits and/or cell types where they can be stable for weeks to months. Other in vivo technologies do not have repeated recordings over such long periods of time. This technology has already enabled unprecedented insights into how group activity in specific cell populations is associated with complex behaviors, including movement, memory, motivation, appetite and aversion learning, among others.[25–28]

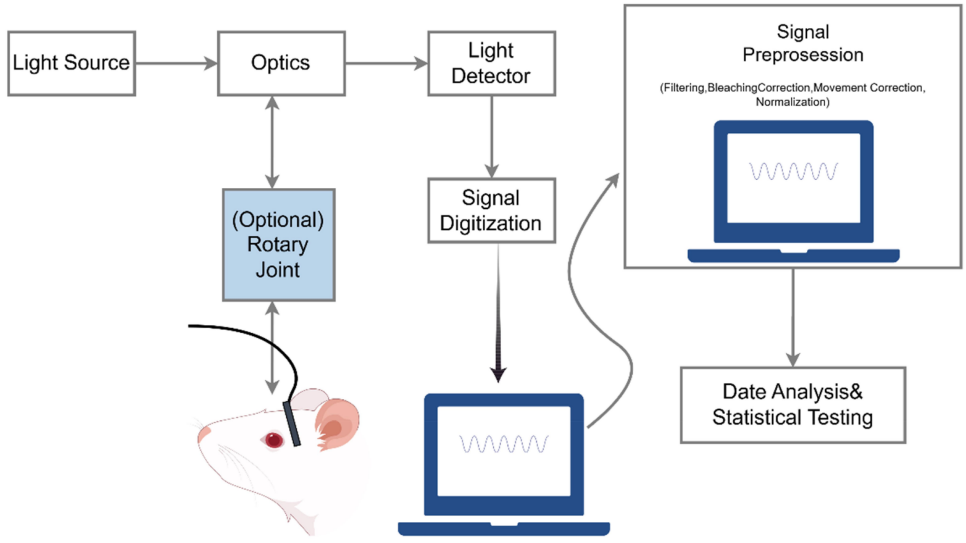

Figure 1. Schematic of the setup of a generic rodent in vivo fiber photometry experiment.

Another advantage of fiber-optic photometry over other in vivo techniques is the relatively low size and complexity of the raw data compared to electrophysiological, two-photon or micro microscopic imaging. As summarized by Eleanor H Simpson et al, the processing of photometric data requires in-depth knowledge of the technique and careful consideration of possible confounding factors, but does not require computationally demanding spike sorting or single-cell extraction.[18] This low dimensionality means that there are no barriers or limitations to data sharing between populations and, if widely adopted, will facilitate replication and reproducibility. Arguably, the widespread popularity of fiber optic photometry lies in its potential for new applications. We therefore provide here a guide to the choices end-users need to make when using fiber-optic photometry to collect, analyze and interpret information to the best of their ability.

Fluorescent class of GECIs

Calcium ion fluorescent probes constitute a class of photon-excitable molecules that specifically bind to Ca^{2+} ions. These probes generate quantifiable fluorescence intensity variations corresponding to intracellular calcium dynamics, thereby enabling real-time monitoring of calcium signaling events in living cells. This functional characteristic renders them indispensable tools for guiding the selection of calcium indicators in fiber photometry studies. Calcium indicators are categorized into two main groups according to their structure, including GECIs and Chemical Indicators (CIs).[29,30] GECIs are genetically encoded proteins that can be stably expressed in cells over extended periods. Compared to chemical calcium indicators, they exhibit superior resistance to photobleaching and more stable cellular localization due to their genetic encoding, which avoids issues such as dye leakage or uneven loading. Both GECIs and chemical indicators (e.g., OGB-1) can detect calcium transients from single action potentials, but GECIs are particularly advantageous for longitudinal studies requiring repeated stimulation over days to weeks.[31,32] In addition to the aforementioned methods, various other sensors are utilized in fiber photometry-based in vivo imaging technologies. For instance, GRAB sensors (G-protein-coupled receptor activation-based sensors) directly convert dynamic changes in target neurotransmitters into fluorescent signals,[33] and in recent years, this approach has gained increasing recognition as a preferred method for measuring in vivo neurotransmitter dynamics in rodents.[34] GECIs can be classified into two categories based on their signal generation mechanism: bioluminescent indicators that rely on enzyme-substrate reactions and fluorescent indicators that emit light upon calcium-dependent conformational changes. This section focuses on fluorescent GECIs due to their

