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Review

Small-Molecule Fluorescent Probes for Visualizing Biological Processes: Nanoarchitectonics from Molecular Imaging to Clinical Theranostics

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Yue Chuai, Kewei Sun, Katsuhiko Ariga, Yazhou Chen,* and Linawati Sutrisno*

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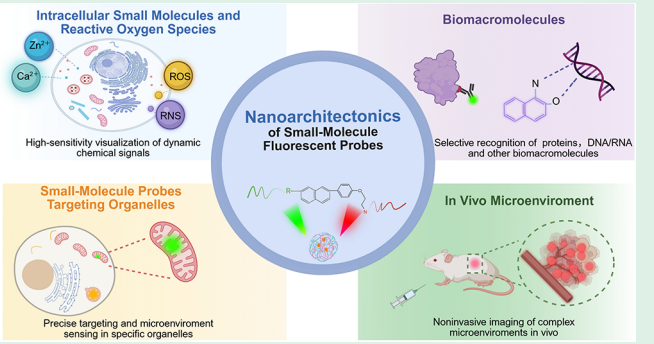
ABSTRACT: In recent years, molecular imaging techniques, particularly fluorescence imaging and photoacoustic imaging, have attracted increasing attention owing to their noninvasive nature, high sensitivity, and capability for real-time monitoring. Among various imaging agents, small-molecule fluorescent probes have emerged as powerful tools due to their high sensitivity, structural versatility, and compatibility with multiple imaging modalities. This review summarizes recent advances in the application of small-molecule fluorescent probes for molecular imaging, focusing on probe design strategies and imaging approaches targeting biomolecules, molecular events, and subcellular microenvironments. We further highlight their potential in disease diagnosis, fluorescence-guided surgery, and pharmacological evaluation. Overall, this review provides an overview of the unique advantages and broad applications of small-molecule fluorescent probes and discusses their prospects for precision medicine.



KEYWORDS: *small-molecule fluorescent probes, precision medicine, molecular imaging, fluorescence imaging, disease diagnosis, fluorescence-guided surgery*

1. INTRODUCTION

Humankind has continuously advanced diverse branches of chemistry, enabling the creation of increasingly sophisticated molecules and materials with broad functionality.[1−3] Simultaneously, developments in nanotechnology have provided unprecedented access to structures at atomic, molecular, and nanometer scales, allowing precise characterization of their properties and functions.[4−10] These advances have driven innovations in energy, environmental science, information technology, and medicine.[11−23] Among these fields, biomedical applications are particularly important because they directly contribute to understanding, diagnosing, and treating human diseases.

Recent progress in bio-recognition technologies and molecular imaging has enabled the detection of subtle changes in biomolecule concentrations, signaling pathways, and local microenvironments within living systems.[24−26] Such capabilities provide valuable insights into cellular metabolism, disease progression, and therapeutic responses. In particular, small-molecule fluorescent probes have emerged as versatile tools. This is attributed to their high sensitivity, structural tunability, and compatibility with diverse imaging modalities. For example, fluorescent chemogenetic labels have been widely used in recent years due to their high specificity and functional versatility. Green fluorescent protein (GFP), as a classic gene-encoded fluorescent marker, enables the direct visualisation of specific targets or the real-time monitoring of cellular events, thereby effectively addressing fundamental challenges in molecular and cell biology, such as imaging and detection.[27] These probes enable the visualization of biological processes with high spatial and temporal resolution, ranging from molecular interactions and subcellular dynamics to tissue-level pathological changes.

At the core of many recent advances lies the concept of nanoarchitectonics (Figure 1), which integrates molecular design, nanotechnology, and materials science to construct

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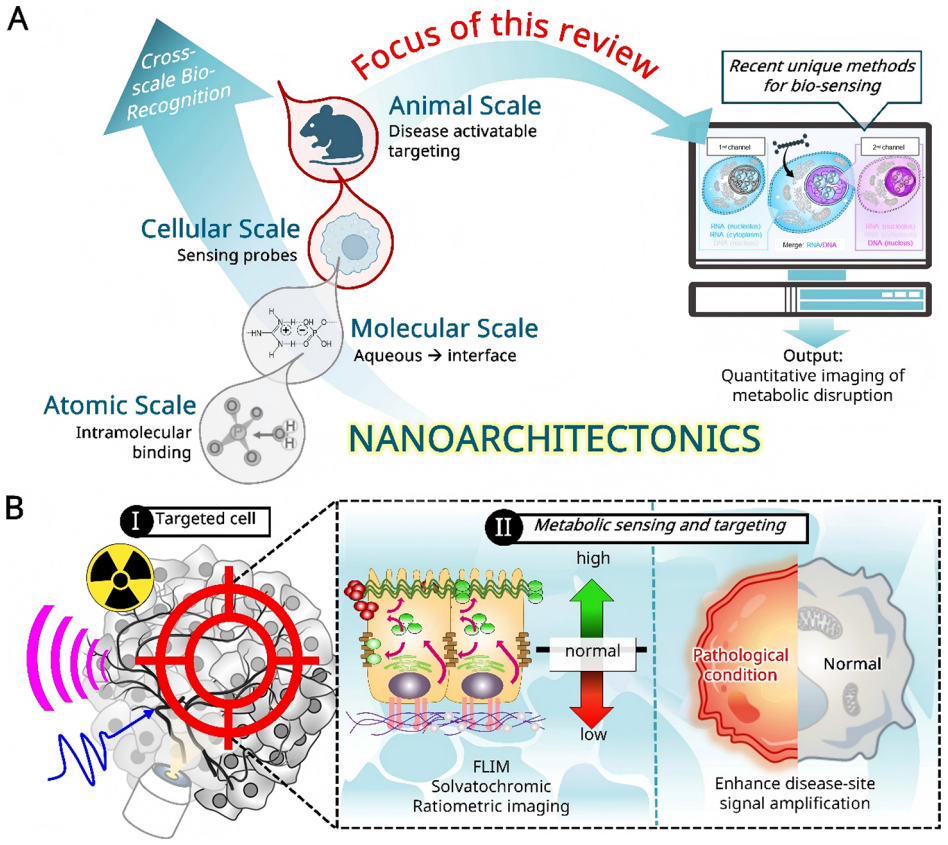

Figure 1. Summary of the broad scope of molecular recognition across multiple scales and its essential roles in enabling selective and sensitive biosensing applications. (A) Schematic overview illustrating the diverse scope of molecular recognition across different spatial and structural scales, ranging from the atomic scale to complex biological systems. (B) Representative applications in biosensing, including selective detection, signal transduction, and real-time monitoring of biological targets.

functional systems from atoms, molecules, and nanoscale building blocks.[28–36] From this perspective, fluorescent probes are not merely sensing molecules but carefully engineered systems that combine recognition units, signal transducers, targeting motifs, and delivery components to achieve selective biological information processing. Nanoarchitectonic design strategies have accelerated the development of probes that monitor biological events across multiple spatial scales and expanded opportunities for disease diagnosis, therapeutic evaluation, and precision medicine.

