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REVIEW ARTICLE

State-of-the-art: functional fluorescent probes for bioimaging and pharmacological research

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Cardiovascular diseases, neuropsychiatric disorders, and cancers seriously endanger human health. Mechanistic and pharmacological mechanisms of candidate drugs are central to the translational paradigm. Since many signal transduction and molecular events are implicated in these diseases, a novel method to interrogate the key pharmacological mechanisms is required to accelerate innovative drug discovery. Much attention now focuses on the real-time visualization of molecular disease events to yield new insights to the pathogenesis of the diseases. This review focuses on recent advances in the development of chemical probes for imaging pathological events to facilitate the study of the underlying pharmacodynamics and toxicity involved. As reviewed here, optical imaging is now frequently viewed as an indispensable technique in the field of biological research. Promoting interdisciplinary collaboration among chemistry, biology and medicine, is necessary to further refine functional fluorescent probes for diagnostic and therapeutic applications.

Keywords: fluorescent probe; bioimaging; pharmacological research; molecular events

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INTRODUCTION

Cardiovascular diseases, neuropsychiatric disorders, and cancers remain the major global causes of death and the major burden on public healthcare systems [1–4]. Accordingly, much research is focused on identifying and validating novel drug targets [5]; however, most aspects of the links between disease etiology and progress and the development of clinical symptoms remain unknown [6–8]. Studying the signal transduction process is necessary to clarify the links, but this is highly challenging. Indeed, identifying the molecules involved in intracellular signal transduction is only the first step [9–11]. This must be followed by a study of the concentration and location of these signaling molecules in their native environments to reveal the operation of the signal transduction cascades and their key points that could be a focus for drug discovery and development [12–14].

Biological signaling molecules have been identified by biochemical assays; however, these traditional methods are limited by their time-consuming operation, nonspecific and low test volume. Novel tools to clarify the key molecules that link the pathological cascade are becoming a new research focus [15], for example, the development of fluorescent sensors. More recently, chemistry has been integrated with biology to provide chemical fluorescent probes capable of tracking biological events [2, 4, 16]. These novel chemical biology tools may even deliver tailored therapeutic agents while the targets are imaged to permit a real-time analysis of the drug/target interaction.

This review will focus on recent advances in the development of chemical probes for imaging key molecules involved in pathological events. Functional fluorescent dyes have been established that are nondestructive and relatively easy to use. Some probes are even capable of the real-time tracking of pathophysiological events with a high spatial resolution to detect specific molecular steps in signal transduction. Fluorescent probes are also becoming indispensable tools for acquiring important information in biological systems to explore the nature of various pathological processes and in the development of high-throughput assays. Thus, these probes have an important role in the design, screening and validate novel drug targets.

PATHOLOGY AND TOXICOLOGY OF MOLECULAR EVENTS AND THEIR IMPLICATIONS

Pharmacology and toxicology are the key disciplines for new drug development to provide the necessary information on the pharmacological efficacy, mechanisms, toxicity, and clinical applications. Fluorescent probes are valuable on the evaluation of each of these steps.

The link between signaling pathway and disease pathway analysis illuminates the mechanism of pharmacology and toxicology. Cell signal transduction refers to the passage of information molecules through the cell membrane, intracellular receptors or biochemical reactions. This information is converted by intracellular signal transduction systems, thereby affecting cell function [17]. A defect in molecular signal transduction often forms the basis for the aberrant expression or localization of biomolecules (proteins, lipids, nucleic acids and small metabolites) in the pathogenic process of diseases [18–21]. These biomolecules are diverse and greatly adding to the complexity of designing appropriate fluorescent probes. Signaling pathways, such as a G protein-coupled receptor (GPCR), nuclear receptor, MAPK/ERK, the