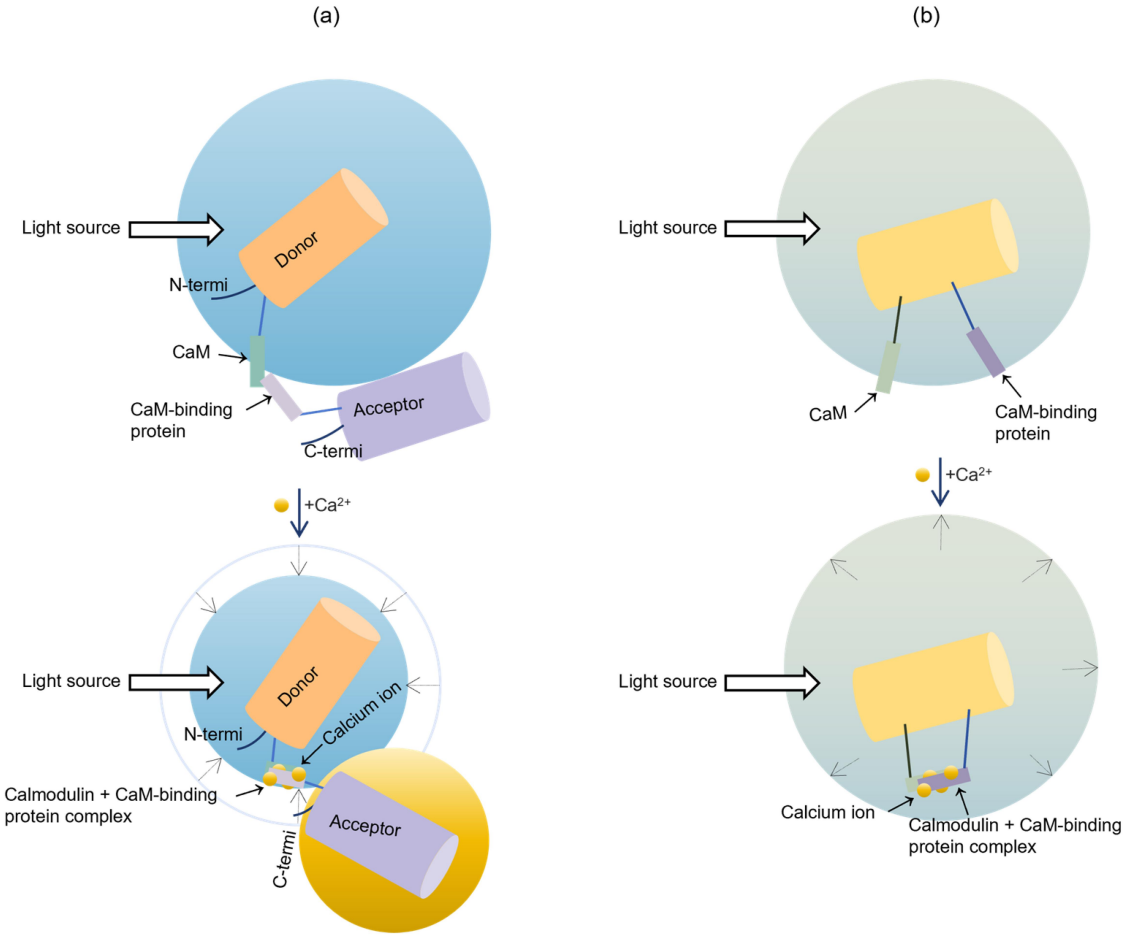

Figure 2. Genetically encoded calcium indicators. (a) Fluorescence resonance energy transfer (fret)-based GECI. Calcium ion binding brings the donor into close proximity with the acceptor, thereby inducing FRET. (b) Monofluorophore GECI. Calcium ion binding to the indicator results in a conformational change in the indicator, leading to an increase in emitted light at fluorescent wavelengths. CaM, calmodulin; termi, terminal.