Against this backdrop, this review highlights recent advances in small-molecule fluorescent probes for visualizing biological processes and metabolic disruption across scales. We first discuss imaging strategies and probe design principles for detecting molecular targets, biomacromolecules, and subcellular microenvironments. Subsequently, we summarize emerging translational applications, including disease diagnosis, fluorescence-guided surgery, and pharmacological evaluation. Finally, we discuss current challenges and future opportunities for integrating molecular imaging, nanoarchitectonics, and precision medicine.

2. MOLECULAR DESIGN FOR FLUORESCENT IMAGING PLATFORM

To understand metabolic disruption across biological scales, imaging technologies must provide both spatial information and molecular specificity. Metabolic homeostasis is essential for cellular and organismal health, while its disruption is a hallmark of diseases such as cancer, neurodegeneration, cardiovascular disorders, diabetes, and inflammation.[37] Early detection of metabolic perturbations enables disease diagnosis before structural damage, monitoring of therapeutic responses, and investigation of cellular stress, apoptosis, signaling, and organ-level dysfunction.[38] Because metabolism spans from molecular assemblies to whole organs, biomedical imaging must cover multiple spatial scales.

Multiscale biomedical techniques are essential for detecting metabolic disruption because metabolic processes span from molecular assemblies to whole tissues.[39] At the organelle level, confocal microscopy remains an important toolbox in metabolic research.[40–42] It enables measurement of calcium signaling,[43] live-cell metabolic tracking,[44] localization of metabolic enzymes,[45] and monitoring of mitochondrial dynamics, such as fusion and fission.[46] Although its spatial resolution is limited to approximately 200 nm, it is well suited for studying organelle morphology, protein colocalization, trafficking, and inter-organelle communication. For metabolic studies requiring preservation of native ultrastructure, cryofixation has also gained increasing attention.[47]

Beyond the cellular level, tissue-scale imaging enables metabolic mapping across wider biological contexts. Techniques such as multiphoton microscopy and fluorescence lifetime imaging microscopy (FLIM) allow biologists to assess metabolic cofactors including NADH and FAD.[48] For whole-tissue and in vivo imaging, deeper penetration is required, and

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techniques including photoacoustic imaging, X-ray imaging, ultrasound, magnetic resonance imaging, and nuclear imaging provide complementary capabilities for mapping metabolic changes in intact organisms.[49−52] Together, these modalities bridge molecular, cellular, and organ-level metabolic alterations and facilitate the study of disease progression and therapeutic responses.

However, imaging platforms primarily provide the technical framework for visualization, whereas molecular probes determine the specificity and sensitivity of biological signal detection. Small-molecule fluorescent probes have therefore emerged as powerful tools for monitoring metabolic disruption with high sensitivity and spatiotemporal resolution. From a nanoarchitectonics perspective, organelle-targeted probes can be viewed as molecular systems that spatially integrate recognition units, fluorophores, and targeting groups, enabling selective processing and visualization of metabolic information.

3. PROBING BIOLOGICAL PROCESSES WITH SMALL-MOLECULE FLUORESCENT PROBES

While imaging modalities determine spatial resolution and penetration depth, molecular probes determine the specificity and sensitivity of biological detection. Consequently, the development of small-molecule fluorescent probes has become a central strategy for visualizing biological processes and metabolic disruption across multiple spatial scales. Through rational molecular design, these probes can convert otherwise invisible biochemical events into detectable optical signals.[53] In this section, we present selected examples of recent advances in small-molecule probes to detect intracellular activities, including small molecules, biomacromolecules, sub-organelles, and the microenvironment (Table 1).

3.1. Molecular Imaging Targets: Ions, ROS, Neurotransmitters

Trace ions and reactive species within cells are core participants in signal transduction and homeostasis maintenance. Small-molecule fluorescent probes can capture their transient changes with high sensitivity and selectivity.[54] For example, metal ion probes targeting Zn^{2+}, Ca^{2+}, Fe^{2+}, and Cu^{2+}, which contribute to neural signal transduction and apoptosis, have been widely developed to track the spatiotemporal dynamic distribution of these ions in real time.[55] As one of the earliest and most extensively investigated biomarkers, reactive oxygen species (ROS) participate in regulating physiological homeostasis and disease progression. Consequently, ROS-responsive fluorescent probes have become one of the most mature and representative categories in molecular imaging. Furthermore, the detection of reactive nitrogen species (RNS) and reactive sulfur species (RSS) is of paramount importance.[56] Molecules such as hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), nitric oxide (NO), and hydrogen sulfide (H_2S) are closely related to pathological processes including oxidative stress, inflammation, aging, and cancer; specific fluorescent probes serve as powerful tools for studying redox signaling pathways in these processes.[57,58]

Similarly, probes targeting neurotransmitters like dopamine and glutamate make it possible to monitor neuronal activity and synaptic transmission in real time. Li et al. have successively developed fluorescent probes for neurotransmitters such as acetylcholine, dopamine, norepinephrine, adenosine, serotonin, and neuropeptides. Such probes can accurately detect the dynamic changes of neurotransmitters in real time.[59] By utilizing methods such as transfection, viral injection, and the generation of transgenic animals, researchers can express these probes in various cultured cells, mouse brain slices, or model organisms like living Drosophila and mice. By rationally engineering nanostructures with specific functions, nanoarchitectonics can modulate probe accessibility, local concentration, intermolecular interactions, and the reaction microenvironment, thereby enhancing the sensitivity, selectivity, stability, and spatio-temporal resolution of fluorescent sensing systems. This converts chemical signals into intuitive, easily measurable fluorescence signals, which significantly simplifies the monitoring of dynamic neurotransmitter changes and provides a new pathway for future precision medicine and novel drug development.[60]

3.2. Biomacromolecules

Beyond the detection of small molecules, the application of fluorescent probes at the level of biological macromolecules and subcellular organelles possesses greater depth. By designing "Turn-on" response modes, researchers have developed a plethora of probes that can be illuminated by specific enzyme-catalyzed reactions. By organising and assembling probe molecules, recognition units, and functional components in an ordered and coordinated manner at the nanoscale, it is possible to construct sensing systems with specific spatial configurations and local microenvironments. This nanoscale structural control not only optimises the spatial alignment and intermolecular interactions between probes and biomacromolecules, but also enhances the probes' recognition capacity and signal response efficiency for specific proteins, nucleic acids, and other

Table 1. Comparative Analysis of Representative Small-Molecule Fluorescent Probes for Biological and Metabolic Imaging

probe	target	response mode	targeting strategy	imaging depth	major advantages	major limitations
NucATP	ATP	turn-on	nuclear targeting	cellular	high selectivity; nuclear ATP imaging	limited deep-tissue application
Te-BOD	GSH	activatable	ER targeting	cellular	ER localization; high selectivity	potentially limited in vivo penetration
DPXBI	viscosity	turn-on	microenvironment-responsive	tissue	early liver-injury detection	quantitative accuracy affected by environment
Hcy-BOH/Cy-Dopa	H_2O_2	ratiometric	lesion-responsive	tissue	reduced concentration/depth interference	more complex signal analysis
ANB-SHQ-6-pep	ClO^-	activatable	BBB/peptide targeting	deep tissue	BBB penetration; NIR-II imaging	more complex molecular architecture
FANQ	tumor biomarkers	dual-activatable	tumor targeting	in vivo	high tumor specificity	multi-step activation

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biomacromolecules by modulating local polarity, hydrophilicity and hydrophobicity, steric hindrance, and molecular enrichment effects.[61] This not only maps the spatial distribution of enzymes but also enables real-time monitoring of their activity changes, exhibiting tremendous research value in the study of tumor invasion and inflammation.[62] Yao's team successfully developed a series of two-photon (TP) small-molecule fluorescent probes capable of sensitively detecting general nitroreductase activity in various biological tumor models. Compared to other probes, these features include rapid response, high sensitivity, and excellent selectivity, and they are widely used for evaluating the tumor microenvironment.[63] Overall, biomolecularly activated fluorescent probes demonstrate exceptional specificity and signal amplification capabilities, making them powerful tools for disease diagnosis and image-guided surgery. Nevertheless, increasing imaging depth and reducing off-target activation remain important areas of research.

