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Genetically encoded sensors illuminate *in vivo* detection for neurotransmission: Development, application, and optimization strategies

Xiaoyu Zhong [a,1], Hengyu Gu [a,1], Juyao Lim [b,1], Peng Zhang [a,c,d,e,f,*], Guangfu Wang [g,**], Kun Zhang [a,c,d,e,f,*], Xiaowan Li [g,**]

[a] Department of Pharmacology and Chemical Biology, Shanghai Jiao Tong University School of Medicine, Shanghai, China
[b] Malaysian Medics International-Hospital Raja Permaisuri Bainun, Ipoh, Malaysia
[c] Shanghai Key Laboratory of Emotions and Affective Disorders (LEAD), Shanghai Jiao Tong University School of Medicine, Shanghai, China
[d] Shanghai Key Laboratory of Tumor Microenvironment and Inflammation, Shanghai Jiao Tong University School of Medicine, Shanghai, China
[e] Shanghai Frontiers Science Center of Cellular Homeostasis and Human Diseases, Shanghai Jiao Tong University School of Medicine, Shanghai, China
[f] Key Laboratory of Cell Differentiation and Apoptosis of Chinese Ministry of Education, Shanghai Jiao Tong University School of Medicine, Shanghai, China
[g] HIT Center for Life Sciences, School of Life Science and Technology, Harbin Institute of Technology, Harbin 150001, China

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ABSTRACT

Limitations in existing tools have hindered neuroscientists from achieving a deeper understanding of complex behaviors and diseases. The recent development and optimization of genetically encoded sensors offer a powerful solution for investigating intricate dynamics such as calcium influx, membrane potential, and the release of neurotransmitters and neuromodulators. In contrast, traditional methods are constrained by insufficient spatial and/or temporal resolution, low sensitivity, and stringent application conditions. Genetically encoded sensors have gained widespread popularity due to their advantageous features, which stem from their genetic encoding and optical imaging capabilities. These include broad applicability, tissue specificity, and non-invasive operation. When combined with advanced microscopic techniques, optogenetics, and machine learning approaches, these sensors have become versatile tools for studying neuronal circuits in intact living systems, providing millisecond-scale temporal resolution and spatial resolution ranging from nanometers to micrometers. In this review, we highlight the advantages of genetically encoded sensors over traditional methods in the study of neurotransmission. We also discuss their recent advancements, diverse applications, and optimization strategies.

1. Introduction

Synapses are specialized structures that form the foundation of neural networks, enabling billions of neurons to process and transmit information. Classical synaptic transmission begins when an action potential (AP) triggers calcium (Ca^{2+}) influx at the presynaptic terminal, leading to the release of neurotransmitters (NTs) or neuromodulators (NMs) within milliseconds. Neurotransmitters such as acetylcholine (ACh), glutamate, and γ-aminobutyric acid (GABA) act rapidly by binding to diverse receptors on the postsynaptic membrane, primarily ligand-gated ion channels and G protein-coupled receptors (GPCR). In contrast, neuromodulators, including monoamines, nucleotides, neurolipids, and neuropeptides, function predominantly through GPCRs to initiate molecular signaling cascades in a slower, more diffuse and longer-range manner. Historically, this process was thought to occur on a broader spatial scale than classical synaptic transmission. Neuromodulators are typically released from extra-synaptic structures called varicosities, diffusing locally across distances of several hundred nanometers (~0.75 μm for ACh and monoamines, and ~0.80 μm for neuropeptides) (Zhu et al., 2020). Their activity is regulated by reuptake transporters and deactivating enzymes. The receptors for NMs, primarily metabotropic GPCRs, are distributed on nearby neurons, allowing NMs to modulate pre-synaptic, post-synaptic, and extra-synaptic components of neural circuits simultaneously. Chemical neurotransmission drives a broad range of physiological processes, including arousal, attention, perception, learning, and other complex behaviors. Dysregulated

* Corresponding authors at: Department of Pharmacology and Chemical Biology, Shanghai Jiao Tong University School of Medicine, Shanghai, China.
** Corresponding authors.
E-mail addresses: benzhp@shsmu.edu.cn (P. Zhang), wangguangfu@hit.edu.cn (G. Wang), zhang_kun@sjtu.edu.cn (K. Zhang), lixiaowan@stu.hit.edu.cn (X. Li).
[1] These authors contributed equally to this work.

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neurotransmission has been implicated in numerous brain disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), autism, addiction, schizophrenia, and depression (Francis et al., 1999; Sarter et al., 2007; Yadav and Kumar, 2022). Probing the intricate mechanisms of NTs and NMs transmission is essential for understanding the processes and modulation underlying higher brain functions. However, studying these rapid, subcellular events requires methods with high spatial and temporal resolution, which traditional approaches often fail to provide. Over the last few decades, advances in electrophysiological methods and analytical chemistry methods have expanded our ability to investigate neurotransmission. The patch clamp technique, for instance, monitors changes in membrane potential and ionic currents using electrodes. However, its application is limited by its invasiveness, low throughput, high technical requirements, and lack of high spatial resolution (Hill and Stephens, 2021). Moreover, this technique is unsuitable for cases involving minimal or no changes in potential (Marcott et al., 2014; Mamaligas and Ford, 2016). Analytical methods like microdialysis and electrochemistry have also been extensively employed, providing precise measurements of NTs and NMs in the extracellular fluid with sampling probes. Despite their accuracy, these methods are constrained by insufficient spatial resolution and/or slow temporal response, hindering their utility for capturing dynamic neural events. Emerging methods, such as fluorescent sensors based on nanomaterials and techniques leveraging the properties of GPCRs, offer unique advantages in specific contexts. However, many of these approaches still face challenges in effectively monitoring the dynamic fluctuations and complex spatio-temporal patterns of neural signaling. Recent advances in genetically encoded sensors have marked a significant breakthrough, enabling real-time monitoring of neurotransmission with submillisecond temporal and nanometer-scale spatial resolution. For example, periplasmic binding protein (PBP)-based sensors for ACh, iAChSnFR, demonstrate exceptional performance, with fluorescence changes of approximately 1200 % and rapid kinetics across diverse organisms. These sensors enable faithful *in vivo* detection of ACh and provide insights into clinical observations, such as the effects of acetylcholinesterase inhibitors in treating Alzheimer's disease (Borden et al., 2020). With the integration of machine learning algorithms, high-resolution imaging techniques, and further understanding of iterative protein evolution, genetically encoded sensors serve as a rapid optimizing toolkit for studying neurotransmission.

In this review, we examine the limitations of both current and emerging detection methods, with a particular focus on the development and application of genetically encoded sensors. Additionally, we highlight key optimization strategies, aiming to inspire innovative designs and future advancements in this field.

2. Merits and drawbacks of current methods for *in vivo* detection

2.1. Electrophysiological methods

First invented in the early 1950s and having been continually refined, electrophysiology has been a pivotal tool for studying neurotransmission, offering high temporal resolution and an excellent signal-to-noise ratio (SNR) (Hodgkin and Huxley, 1952; Dunlop et al., 2008; Rubaiy, 2017) (Fig. 1a). The initial breakthrough by Alan Hodgkin and Andrew Huxley involved penetrating the cell membrane with a sharp glass microelectrode to record intracellular membrane potentials. A later refinement replaced these sharp electrodes with large-bore pipettes, eliminating leakage currents and enabling recordings from ion channels in either attached or excised membrane patches (Neher and Sakmann, 1976; Neher et al., 1978). This advancement laid the foundation for the "patch clamp" technique. The patch clamp method is a powerful tool for dissecting synaptic transmission and is considered the gold standard for studying glutamatergic and GABAergic synapses (Neher, 2015; Jackman and Regehr, 2017). Whole-cell electrophysiology, in particular, provides detailed insights into these transmission mechanisms. However, its application in neuromodulation is limited, as binding of neuromodulators to metabotropic GPCRs typically induces

