Molecular Fluorophores for Deep-Tissue Bioimaging
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ABSTRACT: Fluorescence imaging has made tremendous inroads toward understanding the complexity of biological systems, but in vivo deep-tissue imaging remains a great challenge due to the optical opacity of biological tissue. Recent improvements in laser and detector manufacturing have allowed the expansion of nonlinear and linear fluorescence imaging to the underexplored “tissue-transparent” second near-infrared (NIR-II; 1000−1700 nm) window, opening up new opportunities for optical access deep inside opaque tissue. Molecular fluorophores have historically played a major role in fluorescence bioimaging. It is increasingly important to design new molecular fluorophores to fully unlock the potential of NIR-II imaging techniques. In this outlook, we give an overview of the novel molecular fluorophores developed for deep-tissue bioimaging in the past five years and discuss their pros and cons in applications. Guidelines for designing new molecular fluorophores with the desirable properties are also provided.

1. INTRODUCTION
Fluorescence imaging has been widely used to investigate the mechanisms underlying various biological phenomena, as this approach can afford much detailed spatiotemporal information in living cells and ex vivo tissue sections than other imaging modalities, such as magnetic resonance imaging, ultrasound, and positron emission tomography. A more complete understanding of biological mechanisms involved in physiology and disease increasingly requires imaging exploration at deeper or intact tissue of living organisms. However, this presents a major challenge for conventional fluorescence imaging that operates in the visible spectrum, because tissues are extremely heterogeneous, and the strong scattering of visible light fundamentally limit the penetration capacity of fluorescence imaging. In recent decades, new windows for deep-tissue biological exploration have been opened in the near-infrared (NIR; 700−1700 nm) spectrum owing to suppressed photon scattering and diminished tissue autofluorescence.1,2 Fluorescence imaging techniques based on NIR nonlinear (multiphoton) excitation and NIR linear (one-photon) emission have enabled unprecedented dynamic and high-contrast exploration of neural activity,3,4 cerebral blood flow5,6 and tumor microenvironment7−9 in living mice. Very recently, NIR fluorescence imaging of human patients has emerged as a cheap, safe, and real-time navigation modality for surgeons to localize lesions, ensure clear resection margins, and find small metastases during cancer surgery.10 Yet where there are opportunities there are challenges. The emerging deep-tissue fluorescence imaging scenarios call for the development of novel fluorophores with improved photophysical and chemical properties such as longer wavelengths, better brightness, excellent photo-/chem-stability, and diverse chemical function. As once king of fluorescence imaging, molecular fluorophores are undergoing a renaissance to meet the needs of modern biological and biomedical researches.11 In this Outlook, we discuss the recent progress and challenges in the development of novel molecular fluorophores, especially focusing on those operate in the underexplored second near-infrared window (NIR-II; 1000−1700 nm). We have grouped them into two categories, one for NIR-II-excited multiphoton microscopy, and another for NIR-II-emitted one-photon imaging.

2. PUSHING THE PENETRATION LIMIT WITH NIR-II LIGHT
When considering a photon traveling through tissue, the penetration limit for fluorescence imaging is governed by a physical parameter called the transport mean free path (TMFP) of a photon (Figure 1a).12 TMFP describes the mean propagation distance before the photon’s direction becomes randomized. Imaging beyond TMFP with fluorescence methods results in image blur. In general, TMFP represents an upper limit for the penetration of microscopic imaging. Alternatively, when sacrificing resolution is accept-
able, the penetration limit could be equivalent to several times the TMFP, such as for in vivo small animal imaging. For most mammalian tissues, TMFP can be simply defined as $1/\mu_s(1-g)$ (where $\mu_s$ is the tissue scattering coefficient, $g$ is the anisotropy function defining the degree of forward scattering, $g = 0.8−1$), when photon scattering is dominant over photon absorption. Reducing the tissue scattering coefficient for fluorescence excitation and/or emission would improve the penetration limit, which requires the exploration of an optimum spectral window.

Previous studies on light-tissue interactions revealed that photon scattering nearly scales with $\lambda^{-\alpha}$, where $\lambda$ denotes the wavelength, and the exponent $\alpha = 0.2−4.0$ depends on tissue types (Figure 1c).