3.3. Organelle-Targeted Metabolic Imaging

Building upon molecular-level imaging, subcellular organelles provide another important dimension for understanding intracellular functions. Fluorescent probes capable of targeting specific organelles have therefore become indispensable tools for elucidating cellular physiology and pathology. At the same time, nanoarchitectonics can enhance the interaction between probes and organelle membranes, membrane proteins, or specific biomolecules by creating nanostructures with specific interfacial properties and local microenvironments, thereby promoting the selective enrichment of probes within the target organelles. This enables in situ monitoring of microenvironmental changes (such as pH and viscosity) and functional states within specific organelles, thereby elucidating the pathogenesis of related diseases and promoting disease prevention (Figure 2A,B).[64,65] For instance, Tamima and co-workers reported the first

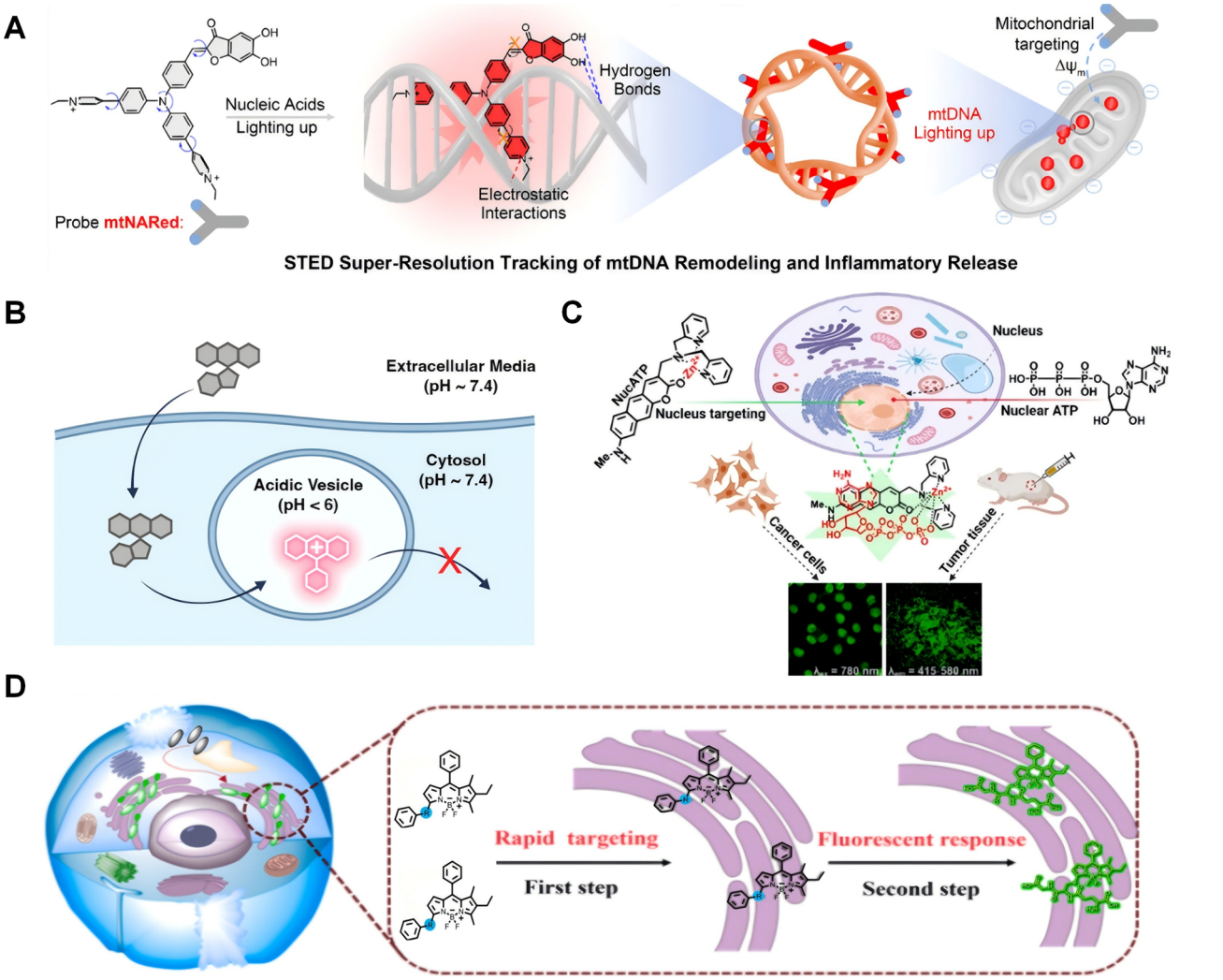

Figure 2. Small-molecule fluorescent probes targeting different organelles. (A) mtDNA-selective probe for super-resolution imaging that enables nanoscale visualization and long-term tracking of mtDNA in living cells. Adapted with permission from ref 65. Copyright 2026, John Wiley and Sons Ltd. (B) Graphic shows the strategy for targeting a pH-dependent HMSiR dye to acidic organelles. Adapted with permission from ref 97. Available under CC-BY-4.0 license. Copyright 2024, American Chemical Society. (C) Probe, a Zn^{2+} complex of a push-full type benzocoumarin dye bearing a dipicolylamine ligand, reversibly binds to nuclear ATP with a turn-on fluorescence response. Adapted with permission from ref 66. Copyright 2023, John Wiley and Sons Ltd. (D) Verification of the cell imaging targeting mechanism of the ER. Adapted with permission from ref 67. Copyright 2025, Elsevier.

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small-molecule fluorescent probe NucATP that targets the cell nucleus. This probe binds reversibly to ATP at pH 7.4, Compared with conventional cytoplasmic ATP probes, NucATP provides an additional spatial dimension by selectively reporting nuclear ATP dynamics. It can be applied to distinguish tumor tissues from normal tissues and to investigate nuclear ATP-involved biological processes as well as energy dynamics during the cell cycle (Figure 2C).[66] However, its demonstrated application remains primarily at the cellular and tissue levels, highlighting the challenge of translating organelle-targeted probes to deep-tissue imaging. Yuwen et al. constructed a novel fluorescent probe, Te-BOD, featuring a high response threshold and endoplasmic reticulum targeting. This probe elevates its response threshold toward glutathione (GSH) through the phenyltellurium group and realizes precise targeting to the endoplasmic reticulum by virtue of high lipophilicity, offering a reliable strategy for accurate diagnosis in tumor tissue biopsy and intraoperative image-guided resection (Figure 2D).[67] Collectively, organelle-targeted fluorescent probes have enabled precise visualization of intracellular biochemical events. Future development should emphasize multiplex organelle imaging and real-time monitoring of interorganelle communication.