widespread use in real-time neuronal activity imaging.[35–37] The categories of GECIs are shown in Figure 2.

Förster resonance energy transfer (FRET)-based GECI

FRET refers to the transfer of non-radiative energy from an excited donor to a ground state receptor through interdipole coupling within a specific spectral overlap.[38] FRET GECIs are composed of a fusion between a fluorescent protein and a calcium-binding protein, which includes a pair of donor and acceptor fluorescent proteins connected by a peptide segment that responds to calcium levels, generating FRET signals (Figure 2a). When the concentration of calcium changes, the conformation of the peptide alters, affecting the distance between the donor fluorescent protein and the acceptor fluorescent protein, which in turn influences the FRET efficiency between them.Thus, changes in the FRET signal between the fluorescent donor and acceptor can be used to indicate changes in calcium concentration and calcium signaling.[38–40]

FRET-based GECIs are composed of a myosin light chain-streptokinase-derived Calmodulin (CaM) fused to its target sequence Cameleon, a muscle calcium sensor Troponin C-based TN-XL, and an endoplasmic reticulum calcium indicator protein apoK1 lacking calcium response elements. Cameleon employs cyan fluorescent protein as the donor and yellow fluorescent protein as the acceptor.[41] In addition to the dynamic range, affinity and localization are superior to the

indicators corresponding to chemical calcium indicators in cytoplasmic imaging,[42] organelle imaging[43] both have good application prospects; TN-XL utilizes the calcium sensor Troponin C (TnC) in the muscle as a calcium-binding part, with FRET at both ends to form a donor receptor pair, which plays an important role in calcium imaging of the cone photoreceptors[44–46]; endoplasmic reticulum calcium indicator apoK1 is a modified A Polipoprotein tricyclic domain, which connects CFP and YFP to amino and carboxyl groups, respectively. It is not widely used, but it is of great significance for the detection of calcium ions in the endoplasmic reticulum.[47]

Compared to chemical calcium ion indicators, FRET indicators offer the benefit of optically detecting living cells in a nondestructive and minimally invasive manner. They can also be easily corrected for factors independent of dye loading, photobleaching, and dye leakage heteroplasies, as well as for changes in the focal plane or artifacts related to sample movement.[41,48,49] However, the FRET calcium indicator has certain drawbacks, including wide spectral coverage, a small dynamic range, and inapplicability to transgenic invertebrate and mammalian cells.[50]

Monofluorescent type GECIs

A single fluorescent protein calcium indicator utilizes the change in emission intensity of a single fluorescent protein as an indicator, employing the variation in fluorescence intensity to signal the concentration of surrounding calcium ions (Figure 2b). These calcium indicators include cytoplasmic calcium indicator proteins such as GCaMP, GECO and jRCaMP, as well as organelle localization proteins such as CEPIA1er, GCaMPER and mito-GCaMP.[51] In this paper, the GCaMP series, commonly used for calcium ion imaging, will be used as an example to introduce single fluorescent GECIs.