Longer-wavelength light possesses lower scattering coefficient, and in most cases NIR light penetrates deeper into the scattering medium than visible light (Figure 1b). Nevertheless, in the NIR spectral region, water has a nontrivial contribution to the absorption spectrum of biological tissue (Figure 1c). The trade-off between tissue scattering and absorption sets the boundaries of suitable spectral windows that exclude a narrow water absorption band centered at 1450 nm. In the past decade, fluorescence imaging has focused on the subwindows of 1000−1400 nm and 1500−1700 nm in the NIR-II spectral region, benefiting from the available cost-effective lasers and affordable InGaAs detectors that are tunable or respond in these windows.

3. NIR-II NONLINEAR EXCITATION FOR DEEP-TISSUE BIOIMAGING

3.1. NIR-II Multiphoton Fluorescence Imaging Techniques. Most widely used nonlinear excitation fluorescence imaging techniques are two-/three-photon (2P/3P) microscopies. 2P excitation accesses a given excited state of fluorophore by using photons of half the energy of the corresponding one-photon (1P) transition, and 3P excitation uses one-third. This nonlinear dependence on excitation intensity confines fluorescence generation to the focal volume, which reduces out of focus background and provides intrinsic three-dimensional resolution (Figure 2a). Through a “whole-area” epifluorescence collection, 2P/3P microscopy collects both scattered and ballistic signal photons to form the final images, resulting in surprisingly little signal loss (Figure 2a).

Therefore, the limitation on penetration depth mostly arises from scattering and absorption of excitation light in tissues. Traditional 2P microscopy relies on Ti:sapphire lasers, which have a wavelength maximum near 1000 nm (Figure 2d), and thus restricts imaging depth to superficial cortical layers (<500 μm thick). Driven by advances in laser technology in the past decade, 2P microscopy at a spectral excitation window of 1000−1600 nm have pushed the imaging depth further and visualized the entire mouse cortex (about 1 mm thick) and

Figure 1. (a) Schematic depiction of TMFP by a light propagation model in tissue. (b) Penetration of light of different wavelengths in a tissue phantom (2.5% intralipid). (c) Scattering spectrum of a mouse brain model and water absorption spectrum.

Figure 2. (a) Signal generation and fluorescence collection in 2P/3P microscopy. (b) Jablonski diagram, illustrating the S0-to-S1 and S0-to-Sn 2P absorption transitions. (c) S0-to-S1 and S0-to-Sn 2P absorption transition bands in a typical 2P absorption spectrum. 1P absorption spectrum (black dotted) is presented for comparison. (d) Commercial femtosecond lasers relevant to 2P excitation. (e) Structures of several commercial or reported 2P/3P fluorophores with their maximum 2P/3P absorption wavelengths in the NIR-II range.
beyond with single-cell resolution. 17−19 3P microscopy extends the excitation wavelength up to 1700 nm and is capable of calcium-activity imaging at high spatial (submicrometer) and temporal resolution through the intact skull at >500 μm depth. 3 The development of bright, bioavailable molecular fluorophores that excite in the NIR-II range is a crucial aspect to the advancement of in vivo 2P/3P microscopy. To date, a variety of molecular fluorophores (Figures 2e and 3a) have been used and developed. In this section, we mainly discuss molecular fluorophores designed for NIR-II 2P microscopy, in view of their accomplishments in recent years.

3.2. NIR-II Two-Photon Excitable Molecular Fluorophores. Characterization of molecular brightness at different excitation wavelengths is a crucial first step toward their adoption as fluorophores for 2P microscopy. There are several metrics to evaluate brightness, such as 2P action cross section. 

Figure 3. (a) Reported 2P (purple) and 3P (blue) fluorophores and probes. (b) Core structure and modification positions of rhodamine fluorophores. (c) Structure engineering at the bridging heteroatoms and nitrogen substituents of rhodamine core to increase quantum yield and fine-tuning wavelength; subscripts in e.g. JF535 denote the maximum 1P absorption. (d) 2P peak molecular brightness spectra of JF Fluors, mCherry for comparison. 48 (e) Lactone-zwitterion equilibrium in rhodamine fluorophores (εr: solvent dielectric constant) and the influence of substituent structures on the L−Z equilibrium. (f) Rhodamine fluorophore (JF585-HaloTag, magenta) as fluorogenic label for 2P fluorescence imaging of neurons in layer 5 of visual cortex, combined with GCaMP6s (green). Reproduced with permission. Copyright 2017, Springer Nature. 48 (g) A hybrid chemigenetic probe (Voltron) composed of rhodamine fluorophores (JF Fluors) and a GEVI (rhodopsin). Reproduced with permission. Copyright 2019, Science Publishing Group. 51 (h, i) Calcium probes (h) and voltage indicator (i) based on rhodamine fluorophores. Copyright 2019, American Chemical Society. 62
Although it is a persistent effort goal to improve the absorption cross section, there are other factors—such as quantum yield, photostability, molecular size, functionality, and modification flexibility—that also need to be considered when selecting a fluorophore for 2P applications.
Such “chemigenetic” indicator that combines the genetic targetability and exquisite molecular recognition of proteins with the superb photophysical and sensing properties of synthetic dyes is emerging as a powerful platform for deep-tissue functional imaging.