3.4. Subcellular Microenvironments

Apart from certain biomolecules and subcellular organelles, cellular life activities rely on the stability of physicochemical parameters within their internal microenvironment. Small-molecule fluorescent probes can visualize these parameters. pH probes can intuitively display minute fluctuations in intracellular pH and are particularly widely used in revealing the acidic characteristics of the tumor microenvironment (Figure 3A).[68,69] To this end, Tang's research team designed a highly efficient cascade C–H activation polymerization strategy. This polymer exhibits dual-state strong fluorescence in both solution and aggregated states, and the photoluminescence properties of the material can be conveniently regulated through protonation-deprotonation processes under acidic and basic conditions, successfully applying it to intracellular pH detection. Furthermore, through a simple N-methylation reaction, the polymer can be converted into a novel fluorescent conjugated polyelectrolyte. These polyelectrolytes can efficiently kill cancer cells through the synergistic effect of dark toxicity and phototoxicity.[70]

Additionally, processes such as apoptosis and senescence are often accompanied by a significant increase in cytoplasmic viscosity; highly sensitive viscosity probes provide a powerful visualization tool for this (Figure 3B).[71,72] In eukaryotes, the abnormal accumulation of undegraded biological waste in lysosomes is pathogenic, leading to lysosomal storage diseases (LSDs).[73] An increase in mitochondrial viscosity can reduce the activity of the electron transport chain, promote the release of cytochrome C, and ultimately elevate the risk of atherosclerosis and malignant tumors; utilizing small-molecule fluorescent probes to monitor abnormal increases in viscosity can assist in the diagnosis and treatment of these diseases, For instance,

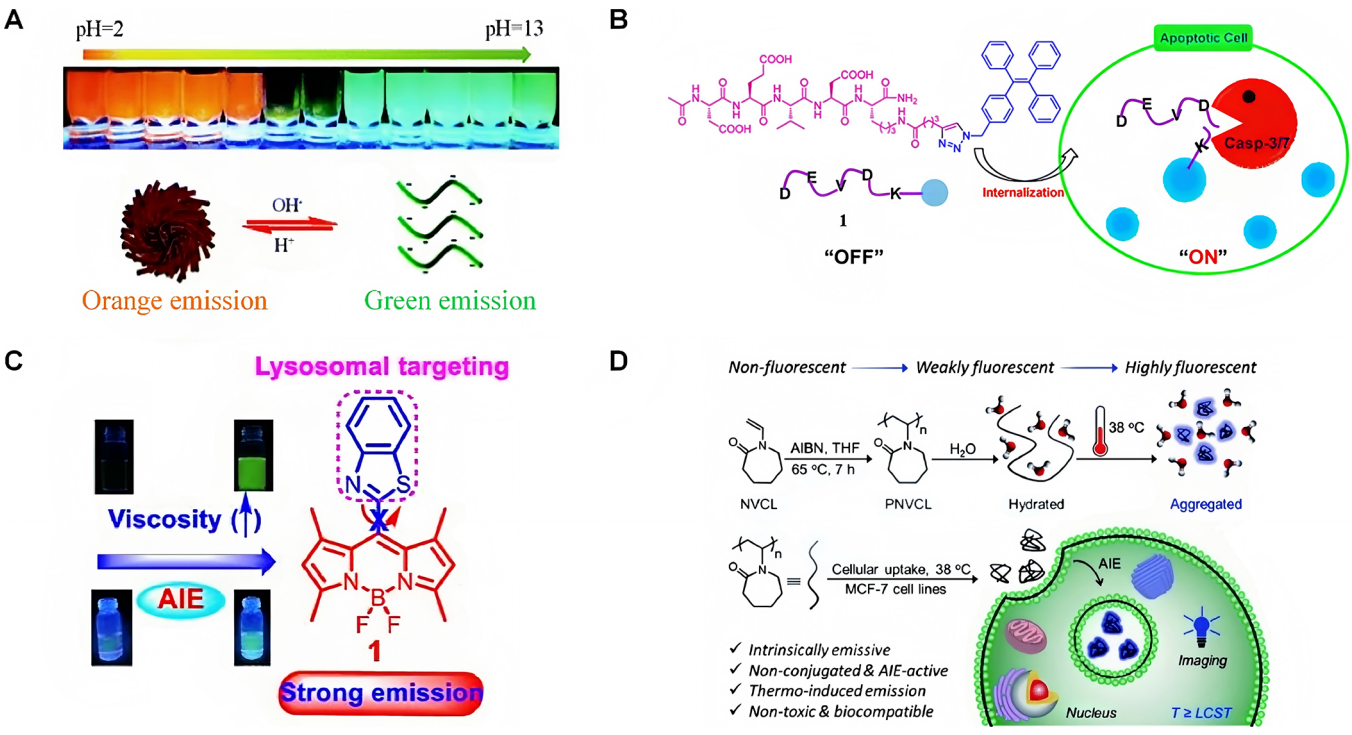

Figure 3. Applications of microenvironment-responsive probes. (A) Dual-emission pH-responsive AIE fluorogen, which contains abundant hydrophobic moieties and tends to aggregate under acidic conditions, generating a strong intermolecular packing to induce strong orange emission and green emission under high pH. Adapted with permission from ref 69. Copyright 2025, Elsevier. (B) Illustration of probe fluorescent light-up probe for real-time cell apoptosis imaging. Adapted with permission from ref 71. Copyright 2012, American Chemical Society. (C) Chemical structure and viscosity-responsive characteristic of the AIE probe 1. Adapted with permission from ref 74. Copyright 2022, American Chemical Society. (D) Schematic depicting the synthetic process and intracellular temperature detection application of PNVCL. Adapted with permission from ref 75. Available under CC-BY-3.0 license. Copyright 2020, Royal Society of Chemistry.

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meso-benzothiazole-functionalized BODIPY-based fluorescent molecular rotors display restricted intramolecular rotation in high-viscosity environments or aggregated states, thereby emitting intense green fluorescence at 534 nm. These probes have been employed to monitor lysosomal viscosity variations in SH-SY5Y neuronal cells induced by lipopolysaccharide, nystatin, low temperature, and dexamethasone, and further applied for the real-time tracking of dynamic lysosomal viscosity changes during the autophagic process. (Figure 3C).[74] Concurrently, molecular probes capable of responding to minute changes in temperature and polarity have also opened up new avenues for studying cellular thermogenesis, mitochondrial metabolic function, and dynamic cell membrane remodeling (Figure 3D).[75] By systematically assembling probe molecules, targeting units, responsive groups and auxiliary functional components at the nanoscale, researchers can regulate the system's size, surface properties, stability, tissue interactions and in vivo transport behaviour, thereby improving the probe's circulatory stability, tissue penetration and ability to retain locally, allowing fluorescent probes to identify features such as pH, ROS/RNS, enzyme activity and metabolic abnormalities in lesion areas with greater precision within complex physiological contexts.