GCaMP series is a single fluorescent GECI, and the change of the emission intensity of single fluorescent protein is used as an index to indicate the content of calcium ions in the environment.[52] The GCaMP series includes GCaMP1, GCaMP1.6, GCaMP2, GCaMP3, GCAMP-HS, GCaMP5, GCaMP6, jGCaMP7 and other series. Although before the GCaMP5G series (including the GCaMP5G series), the sensitivity and reaction speed of the GCaMP series are not as good as the commonly used indicators of chemical synthesis of calcium ions (such as OGB-1AM).[53,54] After the improvement of GCaMP series folding efficiency, signal amplitude, calcium ion affinity and sensitivity, signal discrimination ability and signal retention time, the advantages of GCaMP series have been gradually reflected. In the GCaMP6 series: GCaMP6s has high sensitivity and is suitable for detecting low-frequency signals[53]; GCaMP6m has moderate binding activity and offers the broadest applicability[55]; GCaMP6f features fast kinetic properties and the quickest dissociation, making it suitable for high-frequency signal detection. However, the latest jGCaMP7 series provides a better reflection of calcium signals triggered by a single action potential than chemical calcium indicators. For instance, jGCaMP7b has a brighter baseline fluorescence and is three times more sensitive than GCaMP6s, making it suitable for detecting neuronal processes or nerve fibers. jGCaMP7c, on the other hand, has low background fluorescence, high contrast, and clear signals, with a sensitivity 2.7 times that of GCaMP6s, making it ideal for large-scale imaging.[56] In a recent study with fiber-photometry, the high frequency GCaMP6 series can clearly detect calcium signals at the volume level triggered by a single action potential of neurons, and it is similar to commonly used chemical synthesis indicators in terms of sensitivity, response speed.[53,57] The latest jGCaMP7 series reflects calcium signals triggered by a single action potential far better than chemical calcium-like calcium ion indicators, which can better track the dynamic changes of calcium signals in nerve cells.[56]

Compared with FRET GECIs, single-fluorescent GECIs represented by GCaMPs have higher signal-to-noise ratio and sensitivity, better light resistance, faster kinetic speed and shorter fluorescence delay time, which provides more choices for calcium imaging experiments including fiber-photometry in the future.[56,58] In the case of the GCaMP family, GECIs can be used to detect changes in signals in the structure of specific types of cells or their subcellular structures,[59] the animal behavior under the control of neural network was studied,[60] mechanism of disease occurrence and development,[61]

mechanism of drug action.[62] However, it still has limitations, such as it is difficult to detect non-excitatory cell signals and difficult to detect subcellular organelles, which need further in-depth research.

Viral vector selection

The choice of fiber-photometry indicator can be made in terms of the viral vector system, measures of specific expression of fluorescent proteins in a given tissue, and the type of fluorescent probe.[29,63] Fluorescent probe types have been mentioned above, and the following focuses on viral vector systems.

Viral vector system belongs to the category of gene introduction system, which is a carrier tool to guide the target gene into the cell. A viral vector is a gene carrier built on top of a virus. Using the molecular mechanism of viruses to transfer their genes to other cells to be infected, the viral genome is modified and encapsulated into viral particles with exogenous genes and related gene elements, so as to carry the genetic material into cells or tissues to form a complete gene introduction system in vivo or under cell culture conditions.[64,65] The four commonly used viral vectors are lentivirus (LV), adenovirus (AD), retrovirus (RV) and adeno-associated virus (AAV), of which AD belongs to the family of adenoviruses and AVV belongs to the family of Parvoviridae (specifically the subfamily Dependoparvovirus), RV and LV belong to the Retroviridae family.[66]