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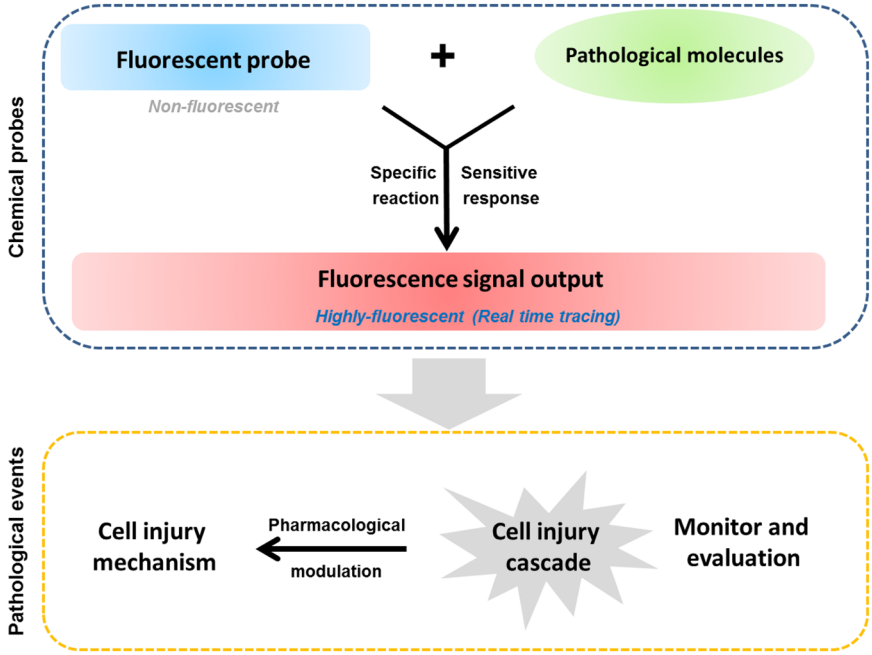

Fig. 1 The schematic figure illustrates the design strategy for using fluorescent probes to evaluate intracellular signaling cascades

transcriptional or ubiquitin-proteasome pathway, are frequently targeted [22–24]. For example, a loss of control over cell cycle, apoptosis, growth, and signal transduction pathways, such as Wnt or MAPK signaling, implicates the hallmarks of cancer and metastasis [25]. Targeting specific signaling pathways has enabled the discovery of many marketed drugs, such as Bcr/Abl kinase inhibitors (imatinib mesylate and dasatinib) or inhibitors of interleukin 1 (IL-1), which have been used for the clinical treatment of rheumatoid arthritis [26]. Identifying the signaling pathway panel in live cells provides important information about the laws and mechanisms of the underlying transduction process, which is required for developing targeted drugs. Marcelo Behar and colleagues pioneered the development of stimulus-specific drug targeting in the IKK-NFκB signaling hub, both in silico and in vivo. They concluded that the dynamics of molecules at the pleiotropic signaling hub may serve as a pharmacological target [27]. Such breakthrough technology platforms are required to establish a signaling pathway panel strategy for drug discovery.

USING FLUORESCENT PROBES TO EVALUATE INTRACELLULAR SIGNALING CASCADES

The visualization of biological events can provide critical insights to clarify pharmacological and toxicological mechanisms. Chemical fluorescent probes are now used widely to locate and quantitate key biomolecules. The method utilizes a small-molecule fluorescent probe as a detection agent. It must be capable of undergoing a specific chemical reaction with a target molecule in a complex biological environment to provide a unique alternation of fluorescence signal (such as intensity and color), which indicates the functional response of the target biomolecule. Chemical fluorescent probes are generally nondestructive and have excellent sensitivity, high selectivity, low cytotoxicity, and time-efficient. Accordingly, they are highly desirable for the real-time tracking of trace metabolites in live cells to monitor pathophysiological processes and to study the role of biomolecules both in vitro and in vivo. Classical approaches to study the function/activity of cell signaling transduction mainly rely on biochemical endpoint assays, which do not allow for characterization of their dynamic activity with a high spatial resolution in their native environment. The development of fluorescent probes has provided a whole new avenue of research for studying cell signaling transduction and regulation in living cells in real-time with high spatial and temporal resolution [28–30]. The use of fluorescent probes to identify pathway and cell signaling analysis, including reactive oxygen and reactive nitrogen species (ROS/RNS) detection [30, 31], cell viability and apoptosis [32, 33], and organelle functional dynamics [34, 35], is well established (Fig. 1). For instance, 2,3-naphthotriazole (NAT) serves as a powerful imaging tool to monitor the generation of nitric oxide (NO) and superoxide, which trigger nitrosative stress [36]. Fluorescence probes are practical tools to detect specific molecules involved in signal transduction. They are becoming indispensable for understanding pathological and toxicological cellular processes.

