Near-Infrared-II fluorescence imaging

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Abstract | Fluorescence imaging in the second near-infrared (NIR-II) window enables deep-tissue imaging with high resolution and improved contrast by taking advantage of the reduced light scattering and tissue autofluorescence in this region of the spectrum. NIR-II fluorescence imaging uses photoluminescent contrast agents — including carbon nanotubes, quantum dots, rare-earth doped nanocrystals, gold nanoclusters, small molecules and their aggregates — and fluorescent proteins, which all exhibit fluorescence in the 1,000-3,000 nm range. After administration of these fluorophores in vivo, live animals can be imaged with specialized detectors and optical instruments, yielding images with contrast and resolution unparalleled by conventional visible and near-infrared fluorescence imaging. This powerful approach enables dynamic imaging of vascular structures and hemodynamics; molecular imaging and image-guided surgery of tumors; and visualization of deep-seated structures, such as the gastrointestinal system. NIR-II fluorescence imaging has revolutionized biomedical imaging over the past 15 years and is poised to make comparable advancements in cardiology, neurobiology, and gastroenterology. This Primer describes the principles of NIR-II fluorescence imaging, reviews the most used fluorophores, outlines implementation approaches, and discusses specific scientific and clinical applications. Furthermore, the limitations of NIR-II fluorescence imaging are addressed and future opportunities across various scientific domains are explored.

[H1] Introduction

The second near-infrared (NIR-II) window, also known as the short-wave infrared (SWIR) window, is a subregion of the electromagnetic spectrum with wavelengths ranging from 1,000 to 3,000 nm.[1–4] NIR-II fluorescence imaging is a technique that uses fluorescence emission within this window to visualize anatomical structures, biological molecules, and functional activities in biological tissues.[5–7] Compared to traditional fluorescence imaging in the visible (400-700 nm) and conventional near-infrared (NIR-I, 700-1,000 nm) windows, NIR-II photons exhibit deeper penetration in biological tissues owing to reduced scattering **[G]** and autofluorescence **[G]** (**Fig. 1**).[1] This advantage makes NIR-II imaging particularly beneficial for capturing high-resolution fluorescence images at depths of several millimeters, where conventional fluorescence imaging struggles to provide clear visualization.[2,8,9] For this reason, the NIR-II spectrum is an attractive choice for imaging deep tissues, such as subcutaneous lymph nodes, neurons in deep brain regions, deep-seated tumors, and intestines within the abdominal cavities. This deep-tissue imaging ability is especially important in live animals, such as mice,[5,10,11] pigs,[12] non-human primates,[13] and humans.[7]

The underlying principle of NIR-II imaging is to use photoluminescent contrast agents or labels with emission in the 1,000-3,000 nm spectral region.[2,14–16] These agents, when excited by a shorter-wavelength light source (700-1,650 nm), emit light in the NIR-II window.[1,2] Existing NIR-II contrast agents include carbon nanotubes (CNTs),[4,6,10,15,17,18] quantum dots (QDs),[2,19–21] small molecules,[14,22–24] fluorescent proteins,[25,26] rare-earth nanoparticles (RENPs),[16,27–29] and gold nanoclusters (AuNCs).[30–33] Amongst the several modes of NIR-II fluorescence imaging, widefield and raster scanning are currently the most widely used.[34] Widefield imaging, which is common in epifluorescence **[G]** imaging and light sheet microscopy, involves a broad laser beam or light-emitting diode (LED) to simultaneously stimulate NIR-II emitters in a sample.[5,8] The emitted fluorescence signal is then detected by a two-dimensional (2D) indium gallium arsenide **[G]** (InGaAs) camera that produces an image of the spatially distributed NIR-II emitters projected onto a single plane.[35] The other widely used method, raster scanning, employs techniques such as NIR-II confocal microscopy for volumetric three-dimensional (3D) imaging.[2] Raster scanning involves spatial scanning of a focused laser beam and point-by-point emission detection by detectors such as InGaAs photodiodes **[G]** , photomultiplier tubes **[G]** (PMTs), or superconducting nanowire single-photon detectors **[G]** (SNSPDs).[2,36] While NIR-II fluorescence imaging has familiar optical setup designs, it achieves superior signal-to-noise ratio **[G]** (SNR) and spatial resolution at greater tissue depths by harnessing NIR-II photons that have reduced scattering and autofluorescence within the tissue.

Over the past decade, NIR-II fluorescence imaging has advanced. Several new NIR-II contrast agents have emerged, encompassing molecularly engineered NIR-II dyes,[14,37,38] genetically engineered NIR-II fluorophores,[25,39] NIR-II J-aggregates,[40–42]