response, that is, a photoinduced electron transfer (PeT) mechanism between fluorophore and electronic donor. The recently developed and constantly growing toolbox of PeT probes for neural activity, including calcium and voltage sensors, allows precise measurement of these signaling events with high spatial and temporal resolution under 2P microscopy. For example, a family of red-fluorescent calcium indicators, CaRuby and CaRuby-Nano, was created by conjugating a calcium chelator BAPTA [bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid] to an extended rhodamine dye (Figure 3h). Calcium chelation alters the electronics of the BAPTA, resulting in reduced PeT and increased fluorescence. CaRuby-Nano was used for in vivo 2P calcium imaging from neocortical layer 2/3 (about 200 μm) pyramidal neurons at around 900 nm excitation. Extending excitation wavelength to ~1050 nm by using a commercial calcium probe Cal-590 is capable to record calcium transients at depths of up to ~900 μm below the pial surface. More recently, genetically targetable calcium probes JF-BAPTA-HaloTag based on X-rhodamines were developed by optimizing the relative position of the BAPTA chelator and the fluorophore (Figure 3h). These probes may be useful for 2P calcium imaging experiments with cell-type specificity. The Miller group has developed a family of voltage sensitive indicators (RhoVR) by coupling with a molecular wire to the pendant phenyl ring of X-rhodamine dyes (Figure 3i). These indicators operated in a membrane potential-dependent PeT process, leading to large voltage sensitivities (up to 47% ΔF/F per 100 mV). Coupling with emerging high-speed 2P microscopy, modified RhoVR can provide single-trial resolution of action potentials in neurons under 2P (λex = 1040 nm) illumination.

4. NIR-II LINEAR EMISSION FOR DEEP-TISSUE BIOIMAGING

4.1. NIR-II One-Photon Fluorescence Imaging Techniques. Owing to the low cost and wide-field imaging capability, linear 1P fluorescence imaging techniques remain competitive for a variety of imaging scenarios, such as in vivo cell tracking, small-animal whole-body imaging, and imaging-guided surgery. However, the visible spectrum extensively employed for linear fluorescence imaging has restricted high-resolution optical imaging to thin sections or to superficial layers. In past decades, much effort had been devoted to developing far-red and near-infrared-I (NIR-I; 650−1000 nm) fluorophores to improve the penetration limit by employing the reduced tissue scattering. But the situation did not change substantially until pioneering work published in 2009, which pushed linear fluorescence imaging to the NIR-II regime by utilizing surface-modified carbon nanotubes as fluorophores. Imaging experiments with these materials revealed dramatic benefits in resolution and penetration depth compared with conventional fluorescence imaging at NIR-I wavelengths, for example, achieving sub-10-μm resolution of through-skull imaging of the brain vasculature at a depth of >2 mm in mouse brain. This pioneering work further classified the broadly defined NIR-II spectrum into three optical subwindows such as NIR-IIa (1000−1300 nm), NIR-IIb (1300−1400 nm), and NIR-IIb (1500−1700 nm), which yield improved fluorescence-imaging clarity with increased wavelength. Based on this criterion, a large number of novel NIR-II fluorophores, including organic dyes and lanthanide nanoparticles, and metal clusters, emerged and enriched the NIR-II fluorescent toolbox. Very recently, new NIR-II microscopic imaging setups have been developed such as 3D confocal microscopy and light-sheet microscopy. The use of 1319 nm illumination and fluorescence detection at 1500−1700 nm in noninvasive NIR-II light-sheet microscopic imaging afforded a penetration depth of approximately 774 μm along the tilted light-sheet direction into the head with sub-10-μm resolution. These achievements pave the foundation for deep-tissue biological and biomedical imaging by utilizing NIR-II linear fluorescence imaging techniques.