4. TRANSLATIONAL AND CLINICAL APPLICATIONS

While the examples mentioned above have greatly advanced fundamental studies of cellular function, their impact increasingly extends beyond fundamental research. Recent developments in probe engineering and imaging technologies have accelerated the translation of fluorescent probes toward clinical diagnosis and therapeutic applications. Probes with advantages such as deep tissue penetration and high signal-to-noise ratios in the NIR-II region bring unprecedented opportunities for in vivo imaging and the clinical diagnosis of numerous diseases. For example, Pan et al. proposed a rational design strategy for asymmetric polymethine dyes, successfully realizing specific cellular imaging in vitro and NIR-II in vivo imaging of tumors and bones in mice, thereby providing a novel approach for expanding the molecular diversity and biomedical applications of NIR-II organic luminescent dyes (Figure 4).[76,77] Small-molecule fluorescent probes provide powerful tools for alleviating the symptoms and pathologies associated with CIRI through targeted or microenvironment-responsive labeling for early diagnostic and therapeutic interventions. These advances in molecular engineering and in vivo imaging have broadened the translational potential of small-molecule fluorescent probes, particularly in early disease diagnosis, surgical guidance, and therapeutic evaluation. Below, we highlight representative examples of their clinical applications.

4.1. Disease Diagnosis

Early disease diagnosis remains one of the most important clinical applications of small-molecule fluorescent probes. Many pathological conditions are accompanied by alterations in specific biomarkers before the appearance of overt symptoms or anatomical abnormalities. By converting these molecular events into detectable optical signals, fluorescent probes provide rapid, sensitive, and minimally invasive approaches for disease screening and monitoring. The examples highlighted in this section illustrate three major diagnostic strategies: BBB-penetrating probes for neurological disorders, microenvironment-responsive probes for organ injury, and ratiometric probes for quantitative disease assessment. Neurodegenerative disorders represent an important area for probe development. In Alzheimer's disease (AD), conventional diagnostic approaches are often invasive, expensive, and susceptible to interference from biological background signals. Consequently, considerable

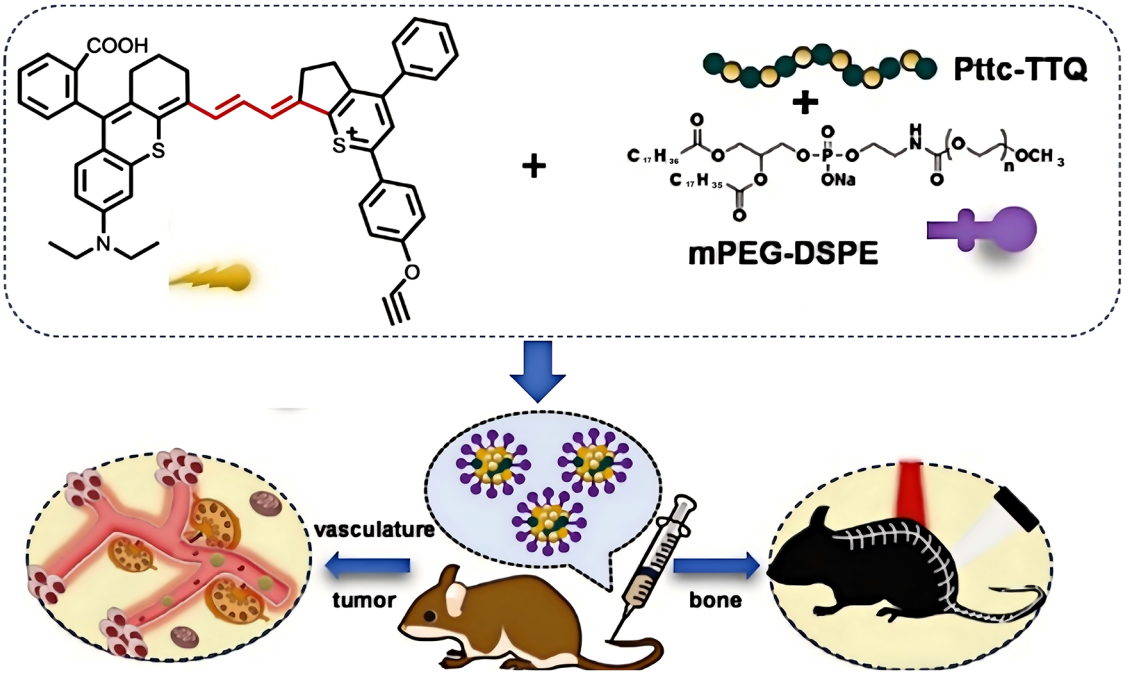

Figure 4. In vivo imaging and disease-targeted diagnosis. The fluorescent polymers, serving as a representative probe for further biological imaging, were intravenously injected into mice. Under 1064 nm laser excitation, the polymers illuminated the entire vascular structure within 5–10 min and delineated the tumor margins. Adapted with permission from ref 77. Copyright 2023, American Chemical Society.

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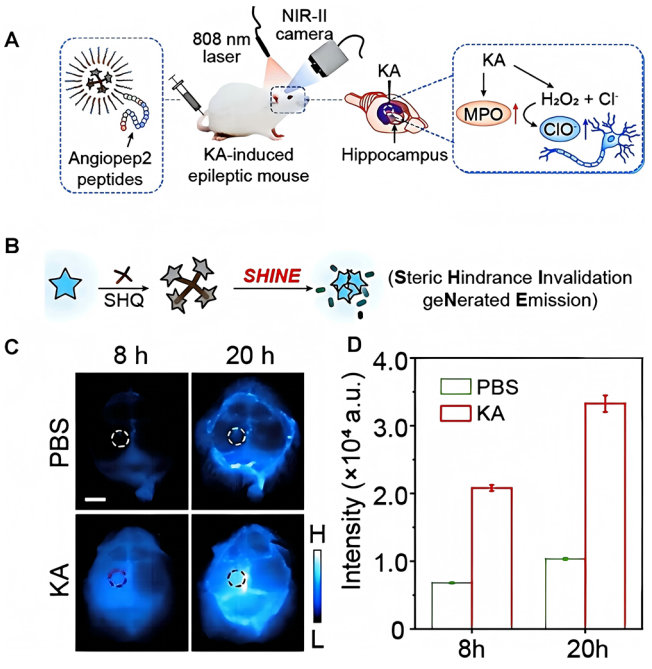

Figure 5. NIR-II fluorescence imaging of epilepsy using an activatable small-molecule probe. (A) Scheme illustration of in vivo visualization of kainic acid (KA) induced epilepsy by the ANB-SHQ-6-pep sensor. (B) Scheme illustration of the SHINE "off-on" sensing mechanism. (C,D) In vivo NIR-II images (C) and signal intensity (D) of normal and epileptic mouse brains at 8 h and 20 h post-injection of the ANB-SHQ-6-pep sensor, respectively. Adapted with permission from ref 78. Copyright 2024, John Wiley and Sons Ltd.