and rare earth down-conversion nanoparticles.[16,29,43] These emerging fluorophores exhibit bright NIR-II emission, enhanced biocompatibility, and a wide range of functionalities. In particular, the FDA-approved contrast agent indocyanine green has been repurposed for NIR-II fluorescence imaging in rodents and humans due to its extended emission spectrum beyond 1,000 nm.[7,22,44] The development of brighter fluorophores, along with faster detectors, has substantially improved NIR-II fluorescence imaging by enhancing its temporal resolution up to ~100 frames per second (fps).[20,45,46] This advancement has enabled dynamic events in living systems to be visualized in real-time, such as quantitative dynamic monitoring of blood perfusion in the cerebral and peripheral vessels,[5,10] time-resolved imaging of cardiac cycles,[47,48] and real-time in vivo imaging of renal clearance dynamics.[14] Alongside high temporal resolution, advances in confocal[2] and light sheet microscopy[8] yield higher spatial resolution, enabling in vivo molecular imaging at the cellular level. Specifically, NIR-II molecular imaging is now possible using clickable dyes to image brain tissue at a molecular level.[37] Expanding on molecular imaging, multiplexed NIR-II molecular imaging was achieved by developing probes with different emission wavelengths in the NIR-II spectrum or with different excited state lifetimes.[16,20] Several subregions of the NIR-II spectrum, such as NIR-IIa (1,300-1,400 nm), NIR-IIb (1,500-1,700 nm), NIR-IIc (1,700-2,000 nm), and NIR-IId (2,100-2,300 nm) have been proposed based on their relation to major water absorption peaks.[2] Lastly, integration with other imaging techniques, such as multiphoton microscopy and structured light illumination, has opened up new possibilities for NIR-II imaging.[49,50] These advances have paved the way for diverse preclinical and clinical applications.[7,32,51]

The focus of this Primer is on preclinical NIR-II fluorescent imaging in animal models and the clinical translatability of emerging small-molecule NIR-II fluorescent agents for imaging in humans. The emphasis on small-molecule NIR-II fluorophores arises from their capability to provide high-resolution imaging in deep tissues, coupled with enhanced molecular targeting precision and specificity, while ensuring rapid clearance from the body. The latest methods for experimentation and interpretation of NIR-II fluorescence imaging are explored in the context of its extensive applications. Furthermore, best practices in reproducibility and data deposition are highlighted, promoting consistency and comparability across different laboratories and experiments, with the goal of setting widely accepted standards. By delving into these specific aspects, the Primer aims to provide a comprehensive understanding of the opportunities and challenges in translating NIR-II fluorescence imaging from bench to bedside.

[H1] Experimentation

In a standard NIR-II fluorescence imaging experiment, an excitation light source is used to stimulate NIR-II contrast agents or labels within biological tissues of live subjects. These agents or labels subsequently emit photons in the NIR-II spectrum, which are captured by a detector. The varied spectral and lifetime properties of the

NIR-II fluorophores enable multiplexed imaging in both the spectral and time domains. NIR-II fluorophores can either fill hollow structures — for example, blood vessels, lymphatic vessels, ureters and intestines — to offer structural contrast or adhere to specific tissues and molecules for targeted labeling. Before experimentation, it is essential to assess the luminescence, biochemical, and pharmacological properties of NIR-II fluorophores. Additionally, factors such as laser safety and ethical handling of animal and human subjects are crucial when implementing NIR-II fluorescence imaging.

[H2] Optical systems for NIR-II fluorescence imaging

[H3] Excitation sources

NIR-II imaging relies on detecting NIR-II photons emitted by luminescent probes post-excitation. Broadly, these NIR-II luminescent probes can be stimulated by various excitation sources, including light, X-rays, pressure, and chemical reactions.[1,11,52–56] Light in the 700-1,650 nm region is the most frequently used excitation source in NIR-II fluorescence imaging.[2,15]

Custom-built and commercially available NIR-II fluorescence imaging systems come with an excitation light source designed to stimulate NIR-II emission from the probes. When choosing the operating wavelength for excitation sources, three critical factors come into play. First, for maximum excitation efficiency, the power distribution spectrum of the excitation source should closely match the excitation spectrum of the NIR-II fluorophore. Notably, the excitation spectrum can be roughly gauged by the fluorophore's absorption spectrum. Second, the excitation wavelength must ensure deep tissue penetration. This requirement ensures efficient excitation of deeply situated NIR-II fluorophores, while minimizing scattering and absorption of excitation photons by the biological tissue. The tissue penetration criterion requires the excitation wavelength to fall within the two optical windows of biological tissues to reduce attenuation of the excitation light.[3] This stipulation establishes a lower bound of 700 nm for excitation.

The third critical factor is that the excitation light should cause minimal damage to the biological tissue. In NIR-II fluorescence imaging, there are two primary types of excitation light-induced damage: photochemical and photothermal. Photochemical damage arises from reactive oxygen species **[G]** that are predominantly generated by endogenous chromophores after they absorb short-wavelength excitation light (< 600 nm).[57–59] This type of damage hinders cellular functions because chemical reactions with vital biomolecules, such as DNA, are initiated by reactive oxygen species. Photothermal damage stems from the conversion of absorbed light into heat, primarily driven by longer wavelengths, particularly red to near-infrared light (> 600 nm).[57] Simplifying for a first-order approximation, the photothermal absorption of tissues can be attributed to water, which is abundant in most soft biological tissues. Since water's

overtone absorption **[G]** bands manifest at 970 nm, 1,200 nm, 1,450 nm, and beyond 1,800 nm,[60] using excitation wavelengths different to these bands is recommended.

