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Past, Present, and Future of Tools for Dopamine Detection

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Abstract—Dopamine (DA) is a critical neuromodulator involved in various brain functions. To understand how DA regulates neural circuits and behaviors in the physiological and pathological conditions, it is essential to have tools that enable the direct detection of DA dynamics *in vivo*. Recently, genetically encoded DA sensors based on G protein-coupled receptors revolutionized this field, as it allows us to track *in vivo* DA dynamic with unprecedented spatial–temporal resolution, high molecular specificity, and sub-second kinetics. In this review, we first summarize traditional DA detection methods. Then we focus on the development of genetically encoded DA sensors and feature its significance to understanding dopaminergic neuromodulation across diverse behaviors and species. Finally, we present our perspectives about the future direction of the next-generation DA sensors and extend their potential applications. Overall, this review offers a comprehensive perspective on the past, present, and future of DA detection tools, with important implications for the study of DA functions in health and disease.

Key words: dopamine, GPCR, sensor.

INTRODUCTION

Neural communication, primarily signaled by chemical neurotransmitters (NTs) and neuromodulators (NMs), is essential for brain functions. NTs, such as excitatory glutamate and inhibitory γ-aminobutyric acid (GABA), are released in the order of sub-second timescale and usually restricted within the synaptic cleft (Nadim and Bucher, 2014). In addition to point-to-point synaptic transmission, NMs can also function in a volume-transmission manner through G protein-coupled receptor (GPCR) to shape synaptic plasticity and neural circuits over a slower timescale ranging from seconds to minutes (Taber and Hurley, 2014; Liu et al., 2021. Among these NMs, dopamine (DA) plays essential roles in regulating reward, learning and movement (Graybiel et al., 1994; Wise, 2004; Björklund and Dunnett, 2007; Schultz, 2016). Dysfunctions in the DA system have been associated with various brain diseases, including Parkinson's disease, schizophrenia, and addiction disorders (Arnsten et al., 2017; Masato et al., 2019; Wise and Robble, 2020).

It is now well-accepted that in the physiological context DA acts via two releasing patterns, namely tonic release and phasic release. Tonic release of DA refers to a "background" DA level maintained by the spontaneous low-frequency activity of dopaminergic neurons; while phasic release of DA is mainly induced by burst firing of dopaminergic neurons, leading to a rapid, transient increase of DA concentration (Gonon, 1988). In the human genome, there are 5 GPCRs responsible for DA. DA receptors are classified into $G\alpha_{s/olf}$-coupled D1-like receptors (D1R and D5R) and $G\alpha_{i/o}$-coupled D2-like receptors (D2R, D3R and D4R), which promote or inhibit the production of intracellular cyclic adenosine monophosphate (cAMP) to differentially regulate behaviors, respectively (Klein et al., 2019).

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Abbreviations: ACh, acetylcholine; BLA, basolateral amygdala; cAMP, cyclic adenosine monophosphate; CNiFER, cell-based neurotransmitter fluorescent engineered reporter; cpGFP, circularly permutated GFP; cpRFP, circularly permutated red fluorescent protein; DA, dopamine; EC50, half-maximal effective concentration; FFN, Fluorescent false neurotransmitter; FLIM, fluorescence lifetime imaging microscopy; FRET, fluorescence resonance energy transfer; FSCV, fast-scan cyclic voltammetry; GABA, γ-aminobutyric acid; GECI, genetically encoded calcium indicator; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; HPLC, high-performance liquid chromatography; LOV, light-oxygen-voltage-sensing; MPOA, medial preoptic area; MS, mass spectrometer; NE, norepinephrine; NIR, near-infrared; nIRCat, near-infrared catecholamine nanosensor; NM, neuromodulator; NT, neurotransmitter; PBP, bacterial periplasmic binding protein; PKA, protein kinase A; PLC-IP3, phospholipase C-inositol triphosphate; REM, rapid eye movement; RPE, reward prediction error; SNR, signal-to-noise ratio; SPN, spiny projection neuron; SWNT, single-walled carbon nanotube; TEV, tobacco etch virus; TM5, fifth transmembrane; TM6, sixth transmembrane; VMAT2, vesicular monoamine transporter 2.

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Expressing genetically encoded calcium indicators (GECI) (Shen et al., 2020) in dopaminergic neurons is a widely used approach to monitor the activities of dopaminergic neurons that has greatly enhanced our understanding of the DA system. However, it is important to note that Ca^{2+} signaling within the dopaminergic neurons is not perfectly correlated with DA release, as DA release in axon termini could be shaped by local modulation (Liu et al., 2022; Lovinger et al., 2022). Moreover, the complex spatial–temporal dynamics of DA in the brain, including its propagation and decay, cannot be accurately modeled by relying solely on the Ca^{2+} signaling of dopaminergic neurons (Sippy and Tritsch, 2023). Thus, to fully understand the role of DA in regulating neural circuits and its impact on behaviors, a direct measurement approach with high spatial–temporal resolution and specificity is warranted.

In this review, we provide an overview of the existing methods for detecting DA, including analytic chemistry techniques such as microdialysis and fast-scan cyclic voltammetry (FSCV), as well as imaging approaches including cell-based, chemical dye-based, and nanomaterial-based sensors (Table 1). We then shift our focus to genetically encoded DA sensors and their applications *in vivo* during behaviors. Finally, we envision future directions for extending the capabilities of DA sensors to gain a better understanding of the DA system.