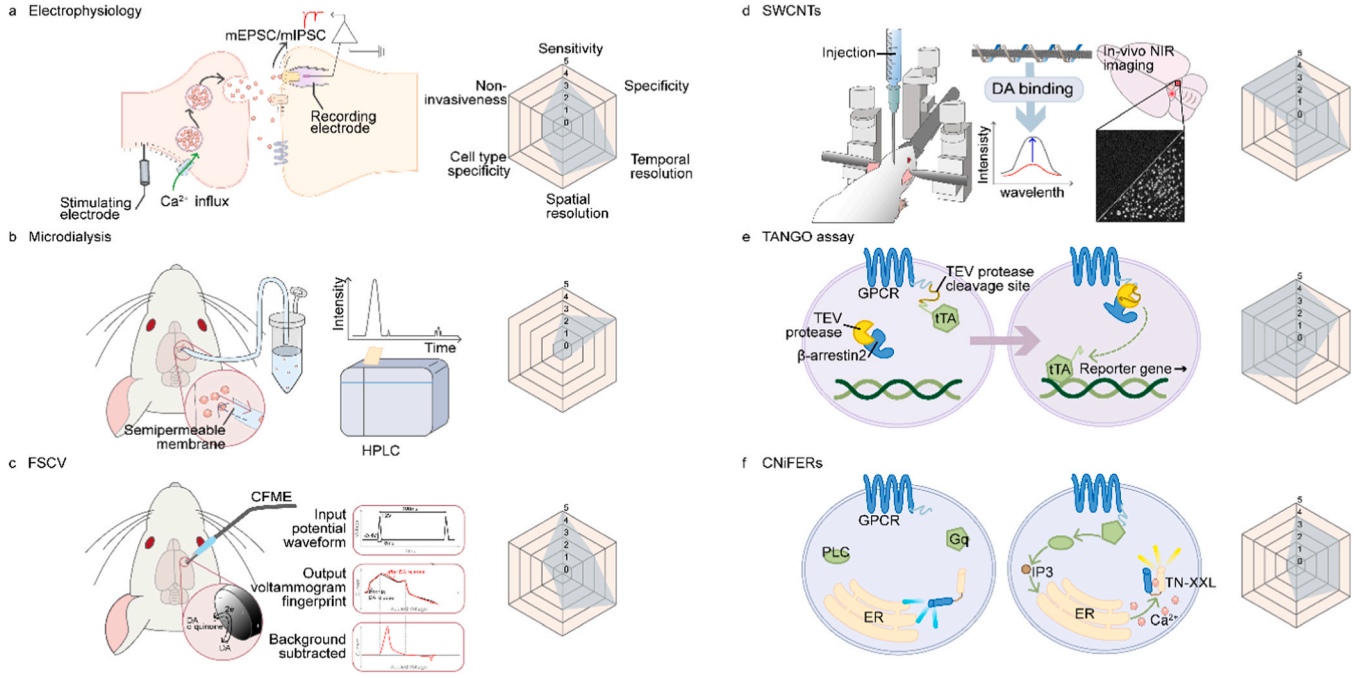

Fig. 1. Comparative analysis of neurochemical detection methodologies. (a) Schematic of cell-attached patch clamp detecting potential changes in postsynaptic membrane. (b) Microdialysis utilizing a semipermeable membrane to sample neurochemicals in the extracellular matrix. (c) FSCV applying cycling voltages (~10 Hz) to generate characteristic voltammograms of specific analytes. (d) SWCNTs injected into brain areas bound to target chemicals and thereby changing their conformation to release fluorescence in the NIR region. (e) TANGO assay illustrating the interaction between GPCRs and β-arrestin2, with TEV protease-mediated genetic editing triggering reporter gene expression. (f) CNiFERs leveraging GPCRs and genetically encoded calcium indicators (e.g., TN-XXL) to monitor neurochemicals in specific brain areas.

minimal or no changes in membrane potential (Marcott et al., 2014; Mamaligas and Ford, 2016). Despite its strengths, the patch clamp technique is invasive, requiring delicate instrumentation and craniotomy procedures. Additionally, the large-bore pipette restricts resolution to single cells. Efforts to improve automation have been made, but challenges in achieving medium-throughput screening, even with microelectrode array assays, continue to limit its broader application in network-level analyses (Gao et al., 2021).

An alternative approach, membrane capacitance recording, provides unique insights into presynaptic endocytosis. This method detects quantized changes in membrane capacitance with high sensitivity and temporal resolution, specifically during the fusion of non-recycling large dense-core vesicles at axonal terminals (Neher and Marty, 1982; Klyachko and Jackson, 2002). However, its invasive nature largely restricts its use to ex vivo applications.

2.2. Analytical chemistry methods

Developed in the 1960s, microdialysis is widely utilized for *in vivo* detection of absolute neurochemical concentrations. This technique involves the implantation of a probe with a semipermeable membrane, which collects molecules from targeted brain regions. These molecules are then separated and quantitatively analyzed using high-performance liquid chromatography-mass spectrometry (HPLC-MS) (Plock and Kloft, 2005) (Fig. 1b). Microdialysis excels in providing precise, sensitive, and specific concentration measurements, enabling the differentiation of structurally similar neurotransmitters (e.g., dopamine [DA] and norepinephrine [NE]). However, its spatial resolution is limited by the probe's relatively large diameter (~150 μm), and its temporal resolution is constrained by the extended sampling duration (5–10 minutes) required to reach equilibrium. These limitations hinder the ability to capture rapid *in vivo* transients. Furthermore, the invasive nature of the procedure restricts its broader application in research (Plock and Kloft, 2005).

Introduced to neuroscience in the 1980s, FSCV has become an indispensable technique for real-time neurochemical monitoring, especially for monoamines like DA, serotonin, and NE, due to its high spatiotemporal resolution and SNR (Wightman et al., 1986; Mena et al., 2021) (Fig. 1c). FSCV uses carbon-fiber microelectrodes (CFMEs) to apply rapid cycling voltages, enabling the oxidation and reduction of target analytes. The resulting voltammograms serve as unique "fingerprints," with waveform shapes and peak positions specific to individual analytes after background subtraction. For instance, DA detection typically involves scanning from −0.4 V to + 1.2 V and back at 400 V/s with a frequency of 10 Hz (Clark and España, 2023). FSCV boasts nanomolar sensitivity, submillisecond temporal resolution, and superior spatial precision with ~10 μm diameter electrodes. However, FSCV faces limitations: it is largely unsuitable for neuropeptides due to their non-electroactive nature, and distinguishing between DA and NE remains challenging due to their similar voltammogram features.

2.3. Optical methods

The non-optical techniques discussed above are inherently invasive to varying degrees. Such invasiveness often results in tissue damage, potentially triggering inflammatory responses and introducing experimental artifacts that may compromise data interpretation. To address these limitations, various optical approaches have been developed, typically consisting of a recognizing moiety and a reporting moiety. Several optical tools, including synthetic FM dyes (Gaffield and Betz, 2006), nanomaterials (e.g., carbon nanotubes and quantum dots) (O'Connell et al., 2002; Bruns et al., 2017; Selvaggio et al., 2020), fluorescent false neurotransmitters (FFNs) (Gubernator et al., 2009), and pH-sensitive fluorescent proteins (Li et al., 2005), enable indirect visualization of processes such as vesicle exocytosis, ligand-receptor interactions, and downstream signaling events. When combined with advanced imaging modalities, these optical methods offer high spatial resolution and considerable temporal resolution for *in vivo* detection while maintaining minimal tissue disruption.

2.3.1. Fluorescent sensors based on nanomaterials

Recently, single-walled carbon nanotubes (SWCNTs) coated non-covalently with DNA have emerged as a complementary approach for neurochemical detection (Fig. 1d). SWCNTs, essentially rolled-up graphene sheets (Saito et al., 1998), possess a nanoscale diameter (e.g., ~0.7 nm) that enables intrinsic fluorescence in the near-infrared (NIR) region (>850 nm). Their emission wavelength is determined by their chirality (O'Connell et al., 2002). When paired with customized surface chemistries, SWCNTs become versatile sensors for various biological applications. For instance, SWCNTs coated with (GT)15-ssDNA detect catecholamine release via conformational changes (Kruss et al., 2014; Nißler et al., 2019), while NIRSer employs specific DNA aptamers to recognize serotonin (5-HT) (Dinarvand et al., 2019). The NIR fluorescence of SWCNTs falls within the biological tissue transparency window, significantly enhancing the signal-to-noise ratio (SNR) by reducing light scattering and minimizing background interference (Spreinat et al., 2020). With spatial resolution superior to electrode-based methods and temporal resolution (~100 ms per frame) comparable to that of FSCV, SWCNTs demonstrate exceptional methodological potential. However, challenges remain, particularly in differentiating between dopamine (DA) and norepinephrine (NE) (Kruss et al., 2017; Mann et al., 2017).

2.3.2. Tango assay

β-arrestin2 plays a critical role in the internalization of GPCRs and G protein-independent signaling cascades (Ferguson et al., 1996; Goodman et al., 1996). Based on this finding, the Tango assay was developed to detect GPCR ligands by utilizing the downstream interaction between GPCR and β-arrestin2 (Barnea et al., 2008) (Fig. 1e). In the Tango assay, a transcription factor (tTA) is tethered to the C-terminal of the GPCR via an engineered TEV protease cleavage site, while β-arrestin2 is fused with TEV protease. Upon ligand binding, β-arrestin2-TEV is recruited to the receptor, resulting in the proteolytic release of tTA. The freed tTA then translocates to the nucleus and initiates the expression of reporter genes, such as green fluorescent protein (GFP). Initially designed as an *in vitro* method, the Tango assay has since been adapted for *in vivo* applications through transgenic technologies (Inagaki et al., 2012; Jagadish et al., 2014) and engineering approaches (Katow et al., 2019). However, a notable limitation of this method is the inherent delay in reporter gene expression, which requires several hours to produce detectable signals, thereby making it unsuitable for real-time monitoring of dynamic neurotransmission.

2.3.3. CNiFERs

Cell-based neurotransmitter fluorescent-engineered reporters (CNiFERs) offer an innovative approach for selective *in vivo* detection of specific neurochemicals (Fig. 1f). This system utilizes cultured cells co-expressing specific GPCRs and genetically encoded Ca^{2+} indicators, which are then implanted into targeted brain regions (Nguyen et al., 2010). Upon the release of neurochemicals into extracellular space, the implanted cells respond immediately, allowing real-time visualization of release events through changes in Ca^{2+} indicator fluorescence. CNiFERs harness the specificity of ligand-binding interactions and the signal amplification provided by Gq-mediated signaling pathways to detect transient neurotransmitter release. This technology has been successfully applied to detect a range of neurotransmitters, including acetylcholine (ACh), serotonin (5-HT), dopamine (DA), and norepinephrine (NE) (Nguyen et al., 2010; Muller et al., 2014; Foo et al., 2021). Despite these advantages, potential tissue disruption and inflammatory responses associated with cellular implantation remain critical considerations in experimental design.

2.3.4. Genetically encoded sensors

Genetically encoded sensors have gained significant popularity due to their unique advantages derived from genetic engineering and optical imaging. These sensors use engineered proteins or their variants as reporters to translate neural activity into visible signals, enabling detailed exploration of various neurobiological processes. These include synaptic vesicle release, membrane potential fluctuations, intracellular calcium dynamics, and neurotransmitter/neurochemical release. The genetic encoding strategy allows for cell-type-specific expression and precise subcellular targeting, facilitating sensor deployment in specific neuronal compartments such as axons, dendrites, or individual synapses. The stable expression of these sensors supports longitudinal studies, making them particularly valuable for investigating experience-dependent plasticity and learning-induced changes in neuronal communication. Moreover, high-resolution optical imaging techniques, such as super-resolution microscopy and deconvolution microscopy, enable non-invasive monitoring of thousands of neurons simultaneously *in vivo*. This capability allows for the study of intact neural circuits in behaving animals. Recent advancements in optimizing genetically encoded sensors have further enhanced their spatiotemporal resolution and kinetic properties. These improvements have cemented their status as indispensable tools for studying complex neural dynamics, as discussed in the following sections.