The excitation wavelength should ideally fall within the 700-1,650 nm range, provided it aligns well with the excitation spectrum of the NIR-II fluorophore and avoids the major absorption bands of water. Conventional NIR-II fluorophores such as CNTs, QDs, and RENPs, excited in the 700-1,000 nm range exhibit minimal photothermal effects but face challenges with lingering scattering and autofluorescence. By contrast, recent fluorophores that absorb above 1,000 nm in the NIR-II region improve image clarity and penetration depth due to reduced scattering and autofluorescence, however, there is a potential to increase tissue heating near water absorption bands.[61–64] Selecting wavelengths within the 700-1,650 nm range for excitation is justified by the availability of semiconductor diode lasers **[G]** and LEDs that operate within this spectrum.[65] Specifically, AlGaAs lasers, which commonly have output wavelengths at 785 nm and 808 nm, emerge as primary choices for excitation in NIR-II fluorescence imaging.[6,7] In addition, Nd-doped yttrium aluminum garnet (Nd:YAG) lasers with a typical emission band at 1,064 nm offer deep penetration with minimal photothermal effects in biological tissues.[63,66,67] Lasers typically have a narrow spectral distribution of emitted power, making them suitable for optically exciting NIR-II fluorophores with a narrow absorption spectrum. However, NIR-II fluorophores with wider absorption bands are more effectively excited by LEDs. This is because LEDs typically have a broader power distribution spectrum, ensuring maximal overlap between the light source's excitation power and the fluorophore's absorption.

The spectral width of a specific excitation light source has another important implication. Since no light sources emit an ideal single-wavelength, they have a residual power distribution in the NIR-II spectrum. Although this residual power is weak compared to the peak power, it can surpass the fluorescence intensity, creating undesired background in the image. Therefore, it is crucial to use a bandpass or shortpass filter — referred to as an excitation filter **[G]** — before the light reaches the subject to eliminate the light source's long-wavelength residual power. Additionally, an emission filter **[G]**, usually a longpass filter, is needed between the subject and detector to eliminate excitation light reflection off the subject. A general guideline for selecting the excitation and emission filters is provided in **Supplementary Note 1**.

Finally, an additional consideration for excitation is the homogeneity of the illumination intensity over the entire field of view. Quantitative analysis of NIR-II fluorescence images requires homogeneous excitation intensity over the whole field of view to establish a correlation between NIR-II intensity and local fluorescent probe concentration. Homogeneous illumination can be achieved by using a multi-fibre array or an optical diffuser **[G]**. Although optical diffusers offer a more uniform intensity distribution, undesired impurities in the diffuser can alter the spectral purity of the laser source. For instance, the glass used to build spatial diffusers may contain neodymium

ions, which absorb the 808 nm excitation light, leading to 1,045 nm fluorescence that causes false contrast in the in vivo image.[68]

Schematic representations of conventional in vivo NIR-II imaging systems (**Fig. 2**) illustrate the importance of the excitation source in NIR-II fluorescence imaging. In widefield NIR-II fluorescence imaging, the excitation appears as a broad plane of photon flux (**Fig. 2a**). In addition, for raster-scan confocal fluorescence imaging in the NIR-II spectrum, the excitation manifests as a focused spot (**Fig. 2b**), which is scanned point-by-point to traverse the entire 3D volume of the sample. Furthermore, for NIR-II light-sheet microscopy, the excitation presents as a slim sheet of illumination, which excites the fluorophores within a single plane at a time (**Fig. 2c**).

[H3] Image formation optics

The optical systems for NIR-II fluorescence images include widefield, confocal, and light-sheet configurations (**Fig. 2a-c**). However, a consistent principle for all setups is that the image formation optics should always have a lens system paired with a detector that is sensitive to photons in the NIR-II spectrum. Beyond these elements, individual NIR-II fluorescence imaging setups may incorporate unique components, such as optomechanics **[G]** like a Galvo scanner, translation stage, and filter wheel. Broadband mirrors and dichroic mirrors **[G]** are also typically added as required.

Detectors commonly used for NIR-II fluorescence imaging include single-pixel detectors and 2D detector arrays. Among the single-pixel detectors, popular choices are InGaAs photodiodes, InGaAs avalanche photodetectors **[G]** (APDs), InGaAs PMTs, and NbTiN SNSPDs.[2,36,37,69] These detectors capture the NIR-II fluorescence intensity from a single point in space at a time. To generate full 2D or 3D images, the pixel data is assembled using raster scanning enabled by optomechanical mechanisms. For widefield imaging and light-sheet microscopy within the NIR-II spectrum, a 2D array of InGaAs detectors is typically used.[5,7,8,10,11,25,70] This array can simultaneously create a 2D image without requiring pixel-by-pixel signal capture. Several examples of NIR-II detectors and their characteristics are summarized in **Supplementary Note 2**.

In NIR-II fluorescence imaging, the lens system performs two primary functions. First, it shapes the excitation light into the desired illumination pattern. Second, it gathers and directs the NIR-II fluorescence photons toward the detector to form an image. It is crucial for the lens material to exhibit low absorption in the NIR-II range. Additionally, each lens should feature an antireflective coating tailored to the NIR-II spectrum. For generating the desired excitation patterns, the lens system might include a collimator for widefield imaging, an objective lens for focused excitation in confocal imaging, or a cylindrical lens for creating a light sheet. For widefield fluorescence imaging, the image-formation lens system (**Fig. 2d**) has several important parameters — such as focal length (*f*), working distance (*WD*), and back focal distance (*BFD*) — which are

connected to the camera's horizontal dimension (*H*) and the horizontal field of view (*FOV*) as follows:

$$\frac{1}{WD} + \frac{1}{BFD} = \frac{1}{f} \quad (1)$$

and

$$M = \frac{H}{FOV} = \frac{BFD}{WD} \quad (2)$$

where *M* is the magnification of the imaging system. In widefield NIR-II fluorescence microscopy that has an infinity-corrected objective **[G]**, *WD* is the same as the objective's working distance, while *BFD* equals the focal length of the tube lens.