THE PAST: EXISTING METHODS TO DETECT DOPAMINE RELEASE

Analytic methods (microdialysis and FSCV)

Microdialysis has been a popular method to measure DA concentration *in vivo* since 1990s (Olson and Justice, 1993). The technique involves implanting a dialysis microprobe into the brain region of interest to continuously perfuse a cerebrospinal fluid-like solution while simultaneously collecting chemicals diffusing through a semi-permeable membrane. The collected samples can then be separated and analyzed using high-performance liquid chromatography (HPLC) and mass spectrometer (MS). Microdialysis is featured for its high sensitivity, which enables detection of DA at nanomolar concentrations, and its high molecular specificity, which allows for

Table 1. Summary of existing methods used to detect dopamine

	Advantages	Disadvantages	Suitable conditions
Microdialysis	• High sensitivity (use HPLC-MS) • High molecular specificity • Quantitative measurement of absolute DA concentration • Simultaneous detection of multiple neurochemicals	• Low spatial and temporal resolution (probe size of 100–200 μm and sampling rate of minutes) • Invasive (probe insertion causes tissue damage) • No cell specificity	Suitable for quantitative detection of tonic DA release and long-term monitoring of multiple neurochemicals simultaneously *in vivo*
FSCV	• High sensitivity • Quantitative measurement of absolute DA concentration • High temporal resolution (sub-seconds)	• Relatively low spatial resolution (probe size of 5–10 μm) • Unable to effectively discriminate DA from NE • Cannot be used to detect none-electrochemically active molecules • No cell specificity	Suitable for quantitative detection of both tonic and fast phasic DA release *ex vivo* and *in vivo*
FFN	• High spatial resolution (Sub-cellular resolution)	• Relatively low temporal resolution (seconds) • Challenge for *in vivo* (due to dye delivery and less optimal spectrum) • No cell specificity	Suitable for imaging DA *in vitro* and *ex vivo* with single synapse resolution
Nanosensor	• High sensitivity • High spatial and temporal resolution (sub-seconds and sub-cellular resolution) • Good tissue penetrance with NIR emission	• Challenge for *in vivo* (requires intracranial injection) • Unable to discriminate DA from NE • No cell specificity	Suitable for imaging DA *in vitro* and *ex vivo* without additional gene expression
CNiFER	• High sensitivity • High molecular specificity	• Requires exogenous cell implant (may cause tissue damage) • Relatively low temporal resolution (seconds) • No cell specificity	Suitable for detection of volume DA transmission *in vivo*
Tango assay	• High sensitivity • High molecular specificity • Single-cell resolution	• Low temporal resolution (hours) • Irreversible detection	Suitable for mapping DA release event over a period of time
GRAB/ dLight sensor	• High sensitivity • High spatial and temporal resolution (sub-seconds and sub-cellular resolution) • High molecular and cell type specificity	• Can not quantitatively measure absolute DA concentration	Suitable for DA detection in multiple animal models among both physiological and pathological conditions

differentiation between DA and other NMs with similar structure, such as norepinephrine (NE). However, microdialysis has its own limitations, including a low sampling rate (on the order of minutes) that limits its ability to capture rapid DA release events and a constrained spatial resolution due to the size of probe (typically around 100–200 microns).

FSCV was developed and popularized by Millar and Wightman in the 1980s to measure DA release (Armstrong-James and Millar, 1979; Stamford et al., 1984). It is based on the redox properties of DA that can be reversibly oxidized to dopamine-o-quinone at the surface of carbon-fiber microelectrodes, resulting in a electrochemical current with a characteristic shape (Venton and Cao, 2020). Compared to microdialysis, FSCV provides exceptional temporal resolution on the order of sub-seconds (Phillips et al., 2003; Stuber et al., 2008; Flagel et al., 2011) and uses smaller probes with a diameter of less than 10 μm, which minimizes tissue damage. Recently, a new sensor called Neurostring has been developed by combining tissue-like graphene-elastomer composite with FSCV. This sensor enables long-term and real-time measurement of DA *in vivo*, holding great potential for translational use in humans (Li, 2022). Despite these advantages, FSCV suffers from poor molecular specificity and is unable to effectively discriminate DA from NE, limiting its application in brain regions where these two molecules are interwoven (Schwarz et al., 2015). In addition, unlike imaging-based approaches as described below, FSCV does not provide detailed spatial information.

Chemical dye-based methods

Fluorescent false neurotransmitters (FFNs) developed by Sulzer offer an indirect approach to measuring DA release. The first reported FFN is FFN511, designed as a fluorescent substrate for the neuronal vesicular monoamine transporter 2 (VMAT2), which can be taken up into the vesicles and discharged during exocytosis (Gubernator et al., 2009). FFNs have been improved with different properties. For example, pH-sensitive FFN102 exhibits greatly improved signal-to-noise ratio (SNR) (Rodriguez et al., 2013; Meszaros et al., 2018). FFM200 has higher selectivity in labeling dopaminergic neurons, and it realizes the imaging of exocytosis from single presynaptic bouton of a dopaminergic neuron (Pereira et al., 2016). FFNs has excellent spatial resolution for tracking DA release, but relatively low temporal resolution (seconds). Notably, the *in vivo* application of these dyes is challenging due to the difficulty of dye delivery, and a less optimal spectrum mainly located in blue-green range.