EXISTING PROBES FOR DETECTING MOLECULAR EVENTS IN PATHOLOGY AND TOXICOLOGY

Probes for the detection of energy metabolism and metabolites

The normal homeostasis of living organisms relies on the balance between cellular energy intake and cellular energy expenditure. A comprehensive study of the metabolic network requires the use of sensitive fluorescent probes. Adenosine triphosphate (ATP) is the principal energy source for cellular metabolism. Kim et al reported a novel fluorescent method to measure cellular ATP based on a dansyl fluorophore and a long cetyl chain [37]. The probe forms self-assembled, micelle-like aggregates at low concentrations in aqueous solution that can selectively recognize ATP among various bioactive anions and can be detected by an enhancement in fluorescence emission to image cellular ATP in live cells. Wang and colleagues developed an affinity profiling strategy by taking advantage of the quantitative capability of an isotope-coded probe [38]. This strategy revealed not only information about novel ATP-binding proteins but also a previously unknown ATP-binding site. Furthermore, a novel, dual-function, fluorescent probe has been developed and shown capable of detecting the two hypoxia-sensitive species nitroreductase and ATP [39].

Recently, an ADP-selective fluorescent probe that can monitor and quantify the consumption/production of ATP/ADP during phase I of glucose metabolism was identified [40]. The probe can

indicate the biological and medicinal significance of glucose metabolism through monitoring the enzymatic phosphorylation of glucose, the phosphorylation of fructose-6-phosphate, the formation of 3-phosphoglycerate and the conversion of phosphoenolpyruvate to pyruvate [40]. Additionally, Yue and colleagues developed a coumarin derivative that is a dual-site fluorescent probe for visualizing the metabolism of Cys and, thereby, quantifying the redox dynamic in living cells. This probe was validated by demonstrating a reversible response toward thiols with the addition of a thiol-scavenging reagent [41]. This may be a major advance in quantifying intracellular redox balance.

Probes for detecting ionic homeostasis

Changes in the intracellular ion concentration are a critical component of numerous diseases [2, 7, 42]. Pathological changes in intracellular Ca^{2+} homeostasis underline many pathophysiological signaling pathways. Two 1,2-bis(2-aminophenoxy) ethane-N, N,N′,N′-tetraacetic acid (BAPTA)-based fluorescent chemosensors for Ca^{2+} were developed by the Nobel Laureate Roger Y. Tsien in 1980. These probes greatly improve the selectivity and sensitivity of chemosensors [43] that have been a key step in advancing calcium signaling research. Various Ca^{2+} fluorescent probes based on this theory have been developed, greatly expanding the choice in photophysical properties and organelle-targeting ability [44]. Probe 1 was developed for the visualization of cytoplasmic Ca^{2+}, which is suitable for multicolor imaging for the simultaneous detection of metal ions or proteins in the monitoring of cytoplasmic Ca^{2+} oscillation in cultured cells [45]. The Ca^{2+}-sensitive fluorescent probe fura-2 [46]. has been used to detect the anti-inflammatory compound NPC-14686, which can induce a Ca^{2+} increase, and the inhibition of phospholipase C can abolish NPC-14686-induced Ca^{2+} rise. This has illuminated a pathway whereby NPC-14686 induces cellular Ca^{2+} releases from the endoplasmic reticulum and Ca^{2+} stores via protein kinase C-regulated Ca^{2+} channels [46].