3. Development of genetically encoded sensors

3.1. Genetically encoded calcium indicators

Genetically encoded calcium indicators (GECIs) have become indispensable tools for visualizing neural activity in intact brains (Chen et al., 2013). The molecular mechanism is based on calcium-dependent conformational changes between calmodulin (CaM) and specific CaM-binding peptides, such as M13 or RS20. By fusing these calcium-sensing domains with various fluorescent proteins (FPs), Förster resonance energy transfer (FRET) pairs, or single circularly permuted FPs (cpFPs), GECIs provide either ratiometric or intensiometric measurements of intracellular calcium levels. The first GECI, cameleon, was developed by Miyawaki et al. in 1997. It features a tandem arrangement of four key components: cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), CaM, and M13 (Miyawaki et al., 1997). During action potentials, Ca^{2+} binding induces CaM to wrap around M13 peptide, narrowing the distance between the flanking CFP (donor) and YFP (acceptor) and increasing FRET efficiency (Miyawaki et al., 1997). This allows the CFP/YFP fluorescence ratio to serve as a readout for intracellular Ca^{2+} concentrations while reducing noise from motion artifacts (Michikawa et al., 2021) (Fig. 2a).

The introduction of circular permutation in GFP revolutionized GECI

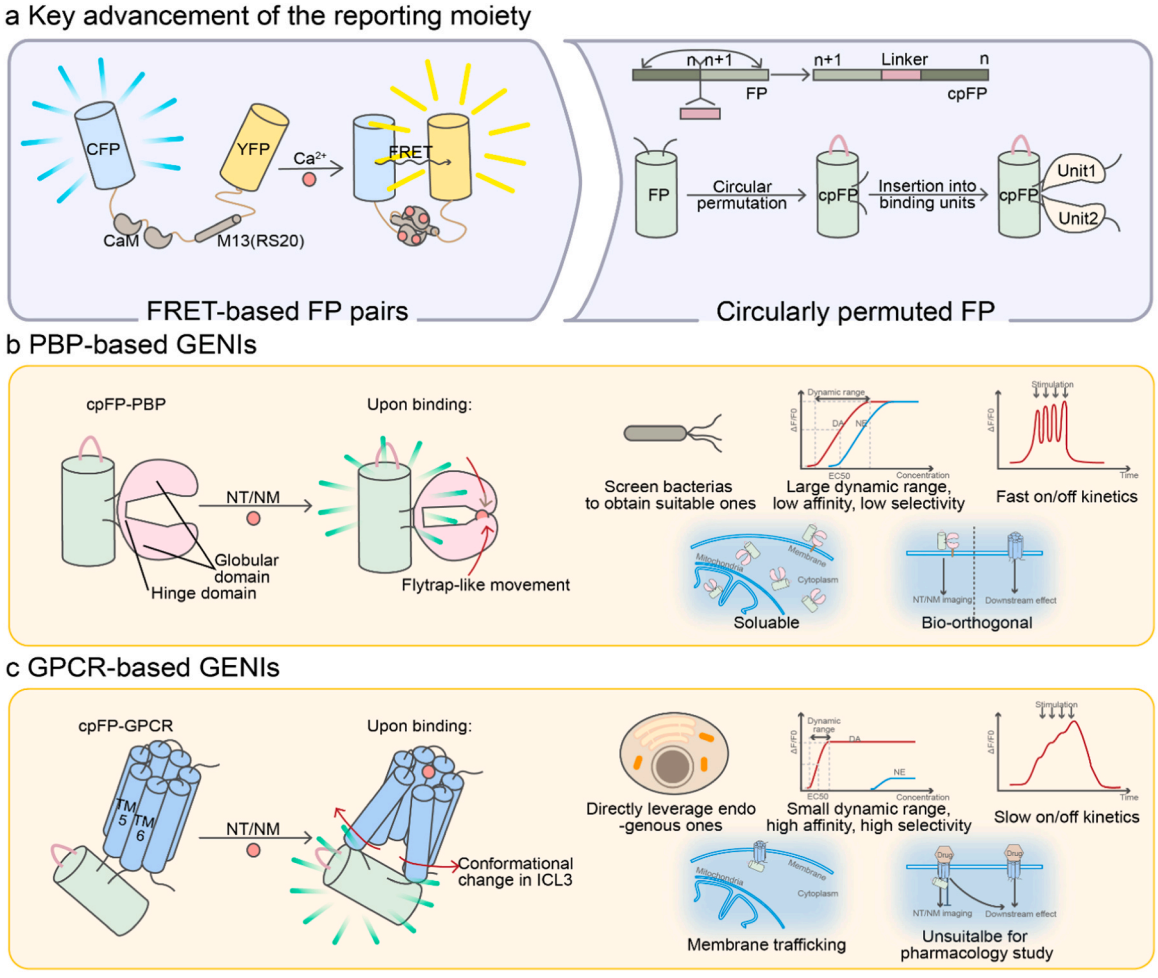

Fig. 2. Schematic of the development and classification of GENIs. (a) The reporting moiety of GENIs following the same optimization strategy as GECIs do, from the FRET-based pairs to cpFP. (b-c) GENIs classified into two primary groups based on their recognition scaffold: PBP-based and GPCR-based sensors. (b) PBP-based GENIs bound to NT/NMs like a flytrap, and they have a large dynamic range, fast on/off kinetics, solubility, and bio-orthogonal characters. (c) Binding of NT/NMs resulting in a large conformational change in ICL3 of GPCR, which causes cpFP to fluoresce. GPCRs are from eukaryotes, making it easy to find one just right for target endogenous chemicals. Meanwhile, it harbors high affinity, high selectivity, and perfect membrane trafficking ability. GPCR-based GENIs' ability to image NT/NMs activity would be impaired when drugs are applied.

development, leading to more sensitive variants like camgaroo, GCaMP, and Pericam (Baird et al., 1999; Nagai et al., 2001; Nakai et al., 2001). Crystallographic studies revealed that cpGFP consists of an eleven-stranded β-barrel structure and a centrally positioned chromophore. The native N- and C-termini are covalently linked, while new termini are engineered near the chromophore for fusion with CaM and M13/RS20 domains. This structural modification increases the flexibility of the β-barrel, improving conformational coupling between the calcium-sensing domains and the fluorescent protein structure. Consequently, conformational changes in the β-barrel alter the chromophore environment, affecting photophysical properties such as solvent accessibility, pKa, absorption, and quantum yield. These advances make cpFPs more environmentally sensitive and better suited as reporter units compared to FRET-based FP pairs (Fig. 2a). cpFP-based GECIs, particularly GCaMP6, offer greatly improved signal response, sufficient signal-to-noise ratio (SNR), and the ability to target specific subcellular locations, such as mitochondria (Nagai et al., 2001; Nakai et al., 2001; Chen et al., 2013). Variants like GCaMP6 and jGCaMP7, developed by the Janelia Research Campus, cater to diverse research needs with enhanced SNR, high sensitivity, and features such as brighter baseline fluorescence (Dana et al., 2019). Additionally, XCaMP variants, engineered by replacing M13 with peptides derived from CaMKK, demonstrate greater sensitivity and linear responsiveness (Inoue et al., 2015; Inoue et al., 2019). Similarly, the latest jGCaMP8 incorporates peptides from endothelial nitric oxide synthase (eNOS) in place of M13, achieving fast kinetics (Zhang et al., 2023b). For subcellular specificity, GCaMP6 can be targeted to axons or somas by fusing it with specific localization proteins, thereby minimizing contamination from surrounding Ca^{2+} signals (Petreanu et al., 2012). Despite all these advancements, it is important to recognize that all calcium indicators act as calcium buffers, which inevitably influence downstream signaling cascades.

3.2. Genetically encoded voltage indicators

While GECIs excel in monitoring neuronal calcium dynamics, genetically encoded voltage indicators (GEVIs) provide complementary capabilities by directly measuring membrane potential changes. GEVIs enable detailed investigation of neuronal firing patterns and are broadly classified into two major types: those utilizing the voltage-sensing domain (VSD) from voltage-sensitive phosphatases and those based on microbial rhodopsins. Recent advancements in GEVIs, including VSFR2.1 (Dimitrov et al., 2007), ArcLight series (Bando et al., 2019; Nakajima et al., 2021), JEDI-2P (Liu et al., 2022b), QuasAr series (Adam et al., 2019), Voltron (Abdelfattah et al., 2019), and ASAP series (St-Pierre et al., 2014; Chamberland et al., 2017; Bando et al., 2019), have significantly improved the efficiency of voltage-related response intensity, precision, and real-time reporting. These innovations, coupled with a high signal-to-noise ratio (SNR), have enabled GEVIs to detect single action potentials within neuronal populations in behaving animals. This capability facilitates detailed explorations of neural circuit dynamics and spiking activity. Despite these remarkable advances, several challenges remain in GEVI technology. These include limited sensitivity, temporal delays in signal reporting, and the non-linear relationship between fluorescence intensity and membrane potential changes. Addressing these limitations will be crucial for further enhancing the utility of GEVIs in neuroscience research.