Nanomaterial-based methods

Synthetic nanosensor is a composite material which has fluorescence increase upon binding with DA. It was synthesized by wrapping the fluorescent single-walled carbon nanotubes (SWNTs) with designed DNA oligonucleotides (Kruss et al., 2014; Del Bonis-O'Donnell et al., 2021). An improved nanosensor, called near-infrared catecholamine nanosensor (nIRCat), has been generated by optimizing the length of DNA oligo (Beyene et al., 2019), which enables the detection of DA release elicited by electric and optogenetic stimuli in brain slices with sub-second temporal kinetics and micrometer spatial resolution. Recently 2D films, including DopaFlim and AndromeDA, are engineered by coating nanosensors on the surface of glasses, which have the potential to push the detection limits to synaptic resolution and quantal sensitivity (Bulumulla et al., 2022; Elizarova et al., 2022). These nanosensors show fluorescence emission in a range of 1000–1400 nm, which is well-suited for *in vivo* imaging because of good tissue penetrance. Besides, nanosensors are more resistant to photobleaching than chemical dyes and fluorescent proteins. Despite these advantages, nanosensors are unable to distinguish between DA and NE, and their functionality in living animals has not yet been validated.

Downstream signal-based methods

Kleinfeld et al. developed cell-based neurotransmitter fluorescent engineered reporters, known as CNiFERs, to detect DA release (Muller et al., 2014; Foo et al., 2021). It is a HEK293 cell line that stably expresses a D2 receptor, a chimeric G protein G_{qi5} and a Ca^{2+} sensor TN-XXL. When DA binds to the D2 receptor, G_{qi5} is recruited to activate the phospholipase C-inositol triphosphate (PLC-IP3) pathway, and the resulting increase of cytosolic Ca^{2+} level is reported by the Ca^{2+} sensor TN-XXL. This method has a relatively low temporal resolution in a range of seconds due to the delay of downstream signal transduction, and its spatial resolution can only resolve the volume-averaged transmission event without synaptic specificity. Moreover, implanting exogenous HEK cells into the brain of living animals will cause tissue damage and unwanted side effects.

The Tango assay is another strategy that relies on the transduction of downstream signals to report the activation of a GPCR to its specific ligand (Barnea et al., 2008). It was first developed by Lee *et al*. and later applied to record DA signaling in the *Drosophila* brain (Inagaki et al., 2012). In this assay, a transcription factor is linked to the DA receptor via a tobacco etch virus (TEV) cleavage site. When DA binds to the engineered receptor, a chimeric protein composed of beta-arrestin and TEV protease is recruited to cleave the tethered transcription factor, allowing it to enter the nucleus and activate the transcription of a reporter gene. This system has nanomolar sensitivity and single-cell resolution. Light-controllable Tango assays (Lee et al., 2017) were developed by adding the light sensitive light-oxygen-voltage-sensing domain (LOV domain) to improve the temporal specificity and SNR. Given that several hours are required for the transcription and translation of the reporter gene, these Tango assays suffer from low temporal resolution and are irreversible.

THE PRESENT: GENETICALLY ENCODED DOPAMINE SENSORS

The discovery and cloning of green fluorescent proteins (GFP) have revolutionized the way that researchers

study biological processes from cell to behaving organisms (Shimomura et al., 1962; Chalfie et al., 1994). The creation of circularly permutated GFP (cpGFP) further advanced the development of genetically encoded fluorescent sensors (Baird et al., 1999; Topell et al., 1999). Given that the cpGFP is sensitive to conformational changes, coupling the cpGFP with a ligand-sensing domain can generate a fluorescent sensor. Compared to sensors relying on the fluorescence resonance energy transfer (FRET) between two FPs, cpGFP-based sensors typically have a larger dynamic range so that they are more amenable for *in vivo* imaging. This strategy was first used to develop GECIs including the widely used GCaMP6 (Chen et al., 2013), and was later extended to the development of voltage sensors (Knöpfel and Song, 2019; Lazzari-Dean et al., 2021). These successful experiences have paved the way for engineering NT/NM sensors.

The first genetically encoded cpGFP-based NT/NM sensor is the glutamate sensor iGluSnFR, which is developed by inserting the cpFP into a bacterial periplasmic binding protein (PBP) (Marvin et al., 2013; Aggarwal et al., 2023). This designing strategy has been used to develop sensors for other NT/NMs, such as acetylcholine (ACh) (Borden et al., 2020), GABA (Marvin et al., 2019), ATP (Lobas et al., 2019) and serotonin (Unger et al., 2020). However, the development of a PBP-cpFP-based DA sensor has been impeded due to the lack of an appropriate PBP selective for DA.

The design of dopamine sensors

The nature has evolved a large family of transmembrane proteins, known as GPCRs to sense NT/NMs with high specificity. Resolved structures have shown that ligand-binding caused a conserved conformational change in class A GPCRs, where the fifth transmembrane (TM5) and sixth transmembrane (TM6) domains undergo an outward movement (Manglik et al., 2015; Yin et al., 2020; Zhuang, 2021). By tapping into this structural feature, our group and Tian's group have independently established a novel GPCR-cpGFP-based design, and developed the $GRAB_{DA}$ sensors and dLight sensors, respectively (Patriarchi et al., 2018; Sun, 2018).