Combining Eq. (1) and (2) shows that the FOV can be expressed as:

$$FOV = \frac{H}{\frac{BFD}{f} - 1} \quad (3)$$

where the *FOV* is directly proportional to *H*. Eq. (3) also shows that for a lens system with a fixed *f*, moving the entire system towards the imaged object magnifies the image since the FOV is reduced. Second, when $WD \gg BFD$, FOV can be approximated as

$$FOV = H \cdot \frac{WD}{f} \quad (4)$$

which is usually a good approximation for whole-body NIR-II fluorescence imaging of rodents. The real-space pixel resolution d_{rs} is proportional to the pixel size in the camera d_{ca}:

$$d_{rs} = d_{ca} \cdot \frac{WD}{f} = d_{ca} \cdot \frac{FOV}{H} \quad (5)$$

Additional components can be incorporated into the image formation optics as needed to achieve specific functions, such as NIR-II confocal microscopy, NIR-II fluorescence tomography, or NIR-II fluorescence imaging in time and spectral domains. A detailed discussion of these specialized methods can be found in **Supplementary Note 3**.

[H2] Fluorescent probes for NIR-II imaging

The requirements of minimum tissue damage and maximum tissue penetration dictate that the ideal fluorescent probes for NIR-II fluorescence imaging should be excited in the 700-1,650 nm range, with emission wavelengths beyond 1,000 nm. Several NIR-II fluorescent probes have been developed that meet these criteria. These NIR-II fluorophores can be grouped into three main categories: inorganic nanoparticles, organic molecules (and their aggregates), and infrared fluorescent proteins (**Fig. 3**).

[H3] Inorganic NIR-II nanoparticles

Inorganic nanoparticles that emit in the NIR-II range are nanostructures with at least one dimension measuring less than 100 nm. Common NIR-II emitting fluorophores include CNTs, QDs, RENPs, and AuNCs (**Table 1**). The unusual NIR-II emission of these inorganic nanoparticles is closely tied to their chemical composition, structure, and size. Different materials obtain their NIR-II fluorescence emission via different mechanisms. The NIR-II fluorescence of CNTs, QDs, and AuNCs arises from quantum confinement effects due to their small size.[71–73] The NIR-II fluorescence of RENPs is

governed by the energy levels and f-f transitions in doped lanthanide ions.[43] Inorganic NIR-II fluorophores benefit from sharp and intense emission peaks, tunable emission wavelengths up to a few micrometers, and excellent photostability. On the downside, their biocompatibility and pharmacokinetics are often limited due to the inclusion of heavy metal ions and relatively large sizes.

Among all NIR-II-emitting fluorophores, CNTs have served as pioneers.[3,4] Their NIR-II emission spectrum has narrow bandwidths (35–80 meV), enabling sensitive and precise detection of local environments through quenching and solvatochromic responses, as well as emission modulation via quantum defect sites.[6,15,18] Their resistance to photobleaching is an additional advantage for long-term imaging. However, the relatively low quantum efficiency of CNTs causes a photothermal effect that has limited broader applications. Recently, copper–indium–selenium (CISe) nanotubes have emerged as a promising alternative with a high quantum yield of 12.4% and an extended lifetime of 336.1 μs. CISe nanotubes facilitate phosphorescence imaging with minimal interference from background autofluorescence.[74]

NIR-II fluorophores based on QDs have also gained prominence. Among the first examples are silver sulfide (Ag_2S) QDs, renowned for high brightness, long circulation half-life, and high tumor-targeting specificity.[19,75] Ag_2S QDs have a temperature-dependent NIR-II fluorescence intensity, making them useful as nanothermometers for non-contact brain temperature measurements.[76,77] More recently, lead sulfide (PbS) QDs have gained attention due to their extremely long emission wavelength, extending into the NIR-IIc window (1,700-2,000 nm), offering unparalleled tissue imaging depths.[2] Another notable development is indium arsenide (InAs) QDs, which have an exceptionally high fluorescence quantum yield, enabling fast image acquisition for dynamic NIR-II fluorescence imaging.[20] However, a major concern of inorganic quantum dots (QDs) is the potential for heavy metals, such as lead and arsenic, to be toxic in biological systems.

A newer class of inorganic NIR-II fluorophores is RENPs. These particles are beneficial for advanced imaging because they offer discrete, narrow emission bands and adjustable fluorescence lifetimes, enabling spectral-domain and time-domain multiplexing techniques, respectively.[16,78] The emission wavelengths of RENPs in the NIR-II region can be tailored by selecting specific lanthanide ions, extending up to 2,842 nm.[79] Moreover, RENPs can be engineered to offer the unique ability of being excited by X-rays. X-ray excited RENPs produce persistent luminescence, which continues for minutes or hours after the excitation source ceases.[11] This long-lasting luminescence delivers better SNR in deep-tissue imaging (up to 4 mm) compared to traditional NIR-II fluorescence imaging, while enhancing the precision of in vivo multiplexed encoding and multilevel encryption.