3.3. Genetically encoded neurochemical indicators

Genetically encoded neurochemical (including NTs and NMs) indicators (GENIs), previously referred to as genetically encoded transmitter indicators (GETIs) in earlier studies, are highly specific, non-invasive biological sensors designed to report directly on processes such as vesicle release and fusion, neurochemical transport and distribution, neuron-associated calcium activity, and membrane potential dynamics (Lin and Schnitzer, 2016). Mirroring the optimization strategies used by genetically encoded calcium indicators (GECIs), GENIs have become versatile tools with applications ranging from acute brain slice studies to disease models (e.g., Alzheimer's disease), social behavior analysis, and autism research. Structurally, GENIs comprise two essential components: a recognition domain for specific neurochemical binding and a reporter domain for signal transduction. The recognition domain determines sensor specificity, while fluorescent proteins (FPs) in the reporter domain convert chemical signals into optical outputs. The linker sequences connecting these domains play a pivotal role in sensor functionality, facilitating conformational changes and optimizing signal output. Based on their recognition domain scaffolds, GENIs are categorized into two main classes: periplasmic binding protein (PBP)-based sensors and G-protein-coupled receptor (GPCR)-based sensors (Wan et al., 2021). These classes differ in their mechanisms, strengths, and limitations, making them suitable for distinct applications. The following sections detail their development, current capabilities, and workflows for production, alongside a comparative analysis to highlight their respective advantages for specific research contexts.

3.3.1. Periplasmic binding proteins (PBPs)-based GENIs

Periplasmic binding proteins (PBPs) are a diverse superfamily of bacterial receptors that facilitate nutrient sensing and uptake by binding various chemicals, including amino acids, lipids, and neurotransmitters (Felder et al., 1999). Structurally, PBPs consist of two globular domains connected by a hinge region, forming a dynamic scaffold. Upon ligand binding, the two domains close together in a motion resembling a Venus flytrap. The hinge region is particularly significant, as it contains multiple ligand-binding sites and serves as a versatile structural platform for integrating additional functional elements. These may include zinc finger structures and other regulatory domains, enhancing the flexibility and applicability of PBPs for diverse biological and biochemical processes (Dwyer and Hellinga, 2004).

Evolutionary gene fusion events have given rise to numerous mammalian receptors, including NMDA-type glutamate receptors, which possess ligand-binding domains homologous to bacterial PBPs (Felder et al., 1999). This evolutionary relationship inspired the development of FLIPE, the first PBP-based neurochemical sensor. FLIPE utilizes GltI (also known as ybeJ), a periplasmic glutamate-binding protein from *Escherichia coli*, as the recognizing moiety, coupled with a CFP-YFP FRET pair to provide optical readouts of the conformational change of GltI *in vitro* (Okumoto et al., 2005). Through refinements such as linker truncation screening and rational mutagenesis of GltI, an improved variant, SuperGluSnFR, was developed. This sensor demonstrates a 44 % ratio change and a dissociation constant (K_d) of 2.5 μM (Hires et al., 2008). However, the SNR of SuperGluSnFR was insufficient for detecting single action potential events. Significant progress was achieved by integrating cpFP with GltI, leading to the development of an intensity-based fluorescent glutamate sensor (iGluSnFR) (Marvin et al., 2013) (Table 1). This sensor exhibits remarkable performance characteristics, including a large dynamic range ($\Delta F/F_{max} = 1.0$) and a physiologically relevant affinity K_d of ~4 μM in neurons, capable of capturing the synaptic glutamate transient. The robustness of iGluSnFR has been validated *in vivo* across various model organisms, including mice, establishing it as the gold standard for glutamate biosensing. Importantly, the design and optimization of iGluSnFR laid a solid foundation for creating additional robust PBP-based sensors for key neurochemicals, such as iGABASnFR (Marvin et al., 2019) for GABA, iAChSnFR (Borden et al., 2020) for ACh, and iSeroSnFR (Unger et al., 2020) for serotonin (Table 1).

GABA, like glutamate, functions primarily as a rapid synaptic neurotransmitter, posing significant challenges for high-fidelity detection of its transient dynamics. The development of the iGABASnFR sensor followed a workflow similar to that of iGluSnFR, beginning with the identification of a suitable bacterial protein capable of GABA uptake (Pf622 from *Pseudomonas fluorescens* in this case). Circularly permuted

Table 1
Key characteristics of single FP-based genetically encoded sensors.

Sensors	Backbone	EC50 (nM)	$\Delta F/F_{max}$	SNR (puff)	τ_{on}/τ_{off} (s)	Specificity	Ref.
Glutamate sensors							
iGluSnFR	GItl	4 (μM)	3.9	~6.5	0.015/0.092	high	(Marvin et al., 2013)
SF-iGluSnFR series	GItl	7–40 (μM)	2–4	~4	0.6–6/5–108	high	(Marvin et al., 2018)
iGluSnFR3[b] series	GItl	1.8–9.6 (mM)	13.1–54	~29	0.02/NA	high	(Aggarwal et al., 2023)
GABA sensors							
iGABASnFR[b]	Pf622	6.2–6.6 (μM)	0.45–0.55	NA	NA	high	(Marvin et al., 2019)
ACh sensors							
$GRAB_{ACh2.0}$	M3R	1 (μM)	0.8	~14	0.25/0.7	high	(Jing et al., 2018)
$GRAB_{ACh3.0}$	M3R	2 (μM)	2.8	NA	0.1/0.9	high	(Jing et al., 2020)
iAChSnFR	OpuBC	1 (μM)	1.2	~50	k_{on}/k_{off} = 0.62/0.73 ($\mu M^{-1}s^{-1}$)	K_d = ~45 μM (choline)	(Borden et al., 2020)
NE sensors							
$GRAB_{NE1m}$	α2AR	83	1.3	~75	0.04/1.89	EC_{50} = ~1400 μM (DA)	(Feng et al., 2019)
$GRAB_{NE1h}$	α2AR	930	2.3	~10	0.07/0.68	EC_{50} = ~0.6 μM (DA)	(Feng et al., 2019)
$GRAB_{NE2m}$	α2AR	380	4.15	NA	0.12/1.72	EC_{50} = 20 μM (DA)	(Feng et al., 2024)
$GRAB_{NE2h}$	α2AR	190	3.81	NA	0.09/1.93	EC_{50} = 9 μM (DA)	(Feng et al., 2024)
nLightG	α1AR	755	10	NA	0.02/0.19	EC_{50} = 20 μM (DA)	(Kagiampaki et al., 2023)
nLigthR	α1AR	654	1.6	NA	NA	EC_{50} = 18.5 μM (DA)	(Kagiampaki et al., 2023)
5-HT sensors							
$GRAB_{5\text{-}HT1.0}$	2CR	22	2.5	~1	0.16/2.81	high	(Wan et al., 2021)
$GRAB_{5\text{-}HT3.0}$	2CR	150	13	~7	0.25/1.39	high	(Deng et al., 2024)
$rGRAB_{5\text{-}HT1.0}$	2CR	790	3.3	~2	0.06/0.68	high	(Deng et al., 2024)
PsychLight1	2AR	26	0.8	NA	NA/5.4	high	(Dong et al., 2021)
sDarken	1AR	127	−0.64	~2	0.04/0.32	high	(Kubitschke et al., 2022)
iSeroSnFR	iAChSnFR	310	17	NA	0.0005/5 (fast) 0.01/18 (slow)	high	(Unger et al., 2020)
DA sensors							
$GRAB_{DA1h}$	D2R	10	0.9	NA	0.14/2.5	EC_{50} = ~0.1 μM (NE)	(Sun et al., 2018)
$GRAB_{DA1m}$	D2R	130	0.9	NA	0.06/0.7	EC_{50} = ~1.5 μM (NE)	(Sun et al., 2018)
$GRAB_{DA2h}$	D2R	7	2.8	NA	0.05/7.3	EC_{50} = ~0.07 μM (NE)	(Sun et al., 2020)
$GRAB_{DA2m}$	D2R	90	3.4	~100	0.04/1.371)	EC_{50} = ~1.2 μM (NE)	(Sun et al., 2020)
$GRAB_{DA3h}$	D1R	22	12.4	~300	0.05/1.85	EC_{50} = ~μM (NE)	(Zhuo et al., 2024)
$GRAB_{DA3m}$	D1R	89	10	~300	0.07/0.56	EC_{50} = ~μM (NE)	(Zhuo et al., 2024)
$rGRAB_{DA1h}$	D2R	4	1.0	NA	0.06/2.15	EC_{50} = ~0.06 μM (NE)	(Sun et al., 2020)
$rGRAB_{DA1m}$	D2R	95	1.5	~1r[a]	0.08/0.77	EC_{50} = ~2.2 μM (NE)	(Sun et al., 2020)
$rGRAB_{DA2h}$	D2R	9.8	2.4	~4r[a]	0.05/3.35	NA	(Zhuo et al., 2024)
$rGRAB_{DA2m}$	D2R	210	5.3	~7r[a]	0.05/2.24	EC_{50} = ~150 nM (DA), ~9 μM (NE)[b]	(Zhuo et al., 2024)
$rGRAB_{DA3h}$	D1R	22	14.2	~9r[a]	0.06/3.6	NA	(Zhuo et al., 2024)
$rGRAB_{DA3m}$	D1R	140	14.6	~9r[a]	0.06/0.61	EC_{50} = ~35 nM(DA), ~710 nM (NE)[b]	(Zhuo et al., 2024)
$dLight_{1.1}$	D1R	330	2.3	~10	0.01/0.1	Kd = ~20 μM (NE)	(Patriarchi et al., 2018)
$dLight_{1.2}$	D1R	770	3.4	~20	0.01/0.09	Kd = ~20 μM (NE)	(Patriarchi et al., 2018)
$dLight_{1.3b}$	D1R	1.6	9.3	~30	NA	Kd = ~20 μM (NE)	(Patriarchi et al., 2018)
$dLight_{1.4}$	D4R	4	1.7	~10	NA	Kd = ~20 μM (NE)	(Patriarchi et al., 2018)
$RdLight_1$	D1R	859	2.5	NA	0.141/0.398	Kd = ~20–100 μM (NE)	(Patriarchi et al., 2020)

[a] SNR of $rGRAB_{DA1m}$, $rGRAB_{DA2m}$, $rGRAB_{DA2h}$, $rGRAB_{DA3m}$, and $rGRAB_{DA3h}$ were compared by relative SNR (relative to $rGRAB_{DA1m}$).
[b] All data were measured in HEK293 cells, except for iGluSnFR3 (neuronal culture, from neonatal Sprague Dawley rat, cortical and hippocampal), iGABASnFR (LNCaP), and specificity of $rGRAB_{DA2m}$ and $Rgrab_{DA3m}$ were compared in neurons.

fluorescent proteins (cpFPs) were inserted at optimal sites, followed by library screening and structure-guided mutagenesis. These steps refined the sensor's performance, achieving the desired balance of affinity, brightness, and SNR (Marvin et al., 2019). iGABASnFR represents the most advanced and, importantly, the only genetically encoded tool currently available for tracking GABA *in vivo*, filling a critical gap in monitoring inhibitory neurotransmission. However, its performance may be influenced by glutamate cross-reactivity and elevated pH levels, which can limit its applicability in certain experimental contexts (Marvin et al., 2019). Despite these limitations, iGABASnFR remains an invaluable tool for investigating inhibitory synaptic activity.