In order to transmit the ligand-induced conformational change within the GPCR to the cpGFP, we inserted the cpGFP to the intracellular loop 3 situated between TM5 and TM6 of D2R to generate the first generation $GRAB_{DA}$ sensors (Sun, 2018) (Fig. 1A). These $GRAB_{DA}$ sensors show a 90% maximal response ($\Delta F/F_0$) to DA, and the EC_{50} of high and medium affinity versions are 10 nM and 130 nM, respectively. Systematic site saturation mutagenesis on cpGFP was used to obtain the optimized second-generation $GRAB_{DA}$ sensors, which exhibit an improved maximum dynamic range up to 340% (Sun et al., 2020). Using a similar strategy, Tian's group developed the dLight sensors (Patriarchi et al., 2018). The dLight1.2 and dLight1.3b sensors are based on D1R, and they exhibit large dynamic ranges of 340% and 930% $\Delta F/F_0$ with affinities of 770 nM and 1600 nM, respectively.

Red-shifted DA sensors have been developed to allow for simultaneous imaging with other green sensors. These sensors use circularly permutated red fluorescent protein (cpRFP) as the fluorescent module, such as cpmApple. Using similar designing strategy, our group and Tian's group developed two series of red DA sensors, named $rGRAB_{DA}$ sensors and the RdLight sensor, respectively (Patriarchi et al., 2020; Sun et al., 2020) (Fig. 1B). The $rGRAB_{DA}$ sensors are based on D2R with responses of 100–150% $\Delta F/F_0$ and affinities of 4–95 nM, while the RdLight sensor is based on D1R with a response of 300% $\Delta F/F_0$ and an affinity of 860 nM.

Using GPCRs as the scaffolds has been proved to be a scalable strategy in developing biosensors to detect a wide range of NTs and NMs, including ACh (Jing et al., 2018, 2020), NE (Feng et al., 2019), serotonin (Dong et al., 2021b; Wan et al., 2021; Kubitschke et al., 2022; Deng et al., 2023), histamine (Dong et al., 2023), adenosine (Peng et al., 2020), ATP (Wu et al., 2022a), endocannabinoids (Dong et al., 2021a)and neuropeptides (Abraham et al., 2021; Duffet, 2022; Ino et al., 2022; Melzer et al., 2021; Qian et al., 2023; Wang, 2022).

The advantages and limitations of DA sensors

Both $GRAB_{DA}$ and dLight sensors have the advantage of being entirely genetically-encoded, which offers several benefits. Firstly, when combined with specific promoters, it becomes feasible to express these sensors with cell type specificity. Additionally, these sensors can be used in diverse model animals, either through virus-mediated expression or generating transgenic lines. Moreover, the sensor constructs can be conveniently packaged into adeno-associated virus and expressed through virus injection into the mouse brain region of interest, allowing for widespread use *in vivo*. Finally, the genetically-encoded property also facilitates the dissemination of

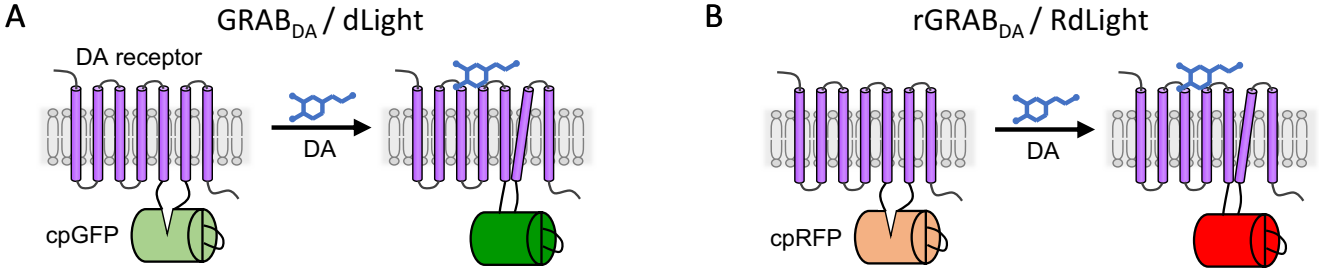

Fig. 1. Genetically encoded dopamine sensors. Schematic representation of genetically encoded green dopamine (DA) sensor (A) and red DA sensor (B). The circularly permutated green fluorescent proteins (cpGFP) or red fluorescent proteins (cpRFP) are inserted into the intracellular loop 3 (ICL3) of DA receptors. Upon DA binding, the DA sensors undergo a conformational change, leading to an increase in fluorescence.

these sensors worldwide, as related plasmids can be readily obtained from the developers or Addgene, and their viruses are also accessible from virus companies or can be produced by custom packaging.

The DA sensors provide exceptional spatial–temporal resolution for *in vivo* DA imaging. Analytic methods such as microdialysis lack high spatial and temporal resolution, and although nanomaterial and chemical dye-based methods offer excellent spatial–temporal resolution, they are unsuitable for *in vivo* recording. In contrast, the DA sensors have subsecond kinetics and high sensitivity, making them an ideal choice for *in vivo* DA imaging. Combining these sensors with advanced imaging modalities enables cellular spatial resolution and subsecond temporal resolution. For example, by using two-photon imaging, the DA sensors were able to spatially resolve fast DA transients in the cortex of living mice during learning behavior (Patriarchi et al., 2018).