When selecting viral vectors, the following key factors need to be taken into account: whether the selected virus has tissue affinity for the target cell; whether the cell is in the stage of division when the virus is introduced; and whether the exogenous gene can be induced to integrate into the host cytosol after transduction. Whether the transduction of the target gene is transient or continuously stable in the genome of the host cell? whether the exogenous gene can be integrated into the target protein? Does the transduction capacity of the virus meet the requirements for expression of the exogenous gene in vivo? Can the exogenous protein integrate into the target protein? Is it necessary to use a specific promoter to drive the expression of the target gene; what is the transduction mechanism? Will this vector be used for cell culture or for experimental studies in vivo? Can the exogenous target protein be efficiently integrated into the target cells within the cell? It is also necessary to consider whether the host's immune response to the virus will have an impact on the experiment.[67,68] Thus, when selecting AAV (adeno-associated virus) for calcium imaging in vivo, it is essential to consider not only the tissue affinity of AAV but also the in vivo delivery barriers and the choice of specific promoters.[69,70] Additionally, the selection of AAV may be influenced by different delivery methods, such as stereotaxic injection into the brain or intravenous injection. In contrast, when working with cultured cells in vitro, there are fewer limitations in selecting AAV viruses, as they can directly contact the cells. Low titers can be used for transduction, and there is no need to consider in vivo delivery barriers.[71,72]

Different AAV serotypes exhibit distinct tissue tropism: AAV2 primarily targets the nervous system,[71] AAV9 efficiently crosses the blood-brain barrier for central nervous system delivery,[73,74] and AAV8 demonstrates high transduction efficiency in the liver.[75] The selection of an appropriate serotype is crucial for achieving cell-type-specific or tissue-specific gene delivery in experimental applications. When studying the nervous system, different promoters are often selected based on various neuronal cell types, experimental conditions, or research objectives. For example, hSyn is commonly used specifically for neurons, CaMKIIa is used for excitatory neurons, and GFAP is used for astrocytes, allowing for specific AAV transduction in the target cell types to reduce background noise.[76–78]

LV vectors are based on human immunodeficiency type I virus, a vector technology for gene therapy,[79] LV is suitable for cell lines and primary neuronal cells, and during cell introduction it has the potential to infect both cells in the dividing and non-dividing stages; it has no effect on embryonic stem cells and does not induce differentiation. It has a payload of 8 kb and is expressed at moderate levels, but its efficiency in infecting the heart in vivo is also relatively low due to the low titer; it is unsuitable as a gene transduction vector for the receptor.LV has the ability to integrate with the

genome, resulting in stable overexpression or disruption, but it is deficient in that it needs to be inserted immediately, resulting in targeting that becomes difficult; furthermore, it is not able to form a complete gene delivery chain. At the same time, there is a potential for tumor induction when LVs enter the host's genome.[79–81] The AD vector system is actually an adenoviral vector system with replication defects. It has no effect on embryonic stem cells and does not induce differentiation. Compared with somatic cells, Ad prefers primary cells and is more likely to infect nerve endings; it also has good immunogenicity and specificity, and can act by binding to the corresponding proteins on the host cell membrane, and exerts anti-tumor effects by mediating antiviral action. The substance has a large package capacity, in which the payload of canine diadenovirus can be as high as 30 kb, and its expression level is very high, the infection rate can even reach 100%, and it can be expressed in vitro and in vivo; it has the advantages of good immunogenicity and stability, and it can induce the organism to produce specific antibodies against specific genes. However, its shortcoming is that the immune response is too strong, which may lead to a strong immune response in animals or adenoviruses.[82,83] Although both RV and LV are members of the Retroviridae family, RV lacks viral packaging genes, so it can only infect cells in the dividing stage. Since the size of RNA that can be effectively packaged by RV is limited, it cannot transduce DNA fragments larger than 11 kb.[84,85] AAV is a virus that can only be produced infectively by the host with the assistance of a helper virus such as an adenovirus or herpesvirus.AAV is highly specific and has good affinity for different tissues; induces cell cycle arrest and inhibits apoptosis. Like LV, it can infect both cells in the dividing and non-dividing phases; it is not dependent on other antigens and is present in blood and body fluids. The maximum payload of this device reaches 4.7kb, and its expression level is medium-high, with strong specificity and good affinity for different tissues, making it ideal for transfection in vivo; it is very suitable for transfection in vivo due to its small molecular size, ability to spread evenly, and low immunogenicity. However, it has a relatively long expression cycle, which takes about 1–2 weeks.[81,86–88] Although a variety of virus types are frequently used in experiments, the targeting of LVs becomes difficult due to the excessive immunogenicity of Ad and the homogeneity of the cell types infected by RVs, which allows for the application of AAVs with very low immunogenicity and a wider selection of specific serotypes.