Potassium ion (K^+) is the most abundant cellular metal cation and, thereby, plays essential roles in cardiac and neuronal excitability and cellular ionic homeostasis. Photoluminescent Carbon dots have been used as a fluorescent probe for the label-free detection of the potassium level in serum and red blood cells [47]. This fluorescent probe was reported to have an outstanding selectivity for K^+/Na^+ and to be a suitable fluorescent tool to measure the physiological level of K^+ levels in the range of 10–80 mmol/L in vitro [48]. Another probe, TLSHalo, is useful to examine the K^+ transition in cells with high resolution and sensitivity, capable of visualizing dramatic changes of K^+ in cells [49]. The fluorescent signal generated by TLSHalo increased abruptly in the presence of ionomycin, whereas it was abolished by preincubation with the cytosolic Ca^{2+} chelator BAPTA-AM or the selective BK channel inhibitor iberiotoxin (IBTX) [49].

PROBES RELATED TO CELL DAMAGE

Redox signaling and oxidative stress

Oxidative stress refers to an imbalance between intracellular free radicals and antioxidants, which are linked to a myriad of pathologies. The dynamic cellular change of ROS provides abundant physiological and pathological information [30]. Therefore, analytical methods for ROS have received increasing attention.

Redox chemistry underlies much of the progression of vascular diseases [7, 9, 50]. Hydrogen peroxide (H_2O_2) is generated by superoxide spontaneously and is a primary product of NADPH oxidase/NOX4 following dismutation (or partitioning) by superoxide dismutase [51]. Accumulating evidence shows that H_2O_2 induces a severe oxidative stress that leads to cell injury [50, 51]. Recent findings have implicated it in pro-survival cell signaling [52]. In addition to vascular disease, abnormal concentrations

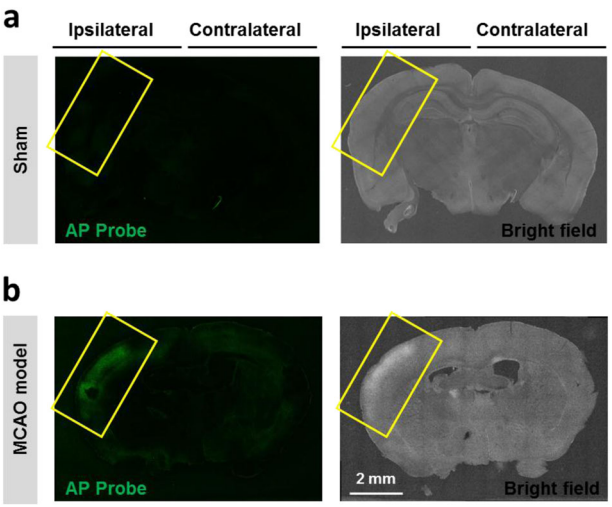

Fig. 2 Visualizing endogenous H_2O_2 formation using a functional fluorescent probe in a mouse with brain ischemia. Adapted from Ref. [52]

of H_2O_2 may accelerate aging, Alzheimer's disease, and cancer [53, 54]. The gold standard for H_2O_2 detection mainly depends on electrochemical methods, electron spin resonance (ESR) and HPLC. By contrast, fluorescence probes have the advantages of high sensitivity, good selectivity, and suitability for live cell detection [55–57]. Following the pioneering report of the use of arylboronate-based fluorescent probes for imaging H_2O_2 in live cells [58], various detection methods have been developed. Theranostic probe AP established new experimental strategies that not only detects H_2O_2 with high specificity but is also capable of reducing the H_2O_2–initiated oxidative damage (Fig. 2). Cells normally emit only a low level of fluorescence, but during H_2O_2 overproduction, the bond between the aspirin moiety and the fluorophore is cleaved, releasing both to fulfill their therapeutic or signaling function. The protective effect of probe AP against H_2O_2-induced endothelial cell apoptosis was confirmed by reduced phosphorylation of JNK, ERK, and p38 in H_2O_2-AP-treated cells [52]. Notably, the probe also protected the vasculature from thrombotic damage in zebrafish [52].