The scaffold of GPCRs offers high specificity and appropriate affinity to DA sensors. Compared to other detection methods, such as FSCV, chemical dyes, and nanosensors, the DA sensor can effectively discriminate DA from other neurotransmitters, including NE, with over 10-folds difference in affinities. Engineered DA sensors have been shown to exhibit an affinity closer to that of native DA receptors, making them suitable for detecting DA within its physiological range. Additionally, protein engineering allows easy tuning of the sensors' affinity, enabling broad detection ranges. For instance, medium or low-affinity versions can be used for DA-abundant brain regions, while high-affinity versions can be used for brain regions with less DA innervation. These abilities make the DA sensor a valuable tool for investigating the effects of DA on the brain.

Despite the advantages of DA sensors mentioned above, there are some considerations that researchers should keep in mind when using them. Firstly, these DA sensors may cause the buffering effect, which is a common issue encountered by existing sensors. This occurs when endogenous ligands binding to sensors, thereby reducing the binding of ligands with native receptors (McMahon and Jackson, 2018). This can be partially avoided by using sensors with relatively low affinity or titrating the sensor expression level (Dong et al., 2022). Secondly, the recorded signals cannot identify the cell types from which DA is released, nor can they reflect whether DA binds to endogenous receptors to exert its functions. This is because DA is usually released broadly with volume transmission and may diffuse far outside the synapse that lacks endogenous receptor expression (Liu et al., 2021). Thirdly, the kinetics of recorded signals *in vivo* do not accurately represent the actual kinetic of DA release but rather a convolution of sensor kinetics and DA release kinetics. Therefore, selecting a sensor with fast kinetics and appropriate affinity can provide a more accurate temporal dynamic of DA release. Fourthly, cpGFP-based sensors are typically pH sensitive, and the firing of neurons usually results in a reduction in intracellular pH, which may cause artifacts on the recorded signals (Zhang et al., 2010). Additionally, like other sensors for imaging, moving artifacts and photobleaching should be taken into consideration. Thus, rigorous control experiments are usually necessary to confirm the fidelity of recorded signals.

Compared to previous methods, genetically encoded DA sensors are the preferred choice for end-users to perform DA detection in most conditions. These sensors are easily accessible and compatible with common imaging equipment and methods. It is worth noting that different DA sensors vary in terms of their dynamic range, brightness, affinity, kinetics, pharmacological profile, and there is not a 'one-size-fits-all' sensor. When designing an experiment, it is crucial to select an appropriate sensor based on specific behavior, brain region, and pharmacological manipulation. For more detailed information, refer to the published reviews (Labouesse et al., 2020; Wu et al., 2022b). However, it should be noted that genetically encoded DA sensors may not always meet the requirements of certain experimental conditions. For instance, the current DA sensors are unable to provide quantitative measurements of DA concentration *in vivo*. In such cases, alternative methods like microdialysis and FSCV can be considered to determine absolute DA concentration. Moreover, in situations where genetic manipulation is not feasible or permitted, other methods such as FFNs and nanosensors can serve as viable alternatives for DA detection. In summary, genetically encoded DA sensors are generally suitable for most conditions, but careful selection is necessary for optimal performance. In cases where genetically encoded sensors are not appropriate, traditional methods like microdialysis and FSCV can be employed to fulfill the experiment's needs.

The application of dopamine sensors

Nobel laureate Sydney Brenner once said (Robertson, 1980) that "Progress in science depends on new techniques, new discoveries and new ideas, probably in that order." With the emergence of genetically encoded DA sensors, we can now explore DA dynamics *in vivo* with unprecedented spatial–temporal resolution. These new discoveries have broadened our understanding of DA functions at the cellular, circuit and system levels.

DA sensors have revealed valuable insights into reward and learning behaviors (Jong et al., 2019; Lutas et al., 2019; Mohebi et al., 2019; Yuan et al., 2019; Iino et al., 2020; Kim et al., 2020; Deng et al., 2021; Hamid et al., 2021; Hamilos et al., 2021; Hu et al., 2021; Kutlu et al., 2021; Lee et al., 2021; Chen et al., 2022; Jeong et al., 2022; Kalmbach et al., 2022; Liu et al., 2022; Gyawali et al., 2023). The well-accepted reward prediction error (RPE) model explains how phasic DA release reflects the difference between experienced and expected reward (Schultz et al., 1997), but whether the slowly ramping DA dynamics also encode RPE remains controversial (Mohebi et al., 2019). Researchers used DA sensors to monitor DA dynamics via fiber photometry in a virtual reality paradigm, and their results confirmed that the ramping DA signals were still consistent with the RPE model, providing a unified understanding of the rapid phasic and slowly ramping DA signals in encoding RPE

(Kim et al., 2020). Using widefield imaging with DA sensors, researchers also observed wave-like DA dynamics across the dorsal striatum. Interestingly, the wave directions varied across different tasks, indicating that both the spatial and temporal DA dynamics encode the reward-related information (Hamid et al., 2021).