4.2. NIR-II One-Photon Emitted Molecular Fluorophores. Organic dyes historically play a major role in fluorescence imaging as fluorescent labels, stains, and reporters, mainly due to their advantages of low molecular mass, good biocompatibility, short organism retention time, and ease of modification through chemical design, compared with inorganic contrast agents. In particular, the achievements of two clinical approved NIR-I dyes, indocyanine green (ICG) and methylene blue (MB), greatly motivated the development of NIR-II organic dyes to facilitate clinical translation of the NIR-II fluorescence imaging technique. However, making bright NIR-II dyes with stable photophysical and chemical properties in a biological environment is a challenging problem for organic chemists. An efficient and convenient approach to obtain NIR-II dyes is to perform structure modification on the available dye chromophores. To date, the reported NIR-II fluorophores mainly include donor−acceptor−donor (D−A−D) chromophores and polymethine cyanine dyes. To improve the biomedical applications of NIR-II dyes, key factors including absorption and emission wavelength, quantum yields, bioavailability, and biocojugation capacity is discussed in this section.

4.2.1. D−A−D Chromophores. D−A−D chromophore is a typical NIR-II organic molecular structure which contains three parts: one acceptor, two donors, and π bridging groups. The spectral properties of D−A−D dyes depend on selecting an electron donor and acceptor as well as π bridging groups. Introducing large π bridging moieties as well as stronger electron acceptors and donors is the most commonly used method to obtain new D−A−D dyes with a longer absorption and emission wavelength. To date, various symmetrical NIR-II D−A−D dyes have been reported with strong electron-withdrawing unit benzobisthiadiazole (BBTD) derivatives as the central acceptor and variable groups including thiophene, benzene, triphenylamine, and fluorene as donors, e.g., CH105S, IR-Fe, Q4, H1, CH-4T, IR-F8P, and IR-E1. Recently, replacing a sulfur atom by a selenium atom in the BBTD moieties could lead to bathochromically shifted emission for D−A−D dyes, such as SY1100 (Figure 4a).
transfer (TICT) characteristic in D−A−D chromophores significantly lowers the energy gap between the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO), yielding an emission peak beyond 1000 nm and a large Stokes shift (usually >200 nm).

In 2015, a small-molecule NIR-II fluorophore CH1055 was synthesized by introducing four carboxy groups into the triphenylamine donors of the D−A−D skeleton (Figure 4a).72 This modification not only facilitates PEGylation to impart the molecule with high aqueous solubility and rapid renal clearance but also allows for bioconjugation to targeting ligands, such as antibody, to ultimately produce a molecular imaging agent for tumor diagnostics. The results highlight the potential of D−A−D chromophores for NIR-II bioapplication, in particular, in wide-field through-skull high-resolution brain vasculature imaging (Figure 4b) and high-contrast tumors diagnostic (Figure 4c). However, the quantum yield loss is massive from toluene (7%) to water (0.03%, IR-26 = 0.05% as reference, similarly hereinafter), leaving ample room for further improvement. The fluorescence quenching of D−A−D chromophores in water is associated with the formation of the dark TICT state, in which intramolecular rotation results in nonradiative deactivation of the excited state.102 TICT is governed by a driving force, which depends on solvent polarity, steric hindrance, and the electron-donating strength of the donor groups. Restricting molecular rotation or encapsulating the molecule into a nanomicelle to prevent water contact can straightforwardly produce a brilliant increase in fluorescent brightness. For example, a sulfonate-substituted derivate of CH1055, CH-4T, which forms supramolecular assembly with plasma protein, results in an increased quantum yield of 1.8%.

Another strategy to improve quantum yield at a single molecule level is to choose a weak, rigid donor and introduce a hydrophobic shielding unit to protect the molecule from solvent polarization, making the TICT state energetically unfavorable.106−108 In recent years, substantial efforts have...
been made to reveal how changes to donor structures and components influence absorbance, quantum yield, and emission wavelength. Dai and Liang and co-workers introduced shielding units (S) into the D–A–D skeleton to form S–D–A–D–S organic dye. The dialkoxy modified fluorene was hired as shielding units to construct new NIR-II dye IR-FTAP, which afforded reduced molecule–molecule interaction, leading to a high signal-to-noise ratio (SBR) of 4.9.
Moreover, these efforts ultimately yield a highest quantum yield record of 0.6% in water for fluorophore IR-FP8P with emission around 1040 nm. The chromophore of IR-FP8P employs a clickable azide-functionalized fluorene as a weak, rigid donor, 3,4-propylenedioxy thiophene as the π-bridge, and the side alkyl chains as protection for the conjugated backbone. The higher quantum yield and absorption coefficient provided an SBR around 2.5 for ovary NIR-II imaging (Figure 4f).81 The improvements in functionalization and brightness at a single molecule level make D–A–D fluorophores a good choice for use in molecular fluorescent labeling in various applications such as targeting imaging of organ/tumor, immune checkpoint, and gut microbiota.