Measures of fluorescent protein specific expression in specific tissues

After selecting the appropriate viral vector system, ensuring that the virus is expressed only in the study tissues is central to obtaining experimental data and influencing the accuracy of the experiment, depending on the specific tissue or cellular characteristics of the study population.[85,89] The target protein must therefore be screened to avoid interference from exogenous genes. To ensure specific expression of the virus, three methods are often employed: localized injections, the use of specific promoters, and the use of the Cre-loxp system.[90] The relevant experimental steps are shown in the Figure 3.

Brain stereotactic technique

Brain stereotaxic localization technique is regarded as a key research tool in modern neuroscience, which can be used for a variety of operations such as targeted injection of neural structures, injury,[91,92] and guided potentials[93] as well as for studies related to psycho-behavioral epilepsy[91, 94] and learning memory[93] and other related studies. In the latter fiber-photometry, the previously performed brain stereotaxic localization is the key operation to achieve precise localization of specific brain regions, and the advantage of this technique is that even without direct observation of the experimental animal's brain, the brain stereotaxic localization can be achieved through the brain stereotaxic localizer and the brain stereotaxic localization maps of the experimental animals.[95] However, when this technique is used to administer drugs directly in the brain regions of experimental animals, surgical operations such as craniotomy drilling and brain-area needle insertion must be performed on the animals after anesthesia, and these operations lack specificity and

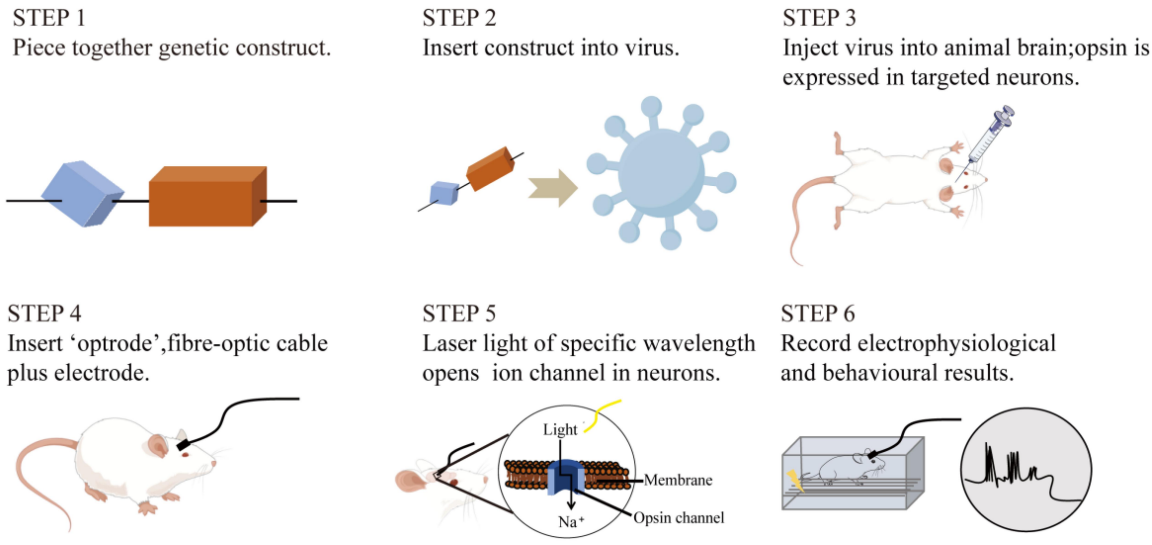

Figure 3. Experimental procedures in Optogenetics.

reversibility,[96] In identifying structures near the midline of the brain, pinpointing those hard-to-reach brain regions remains a challenging task.[97]