effort has been devoted to developing probes with high biocompatibility, blood-brain barrier (BBB) permeability, and real-time imaging capability. Representative examples include NIR-II activatable probes that employ stimulus-responsive fluorescence switching to visualize pathological biomarkers in the brain. Zhao et al. developed a novel NIR-II "off–on" fluorescence sensing strategy based on steric hindrance-regulated molecular packing. Upon response to the biomarker hypochlorous acid (ClO^-), the steric hindrance effect of the steric hindrance quencher (SHQ) is abolished, allowing the fluorophore ANB to re-aggregate and restore intense fluorescence. The sensor constructed via this strategy successfully crosses the blood–brain barrier and enables real-time NIR-II fluorescence visualization of ClO^- in the hippocampus of living epileptic mice (Figure 5A).[78] The researcher established a mouse model of acute epileptic seizures by injecting kainic acid (KA) into the hippocampus. This led to upregulation of myeloperoxidase (MPO) expression and mediated the generation of ClO^-. To enable the sensor to cross the BBB, the Angiopep2 peptide was conjugated to the ANB-SHQ-6 sensor, forming the ANB-SHQ-6-pep sensor (Figure 5B). Following intravenous injection of the ANB-SHQ-6-pep sensor, within 8 h, the signal intensity on the damaged side of the mouse brain was 3.4 times higher than on the unaffected side, with the region appearing significantly brighter. Over the course of 12 h, the signal intensity on the damaged side gradually increased and remained consistently higher than that on the unaffected side, indicating that ClO^- continued to be produced at the site treated with KA (Figure 5C,D). The research findings were highly consistent with the visualisation results from real-time NIR-II imaging, demonstrating that the SHINE sensor, constructed using SHQ, can monitor the formation of ClO^- in live epileptic mice. Biomarkers such as protein aggregates, reactive oxygen species, metal ions, gasotransmitters, cholinesterases, cysteine, and other newly discovered biomarkers can be detected via targeted diagnostic mechanisms and by leveraging the relationship between probe structure and properties. The development of small-molecule fluorescent probes for AD imaging offers a number of advantages over traditional diagnostic methods.[79] Small-molecule fluorescent probes are accelerating the clinical evaluation of AD diagnostic tools, ultimately driving towards accurate early detection of AD (Figure 6A).[80] This study exemplifies how BBB-permeable NIR-II probes are enabling noninvasive neurological diagnostics. The incidence of AD tends to rise with age. Therefore, investigating the accumulation of $ONOO^-$ in the brains of AD patients of different ages may provide potential clues to understanding the age-related pathogenesis of AD. To this end, the researchers injected Rd-DPA3 into 3-month-old, 8-month-old, and 12-month-old APP/PS1 mice and recorded real-time imaging of the mice (Figure 6B). The fluorescent signal from the brain exhibited a marked age-dependent increase (Figure 6C,D), providing in vivo evidence for the age-dependent accumulation of $ONOO^-$ in the AD brain.

Many major diseases are accompanied by the abnormally high expression of specific biomarkers in their early stages.[81] Small-molecule fluorescent probes can be utilized to develop test kits for biomarker detection in body fluid or tissue biopsy samples, thereby providing novel, rapid, and low-cost strategies for early disease screening and diagnosis.[82] For example, to address the lack of reliable, minimally invasive visualization methods for the early diagnosis of liver injury, a research team reported a small-molecule fluorescent probe named DPXBI. The internal rotor structure of this molecule makes its fluorescence intensity highly sensitive to environmental viscosity; when viscosity increases, intramolecular rotation is restricted, thereby emitting a strong fluorescence signal. This not only allows the detection of liver injury hours earlier than traditional clinical biomarkers but also enables the real-time, dynamic tracking of liver damage (Figure 7A).[83] Such viscosity-responsive probes represent an emerging strategy for early organ injury detection. Furthermore, with regard to the detection of liver fibrosis, researchers have developed a variety of therapeutic and diagnostic probes, including those that directly target liver fibrosis biomarkers and enzyme-activated probes. These fluorescent probes have significantly advanced the detection of liver fibrosis, enabling real-time, non-invasive visualisation of biomarkers and changes in the microenvironment.[84] However, clinical application still faces challenges such as poor tissue penetration and insufficient biocompatibility. Consequently, future research in this field will focus on the discovery of biomarkers in biological fluids and the design of novel therapeutic interventions.

Furthermore, to overcome issues such as the delayed response of traditional biomarkers in the early diagnosis of acute kidney injury, as well as the susceptibility to background interference and insufficient targeting of existing fluorescent probes, Zhang's research team developed specifically responsive near-infrared small-molecule fluorescent probes: Hcy-BOH and Cy-Dopa. Under the action of H_2O_2, the fluorescence signals of these two probes undergo opposite changes, manifesting as fluorescence enhancement for Hcy-BOH and fluorescence

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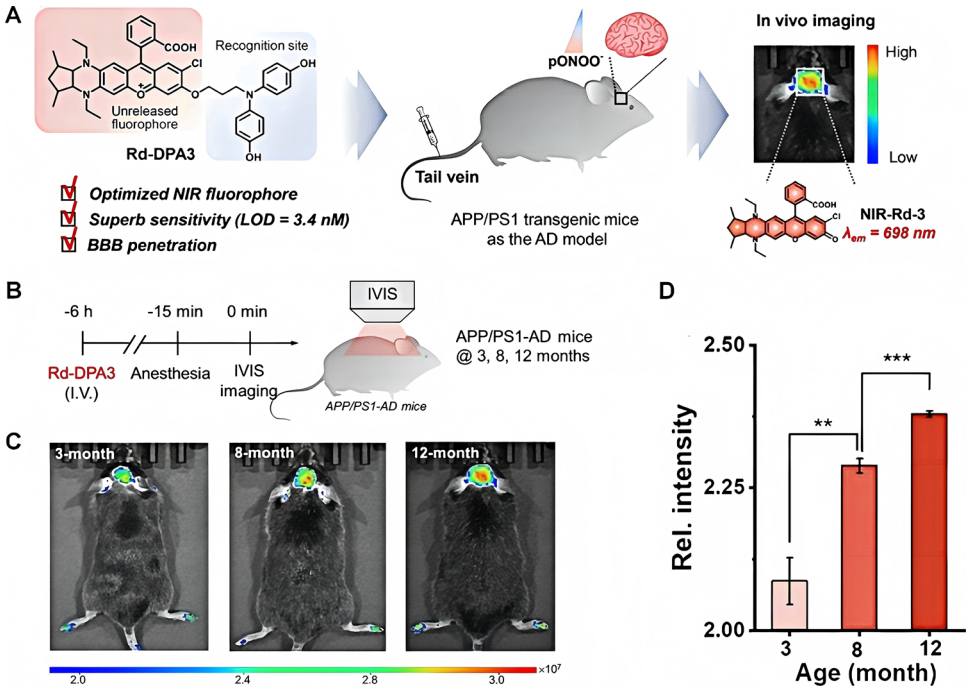

Figure 6. NIR fluorescent probe for in vivo imaging of Alzheimer's disease. (A) Combining the $ONOO^-$-initiated cascade reactions of a novel chemical trigger and a new NIR-emissive Rhodol fluorophore, an activity-based NIR fluorescent probe for $ONOO^-$ in AD brains was constructed. (B) Schematic diagram for in vivo imaging $ONOO^-$ signals in live mice brains. (C) In vivo fluorescence imaging for AD mice of different ages (3, 8, and 12 months) with the tail injection of 100 μL 0.2 mM Rd-DPA3. (D) Changes of relative fluorescence intensities in AD mice of different ages (3, 8, and 12 months) after tail injection of Rd-DPA3. Adapted with permission from ref 80. Copyright 2022, John Wiley and Sons Ltd.