Attempts to extend the emission wavelengths of D–A–D fluorophores remain ongoing, but there exists a trade-off between gently red-shifted emission and a steeply descending quantum yield due to the TICT process. To address this issue, recent efforts have focused on the aggregation induced emission (AIE) effect of D–A–D chromophores such as HLZ-BTED and 2TT-oC26B (Figure 4g and h).82,83 While the intramolecular donor rotation in TICT contributes to red-shifted emission, AIE requires restricting intramolecular motion to enhance fluorescence. Very recently, the two seemingly contradicting individuals were simultaneously realized in the aggregates of the rationally designed fluorophore 2TT-oC26B, thus obtaining a broad emission spectrum with a tail extended to the NIR-Ib window and a high quantum yield of 1.15%. Key elements of the molecular design focus on the branched alkyl chain substituents in thiophene groups, which cause a large distortion of the thiophene-BBTD-thiophene backbone. With 2TT-oC26B administration, a tiny vessel of 10 mm width could be distinguished with an SBR around 2. Furthermore, the small bowel diverticula (~1 mm) could be observed under 5 mm tissue penetration with an SBR of 2.7 (Figure 4h).102 Such distortion not only prevents intermolecular interactions but also provides certain spatial isolation of the molecules in aggregates to promote the intramolecular rotation of triphenylamine units, which were conducive to the formation of the TICT state.

4.2.2. Polymethine Cyanine Dyes. Polymethine cyanine dyes are other typical small organic NIR-II-emitted chromophores. The dyes generally consist of two heterocyclic terminal dyes are other typical small organic NIR-II-emitted chromophores. The dyes generally consist of two heterocyclic terminal amine units, which were conducive to the formation of the conjugated system.113–115 The terminal group structures could be heterocyclic ring containing heteroatoms, like sulfur, oxygen, or nitrogen. The maximum absorption and emission wavelength of polymethine cyanine dyes could be tuned by the terminal groups and conjugated chain length. Lengthening the conjugate chain can increase the extent of delocalized π electrons, which is a classic method to afford red-shifted dyes. Extending the π-system in terminal groups, such as adding π-donor substituents or benzene ring groups, could lead to a bathochromic shift of absorption and emission. Replacing strong electronegative heteroatoms by weak electronegative heteroatoms in a similar scaffold results in bathochromic shifted absorption.

Typical cyanine dyes, including IR-1048, IR-1061, Flav7, and IR-26, show intense absorption and emission, both over 1000 nm in nonpolar solvent such as dichloromethane, but are easy to quench when transferring to polar solvents such as dimethyl sulfoxide, methanol, and water.75 Previous studies suggested three major reasons for explaining this phenomenon: (1) Large, hydrophobic conjugated structures favor these fluorophores to form random oligomers or parallel-oriented H-aggregates in water, leading to the fluorescence quenching.78,116 (2) Increased solvent polarity leads to the symmetry breaking effect of electronic structure in ground/excited state, resulting in solvatochromic-induced quenching. This effect connects with the length of polymethine chain and the donor strength of terminal groups, and is generally intensified with the increase of these two factors.75,117,118 (3) Fluorophores like IR-1048, IR-26, and Flav7 appear to be more electron deficient than common cyanine fluorophores and, thereby, are prone to be attacked by nucleophiles. This could even be done by some weak nucleophiles such as methanol, water, and other protic solvents, leading to the break of the π-conjugated system.76,77 A study on IR-1048 suggested that the connected C-position between the heterocycles and the methane chain would be more likely to be the target of nucleophilic attack.119