Specific promoters

The promoter is regarded as a key cis component in the regulation of gene expression, and it provides an invaluable tool for the study of gene expression and regulation in theory; in practice, it is a key component of expression vectors for genetic engineering and gene therapy.[98,99] There are three types of promoters: constitutive promoters, inducible promoters, and tissue-specific promoters. Because of the differences in expected research outcomes, the choice of specific promoters varies from study to study. For example, one study on projection function used a CaMKII promoter such as unilateral cerebral localized injection of cis-tracer virus (AAV-CaMKII-mCherry) to specifically label the glutamatergic neuronal system, thus confirming that neural projections from lateral rein neurons to the caudal part of the ventral tegmental area are effective.[100]

Under the influence of a specific promoter, external fluorescent genes are expressed mainly or only in the expected specific organ or tissue region, which further limits the range of fluorescence expression and thus improves the accuracy and persuasiveness of fiber-photometry experiments. Specific promoters not only reduce the attenuation effect of genes in vectors at various sites.[101,102] In addition, it shows great potential for application in the field of disease research.[102] Nonetheless, there are promoters whose expression shows cross-over properties with tissues. In addition to the need to consider their affinity to the tissues or cells of the viral vector of choice, transcription elements scattered throughout the repeat sequence region may also have an impact on the function of tissue-specific promoters.

Cre-loxP system

The Cre-loxP system was co-proposed by Sternberg and Hamilton,[103] which is a site-specific recombinase technology that targets cellular DNA-specific sites for deletion, insertion, transposition, and inversion operations. This technique triggers the modification process through specific external excitation,[78,104] and utilizes its controllability to ensure that the target gene is expressed only in one specific cell type.[105] In this recombination system, apart from the use of Cre recombinase, there is no need to add other additional proteins or sequences, and the recombination efficacy is fairly high.[78] The Cre-loxP system, a core technology for exploring the link between structure and function of neuronal circuits, has been widely used in mouse experiments as a sparse random labeling on behalf of individual cells. The expression of cell type-specific genes can be effectively assisted to be

realized by specific Cre mice or by injecting Cre viruses into mice.[105–107]

In fiber-photometry experiments, specific DNA modifications in the Cre-loxP system are particularly critical for cell line tracking. The Cre-loxP system is a powerful tool for calcium ion in vivo imaging mediated by AAV (adeno-associated virus) tracing.[108] The Cre-loxP system utilizes the gene recombination properties of Cre recombinase, combined with AAV vectors, to enable precise and effective expression of genetically encoded calcium indicators (GECIs), such as GCaMP, in specific brain regions. This allows for accurate monitoring of neuronal activity in target neurons when used in conjunction with calcium ion fiber imaging systems.[108] Cre-loxP is not only suitable for studying neuroendocrine circuits,[109] function of non-stem cell astrocytes under physiologic and pathologic conditions,[110] it is also possible to selectively remove different components from the tissue microenvironment by combining them with drugs to analyze the cellular and molecular mechanisms of organ damage.[111] Nevertheless, the Cre-loxP system still shows some shortcomings in a real experimental setting. For example, Cre expression in non-target cell species or tissues, and Cre-mediated recombination may vary in different types of target cells.[112,113] In addition, Cre-on FLEX DIO double floxed and others related to indicator viruses have adopted the recombination technology of Cre-loxP. Taken together, whether it is localization injection, the use of specific promoters or the Cre-loxP system, they all achieve specific expression, but each method has its advantages and disadvantages. Localization injection is a physical means to achieve precise expression of a drug or virus, but this method is deficient in terms of reversibility and stability; specific promoters and Cre-loxp systems have a high degree of specific control at the gene level, and they show greater stability compared to localization injection. However, the problems of tissue infection crossover and recombination variability due to cell type differences in these systems are not something we can ignore.