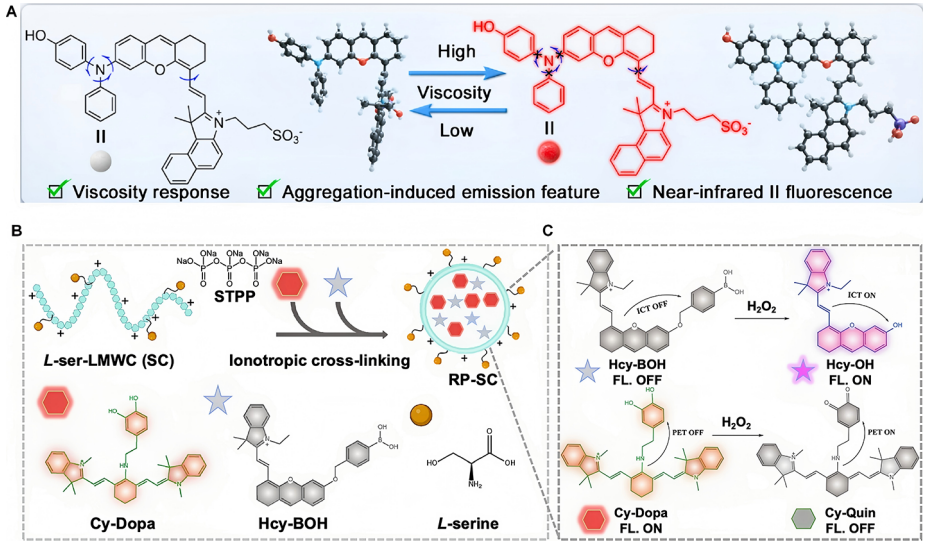

Figure 7. Early diagnosis of liver and kidney diseases. (A) Molecular structure and proposed mechanism of DPXBI in response to viscosity. Adapted with permission from ref 83. Copyright 2023, Biomaterials. (B) Schematic diagram of synthesizing ratiometric nanoprobe RP-SC and (C) reaction mechanism of Hcy-BOH and Cy-Dopa with H_2O_2. Adapted with permission from ref 85. Available under CC-BY-4.0 license. Copyright 2025, American Association for the Advancement of Science.

quenching for Cy-Dopa. By calculating the fluorescence intensity ratio of these two probes, interference from factors such as probe concentration and tissue depth can be effectively eliminated, achieving precise semi-quantitative detection of oxidative stress levels at the lesion site (Figure 7B,C).[85] Ratiometric approaches improve quantitative reliability and reduce imaging artifacts.

4.2. Fluorescence-Guided Surgery

Beyond disease diagnosis, fluorescent probes have also transformed intraoperative visualization by enabling surgeons to identify lesions with higher precision during surgical procedures. FGS is one of the most promising directions for the clinical translation of small-molecule probes. Researchers are

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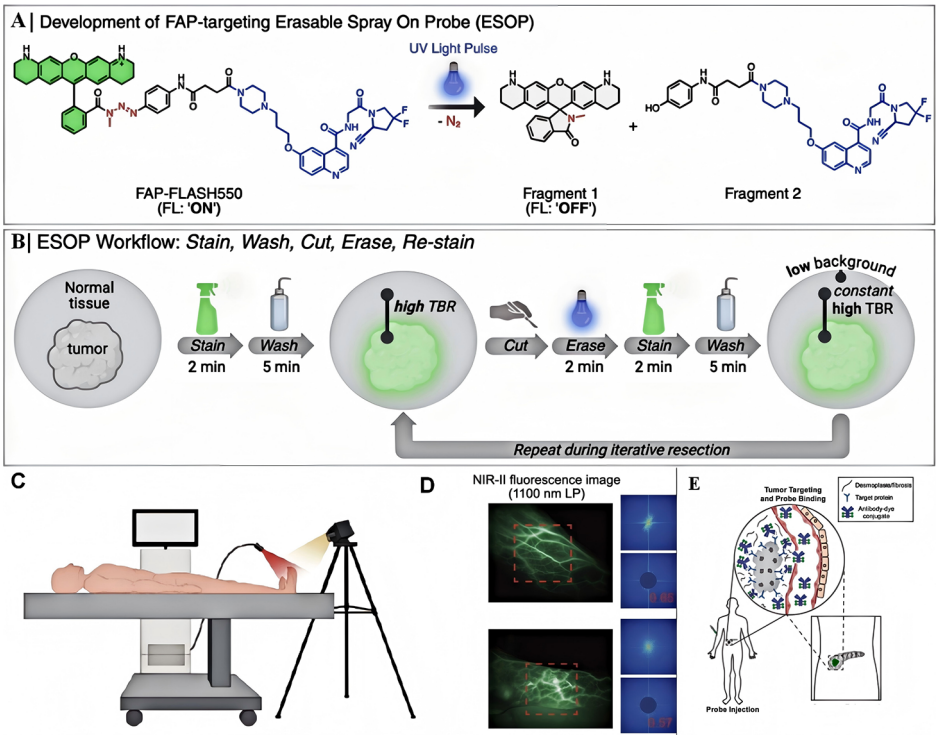

Figure 8. Fluorescence-guided surgery and intraoperative visualization. (A) Structure of FAP-FLASH550 and its reaction with a UV light pulse. (B) After a short topical application procedure, ESOPs could stain tumor margins with highly sensitive tumor-to-background ratios (TBRs). Adapted with permission from ref 86. Available under CC-BY-4.0 license. Copyright 2025, Wiley-VCH. (C) Schematic of NIR-II fluorescence image acquisition during surgical procedure. (D) NIR-II fluorescence images (1100 nm LP) of vessels in the foot dorsum and tibialis anterior regions of the patients, and the corresponding frequency spectral maps of the regions of interest (marked by the red box) after FFT treatment. The numbers in red refer to the proportion of high-frequency information in each map. Adapted with permission from ref 88. Copyright 2024, Engineering. (E) Mucin as a biomarker guides fluorescence imaging in pancreatic cancer FGS. Adapted with permission from ref 90. Copyright 2023, Elsevier Ireland Ltd.

committed to developing near-infrared fluorescent probes that can specifically recognize tumor cells and be activated or "turned on" (Figure 8A,B).[86] Folate Receptor Alpha (FRα)-targeted intraoperative fluorescence imaging has emerged as a promising tool to enhance the recognition of tumor localization. Studies have shown that in patients undergoing resection surgery, the detection rate using fluorescence-guided strategies improved by 29% compared to standard methods, with a sensitivity exceeding 85%. FRα-targeted imaging can enhance lesion detection during cytoreductive surgery and improve the completeness of resection.[87] Furthermore, it also facilitates surgeries such as diabetic foot surgery, calcaneal fracture surgery, and lower extremity trauma surgery (Figure 8C,D).[88] While receptor-targeted probes improve lesion localization, activatable probes further enhance specificity by minimizing background fluorescence and restricting signal generation to diseased tissues.