To address these problems, several methods have been developed. First, encapsulating hydrophobic NIR-II dyes into micelles would protect them from water to some extent, thereby be available for in vivo bioimaging (Figure 5a). Dai and co-workers performed abdominal vessels imaging using a phospholipid nanoparticle (IR-PEG) containing the small molecular dye IR-1061 for the first time, from which the smallest discernible vessel width was measured as 150 μm under >1 mm skin penetration depth (Figure 5b).85 Sletten and co-workers have constructed a new panel of polymethine cyanine dyes with emission from 680 to 1045 nm, utilizing a 7-dimethylamino flavylum heterocycle. NIR-II imaging with a micelle formulation of the longest-wavelength dye Flav7 was capable to penetrate through mouse rib and quantify the heart rate of the anesthetized mouse (Figure 5c).76 Second, the assembly of the molecule–protein complex can prevent molecular aggregation and greatly enhance fluorescence by constraining molecular rotation (Figure 5d). For example, previous studies have demonstrated the conventional NIR-II dyes ICG, IR-783, and IR12-N3 show tail fluorescence into the NIR-II window due to the enhanced quantum yield with the molecule–protein complex (4-fold, 6-fold, and 6-fold quantum yield enhancement for ICG, IR-783, and IR12-N3, respectively) (Figure 5e).2,5,10,109,120,121 NIR-II bioimaging using them provides many advantages over the NIR-I imaging, such as higher imaging contrast (from 0.20 to 0.29) and superior resolution (210 μm) of brain vasculature (Figure 5f). In particular, the first-in-human liver-tumor surgery guided by NIR-II fluorescence imaging was also realized by ICG.10 Third, molecular engineering by shortening the polymethine chain and further introducing electron-donating moieties at terminal heterocycles is an effective way to overcome solvatochromic-induced quenching. Zhang and co-workers have leveraged this strategy to construct a series of antiquenching benzothiopyrylium pentamethine cyanines (BTC fluorophores), which showed a maximum of 44-fold enhanced brightness as compared with their heptamethine analog IR-26. These fluorophores are capable of resolving lymph vessels with a minimum feature size of 84 μm in vivo NIR-II lymphatic imaging.75 Fourthly, large steric hindrance groups and small hydrophilic functional groups, such as sulfonate and carboxyl, are introduced into NIR-II dyes to enhance water solubility and prevent nucleophilic attack by solvent. For example, new NIR-II LZ dyes have large steric hindrance groups (diiodole)
and four water-soluble groups (sulfonate), leading to the superior monodispersity and chemstability in aqueous solution (Figure 5g). These fluorophores show promise for in vivo molecular labeling with less influence by fluorophore–biomolecule nonspecific interaction.

Except for the emission wavelength, the excitation wavelength is also very important to improve the NIR-II 1P fluorescence imaging depth and quality. Zhang and co-workers synthesized a fluorophore FD-1080, which not only resembles ICG but also shows much longer excitation and emission wavelengths at 1064 and 1080 nm, respectively, due to the larger π-system of the benzol[cd]indolium heterocycle (Figure 5h). They performed the first NIR-II excited 1P fluorescence imaging, which demonstrated that imaging under 1064 nm excitation through the scalp and skull can provide higher resolution on brain vessels (0.65 mm) than that under 808 nm excitation (1.43 mm). The same group also used BTC fluorophores and confirmed that excitation at 1064 nm improves the penetration depth, image SBR, and resolution by reducing the signal attenuation coefficient in tissue.

Recently, high-SBR (7.21) abdominal vessels imaging can also be achieved using SHS-1069 under 1064 nm excitation (Figure 5i).

**Excerpt for the emission wavelength, the excitation wavelength is also very important to improve the NIR-II 1P fluorescence imaging depth and quality.**

Most of the existing NIR-II polymethine cyanine dyes serve only as nontargeted fluorophores, such as IR-1061, FD-1080, and Flav7. For correct prognosis and therapeutics for disease, effective targeting NIR-II molecular dyes are in urgent demand. Some of the previously reported NIR-II dyes are modified with functional groups, including alkyne, NHS ester, maleimide, succinimidyl ester, isothiocyanate, azide, and carboxyl. Hong and co-workers reported a clickable NIR-II dye SHS-1069 which bound to integrin αvβ3 targeting peptide c(RGD)3k. Under 1064 nm excitation, the tumor-to-normal tissue ratio (T/NT) of U87 glioma tumor increased 1.7-fold compared to that under 808 nm excitation (from 4.0 to 6.8). Actually, for heptamethine dyes, like IR-1061, FD-1080, and Flav7, a cyclohexene group is introduced to the middle of the conjugated chain to offer a reactive site (Figure 5j). Yan and co-workers have reported a macromolecular NIR-II probe PF by designing Flav7 with covalent linking sulfhydryl groups to realize NIR-II image-guided photothermal therapy. To design covalent linkable fluorophores, a simple method is developed by a nucleophilic reaction at the central chloro group with a thionic or oxygenic substituent. These results provide a basis for bioapplication of NIR-II dyes with conjugation capacity.