The DA sensors have also proven to be valuable tools in uncovering the roles of DA in other behaviors, such as sleep-wake cycle (Dong et al., 2019; Hasegawa et al., 2022), mating behavior (Sun, 2018; Sun et al., 2020; Dai et al., 2022), addiction (Corre et al., 2018; Lefevre et al., 2020; Lin et al., 2020; Nguyen, 2021; Pribiag et al., 2021; O'Neal et al., 2022), thirst (Augustine et al., 2019)and feeding behaviors (Alhadeff et al., 2019; Mazzone et al., 2020). For example, to study the role of DA in sleep-wake cycle, researchers expressed the DA sensors in multiple brain regions, and found a transient DA increase in the basolateral amygdala (BLA) that initiates rapid eye movement (REM) sleep (Hasegawa et al., 2022). Using the DA sensors in mating behavior also leads to new observations. The increased DA release was observed in NAc during different stages of mating, including the mounting, intromission and ejaculation (Sun, 2018; Sun et al., 2020; Dai et al., 2022). In contrast, the DA release in medial preoptic area (MPOA) ramped up as the male mice approached the female mice and terminated at the beginning of sniffing (Zhang et al., 2021), revealing that DA in MPOA participates in regulating mating. More recently, another study used the DA sensors to uncover the involvement of DA in pleasurable touch (Elias et al., 2023).

DA release shapes the synaptic plasticity by binding to DA receptors and triggering downstream signals. By utilizing DA sensors and protein kinase A (PKA) sensors simultaneously, researchers have been able to investigate how DA dynamics modulate PKA activities in spiny projection neurons (SPNs) across learning in real time. This approach revealed an asynchronous modulation of PKA activities in D1R-expressed SPN and D2R-expressed SPN (Lee et al., 2021). By comparing the DA signals with PKA signals, the authors found that the PKA activities in these two groups of neurons respond to different DA dynamics and are not modulated at the same time. These findings revealed by these sensors highlight the complex and dynamic nature of DA signaling in the brain.

The local regulation between NT/NMs has attracted general interests (Lovinger et al., 2022). *In vitro* studies uncovered a local regulation in striatum, where the ACh release from the cholinergic interneurons (CINs) could depolarize the axon of dopaminergic neurons through nicotinic acetylcholine receptors (nAChRs) (Zhou et al., 2001; Threlfell et al., 2012; Kramer et al., 2022), and DA could in turn activate the D2R expressed in CIN to suppress CIN activity (Chuhma et al., 2014; Straub et al., 2014; Wieland et al., 2014. However, the specific mechanisms underlying this regulation remain unknown, and the roles of such reciprocal regulation in physiological conditions *in vivo* are yet to be determined. To address these questions, a recent study used DA and ACh sensors ($GRAB_{DA}$ and $GRAB_{ACh}$, respectively) to track their release and revealed that ACh could induce action potential firing in DA axons to increase DA release (Liu et al., 2022). The authors also validated the regulation in mice *in vivo* during spontaneous movement and found that DA and ACh are coordinated for their roles in motor control (Liu et al., 2022). More recently, two independent studies using dual-color imaging also explored the regulation *in vivo* during reward and decision-making (Chantranupong et al., 2022; Krok et al., 2022).

In addition to their use in mice, DA sensors have also been explicitly employed in other model animals, including zebrafish (Sun, 2018), *Drosophila* (Sun, 2018; Sun et al., 2020; Handler et al., 2019; Zolin et al., 2021), and zebra finch (Tanaka et al., 2018). For example, in *Drosophila*, the DA sensor has been used to report DA release during odor stimulation and associative learning in the mushroom body (Sun, 2018; Sun et al., 2020; Handler et al., 2019). Zebra finch has been an ideal model organism for studying a wide range of neural processes, including those related to vocal learning and social behavior. To uncover the neural circuit underlying the vocal learning, a study expressed the DA sensors in the cortical song nucleus HVC of the juvenile zebra finches, and recorded a significant DA release in the presence of a singing tutor (Tanaka et al., 2018). By contrast, the DA level change was not detected in response to song playback or non-singing tutors or females, revealing a dopaminergic mesocortical circuit that plays an important role in the cultural transmission of vocal behavior.

THE FUTURE: OUTLOOK OF GENETICALLY ENCODED DOPAMINE SENSORS

We are currently experiencing an exciting era for DA detection. The introduction of genetically encoded sensors has revolutionized the approach to neuroscience studies, allowing for investigation of both physiological and pathological conditions. However, there is an urgent need for further optimization of current DA sensors to improve sensitivity and achieve a better SNR *in vivo*. Additionally, current DA sensors still face challenges in terms of multiplex imaging and obtaining quantitative measurements. Addressing these limitations will be a crucial focus for future research in this field (Fig. 2).

Higher sensitivity

While DA sensors are frequently used in the striatum, which has a high density of dopaminergic projections, their sensitivity is currently still inadequate, which limits their applicability in brain regions with sparse DA innervation, such as the amygdala, mPFC, and hypothalamus (Björklund and Dunnett, 2007; Aransay et al., 2015). The low SNR of recorded signals in these areas often necessitates multiple replicates to accurately confirm their reliability. Additionally, the lack of enough sensitivity may exclude the involvement of DA in behaviors where the release of DA is few but significant. Several factors affect the sensors' SNR, including dynamic range ($\Delta F/F_0$), brightness, and affinity. *In vitro* comparison has