MitoHCy-NH2 [59] is a fluorescent probe that can detect the activity of Monoamine oxidase (MAO)-B and the byproduct ROS in replicative aging models. MAO-B and its catalytic production of ROS recognized as an important biomarker for maintaining biogenic amine homeostasis. The pharmacological treatment of pargyline or selegiline could inhibit MAO-B activity as demonstrated by obviously decreasing the fluorescence intensity of the probe. These studies illustrate the use of fluorescent probes for efficacy evaluation and drug screening [59].

Nitrosative stress

Peroxynitrite ($ONOO^-$) is an endogenous reactive molecule that is highly oxidative. It is formed by the diffusion reaction between nitric oxide (NO) and the superoxide radical ($O_2{\bullet}^-$). Due to being extremely active, $ONOO^-$ and its secondary radicals (•OH, CO_3•Pyrene, and NO_2) can react with proteins, DNA, lipids, and amino acids, eventually leading to cell death [1, 5, 60]. $ONOO^-$ is highly challenging to detect, which has limited its understanding. Aminophenyl fluorescein [61] and hydroxyphenyl fluorescein are two fluorescent probes for $ONOO^-$ detection [62]. Tests are limited by poor selectivity for $ONOO^-$ against •OH or ClO^- [61, 63]. $ONOO^-$ can oxidize phenols to quinines, Yang and colleagues developed a series of fluorescent probes with high selectivity based on this reaction [64–66]. These authors also developed PN600, which differentiates hypochlorite from $ONOO^-$ through a multichannel

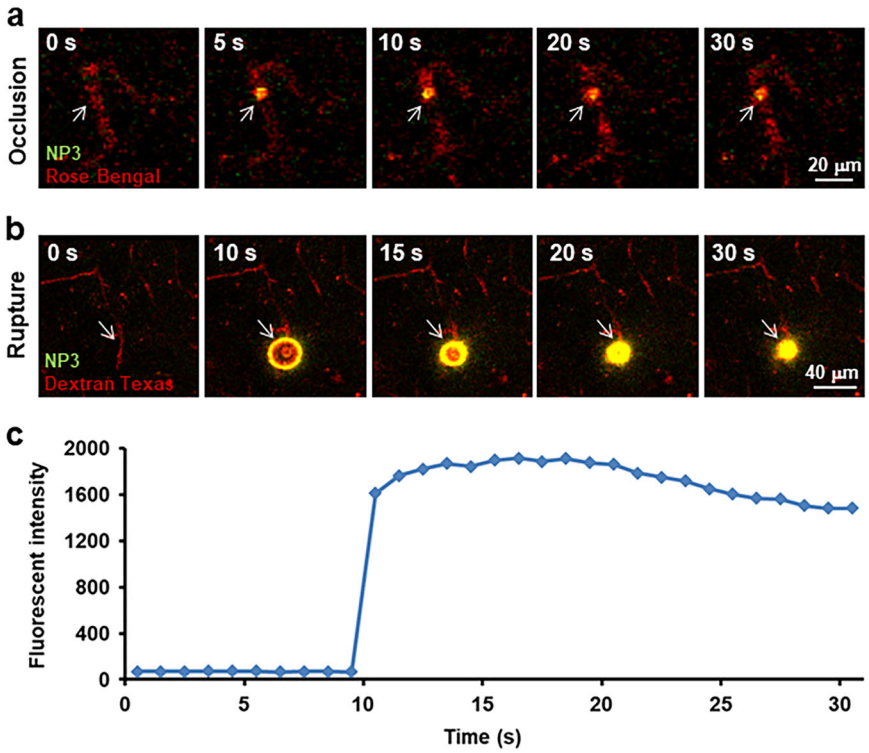

Fig. 3 Real-time visualization of endogenous $ONOO^-$ fluxes after brain microvessel injury with a combination of the NP3 probe and in vivo two-photon laser scanning microscopy. Adapted from Ref. [15]