To further improve the tumor-site specificity of fluorescent probes, Shen et al. innovatively designed a dual-locking activatable probe, FANQ. This probe ingeniously utilizes two essential characteristics of tumor cells—the offensive system and the defensive system—as logic gates for cascade activation. This ensures that the probe is only activated in tumor regions where both biomarkers coexist, achieving highly specific recognition of tumor cells.[89] In summary, during surgical procedures, surgeons can leverage dedicated fluorescence imaging systems to clearly identify tumor boundaries in real time, thereby achieving a more thorough and precise resection of cancerous tissues while maximizing the preservation of surrounding healthy tissue, which significantly improves patient prognosis (Figure 8E).[90] Therefore, this area has become a major research focus in the realm of precision medicine. However, fluorescence imaging (FI) in the visible light spectrum is now considered obsolete due to its numerous limitations. Recent research has identified the near-infrared (NIR) band, ranging from 700 to 1700 nanometres, as a suitable range for fluorescence imaging. The near-infrared spectrum is further subdivided into two regions: NIR-I and NIR-II. Among these, ICG is the only fluorescent marker approved by the US Food and Drug Administration (FDA) for use in the NIR-I region. Compared with visible-light tracers, the use of NIR-I fluorescent tracers effectively reduces issues such as autofluorescence, photobleaching, invasiveness and toxicity, whilst enhancing the contrast between tumours and the background.[91] But due to their larger size, these tracers are unable to cross the blood-brain barrier, making them difficult to use for the diagnosis of deep-seated brain tumours. Future efforts should focus on improving imaging depth, specificity, and regulatory approval for clinical implementation.

4.3. Drug Discovery and Pharmacological Evaluation

In the process of drug development, rapidly evaluating the efficacy of drug candidates is crucial. Small-molecule fluorescent probes provide a high-throughput visual screening platform for this purpose.[92] By applying probes to cellular or animal

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models, researchers can intuitively and dynamically monitor changes in specific target activity or overall cellular state alterations before and after drug intervention. For example, regarding the development of small-molecule fluorescent probes used as imaging biomarkers for epilepsy, researchers have designed molecular tools that can target a range of pathological biomarkers, including various ROS, RCS, enzymes, ions, neurotransmitters, and lipid droplets. These molecular tools possess high selectivity and sensitivity, and in some cases, blood-brain barrier permeability. This serves as a complement to traditional methods, enabling real-time, non-invasive observation of dynamic biomarker fluctuations in living cells and animal models of epilepsy, providing deep insights into the oxidative stress, neuroinflammation, metabolic dysfunction, and synaptic imbalance associated with the condition.[93] This approach not only allows for the rapid screening of effective lead compounds but can also be used to evaluate the dose-response, duration of action, and off-target effects of drugs, thereby accelerating the drug research and development process. Despite significant progress, the clinical translation of fluorescent therapeutic and diagnostic agents remains limited, primarily due to a lack of understanding of their pharmacokinetics, metabolism, biodistribution, and long-term biosafety. According to previous studies, even a fluorescent probe with high selectivity and good responsiveness may still be subject to non-specific binding to proteins or rapid clearance by the immune system in vivo. This is primarily because the in vivo environment is far more complex than that of cellular experiments, and small-molecule fluorescent probes, typically having a low molecular weight, are easily filtered by the glomeruli; furthermore, their hydrophobic nature often leads to abnormal accumulation of these probes in the liver.[94,95] Due to various limitations, only a few tracers, such as ICG, 5-ALA, and methylene blue, have gained clinical approval.[96] Consequently, there is still a long way to go before small-molecule fluorescent probes can be used for therapeutic applications in vivo.

5. CONCLUSION, CHALLENGES AND FUTURE PERSPECTIVES

As a class of highly efficient and customizable chemical tools, small-molecule fluorescent probes have demonstrated unique advantages in intracellular signal transduction tracking, molecular functional evaluation, and cellular microenvironment monitoring, exhibiting broad prospects in clinical applications such as early diagnosis, surgical guidance, and drug evaluation. This review summarized the progress of small-molecule fluorescent probes in biological research, emphasizing that through precise chemical design, they can visualize the complex intracellular chemical environment and its dynamic changes, providing a new perspective for revealing the molecular mechanisms of life activities. Although the design of multifunctional probes has been extensively realized, it is still necessary to improve their biocompatibility, reduce non-specific binding, and enhance clearance efficiency for long-term *in vivo* applications to ensure safe and controllable clinical translation. In the future, as artificial intelligence (AI) technology has gradually permeated the medical field, AI is being utilised to enhance the performance of fluorescence imaging. By combining it with clinical ultrasound and photoacoustic imaging to assist with data computation, image analysis, and pattern recognition, it has significantly improved imaging quality and compensated for the shortcomings of fluorescent markers. This combined approach is expected to overcome technical barriers and accelerate the clinical translation of fluorescence imaging. In conclusion, through collaborative innovations in design strategies, material optimization, and imaging technologies, small-molecule fluorescent probes are expected to play an increasingly critical role in life science research and precision medicine.

AUTHOR INFORMATION

Corresponding Authors

Yazhou Chen – *Henan Institute of Advanced Technology, Zhengzhou University, Zhengzhou 450003, PR China;* Email: yzchenbio@zzu.edu.cn

Linawati Sutrisno – *State Key Laboratory of Radiation Medicine and Protection, School of Radiation Medicine and Protection, Collaborative Innovation Center of Radiological Medicine of Jiangsu Higher Education Institutions, Soochow University, Suzhou 215123, P.R. China; International Center for Young Scientists (ICYS), National Institute for Materials Science (NIMS), 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan;* orcid.org/0000-0003-3085-9660; Email: SUTRISNO.Linawati@nims.go.jp

Authors

Yue Chuai – *Henan Institute of Advanced Technology, Zhengzhou University, Zhengzhou 450003, PR China*

Kewei Sun – *Vacuum Interconnected Nanotech Workstation, Suzhou Institute of Nano-Tech and Nano- Bionics, Chinese Academy of Sciences, Suzhou 215123, China;* orcid.org/0000-0002-1835-243X

Katsuhiko Ariga – *Research Center for Materials Nanoarchitectonics (MANA), NIMS, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan; Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwa-no-ha, Kashiwa 277-8561, Japan;* orcid.org/0000-0002-2445-2955

Complete contact information is available at: https://pubs.acs.org/doi/10.1021/acsabm.6c01397

Author Contributions

Y.C. planned the manuscript, researched the literature, and wrote original draft. K.A., Y.C., and L.S. co-wrote the original draft. All authors discussed, reviewed, edited, co-wrote, and approved the manuscript.

Notes

The authors declare no competing financial interest.

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