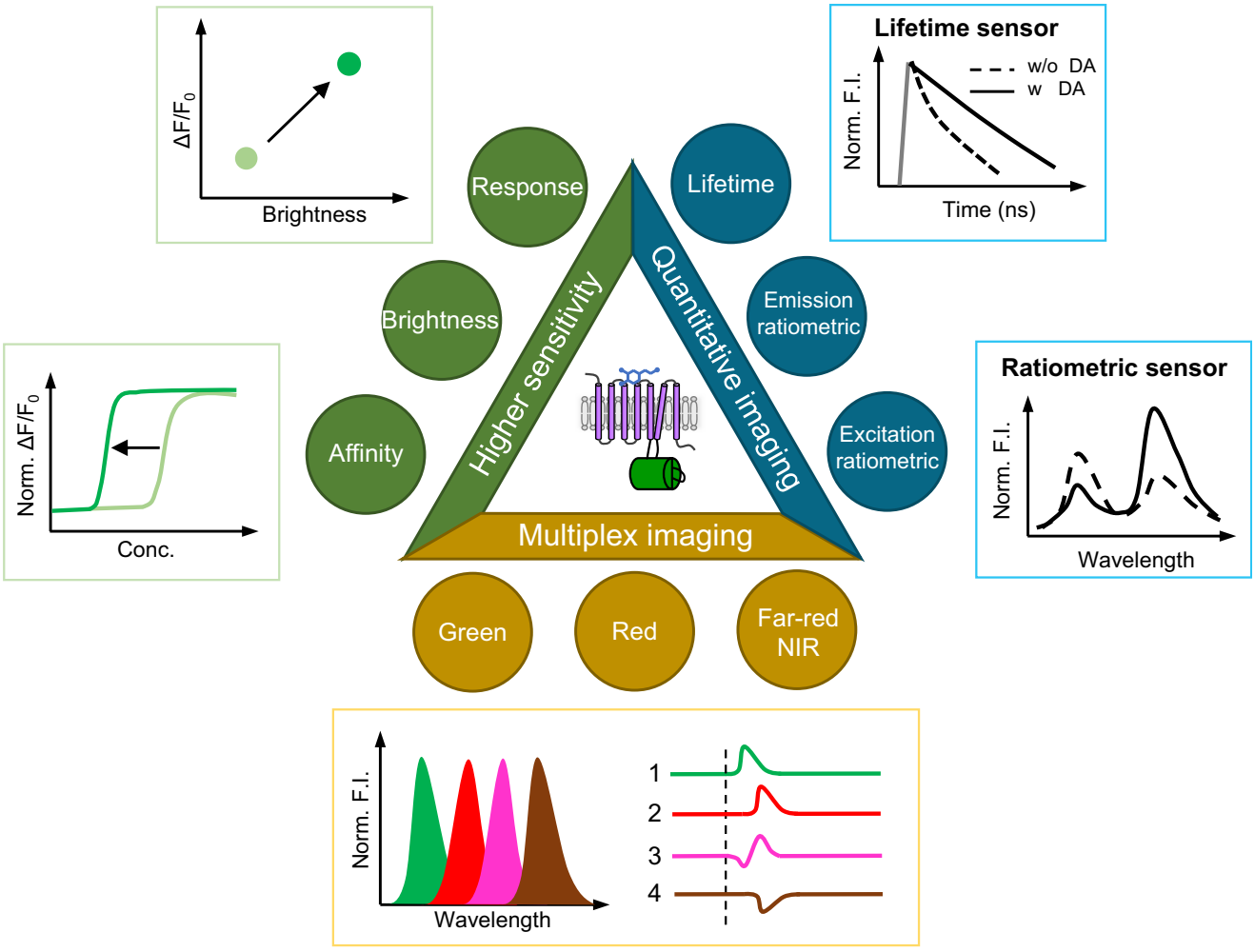

Fig. 2. Proposed future directions for dopamine sensor optimization. Three potential directions for optimizing dopamine (DA) sensors are suggested to enhance their sensitivity, enable multiplex imaging, and facilitate quantitative imaging. To enhance the sensitivity of DA sensors, it is important to optimize various factors including sensor response dynamic range, brightness, and affinity during sensor screening. To enable multiplex imaging, the spectrum of current sensors could be further red-shifted to the far-red and near-infrared (NIR) ranges. Finally, real-time quantitative DA detection could be achieved through the creation of a DA sensor with excitation / emission ratiometric properties or a sensor that undergoes a change in fluorescent lifetime upon binding with DA.

shown that the green $GRAB_{DA}$ sensors have a maximum $\Delta F/F_0$ around 340% and high affinity (10–100 nM), while the dLight sensors have higher $\Delta F/F_0$ but much lower brightness and affinity (over 1 μM). Compared to widely used GECIs that have undergone many iterations and show over 30-fold ΔF/F0 (Chen et al., 2013; Dana et al., 2019), the maximum $\Delta F/F_0$ of DA sensors still has large room for improvement. To reliably detect sparse DA release, high affinity and brightness are also necessary, in addition to $\Delta F/F_0$ improvement.

An efficient high-throughput screening platform can accelerate the optimization of DA sensors. Currently, the GPCR-based sensors can only be screened in mammalian cells, following a workflow of mutation library generation, cell transfection and fluorescent imaging. More efficient methods including ultra-widefield imaging combined with phenotype-activated cell sorting or the CRISPR/Cas9-based directed evolution have been applied to evolve voltage sensors or FPs (Piatkevich, 2018; Griesbeck, 2021; Tian et al., 2023. These methods can be adapted, in principle, for screening DA sensors. Furthermore, the performance of FP and sensors under the one-photon or two-photon microscope are poorly correlated (Adhikari et al., 2021; Liu, 2022), as they are governed by different photophysical mechanisms. Therefore, improving the two-photon performance of DA sensors requires a two-photon imaging based screening system which has been established for optimization of voltage sensors and glutamate sensors (Liu, 2022; Aggarwal et al., 2023).