Lastly, AuNCs represent an emerging and promising category of inorganic fluorophores for in vivo NIR-II imaging.[30–33] AuNCs have a metal core containing tens of atoms stabilized by organic ligands. They are small, with sizes under 2 nm. This small size reduces potential toxicity by minimizing accumulation in the body's mononuclear phagocytic system system and enhances renal clearance through the kidneys.[30–32,80]

[H3] Organic NIR-II molecules

In contrast to inorganic fluorophores, organic NIR-II molecules are developed to enhance pharmacokinetics, biocompatibility, and targeting specificity. However, they typically exhibit weaker fluorescence, poorer photostability, and shorter emission wavelengths. To overcome these challenges, several organic molecules were specifically tailored for NIR-II fluorescence imaging. Dyes, such as indocyanine green (ICG) and IRDye 800CW, which have peak emission in the NIR-I spectrum, have been repurposed for NIR-II applications because their spectral tail extends into the NIR-II range. Beyond free-form organic compounds, their protein complexes and aggregates, particularly J-aggregates, are also gaining attention as NIR-II fluorescent agents.

CH1055 was the first organic molecule specifically designed for NIR-II fluorescence imaging. CH1055 is a fluorescent compound with a donor–acceptor–donor (D–A–D) structure that can be made water soluble for targeted delivery by addition of polyethylene glycol (PEG), resulting in CH1055-PEG.[14] CH1055-PEG has a small molecular weight (8.9 kDa) and can be quickly excreted through the kidneys within 24 hours of intravenous administration. To boost the low quantum yield of D-A-D type fluorophores, shielding units have been added to both sides of the D-A-D structure.[81] Besides D-A-D type fluorophores, cyanine dyes are another class of small molecules that exhibit NIR-II fluorescence. For instance, FNIR-1072 is a specific type of cyanine dye known as a nonamethine cyanine, which emits light at 1,103 nm. This emission wavelength is longer than its heptamethine cyanine counterparts, owing to a more extended π-conjugation system.[24] The chromenylium heterocycle has emerged as a promising, red-shifted scaffold for polymethine fluorophores. Recent advances have introduced clickable groups to this structure, creating a modular scaffold that enables tunable solubility and targeted activity.[82] Lastly, tetra-benzannulated xanthenoid is another class of NIR-II fluorophores with intense absorption and emission beyond 1,200 nm.[64]

Besides rationally designed organic NIR-II molecules, well-known cyanine dyes like ICG and IRDye 800CW have been repurposed for in vivo NIR-II imaging due to their tail emission beyond 1,000 nm.[22,44] ICG — FDA-approved since 1959 — and IRDye 800CW are being studied in clinical trials for fluorescence angiography and image-guided cancer surgery.[83,84] This makes them promising candidates for NIR-II imaging in clinical settings, as recently showcased in the first human liver-tumor surgery guided by NIR-II fluorescence.[7]

Using organic molecule aggregates in a micelle or matrix for NIR-II imaging was originally motivated by the need to solubilize highly hydrophobic, water-insoluble cyanine or thiopyrylium dyes.[47,85,86] A sulfonated D-A-D dye (CH-4T) forms supramolecular assemblies with proteins in the serum, resulting in the CH-4T@protein complex with an exceptional NIR-II quantum yield of 11%.[48] Similarly, forming protein complexes with cyanine dyes yields an NIR-II fluorophore with a quantum yield of 21.2%.[87] Another method for enhancing NIR-II fluorescence is via aggregation-induced emission **[G]** (AIE). This approach increases the NIR-II fluorescence of organic molecules that are otherwise non-emissive.[88–94] Notably, zwitterionic isocyanorhodium(I) complexes exhibit intense NIR-II phosphorescence upon aggregation in an aqueous solution.[95] J-aggregates are another promising strategy for shifting the fluorescence of certain organic molecules from the visible and NIR-I ranges into the NIR-II spectrum.[41,96,97]

Another significant research direction involves reducing the molecular weight and size of NIR-II emitting molecules. Size reduction is driven by the need for rapid renal excretion and ability to cross the blood-brain barrier (BBB). A notable development is the creation of boron difluoride (BF2) formazanate NIR-II dyes, with modifications to the aniline moiety to enhance BBB penetration for noninvasive brain imaging.[98] Additionally, styrene oxazolone dyes, inspired by the chemical structure of chromophores in fluorescent proteins, have been synthesized. These dyes exhibit NIR fluorescence and have small molecular weights (<450 daltons), facilitating rapid renal clearance and BBB crossing.[99]

Inorganic lanthanide ions can be combined with organic ligand molecules to produce NIR-II fluorophores with unique spectral properties. For example, a molecular erbium(III) complex coordinated with bacteriochlorin and a Kläui ligand exhibits a large Stokes shift (>750 nm) and exceptionally sharp emission peaks (peak width ≤ 32 nm).[100]