The iAChSnFR was engineered using a hyperthermophilic homologue of *B. subtilis* OpuBC from *Thermoanaerobacter sp. X513* and a circularly permuted Superfolder GFP (SFGFP) (Borden et al., 2020). Optimization efforts involved introducing 21 targeted mutations to key structural elements, including the binding site and its periphery, the hinge region, the proto-interface, and the linker connecting X513 to cpSFGFP. These modifications significantly enhanced the specificity and affinity of iAChSnFR for acetylcholine (ACh). Compared to GPCR-based sensors such as $GRAB_{ACh2.0}$ and $GRAB_{NE1m}$, iAChSnFR demonstrates superior performance with rapid kinetics and an exceptionally large fluorescence change ($[\Delta F/F_0]max$ = ~1200 %) (Borden et al., 2020; Zhu et al., 2020). These properties make it a powerful tool for real-time monitoring of ACh dynamics. However, like $GRAB_{ACh}$ series sensors, iAChSnFR is not immune to interference from structurally similar analytes, such as choline, which can produce nonnegligible interference signals (Nguyen et al., 2010; Jing et al., 2018; Borden et al., 2020). The reliability and utility of iAChSnFR for *in vivo* ACh detection have been validated across multiple model organisms, reinforcing its status as a robust and versatile tool for studying cholinergic neurotransmission.

The development of iSeroSnFR employed an innovative combination of machine learning and computational design to modify the iAChSnFR

binding pocket, eliminating the need for traditional bacterial PBP screening. Through 19 rational mutations targeting the binding site, the optimized iSeroSnFR achieves moderate affinity for serotonin (in the high-μM range) while significantly reducing cross-reactivity with acetylcholine (ACh) and choline. Notably, the sensor exhibits an impressive fluorescence increase ($[\Delta F/F_0]_{max} = 1700$ %) (Unger et al., 2020). iSeroSnFR has been proved to be a reliable tool for *in vivo* monitoring of serotonergic modulation within neuronal populations. Its capabilities extend to investigations of diverse behavioral paradigms, including fear conditioning, sleep-wake cycles, and social interactions. The sensor has demonstrated utility in both brain slice preparations and freely moving animals, making it a versatile choice for studying serotonin dynamics in complex physiological and behavioral contexts.

3.3.2. GPCR-based GENIs

GPCR-based sensors represent a major class of GENIs, harnessing the natural attributes of G-protein-coupled receptors (GPCRs), which are endogenously expressed on neuronal membranes. GPCRs are the largest receptor family for NTs and NMs, featuring a conserved core structure comprising seven transmembrane α-helices (TM1–7), three intracellular loops (ICL1–3), and three extracellular loops (ECL1–3) (Fredriksson et al., 2003; Rosenbaum et al., 2009). As ligand-binding domains of GPCRs have evolved to recognize diverse neurochemicals with high specificity, affinity, and membrane trafficking capabilities, they present an ideal scaffold for sensor development. Ligand binding induces conformational changes, most notably in transmembrane helices 5 and 6 (TM5 and TM6), driven by the movement of ICL3, which forms the basis for optical signal transduction (Fig. 2c) (Manglik et al., 2015). The first GPCR-based neurochemical sensor, α2A-cam, was engineered for norepinephrine (NE) detection by inserting CFP and YFP at designated positions in the ICL3 and C-terminus of the α2A-adrenergic receptor (α2AAR), respectively (Vilardaga et al., 2003). This sensor retained the essential ligand-binding and signaling kinetics of its parental GPCR, achieving rapid response times (~40 ms). However, the sensor's FRET efficiency was relatively modest (<10%), limiting its dynamic range. The development of cpFP-based GPCR sensors was founded on two key observations: cpFPs effectively report conformational changes in coupled sensory domains, as demonstrated in previous GENIs, and ICL3 undergoes the most substantial conformational changes upon ligand binding in GPCRs. This approach has paved the way for the advancement of next-generation GPCR-based sensors with improved performance and broader applicability in studying complex neurochemical dynamics.

The creation of first-in-class GPCR-based genetically encoded neurochemical indicators (GENIs) utilizing circularly permuted fluorescent proteins (cpFPs) relied on two fundamental premises: cpFPs, as previously demonstrated in GENIs, effectively report conformational changes of coupled sensory units, and the intracellular loop 3 (ICL3) of GPCRs undergoes the most substantial conformational change upon ligand binding, making it an ideal site for cpFP insertion. These foundational insights provided a robust theoretical framework for the design strategy of inserting cpFPs into the ICL3 region of GPCRs. The engineering process follows a systematic, three-phase approach. The initial phase involves selecting a specific GPCR subtype as the backbone for the target neurochemical. cpFPs are arbitrarily inserted into the ICL3 to generate diverse receptor-cpFP chimeras. Rigorous characterization of these chimeras is conducted to identify a best-performing candidate with satisfactory baseline performance. The second phase is to optimize the N- and C-terminal linkers between GPCR and cpFP, which improves the sensor's performance. Specifically, random mutagenesis and systematic screening are conducted at around 5 sites in chimera's linkers. An alternative strategy, as demonstrated in the development of PsychLight (Dong et al., 2021), involves grafting pre-optimized linker sequences from successful existing GENIs, followed by targeted re-screening (Dong et al., 2021). In this way, a semi-finished product is created with expected inherited properties, which demonstrate high affinity, superior membrane trafficking, specificity for target neurochemicals, and robust fluorescence increase. Despite these achievements, the initial prototypes often display limited dynamic range, typically below 50%, necessitating further optimization. The third phase addresses this limitation through point mutations across the receptor, linker, and cpFP domains. These modifications have proven instrumental in enhancing not only sensors' dynamic range but also their sensitivity, selectivity, and brightness (Sun et al., 2018; Jing et al., 2019; Wan et al., 2021; Duffet et al., 2022). This comprehensive engineering approach has led to the successful development of two prominent GENI families, i.e., the Light and GRAB series, both of which have demonstrated widespread utility and have become essential tools in neuroscience research.

$GRAB_{DA1}$ and $GRAB_{DA2}$ utilize dopamine D2 receptors (D2R) as backbones, whereas $GRAB_{DA3}$ and dLight1 are based on dopamine D1 receptors (D1R). The $GRAB_{DA}$ family has expanded to include six green and six red versions (Sun et al., 2018; Sun et al., 2020; Zhuo et al., 2024), complemented by dLight's portfolio of four green and two red versions (Patriarchi et al., 2018; Patriarchi et al., 2020) (Table 1). These sensor families exhibit distinct performance. dLight sensors offer superior fluorescence intensity and faster reaction kinetics but are more prone to imaging artifacts and background noise (Patriarchi et al., 2019). In contrast, $GRAB_{DA}$ exhibits enhanced SNR due to reduced endogenous interference (Sun et al., 2018). Recent advancements in green variants, particularly $GRAB_{DA3m}$ and $GRAB_{DA3h}$ (denoting medium and high dopamine affinities, respectively), have significantly improved performance. These variants outperform $GRAB_{DA2m}$ and dLight1.3b, demonstrating an over twofold increase in fluorescence ($[\Delta F/F_0]max \approx 1000\%$) and higher SNR (Zhuo et al., 2024). Their robust performance has enabled unprecedented visualization of dopaminergic transmission across diverse neural circuits, ranging from densely innervated regions such as the striatum and nucleus accumbens to sparsely innervated areas including the medial prefrontal cortex and amygdala. These tools have advanced our understanding of critical processes such as reward processing, learning, motor control, movement, and anxiety formation (Kim et al., 2020; Hamid et al., 2021; Lee et al., 2021; Ney et al., 2021; Liu et al., 2022a). Additionally, CNiFER, a dopamine detection method based on cell transplantation and GPCR modules, offers an alternative approach. Although its temporal resolution is much lower, with delays in the seconds range, it excels in chemical selectivity, sensitivity, and stability against external interference (Muller et al., 2014). This trade-off highlights the ongoing challenge of balancing multiple sensor performance parameters for specific applications.