Near-infrared polymethine cyanine dyes have always been of great interest in in vivo fluorescent biosensing application. Modifying substituents on polymethine chain or exploiting intermolecular photophysical interaction (such as FRET, aggregation-induced quenching) are the two common routes to design cyanine-based fluorescent probes. These strategies have been confirmed in NIR-II cyanine dyes; for example, Fan and co-workers designed dual activatable NIR-II nanoparticle HISSNPs to achieve MCF-7 xenografts cancer imaging through changing the IR-1061 aggregation state. In addition, manipulating the electronic effects of substituents on terminal heterocycles is another promising route to construct novel NIR-II probes. This is because the extended π-conjugated system of NIR-II cyanine dyes greatly increases the contribution of the substituents of terminal heterocycles on the photophysical properties. Zhang and co-workers investigated the position-dependent effect of diethylamino moieties on the spectra and quantum yield of BTC fluorophores, which allowed the creation of an NIR-II fluorescent low-pH sensor BTC1070 (Figure 5k). Protonation of diethylamino moieties changed the electron-donating effect and in turn resulted in ratiometric fluorescence signal output (1065/980 nm). The low scattering and superior sensing accuracy of NIR-II ratiometric signals allowed for noninvasive gastric pH quantification under 4 mm biotissue penetration (Figure 5k).

Furthermore, on the basis of increased understanding of the photophysical and chemical properties of NIR-II polymethine cyanine dyes, various activatable or ratiometric NIR-II fluorescent probes have been created for monitoring physiological events or biomarkers in vivo, such as reactive oxygen/nitrogen species (ROS/RNS, Figure 5l) and enzyme.

It should be noted that polymethine cyanine dyes hold the property to form head-to-tail dipole arrangement J-aggregates in certain conditions. Compared with monomers, J-aggregates exhibit unique photophysical properties of bathochromically shifted absorption and emission bands, enhanced absorption coefficients (ε), and shortened fluorescence lifetimes. J-aggregates have many advantages for in vivo imaging. For instance, red-shifted absorption and emission wavelength are capable of increasing penetration depth, and in addition, the increased ε will enhance brightness. However, it is difficult to obtain stable and highly purified J-aggregates in solutions. At present, nanocarriers are a promising approach to promote and stabilize J-aggregates. Sletten and co-workers reported IR-140 J-aggregates through encapsulating IR-140 into hollow mesoporous silica nanoparticles, which showed high stability in aqueous solution for several weeks. After aggregation, IR-140 J-aggregates showed bathochromically shifted absorption and emission from 862 and 875 nm to 1040 and 1047 nm, respectively (Figure 5m). Holding the longer emission wavelength caused deep tissue penetration; intense signals were observed at lungs, liver, and spleen. Fan and co-workers also reported NIR-II J-aggregate nanoparticles SQR-NPs by encapsulating bispyrrole-sq-bispyrrole (SQP) into PEG-b-PPG-b-PEG (F-127). In addition, NIR-II molecular dye also holds promise in forming J-aggregates. Zhang and co-workers developed FD-1080 J-aggregates formed by self-assembly between FD-1080 and 1,2-dimyr-istoyl-sn-glycero-3-phosphocholine (DMPC) (Figure 5n). FD-1080 J-aggregates showed a high SBR (5.6 and 4.76 for brain and hind limb vessels, respectively) for in vivo dynamic vascular imaging beyond 1500 nm. Thus, stabilizing by nanocarriers is an efficient way to form J-aggregates as NIR-II imaging agents.