To address the complexity of achieving Cre-specific expression in tissues, researchers may opt for mouse strains such as Ai95 that already carry GCaMP and loxP sites. These strains enable Cre-dependent activation of GCaMP expression in specific cell types without requiring additional viral injections, significantly simplifying experimental workflows.[78] However, a key limitation of this approach is the lack of temporal control over GCaMP expression compared to stereotaxic viral delivery, which allows precise timing of transgene activation at specific experimental timepoints.

Discussion

Fiber-photometry is an optogenetic tool for probing the link between the activity of animal neurons and their behavior, which enables the recording of specific neuronal activity in the brains of free-ranging animals by binding to calcium ions.[114] The choice of calcium ion indicator plays a crucial role in fiber-photometry and even has a non-negligible impact in optogenetic research methods. Given the variety of available types of fiber-photometry indicators, it has become particularly important to explore fiber-photometry indicators from the perspective of viral vector systems, measures of specific expression of fluorescent proteins in specific tissues, and calcium-ion fluorescent probes. Advances in fiber-photometry have revealed many possible biological principles, which can help us to study the mechanisms of neural activity from an in vivo perspective in greater depth, and to further utilize the changes in neural activity in vivo to explore effective drugs or treatments. However, a significant drawback of the fiber-photometry technique is that it cannot effectively monitor the behavior of a single cell; in contrast, fluorescence detection is obtained from clusters of neuronal cells in the vicinity of the probe tip.[115] The application of fiber photometry is constrained by the ability of researchers to extract background autofluorescence from calcium signals, due to the fact that the structure of cells is indistinguishable.[7,116]

Based on this review, researchers may consider the following points when conducting related studies[1]: the maintenance effect of calcium indicators after multiple excitation light activations. The excitation light emitted by fiber-photometry can enter the brain through optical fibers, which triggers the fluorescence of fluorescently labeled

cells or neurons to emit fluorescence. For labeled neurons that have already been excited, their fluorescence effect may be affected when they are excited again. How to minimize the decrease of experimental effect caused by multiple excitation light irradiation and to complete single injection experiments with longer cycle time in a wider range, so as to improve the experimental value of experimental animals, is a question that deserves in-depth discussion.[2] On the biological dimension, we need to conduct more in-depth explorations. Fiber-photometry uses changes in calcium ion concentration to observe the activities of brain-active cells in specific nuclei neurons of the animal brain, but this method is limited to the nucleus level and only reflects the behavior of calcium ions, making it difficult to conduct more in-depth studies, such as determining the specific role of a particular subunit in neurotransmission. Therefore, exploring how to conduct studies at the molecular level (e.g., optimizing indicators) will be a key breakthrough point in the development of fiber-photometry.[3] Investigating changes in calcium signaling associated with nonexcitable cells Not only is fiber-photometry able to detect and respond to action-induced calcium ion signaling, but calcium ions also play an integral role in representing nonexcitable cells such as cerebrovascular endothelial cells. Questions about how to reveal changes in calcium signaling related to nonexcitable cell function and how these changes affect physiological processes remain to be explored in depth in future studies.

With the continuous progress of calcium imaging technology, combined with the selection strategy of calcium indicator, the correct selection of gene-encoded calcium indicator may not only change the current research status in the field of calcium signaling mechanism, but also promote the development of related optogenetic technology mainly relying on gene-encoded calcium indicator signaling, represented by fiber-photometry, which will bring significant breakthroughs in the study of pathogenesis of neurological diseases. Taken together, studying the selection of calcium indicators in fiber-photometry not only provides a reference for finding suitable research indicators, but also brings new development opportunities for the relationship between fiber-photometry and neurological diseases, which provides an innovative technological means for in-depth discussion of the causes of psychoneurological diseases.

Disclosure statement

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Author contributions

L.W. and W.S.: Conceptualization, methodology, and original draft writing. Visualization and investigation. L.W. and W.S.: Data acquisition and review, editing, and writing of the manuscript. L.H., L.W., and L.S.: Revision of the article. J.D. and G. L.: Project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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