[H3] Genetically engineered proteins with off-resonance NIR-II emission

Genetically encoded fluorescent proteins are used to address the challenges of exogenous NIR-II probes (**Supplementary Note 4**). Several genetically encoded red-shifted NIR fluorescent proteins have been developed with fluorescence emission in the NIR-II window. MiRFP718nano was developed as a red-shifted NIR fluorescent protein that efficiently binds to biliverdin chromophore, with a tail emission extending well beyond 1,000 nm.[25] Another infrared fluorescent protein, iRFP713, exhibits off-resonance fluorescence in the NIR-II spectrum despite having a peak emission at 713 nm.[26] A genetic engineering method was used to produce a range of albumin fragments and recombinant proteins that form covalent bonds with cyanine dyes to enhance their off-resonance tail emission in the NIR-II spectrum.[39] The recombinant

proteins covalently bind with cyanine dyes, enhancing their brightness and stability, providing water solubility and the potential for further functionalization. The process is similar to covalent integration of the exogenous chromophore biliverdin by genetically encoded fluorescent proteins to achieve ultra-red and infrared emission.[101,102]

[H2] Preparation and administration of NIR-II probes

Once the NIR-II probe has been selected, the following points must be considered when preparing and administering the NIR-II probe in live subjects (**Fig. 4**):

[H3] Evaluation of cytotoxicity and systemic toxicity

The first step when preparing NIR-II probes for in vivo imaging is to assess their cytotoxicity in vitro (**Fig. 4a**). This evaluation should cover a range of concentrations and employ model cell lines, such as human embryonic kidney cells **[G]**; specific cancer cell lines like 4T1 and U87MG cells; cardiomyocytes; and primary hippocampal neurons. The upper concentration bound for in vitro testing should be 2-10 times higher than the intended in vivo concentration.[103] Once a non-toxic concentration is identified, it should be used for in vivo testing (**Fig. 4b**). A concentration proven safe during in vitro testing may not be safe for in vivo studies. Critical metrics — such as mouse survival rate, weight changes, circulation half-life, biodistribution in different organs, excretion pathways, blood panels, and histological evaluation of major organs — should be monitored to assess in vivo toxicity at specific concentrations, usually reported in microgram or milligram per kg body weight.

[H3] Excitation power density evaluation

Once the specific excitation wavelength has been selected, either capillary tubes with a 50-μm diameter for mesoscopic imaging or subdiffraction-sized spherical beads for microscopic imaging should be prepared and loaded with the chosen NIR-II fluorescent probes.[2] The feature sizes and probe loading concentrations of the capillary tubes or beads should match those intended for the in vivo experiments. The samples should then be placed at an equivalent depth in a scattering phantom, such as 5% Intralipid **[G]**, to determine the minimum excitation power density required to achieve an SNR of at least 5 (**Fig. 4c**).[2] The autofluorescence background of the scattering phantom should also be evaluated as it might differ from in vivo conditions.

[H3] Photobleaching and photothermal effects

Before proceeding with live animal experiments, the photobleaching and photothermal effects of the probes need to be characterized. Photobleaching can be assessed by continuously illuminating the sample with the chosen excitation wavelength and minimal excitation power density for 1 hour while monitoring the NIR-II fluorescence intensity. Temperature recording with a thermal camera can be performed simultaneously to understand any photothermal effects during fluorescence imaging (**Fig. 4d**).

[H3] In vivo administration

Upon satisfactory completion of the above steps, the probe solution can be administered into live mice via a suitable route, such as intravenous, retro-orbital, intratumoral, or intraperitoneal injection. To prevent agglomeration, the solution should be sonicated thoroughly before administration. Initial imaging should use the established minimum excitation power density, with adjustment of the excitation power and exposure time as needed to optimize SNR, but without exceeding limits set by the International Commission on Non-Ionizing Radiation Protection.[57] Throughout the imaging procedure, real-time monitoring of the animal's breath rate, skin, and rectal temperatures is essential to ensure comfort during the experiment.

[H2] Data collection for NIR-II fluorescence imaging

The data collection process for NIR-II fluorescence imaging requires carefully coordinated steps to ensure high-quality images and meaningful results. After choosing suitable excitation sources and setting up the imaging system, the imaging process usually begins immediately after administration of the NIR-II fluorescent probe to capture the dynamics of NIR-II fluorescence changes in the body. The excitation power density and exposure time are carefully adjusted, starting at minimal settings and fine-tuning to optimize the SNR. A few key considerations during data collection are summarized in this section.

[H3] Frame rate of widefield NIR-II imaging

In widefield imaging, each image is captured by projecting the entire field of view onto the sensing area of a 2D InGaAs camera. Since all pixels are captured simultaneously, the frame rate is typically determined by the sum of the exposure time and instrument overhead time. Using brighter NIR-II fluorophores can reduce the exposure time, while the overhead time can be reduced with buffered capture, batch operation, and pixel binning.

[H3] Frame rate of confocal NIR-II imaging

Confocal NIR-II fluorescence microscopy is based on raster scans rather than simultaneous projection of the entire 2D field view. As a result, the frame rate of confocal NIR-II microscopy is less affected by the photodetector overhead time. In confocal NIR-II microscopy, the frame rate for a 2D optical section is determined by 1/(dwell time/pixel x number of pixels/frame). As InGaAs photodetectors usually have rise and fall times and pulse widths on the order of nanoseconds, a dwell time on the order of microseconds is typical for exposure times that enable enough fluorescence photons to be collected. In this case, the frame rate can be roughly estimated based on the brightness and desired image size.