signaling mechanism [67]. To track the in situ production of $ONOO^-$ during acute brain vascular injury, one group has recently reported that NP3 is a two-photon, fluorescent, "switch-on" probe based on a brain-blood-barrier (BBB)-permeable fluorophore. The oxygen-glucose deprivation-induced increase of NP3 fluorescence was reduced in the presence of either the calmodulin inhibitor or BAPTA, suggesting its specificity for monitoring $ONOO^-$ fluxes during ischemia [15]. The good BBB permeability and two-photon absorption provide the probe with excellent temporal and spatial resolution to track $ONOO^-$ in live mice after ischemia-induced neurovascular damage [15] (Fig. 3). Low-glucose stress induced a pronounced increase in the formation of $ONOO^-$, which is confirmation of its sensitivity for detecting nitrosative stress [50]. This signal was abrogated by melatonin treatment [50]. Therefore, this study led to the finding that dysfunction of the TP53-induced glycolysis and apoptosis regulator (TIGAR) resulted in tight junction injury during low-glucose injury via the generation of $ONOO^-$. Based on the fluorescence resonance energy transfer (FRET) mechanism, Yuan et al developed a new two-photon, radiometric, fluorescent probe (MITO-CC) to image mitochondrial $ONOO^-$ [68]. MITO-CC exhibited highly specific targeting of mitochondrial $ONOO^-$ with a fast response rate (within 20 s) and high sensitivity (detection limit = 11.30 nmol/L) [68].

Mitochondria damage

Mitochondria-dependent cellular respiration is achieved by a chain of metabolic reactions that convert biochemical energy and O_2 into ATP. A disturbance of oxidative phosphorylation in mitochondria plays a critical role in the progression of human diseases. This disturbance usually initiates the apoptosis signaling pathway, leading to damage to proteins and nucleic acids [5, 52, 69, 70]; however, the complex link between mitochondrial damage and cellular pathology has not been fully elucidated. Methods for detecting dynamic changes in mitochondria damage have been helpful for elucidating the role of mitochondria in pathophysiology.

A fluorescent carbon-dot (C-dot)-based nanoprobe was developed that was then covalently conjugated to a mitochondrial-targeting moiety. The probe was successfully used to detect and image mitochondrial $ONOO^-$ [71]. Shchepinova and colleagues designed MitoNeoD as a new, dual-purpose, mitochondrial $O_2{\bullet}^-$ probe comprising a triphenylphosphonium lipophilic cation moiety, leading to a rapid mitochondria accumulation [72]. In addition, Han et al reported that Mito-diNO2 is a mitochondrial-targeting, near-infrared, radiometric, fluorescent probe [73] that can quickly detect the concentrations of selenocysteine (Sec) and disulfide (CS_2) in mitochondria. Sec elicits a protective role against CS_2-induced acute inflammation and liver damage [74]. Exposure to CS_2 activated ERK phosphorylation and other pro-inflammatory factors [75], while supplementation with Sec inhibited this pathway. This validates that the probe is able to monitor Sec treatment of CS_2 liver poisoning.

Rhodamine 123 (RH-123) fluorescence quenching kinetics can be used to evaluate the mitochondrial membrane potential ($\Delta\psi_{mit}$). It provides a method to study mitochondrial proton uptake through the F0-ATPase channel during ATP synthesis [76]. Mitochondrial-targeted redox probe (MitoRP), comprised of a TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy) moiety and coumarin 343, is probe that couples complex I and fluorescence when electron transport is blocked by treatment with rotenone [77].

Autophagy pathway

Macroautophagy is a self-degradative process that delivers cytoplasmic components to lysosomes for degradation and recycling [1, 7, 78]. Measuring autophagic activity is critical for dissecting the molecular mechanism and function of autophagy but remains challenging due to the lack of a definitive method [79]. By staining autophagosomes as they are being formed, chemical probes provide a sensitive and quantitative method to evaluate autophagic flux in cultured cells and whole organisms, providing an advantage over conventional genetic approaches.