5. **SUMMARY AND OUTLOOK**

The “tissue-transparent” NIR-II spectral window has received considerable interest in the past decade in view of the lower scattering, moderate tissue absorption, and few tissue autofluorescence. By taking advantage of these features,
emerging fluorescence methods based NIR-II nonlinear excitation and NIR-II linear emission have revolutionized in vivo bioimaging with unprecedented penetration depth and image resolution. Typical nonlinear methods, 2P/3P microscopies, permit diffraction-limited three-dimensional imaging of tissues in vivo with single-cell or subcellular resolution, but usually need invasive procedures such as implantable chronic windows. Compared with traditional 1P microscopy, the advantages of 2P/3P microscopies are mainly the three-dimensional deep-tissue high-contrast imaging capability due to the nonlinear contrast generation mechanism. Therefore, these methods are preferred for basic biology studies in which interrogating cellular structure and function in thick tissue and living animals is required, for instance, to visualize cell–cell interactions, neurovascular coupling, and neuronal activity in brain research, and to study the dynamics of immune-cell interactions in intact tissue. However, as 2P/3P microscopy operates with small interactions in intact tissue, however, as 2P/3P microscopy operates with small interactions in intact tissue, it does not yield a complete picture of the underlying activity across varying spatial scales, for example, in mice whole body and even larger human organs. For this purpose, the NIR-II linear fluorescence method with mesoscopic and macroscopic imaging capability may become highly complementary. With an acceptable loss of resolution, this method provides a penetration limit several or a dozen times deeper than 2P/3P microscopy and, thus, is well suited for noninvasive imaging of entire organs and animals in vivo and is more typically part of mouse-to-human clinical translation imaging applications. Its applications include, for example, molecular imaging of entire tumors, imaging-guided surgery, visualizing anatomic structure and hemodynamic information, brain imaging, and the study of the movement of stem cells or immune cells at deep locations. With the development of technology, both nonlinear and linear fluorescence methods are constantly overcoming their limitations, allowing investigation of biological systems across a wide range of spatial and temporal scales. Pushing the frontier of deep-tissue fluorescence bioimaging also requires the design of functional fluorophores that adapted to each method. The complementarity of nonlinear and linear fluorescence methods provides a wide spectral range (500–1700 nm) for chemists to develop novel fluorophores. In this Outlook, we outline the molecular fluorophores that have evolved over the past five years, driven by the technological advances of nonlinear and linear fluorescence bioimaging in the “tissue-transparent” NIR-II window. The impressive performance of these fluorophores in structural and functional imaging of the mouse brain, tumor and whole body, and in clinical diagnosis provide a huge motivation for people to go forward. Nevertheless, current efforts are still in their infancy, and there remains a gap regarding people’s high expectations for deep-tissue high-resolution fluorescence bioimaging. Regardless of nonlinear or linear fluorescence, the ideal molecular fluorophores would have exquisite brightness and photostability, small size, improved in vivo bioavailability, and ease of use and functionalization, and their nonlinear excitation or linear emission wavelengths better fall in the outstanding spectral area of NIR-II window, for example, around 1300 nm or 1700 nm. Unfortunately, a molecular fluorophore that fulfills all of these criteria has not yet been developed. Some challenges may be objectively difficult to overcome. For example, a trade-off between wavelength and quantum yield is inevitable, because nonradiative relaxation is enhanced at longer wavelengths of the NIR spectrum, partly induced by the vibrational overtone of surrounding chemical bonds such as C–H, O–H, and N–H. Fortunately, the application of modern synthetic techniques allows further structural refinements to yield dyes with better photophysics, making us believe that the upper limits of quantum yields of most fluorophores have not yet been reached. Chemists also should not be discouraged in the hurdles that will have to be overcome to create a perfect fluorophore. Indeed, some intriguing and unanticipated phenomena in the application of fluorophores may have a significant impact on biology and biomedicine. The fluorogenic properties of X-rhodamine after binding to self-labeling tags are good examples: In-depth studies of this discovery have created a genetically targetable fluorogenic toolbox and chemigenetic sensor platform, making synthetic dyes progressively attractive and accessible to neurobiologists. Likewise, the phenomena that spectra change and fluorescence enhancement caused by the interaction of NIR-II emitted fluorophores (such as CH-4T and FD-1080) and biomolecules (such as albumin and DMPC lipids) may also imply the emergence of a new imaging and sensing platform based on hybrid structures.

Likewise, the phenomena that spectra change and fluorescence enhancement caused by the interaction of NIR-II emitted fluorophores (such as CH-4T and FD-1080) and biomolecules (such as BSA and DMPC lipids) may also imply the emerging of new imaging and sensing platform based on hybrid structures.

Finally, the overview that is provided in this Outlook aims to call on the research community to strengthen cooperation with scientists among diverse research fields, such as biology, biomedicine, and microscopy. We look forward to the next era of fluorescence bioimaging in which the frontiers of NIR-II nonlinear and linear techniques expand with the help of new molecular fluorophores.

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