[H3] Controls in NIR-II fluorescence imaging

Proper controls must be used for all NIR-II imaging experiments. These controls include capturing NIR-II fluorescence images of the same animal before administering the NIR-II probe, under identical imaging conditions — excitation wavelength, power density, excitation and emission filters, exposure time — to assess the level of

autofluorescence. Additionally, it is essential to include a control group that receive an injection of a carrier, for example saline or empty lipids, via the same administration route and check for fluorescence contributed by other molecules in the carrier solution. If the goal is to demonstrate molecular imaging with NIR-II probes targeting a specific molecule, controls involving administration of the same NIR-II fluorophore but without the targeting ligands or with a mismatched control — such as arginine-alanine-aspartic acid (RAD) as a control for arginine-glycine-aspartic acid (RGD) in the $\alpha_v\beta_3$ integrin target[104] — should be considered. Lastly, for fluorescence detection in a disease model, a control group should be included. This group of animals should undergo a sham operation that doesn't induce disease, have the same NIR-II probe injected, and be imaged under identical conditions to the diseased group.

[H1] Results

This section provides results that demonstrate the benefits of in vivo NIR-II fluorescence imaging compared to imaging in the NIR-I spectrum. Various types of data analysis methods specific to NIR-II imaging are explored, detailing the mathematical tools and key equations commonly used. Lastly, the critical role of statistical analysis and error calculation is discussed.

[H2] Representative results

[H3] Comparison of NIR-I and NIR-II fluorescence imaging

As a specific example, a representative image of a live mouse's cerebral vasculature through an intact scalp and skull taken in the NIR-I spectrum (850-900 nm, using IRDye 800 as a label) is shown in **Fig. 5a**. This image is contrasted with another, taken in the NIR-IIb spectrum (1,500-1,700 nm, labeled with CNTs), through both the intact scalp and skull (**Fig. 5b**). Comparing these two cerebrovascular images shows striking differences. The image captured in the NIR-IIb spectrum displays much clearer, detailed features of the brain vasculature, even with the light-scattering scalp and skull above the brain.[5,105] By contrast, the image taken in the NIR-I window appears blurrier due to strong light scattering from the scalp and skull. As a result, it usually requires craniotomy — a surgical procedure to remove the scalp and part of the skull — to effectively visualize these vessels in the visible and NIR-I spectra. The substantial increase in the visibility of deep-tissue structures in the NIR-IIb window underscores its unique properties. Compared to its shorter-wavelength counterparts, the NIR-IIb window benefits from significantly reduced scattering of photons and autofluorescence in biological tissues, making it an effective tool for imaging applications.

[H3] Dynamically enhanced NIR-II fluorescence imaging

Dynamic NIR-II fluorescence imaging enables mapping of cerebral blood flow in deep tissues using principal component analysis (PCA). For example, real-time images of a mouse brain captured at 5.3 fps through an intact scalp and skull, immediately after

injecting NIR-II fluorescent probes (SWCNTs) intravenously.[5] This dynamic imaging produces a 3D dataset, where the first two dimensions are spatial coordinates and the third is a time component, for example frame number. Applying PCA to this dataset reduces its high dimensionality by focusing on a few principal components that account for the most variance.[70] This enables isolation of pixels that show distinct intensity patterns over time to effectively distinguish arterial from venous features.[10] In a study of cerebrovascular hemodynamics, PCA-enhanced images from a healthy mouse showed arterial and venous vessels in both hemispheres of the brain (**Fig. 5c**). By contrast, a mouse with a surgically induced middle cerebral artery occlusion (MCAO), commonly used as a stroke model, exhibited a lack of arterial features in the affected hemisphere (**Fig. 5d**).[5] These findings validate the capability of dynamic NIR-II imaging to differentiate hemodynamics in deep tissues of live mice via image processing techniques such as PCA.

[H3] Deep-learning enhanced NIR-II fluorescence imaging

A neural network–based method was demonstrated to transform a blurred image taken in the less optimal NIR-I or NIR-IIa window to resemble the more effective NIR-IIb window. To validate this approach, researchers experimentally acquired an NIR-IIa image of a mouse's hindlimb vasculature (**Fig. 5e**), along with a deep-learning generated image of the same sample in the NIR-IIb window (**Fig. 5f**). The generated image shows a remarkable resemblance to the ground truth NIR-IIb image (**Fig. 5f**, inset), demonstrating the ability of the neural network to faithfully enhance the contrast and features of the original NIR-IIa images without introducing artifacts.[106] The outcome suggests promising applications in clinical diagnostics and biomedical research. Specifically, the technology could elevate the capabilities of FDA-approved ICG and preclinical dye IRDye 800CW, which primarily emit in the NIR-I range but can be used for NIR-IIa imaging through tail emission.[22,44] This innovation could transform the less invasive and cost-efficient NIR-I and NIR-IIa imaging techniques into robust alternatives to current, more expensive imaging methods.