Multiplex imaging

DA functions in conjunction with other neurotransmitters to regulate many higher brain functions, such as cognition and emotion. Many pathological conditions, including Parkinson's disease, are associated with alterations in DA and several other neurotransmitters (Barone, 2010; Lövheim, 2012). In addition, as previously mentioned, the regulation between DA and other neurotransmitter systems is highly ubiquitous and physiologically relevant (Lovinger et al., 2022). The development

of green and red neurotransmitter sensors has already enabled dual-color imaging of two neurotransmitters (Patriarchi et al., 2020; Sun et al., 2020). To utilize the entire color spectrum and perform multi-color *in vivo* imaging to monitor several neurotransmitters simultaneously, it is necessary to expand the sensor's spectrum further, particularly extend the wavelength to a range of far-red (650–700 nm) and near-infrared (NIR; >700 nm). Importantly, far-red/NIR sensors can also increase imaging depth, with reduced background and phototoxicity (Hong et al., 2017; Karasev et al., 2019).

Circularly permutated far-red/NIR FPs are a preferred consideration for the development of far-red/NIR DA sensors. Two far-red cpFPs, cpmKelly2 and cpMaroon, have been created and used for development of calcium and zinc ion sensors, respectively (Dalangin et al., 2020; Wu et al., 2023), providing potential fluorescent modules for DA sensors. In addition to the GFP-like cpFPs, many biliverdin-binding NIR FPs have also been developed (Shcherbakova et al., 2018; Zhou et al., 2020) and applied for the development of calcium sensors, either in a split form or based on FRET (Qian et al., 2019; Qian et al., 2020; Shemetov et al., 2021). However, unlike GFP-like FPs, these NIR FPs are difficult to circularly permutate effectively. It is worth noting that these existing far-red/NIR FPs suffer from low brightness, which can pose a challenge for the development of sensitive DA sensors.

The chemigenetic strategy provides an alternative approach for development of far-red/NIR DA sensors. Compared to FPs, fluorescent chemical dyes generally offer superior brightness and photostability. Moreover, they can covalently bind with self-labeling proteins through a specific ligand, such as HaloTag (Los, 2008; Gautier and Tebo, 2020; Cook et al., 2023). Some dyes can even cross the blood–brain barrier to achieve *in vivo* brain labeling (Abdelfattah et al., 2019, 2023; Grimm et al., 2020). Recently, researchers have exploited the circularly permutated HaloTag and context-sensitive chemical dyes to engineer far-red calcium sensors and voltage sensors (Wang et al., 2020; Deo et al., 2021). These advances suggest that the chemigenetic strategy has the potential to facilitate the development of far-red/NIR DA sensors.

Quantitative imaging

Current DA sensors mostly monitor fluorescent intensity, but they are often influenced by various factors, such as fluorophore concentration, excitation intensity and photobleaching. These factors limit the accuracy and precision of the measurements and make it difficult to obtain a quantitative measurement of DA levels. While these sensors can report relative changes in DA levels, they cannot provide absolute measurements in concentration, which are crucial for studying DA functions. On the one hand, quantifying DA levels in real time will provide critical insights into the specific physiological role of both phasic and tonic release (Gonon, 1988), the latter of which is difficult to be measured by current DA sensors. On the other hand, different DA receptors, such as D1R and D2R, have significantly different affinities for DA (over 10-fold) and regulate downstream signals in opposite ways to exert distinct functions (Calabresi et al., 2014; Cox and Witten, 2019). Thus, to better correlate DA levels with different physiological conditions, tools that can provide information on absolute concentration are required.

The development of a ratiometric DA sensor holds promise for achieving this goal. Ratiometric methods were applied to measure intracellular calcium concentrations as early as the 1990s (Grynkiewicz et al., 1985). Ratiometric sensors can be categorized into two groups: emission ratiometric and excitation ratiometric. Emission ratiometric sensors can be relatively easy to engineer by fusing another spectrum-distinct FP to existing sensors, even without FRET occurring (Ast et al., 2017; Kim et al., 2022). However, they typically require two channels, which limits multiplex imaging. Additionally, the different mature rates and scattering properties of two FPs may affect accurate quantification. In contrast, the excitation ratiometric sensors are usually based on a single FP and can be engineered by tuning the 405 nm-excited response of green sensors or endowing long Stokes shift property on red sensors (Zhao et al., 2011; Kim et al., 2022).

Fluorescence lifetime has immense potential for use in quantitative measurements. It refers to the average time that a fluorophore remains in its excited state and can be measured quantitatively using fluorescence lifetime imaging microscopy (FLIM) (Yasuda, 2006; Becker, 2012; Datta et al., 2020). As fluorescence lifetime is an intrinsic property of the fluorophore, it is independent of intensity-related factors and has been utilized to measure small intracellular molecules such as calcium (Zheng et al., 2015; van der Linden et al., 2021), lactate (Koveal et al., 2022) and cAMP (Massengill et al., 2022). However, existing NT/NM sensors are primarily screened on fluorescent intensity and exhibit a minimal change in fluorescence lifetime upon ligand binding (Ma et al., 2022). Therefore, there is a need to optimize existing sensors or design new ones that can take advantage of the benefits of fluorescence lifetime for quantifying DA levels.

AUTHOR CONTRIBUTIONS

All authors contributed equally to all aspects of the manuscript.

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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