Discriminating norepinephrine (NE) from dopamine (DA) *in vivo* has posed a significant technical challenge for NE sensors due to their structural similarity, differing by only a single hydroxyl group. Traditional methods, such as microdialysis and FSCV, have proven ineffective in reliably distinguishing these transmitters. However, genetically encoded NE sensors based on native GPCRs have successfully addressed this limitation, offering rapid, precise, non-invasive, cell-type-specific, and *in vivo* recording of NE transmission (Feng et al., 2019). Two primary sensor families have emerged: $GRAB_{NE}$ (Feng et al., 2019; Feng et al., 2024) and nLight (Kagiampaki et al., 2023), engineered from α2A and α1A adrenergic receptors, respectively (Table 1). $GRAB_{NE1m}$ demonstrates excellent performance, including a robust fluorescence response ($[\Delta F/F_0]_{max} = \sim 230$ %), good photostability, nanomolar sensitivity, sub-second kinetics, and remarkable specificity, with a 1,000-fold higher affinity for NE over DA—significantly surpassing the wild-type α2AR's 85-fold specificity (Feng et al., 2019). The next-generation $GRAB_{NE}$ sensors, such as $GRAB_{NE1h}$, achieve a twofold increase in sensitivity and improved selectivity (94-fold higher Emax/EC50 for NE over DA versus 37-fold) through site-directed mutagenesis of the GPCR and cpEGFP domains, combined with cell-based screening (Fig. 3c(i)). These advancements ensure reliable *in vivo* performance in both optogenetic manipulations and behavioral paradigms, further establishing these tools as indispensable for studying

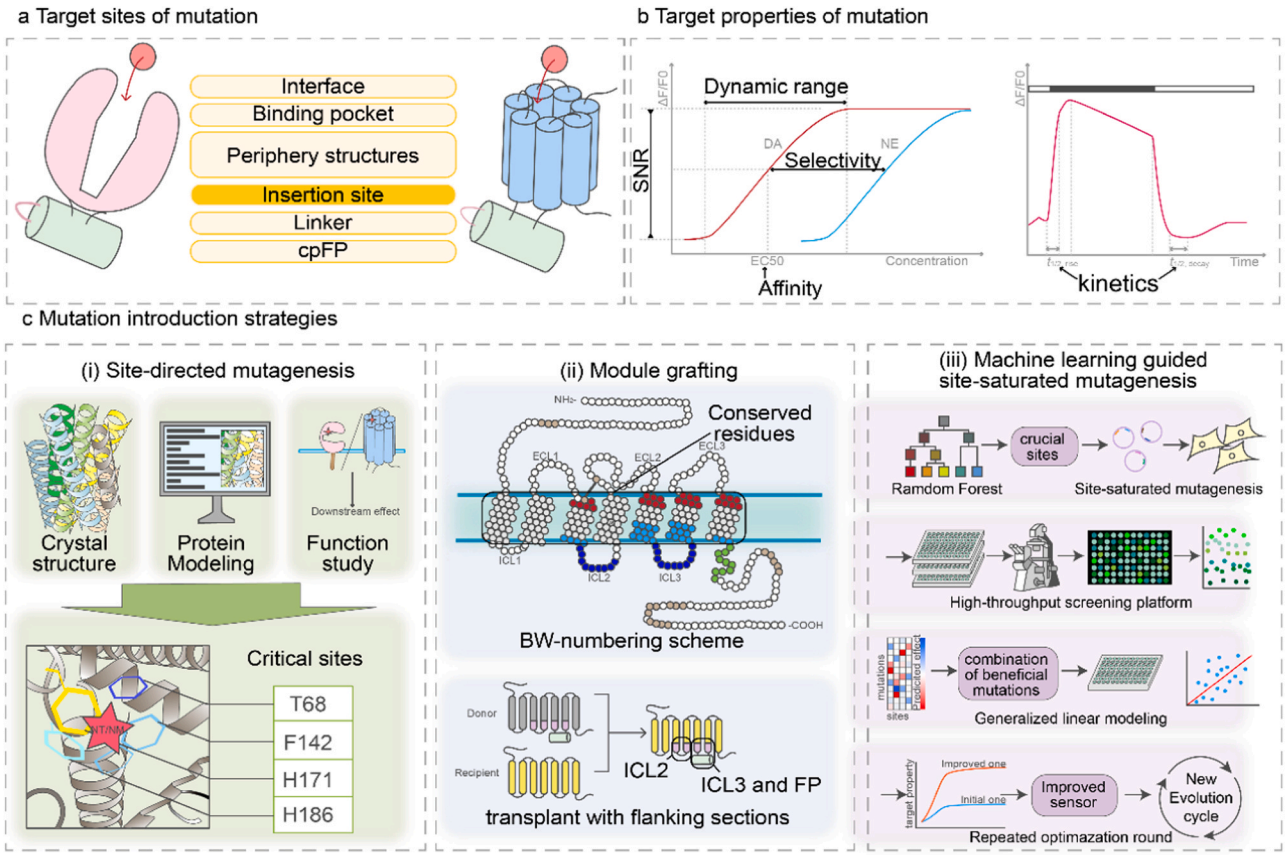

Fig. 3. Schematic of mutation introduction strategies in sensor optimization. (a) Mutation introduction representing the core mechanism of sensor optimization, targeting critical regions including the ligand interaction interface, binding pocket and periphery, linker, and cpFP. The cpFP insertion site emerges as a crucial element requiring meticulous initial optimization. (b) The primary optimization objective focused on modifying five key sensor properties. (c) New strategies accelerating the evolution of GENIs. (i) Site-directed mutagenesis is assisted by crystal structure revealing, protein modeling, and function study, which altogether predict the critical sites of function. (ii) Module grafting utilizes the BW-numbering scheme to align transplanted modules, identifying the most conserved residues at each position, with flanking section transplantation potentially enhancing performance. (iii) Machine learning and high-throughput screening platforms lead the new direction of site-saturated mutagenesis.

NE transmission (Patriarchi et al., 2019).

Serotonin detection has significantly progressed through multiple GPCR-based approaches, including $GRAB_{5\text{-}HT}$ (Wan et al., 2021; Deng et al., 2024), PsychLight (Dong et al., 2021), and sDarken (Kubitschke et al., 2022), which are three distinct GPCR-based sensor series for 5-HT, alongside the previously detailed iSeroSnFRs (Table 1). While the GRAB-based variants demonstrate higher affinity compared to iSeroSnFR, their early versions showed modest fluorescence responses. $GRAB_{5\text{-}HT3.0}$ represents a significant advancement, achieving an optimal balance between affinity ($EC_{50} \approx \sim 150$ nM) and response magnitude ($\Delta F/F_0 = \sim 1300$ %), thereby enabling reliable detection of trace 5-HT concentrations *in vivo*.

Following successful applications in detecting dopamine (DA), acetylcholine (ACh), and norepinephrine (NE), the development workflow for GPCR-based GENIs has rapidly matured, broadening their applicability to other important neurochemicals such as endocannabinoids, adenosine, histamine, nucleotides (e.g., ATP), neuropeptides (e.g., oxytocin) and neurolipids (e.g., N-arachidonoylethanolamine and 2-arachidonoylglycerol), whose features and applications have been fully discussed in the previous review (Qian et al., 2023). In summary, GPCR-based GENIs offer significant potential as versatile tools due to their native structural features, general scaffold designs, and modular adaptability, supporting a wide range of applications in neuroscience research.

3.3.3. *PBP-based sensors vs. GPCR-based sensors*

Both PBP-based and GPCR-based sensors offer superior spatiotemporal resolution compared to conventional methods, yet each class has distinct advantages and limitations that warrant careful evaluation (Fig. 2b; Fig. 2c). GPCR-based sensors benefit from the evolutionary refinement of GPCRs, which naturally exhibit high affinity and selectivity for their endogenous targets. This intrinsic selectivity enables accurate discrimination between structurally similar molecules, such as acetylcholine (ACh) and choline or norepinephrine (NE) and dopamine (DA), without extensive engineering of the binding domain. However, GPCRs inherently exhibit smaller conformational changes and slower kinetics upon ligand binding compared to PBPs (Jing et al., 2018; Patriarchi et al., 2018). Additionally, GPCR-based sensors may trigger undesired downstream signaling, posing challenges for certain applications. Consequently, developing robust GPCR-based sensors often requires iterative engineering to enhance their dynamic range and reduce signaling artifacts. In contrast, PBP-based sensors, though limited in their natural neurochemical binding repertoire due to their bacterial origins, possess several advantageous features. These include large conformational changes that translate into substantial dynamic range, rapid binding kinetics, and orthogonality to endogenous signaling pathways in model organisms. This orthogonality is particularly advantageous for long-term expression studies, as it minimizes interference with cellular signaling processes. Beyond key performance parameters, the distinct distribution patterns of PBP-based and GPCR-based sensors present a double-edged sword. On the one hand, as exogenous proteins, PBPs exhibit limited expression or, more specifically, reduced embedding on the neuronal membrane surface. This characteristic supports high-resolution monitoring of individual

synaptic events but may lead to narrower response range and lower apparent affinity due to insufficient surface expression (Marvin et al., 2019; Borden et al., 2020). On the other hand, GPCR-based sensors benefit from enhanced membrane trafficking and broader distribution, enabling the detection of trace amounts of neurotransmitters (NTs) and neuromodulators (NMs). However, this wider distribution compromises spatial specificity and resolution (Feng et al., 2019; Wan et al., 2021).

Despite the extensive development of GPCR-based sensor families (Light and GRAB series) across diverse neurochemicals, several fundamental limitations highlight the need for parallel development of PBP-based alternatives. Although GPCR-based sensors stemmed from endogenous GPCRs and are highly suitable for screening GPCR-targeting drugs, it is important to note that the possibility of sensors responding to drugs is problematic in certain cases involving drug administration, for example, evaluating the safety of drugs on neurons. Thus, to reflect neurochemical activities faithfully under drug manipulation, GPCR-based sensors need additional consideration or need to be redesigned as selective responding to neurochemical release, while PBP-based alternatives save the efforts. Besides, their subcellular targeting capabilities are notably limited, particularly within organelles and the cytoplasm. In contrast, PBP-based sensors demonstrate greater versatility, accommodating both membrane-associated and soluble applications, which is particularly significant in studies of neurotransmitter transport. For instance, cytoplasmic-localized iSeroSnFR has enabled real-time monitoring of serotonin transport, providing unprecedented insights into 5-HT trafficking and release dynamics (Bukowski et al., 2024). PBP-based sensors also offer technical advantages. Their amenability to purification and crystallization enables detailed structural characterization and rational optimization (Marvin et al., 2011; Borden et al., 2020)—a key advantage over GPCR-based sensors, for which no structural data have been published to date. Moreover, the species-specific localization, export, retention, and binding sequences that GPCR-based sensors contained may lead to unsatisfactory expression in distantly related organisms, yet it does not necessarily occur. Conversely, PBP-based sensors are more broadly applicable across species, making them particularly suitable for neuromodulation studies in model organisms like zebrafish, Caenorhabditis elegans, and Drosophila melanogaster. The screening protocols for these sensors further distinguish the two approaches. GPCR-based sensors require mammalian cell systems (commonly HEK293T), whereas PBP-based sensors can be efficiently expressed in bacterial or yeast systems, streamlining development and scalability.