To assess the translational relevance in treating autophagy-related diseases, it is vital for the real-time and specific tracing of autophagy flux under the pathological context of diseases. MitoTracker Green (MTG) revealed an over two-fold increase of average mitochondrial digestion time after nutrient deprivation in the presence of protease inhibitors (pepstatin A, 5μmol/L) [80]. Pharmacological treatment with autophagy inhibitors suppressed the increase of LysoTracker Red (LTR) uptake, indicating that increased LTR uptake paralleled with autophagy induction. Further observations support the notion that mitochondrial autophagy involves the mitochondrial permeability transition via the PI3 kinase and JNK pathway [80].

Zn-G_4 can monitor late-phase autophagy or endosome/lysosome networks [81], since it stains autolysosomes selectively due to its high affinity for autolysosome-specific hydrophobic lipids or proteins [81]. Lyso-OC is a two-photon probe that detects changes in the polarity of lysosomes [82]. Indeed, the use of fluorescent probes have allowed the monitoring of the membrane dynamics of cellular autophagy without the need for genetic engineering to study autophagic flux [79]. Collectively, the fluorescence measurement and fluorescence imaging with the probe should be useful in screening and characterizing various agents acting on autophagic pathways.

Formaldehyde stress

Formaldehyde (FA) is produced endogenously and has been suggested to function as a physiological signaling molecule [83–85]. FA participates in process of cell proliferation and memory formation; however, excessive FA has an apoptotic influence on cells [4, 86].

Li's team has biochemically characterized a fluorescent probe (PFM) to detect FA in living cells with rapid detection kinetics and a reversible profile [4]. Probe PFM can track dynamic changes of endogenous FA in live cells and brain tissue with high spatial resolution. Aberrant FA was found to accumulate in the cortex and hippocampus of amyloid precursor protein (APP) transgenic mice [4], providing the direct evidence for the involvement of formaldehyde in the pathology of the AD. 1-(4-(1H-phenanthro[9,10-d]imidazol-2-yl)phenyl) but-3-en-1-amine (PIPBA) has been character as FA probe and used to demonstrate indirect oxidative toxicity [87]. The significantly higher fluorescent intensities of PIPBA indicate the excess elevation of FA in the presence of a radical initiator, 2,2-azobis[2-(2-imidazolin-2-yl) propane] dihydrochloride (AIPH, 1mmol/L) [87]. Consistently, remarkably weakened PIPBA fluorescence was observed following the pretreatment of zebrafish with the FA scavenger (HSO_3^-, 500μmol/L) [87]. The probe PFM was endowed with outstanding properties, including conformational restraint, a sensitive radiometric response, and lysosome targeting ability [88]. When combined with a pharmacological strategy, Liang and colleagues highlighted the promise of PFM [4] as a reliable tool for imaging native formaldehyde at the subcellular level, which is useful in exploring the pathological mechanism of FA in protein misfolding and the etiology of neurodegenerative diseases [88].

PROBLEMS AND OUTLOOK

New drug targets are needed for the development of therapies with novel molecular mechanisms of action to improve patient outcomes. Fluorescent probes are the cornerstones of chemical bioimaging. They can be used conveniently for the in situ real-time monitoring of target molecules and biological processes and will be useful in acquiring the necessary information on the pharmacological efficacy, toxicity, mechanisms, and clinical applications.

Despite many advances, remaining challenges include the specificity and sensitivity of the probes. Advancing the field of fluorescent probes requires a collaboration between chemical biology, pharmacological/toxicological sciences and clinical pathology. Furthermore, more research efforts should be directed at expanding the application of these probes to explore pharmacological/toxicological mechanisms and high-throughput screening assays, which would convey the translational impact, since it may provide insights into novel mechanisms and strategies for drug development.

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ADDITIONAL INFORMATION

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