4. Applications of GENIs

4.1. Neuromicroanatomy: delineate transmission features and dynamics

GENIs are advancing iteratively to enable real-time monitoring of diverse neurochemicals, with the ultimate goal of imaging transmission at individual release sites on a submillisecond scale. The tremendous progress achieved in recent years has established a robust foundation for studying fundamental aspects of neurotransmitter transmission, such as the number of release sites, release pool size, release probability, quantal size, diffusion extent, and refilling rate (Borden et al., 2020).

Notably, the integration of top-performing GENIs with deconvolution microscopy has facilitated the first direct measurement of neuromodulator diffusion extent (Zhu et al., 2020). This breakthrough challenges the longstanding volume transmission hypothesis, which posits that neuromodulators diffuse widely to mediate one-to-many transmission, binding to various nearby cell types and allowing neuropeptides to travel even millimeters away (Agnati et al., 1992; Zoli et al., 1999). Instead, high-resolution imaging of endogenous ACh and monoamine release at isolated sites in mouse acute brain slices, using $GRAB_{ACh2.0}$/iAChSnFR, $GRAB_{NE1m}$, $GRAB_{5\text{-}HT1.0}$, and $GRAB_{DA}$ sensors, revealed a strikingly different reality. These experiments captured spots of varying brightness within the imaging field, which were then analyzed using deconvolution to mitigate diffraction effects. By fitting a single exponential decay function to plots of relative maximal $\Delta F/F$ values against distance from the brightest pixel, researchers derived diffusion length constants. Surprisingly, these analyses revealed that the spatial diffusion of ACh and monoamines is highly restricted, with a spread length constant of ~0.75 μm for both ACh and monoamines (Borden et al., 2020; Zhu et al., 2020). This limited diffusion sharply contrasts with the hypothesized millimeter-scale transmission. For the fast-acting neurotransmitter glutamate, a similar analysis revealed a slightly smaller diffusion length constant of ~0.60 μm, attributable in part to its electronegativity (Jensen et al., 2019). These findings fundamentally challenge the volume transmission hypothesis by demonstrating remarkably similar diffusion constraints across neurotransmitter classes. They support an evolutionary model where synaptic architecture has been optimized for efficient transmission at specific sites rather than broad spatial influence.

Beyond revealing the spatial constraints of neurotransmitter diffusion, the integration of GENIs with traditional techniques has fundamentally transformed our understanding of neural circuit function and behavior (Zhang et al., 2023a). For example, by combining iGluSnFR sensors with electrophysiological recordings, researchers have uncovered that learning-induced synaptic plasticity involves precisely coordinated changes in both presynaptic glutamate release dynamics and postsynaptic response properties. Specifically, iGluSnFR sensors revealed increased glutamate release events in acute hippocampal slices following LTP induction, while simultaneous electrophysiological recordings demonstrated how these enhanced release patterns strengthen specific synaptic connections through coordinated postsynaptic receptor activation (Kopach et al., 2020). These findings provide mechanistic insights into how neural circuits are refined during learning, showing that synaptic strength is regulated through both spatial precision (as demonstrated by restricted diffusion) and temporal precision in neurotransmitter release patterns. Moreover, studies have shown that excessive extracellular glutamate can disrupt this delicate balance, potentially contributing to pathological processes in neurodegenerative diseases (Barnes et al., 2020). Such comprehensive analysis, leveraging both genetic sensors and traditional methods, has transformed our view of neurotransmission from a simple point-to-point signal transfer to a sophisticated, spatially and temporally regulated process that fundamentally shapes neural circuit function and behavior.

4.2. Neurophysiology and neuropathology: further our understanding behind behaviors and symptoms

As the renowned molecular biologist and Nobel laureate Sydney Brenner aptly remarked, “Progress in science depends on new techniques, new discoveries, and new ideas, probably in that order” (Robertson, 1980). GENIs represent a revolutionary toolbox that illuminates the brain with unparalleled precision, ushering in a new era of understanding neurological behaviors and disorders.

The application of GENIs has provided crucial insights into various neurological conditions. For example, genetically encoded GABA sensors have transformed our understanding of epilepsy mechanisms. In the mammalian cerebral cortex, inhibitory interneurons regulate the excitatory activities of pyramidal neurons by releasing GABA (Priebe and Ferster, 2008; Cardin, 2018). Epilepsy, characterized by a tendency for hyper-synchronized neuronal discharge (Fisher et al., 2014), has been strongly linked to malfunctions in GABA receptors on interneurons, leading to cortical disinhibition (Simkin et al., 2022). However, the mechanisms by which seizures emerge from abnormal background discharge activity remain elusive. One hypothesis posits that the failure of feed-forward inhibition contributes to seizure onset, a theory that could be validated by observing a rapid collapse of GABA transients at the onset of seizures (Trevelyan et al., 2007). Genetically encoded GABA sensors have facilitated simultaneous recording of GABA transients and electrocorticogram signals in the superficial cortex. Surprisingly, these

studies have not observed significant GABA collapse at seizure onset (Marvin et al., 2019). Further investigations involving various interneuron subtypes may provide deeper insights into the role of GABA during seizure initiation.

Beyond epilepsy, GENIs have revolutionized our understanding of dopaminergic signaling in reward-related behaviors. Studies of the ventral tegmental area (VTA) projections to the nucleus accumbens (NAc) and ventral pallidum (VP) have revealed intricate mechanisms of reward processing and learning (Mohebi et al., 2019; Heymann et al., 2020). Using GENIs to monitor dopamine dynamics, studies revealed that distinct populations of BLA neurons respond selectively to either reward-predicting or aversive stimuli, while most VTA dopamine axons in BA ($VTA^{DA\rightarrow BA}$) are activated by both reward and punishment-predicting cues (Lutas et al., 2019). Notably, under hunger conditions, both BLA neurons and VTA dopamine axons show enhanced responses to food-related cues, suggesting a crucial role in motivational salience and adaptive behavior formation (Lutas et al., 2019).

The versatility of GENIs extends to capturing neurotransmitter dynamics across various experimental contexts, including acute brain slices, intact animals, and freely moving subjects. This capability has advanced our understanding of complex psychiatric disorders and behaviors, including Alzheimer's disease, addiction, depression, and sleep-wake cycles. Comprehensive summaries of these advancements can be found elsewhere (Lin et al., 2021; Wright et al., 2024).

4.3. Neuropharmacology: evaluate and screen drugs

The strategic importance of GENIs in drug development is particularly significant, as neuromodulatory systems are primary targets for neurological and psychiatric therapeutics. Approximately 35 % of all FDA-approved drugs target GPCRs (Hauser et al., 2017). GPCR-based GENIs offer robust capabilities for quantitative drug response characterization, standardized parameter measurement, and precise action site mapping. A landmark application of GENIs in drug discovery is exemplified by PsychLight, a 5-HT2A receptor-based sensor capable of distinguishing between conformational changes induced by hallucinogenic and non-hallucinogenic compounds *in vivo* (Dong et al., 2021). Using cell-based medium-throughput assays, 83 compounds were screened, leading to the discovery of AAZ-A-154, a novel non-hallucinogenic psychedelic analogue that retains antidepressant effects. PBP-based GENIs have also shown utility in drug characterization. For example, machine learning-guided protein redesign of OpuBC, the hyperthermophilic binding pocket utilized in iAChSnFR and iNicSnFR, has enabled recognition of nicotine and the smoking cessation drug varenicline (Shivange et al., 2019). Additionally, other neural drugs that have realized screening with GENIs include the rapidly acting antidepressant S-ketamine (Bera et al., 2019), selective serotonin reuptake inhibitor antidepressants (Mayer et al., 2023), and opioids (Kroning et al., 2021).

5. Future optimization strategies for GENIs

The development of ideal GENIs necessitates a meticulous balance of multiple performance parameters. First and foremost is the need for appropriate binding affinity combined with a high signal-to-noise ratio (SNR), ensuring reliable detection of physiologically relevant concentration fluctuations. Selective molecular recognition must be paired with rapid kinetics to accurately capture dynamic changes in neurotransmitter levels while minimizing interference with native cellular signaling pathways. Furthermore, these sensors should exhibit precise subcellular targeting capabilities and efficient membrane trafficking, ensuring controlled expression and functionality across diverse experimental conditions. Modern neuroscience applications require sensors that are compatible with diverse imaging modalities, including fiber photometry, wide-field epifluorescence, multiphoton imaging, and super-resolution techniques. This versatility facilitates investigations in both freely moving and head-fixed preparations, enabling deep tissue imaging and ensuring long-term stability. However, there is no "one-size-fits-all" sensor; each sensor represents a unique balance of affinity, brightness, selectivity, and kinetics. Therefore, selecting an appropriate tool tailored to specific research needs while continuously developing new sensors and optimizing existing ones is essential for advancing neuroscientific exploration (Fig. 3b). Future optimization strategies for GENIs should prioritize enhancing signal properties, including improved brightness, photostability, and dynamic range, while minimizing background fluorescence. Advanced molecular engineering techniques, such as refined binding pocket design and enhanced protein stability, will be crucial for the development of next-generation sensors. Addressing challenges related to subcellular localization, protein aggregation, and membrane integration is vital for improving targeting strategies. Furthermore, continued advancements in measurement capabilities—such as temporal resolution, spatial precision, and quantification accuracy—will be essential. This section introduces potential optimization strategies aimed at accelerating the development and application of GENIs.

5.1. Mutation introduction strategies

The optimization of GENIs fundamentally depends on strategic mutation introduction at critical interfaces between the binding pocket, linker, and fluorescent protein (FP) (Fig. 3a). While combinatorial full matrix screening of multiple amino acid positions can be highly effective, it is labor-intensive and often leaves a significant portion of sequence space unexplored. Therefore, developing efficient mutation introduction strategies is essential to accelerate sensor optimization. Module grafting harnesses validated properties from previously developed GENIs by transplanting specific modules into a compatible context within the evolutionarily conserved GPCR family, as illustrated by examples above (Fig. 3c(ii)). To enhance grafting success rates, researchers have adopted the Ballesteros-Weinstein (BW) numbering scheme, which identifies conserved residues across the seven transmembrane helices of GPCRs (Ballesteros and Weinstein, 1995). This standardized method facilitates precise sequence alignment while preserving the critical geometry of the GPCR scaffold. Moreover, incorporating flanking sections and critical regions from donor sensors has proven to ensure a seamless transition during grafting (Ino et al., 2022; Kagiampaki et al., 2023). For instance, grafting the ICL2 region from donor indicators—such as dLight1.3b and RdLight1—onto nLightG and nLightR, respectively, has dramatically improved the sensitivity, selectivity, and dynamic range of these sensors (Kagiampaki et al., 2023). Similarly, the transplantation of the entire cpGFP-containing ICL3 from preexisting GRAB sensors has been practiced to develop sensors targeting somatostatin (SST) and corticotropin-releasing factor (CRF) (Wang et al., 2023). This approach, guided by the BW numbering scheme, highlights the potential of module grafting as a powerful strategy for GENI optimization.

The application of machine learning has significantly boosted the sensor optimization cycle. Machine learning guides site-saturated mutagenesis (SSM) by predicting the most crucial sites with the Random Forest (RF) algorithm, and based on the first-round outcome of SSM, it could forecast the most beneficial combination of mutations, with Generalized Linear Modeling for an instance (Fig. 3c(iii)). Machine learning has already facilitated the development of several advanced sensors, including iNicSnFR, iSeroSnFR, and GCaMP (Shivange et al., 2019; Unger et al., 2020; Wait et al., 2024). This approach demonstrates the transformative potential of integrating computational tools with experimental workflows to accelerate sensor development and improve performance.

Executing the results predicted by machine learning still requires a high-efficiency method to screen through thousands of mutations simultaneously, where the development of high-throughput screening platforms has shown their advantages (Fig. 3c(iii)). For GPCR-based genetically encoded voltage indicators (GEVIs), HEK293 cell-based screening assays have been established to automatically record and

analyze the fluorescence response of each mutant expressed stably in microwells (Tian et al., 2023). Opto-MASS, a recently introduced platform, exemplifies this advancement by achieving real-time ranking of sensor variants. This platform offers drastically increased throughput, dynamic performance monitoring, and multi-parameter selection, dramatically enhancing screening efficiency (Rappleye et al., 2023). Through automated systems and advanced parameter analysis, Opto-MASS enables rapid identification of promising sensor variants, significantly accelerating the optimization process. A similar high-throughput strategy has also proven effective for optimizing PBP-based genetically encoded calcium indicators (GECIs) (Lin et al., 2023), demonstrating the versatility and impact of these platforms in sensor development across various classes.

The most effective sensor development strategies often integrate multiple optimization approaches, blending initial rational design with module grafting from validated sensors, machine learning-guided mutation prediction, and high-throughput screening validation. This synergistic methodology maximizes the likelihood of identifying optimal sensor variants while minimizing resource consumption. Future optimization efforts will likely benefit from advancements in computational modeling, the expansion of mutation databases, and the refinement of screening technologies. By improving mutation introduction strategies and incorporating increasingly sophisticated screening and validation methods, the development of next-generation GENIs with superior performance across multiple parameters is poised to accelerate significantly.

5.2. Color expansion

The development of a diverse spectral palette for GENIs marks a significant advancement in neuroscience, enabling simultaneous visualization of multiple neurochemical dynamics within specific locations. This capability is particularly valuable for studying complex phenomena such as co-release, co-transmission, and synergistic interactions. Moreover, longer-wavelength variants, including red- and NIR-shifted sensors, offer advantages such as improved tissue penetration and reduced phototoxicity, facilitating noninvasive deep-brain imaging. The successful spectral diversification strategy employed in GECIs provides an instructive framework for GENI development. GECIs now span an impressive spectral range, including blue (e.g., XCaMP-B (Inoue et al., 2019)), yellow (e.g., XCaMP-Y (Inoue et al., 2019), jYCaMP (Mohr et al., 2020), NEMO (Li et al., 2023)), red (e.g., JRGECO1a (Dana et al., 2016), XCaMP-R (Inoue et al., 2019), RCaMP3 (Yokoyama et al., 2024)), and NIR (e.g., NIR-GECO (Hashizume et al., 2022), iBB-GECO (Qian et al., 2019)). A primary approach for generating shifted variants involves module grafting. For instance, jRGECO1a was developed by replacing cpGFP with the circularly permuted mApple red FP (cpmApple), showcasing a modular strategy for spectral shifts.

Although red-shifted variants with robust fluorescence responses remain less prevalent compared to green counterparts, several dominant ones have proven useful in studying co-release mechanisms. In the domain of PBP-based GENIs, R-iGluSnFR1 marked a pivotal breakthrough as the first red-shifted variant. This sensor, engineered by replacing the cpGFP domain of G-iGluSnFR with the cpRFP domain of R-GECO1, demonstrated the feasibility of color shifting while maintaining functional integrity (Wu et al., 2018). While yellow variants of iGluSnFR were successfully developed through Venus-inspired mutations, the optimization of red variants remains an active focus of research. In the context of GPCR-based GENIs, $rGRAB_{DA1m}$ and $rGRAB_{DA1h}$ pioneered red-shifted designs by employing strategies similar to those used for GECIs. The latest iterations, $rGRAB_{DA3m}$ and $rGRAB_{DA3h}$, have achieved superior brightness, fluorescence responses, and SNR compared to earlier versions like RdLight11 (Zhuo et al., 2024). These advancements have enabled groundbreaking studies, such as the simultaneous application of RdLight1 and iGluSnFR in the striatum. This experiment revealed that dopamine release, accompanied by suppression of glutamate release, is evoked by reward-predictive events in the nucleus accumbens (Patriarchi et al., 2020).

The expansion of the GENI spectral palette marks a pivotal advancement in decoding the intricate interplay of multiple neurotransmitter systems within neural circuits. Future developments in this field are expected to prioritize optimizing spectral separation to enhance multiplexing capabilities, alongside improving the brightness and photostability of red-shifted variants. Efforts will also focus on developing more advanced NIR sensors for deeper tissue imaging, coupled with general enhancements in SNR across all spectral variants. With the continued evolution of colorful GENIs, modeled after the success of GECIs, it is anticipated that simultaneous detection of co-released neurotransmitters—such as dopamine and glutamate, acetylcholine and GABA, or monoamines and neuropeptides—will soon be achievable. These innovations will significantly expand our capacity to monitor multiple neurochemical signals in intact neural circuits, offering unprecedented insights into the complexity of neural communication and regulatory mechanisms.

5.3. Imaging methods

Imaging methods play a crucial role in determining the spatiotemporal resolution of data, even with advanced sensors, and the ongoing advancements in imaging techniques and data analysis are pushing the boundaries of neuroscience. Different imaging methods come with unique merits and drawbacks, so matching the appropriate sensor with the right tool is essential to meet specific research needs. For instance, fiber photometry, a “plug-and-play” system, offers affordability, convenience, and minimal invasiveness, making it highly popular for measuring neurochemical dynamics *in vivo*, particularly in freely moving rodents (Simpson et al., 2024). This technique provides easy-to-read, stable signals deep beneath the skull surface, making it ideal for studying long-term behaviors and social interactions over months in living animals.

However, the fiber photometry readout represents a bulk measurement from the targeted region, which can complicate interpretation, especially when the signals are poorly synchronized or heterogenous (Simpson et al., 2024). To map or observe neural dynamics in more detail, wide-field epifluorescence imaging is often a better choice. This method, using single-photon illumination, is limited in depth penetration but works well in transparent model organisms like Drosophila larvae, zebrafish larvae, and C. elegans. Two-photon microscopy, a significant leap forward, uses infrared photons for excitation, providing superior tissue penetration compared to single-photon methods. While it offers a narrower field of view, two-photon microscopy excels in spatial resolution (sub-micron) and minimizes phototoxicity, making it invaluable for high-resolution imaging of specific neural structures in model organisms like Drosophila. Super-resolution and deconvolution microscopy have further advanced neurochemical imaging by minimizing diffraction effects. These techniques adjust hardware (e.g., specialized local excitation strategies) and/or software (e.g., Gaussian fitting or deconvolution analysis) to enhance the fidelity of imaging, pushing the limits of spatial resolution and providing a more accurate view of volume transmission, as discussed previously (Zhu et al., 2020).

Overall, genetically encoded fluorescent sensors represent a cutting-edge toolkit that surpasses traditional methods in several dimensions, including chemical, spatial, and temporal resolution; noninvasiveness; minimal phototoxicity; and design flexibility. Recent efforts to optimize GENIs have made them ideal for both microscopic and macroscopic studies of neurotransmission. While they continue to evolve toward greater robustness, versatility, and universality, when combined with techniques like optogenetics and/or FSCV, genetically encoded fluorescent sensors have the potential to revolutionize neuroscience, paving the way for the development of clinical treatments for complex neurological and psychiatric disorders.

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Zhang Kun: Writing – review & editing, Validation. **Wang Guangfu:** Writing – review & editing, Validation. **Zhang Peng:** Writing – review & editing, Funding acquisition. **Lim Juyao:** Writing – original draft. **Gu Hengyu:** Writing – original draft. **Zhong Xiaoyu:** Writing – original draft, Visualization, Conceptualization. **Li Xiaowan:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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