[H2] Analysis methods

[H3] Static NIR-II fluorescence imaging

In widefield NIR-II fluorescence imaging, raw images need to be processed with background subtraction and flat-field correction according to the following equation:

$$I(x,y) = \frac{I_0(x,y) - I_{background}(x,y)}{\frac{I_{flatfield}(x,y) - min(I_{flatfield}(x,y))}{max(I_{flatfield}(x,y)) - min(I_{flatfield}(x,y))}} \quad (6)$$

where $I_0(x,y)$ is the raw 2D NIR-II fluorescence image, $I_{background}(x,y)$ represents the background image captured by the camera when the sample is not exposed to the camera lens, achieved by keeping the camera shutter closed while maintaining the same exposure time. $I_0(x,y) - I_{background}(x,y)$ helps correct for non-uniformities,

noise, as well as dead, stuck, and hot pixels of the camera. In the denominator of Eq. (6), $I_{flatfield}(x,y)$ is usually recorded with laser excitation on a uniform material that emits light within the emission window and has an area larger than the field of view. Such a material could be the reverse side of a black laser safety material or a silicon wafer.[24] The denominator in Eq. (6) represents a normalized flatfield image. This normalized image is used to divide the background-subtracted 2D fluorescence image, yielding the flatfield-corrected image.

In confocal NIR-II fluorescence microscopy, an important quantitative parameter is the point spread function (PSF) of the system. The PSF is the impulse response of an optical system that describes how a point source of light is imaged, capturing its spatial resolution and blurring effects. The PSF function of an NIR-II confocal system is given as:

$$PSF_{confocal}(x,y,z)$$

$$= PSF_{excitation}(-x,-y,-z) \cdot \int_{\xi} \int_{\eta} PSF_{emission}(\xi,\eta,z) D(\xi - x, \eta - y) d\xi d\eta$$

$$= PSF_{excitation}(-x,-y,-z) \cdot [PSF_{emission}(x,y,z) * D(x,y)] \quad (7)$$

where ξ and η are integration variables, $PSF_{excitation}$ represents the 3D spatial distribution of the excitation power, $PSF_{emission}$ represents the PSF of a widefield microscope without a pinhole, D is the pinhole function.

In both widefield and confocal NIR-II fluorescence imaging, features such as blood and lymphatic vessels are usually quantified to characterize the smallest discernible structures in the image.[2,5,10,11,23,88,107] A line is typically drawn across the feature of interest, and the resulting intensity along the line is graphed to produce an intensity profile. This profile is fit with a Gaussian function (**Supplementary Note 6**). The parameters derived from the Gaussian fit enable the SNR and the size of a feature, such as vessel width, to be determined. The feature size is often approximated by the full width at half maximum of the Gaussian peak.

In hyperspectral NIR-II fluorescence imaging, each pixel is associated with an emission spectrum of the fluorophore. Emission peaks are typically fit with a Lorentzian function. Lorentzian fitting of NIR-II emission peaks from organic color centers **[G]** in SWCNTs has detected changes in local environments, including acidification and cancer biomarkers, with high sensitivity.[18,108]

[H3] Dynamic NIR-II fluorescence imaging

Video-rate dynamic NIR-II imaging can capture rapid hemodynamic changes in blood vessels, where blood carries the systemically administered NIR-II contrast agent during flow. If the blood flow is slow, as occurs in ischemic reperfusion, the leading edge of the blood, marked by the injected contrast agent, can be tracked after systemic

injection of the NIR-II contrast agent. By plotting the position of the flow front over time and fitting a linear curve to it, one can determine the blood velocity from the slope of this linear equation.[10,63]

However, if the blood flow is very fast, as seen in normal femoral and cerebral arteries, the leading edge often moves too quickly to be identified. In such cases, to determine blood velocity, the fluorescence intensity within a designated region of interest (ROI) is plotted over time. The varying NIR-II fluorescence intensity in a vessel segment reveals the blood flow dynamics.[5,109] In previous studies, this dynamic variation was modeled using a linear flow model that includes axial mixing.[10] This model suggests that the NIR-II fluorescence intensity *I* at a given location (*x*) and time (*t*) depends on the instantaneous blood flow velocity (*v*):

$$I(x,t,v) = \frac{I_0}{1+exp(\frac{x-vt}{A_0+Kvt})} \quad (8)$$

where I_0 represents the fluorescence intensity of the injected contrast agent solution without any mixing, A_0 represents the degree of initial mixing at $t = 0$, and K is the mixing constant ($K = 0$ indicates no mixing, while $K = \infty$ indicates maximum mixing).

Eq. (8) is a logistic function with its S shape — the flow front — moving in the +x direction and becoming less steep with increasing *t*. By applying the Taylor series expansion, it can be demonstrated that, to the first order, $I(x,t,v)$ has a linear relationship with *t*, with the slope directly proportional to *v*:

$$\frac{\partial I(x,t,v)}{\partial t}|_{t=0} \propto v \quad (9)$$

By calibrating the dynamic imaging system using several flow rates of NIR-II fluorescent agents pumped into a catheter tubing filled with water, the slope of $\frac{\partial I(x,t,v)}{\partial t}|_{t=0}$ vs v can be determined. This slope can then be applied to in vivo dynamic NIR-II fluorescence imaging to determine the blood velocity.[5,10,109]

Bright NIR-II contrast agents enable high-speed dynamic imaging — for example, over 20 fps — making it possible to detect individual cardiac cycles from the intensity curve of a specific arterial ROI.[29,47,48] Such variation in the fluorescence intensity curve is possible because arterial blood flow fluctuates, accelerating during the systolic phase and decelerating during the diastolic phase. The deep tissue penetration of NIR-II fluorescence allows researchers to observe fast dynamics in arteries, which are typically deeper than veins and challenging to see with shorter-wavelength visible and NIR-I spectra.

Alongside tracking the flow front or ROI-averaged fluorescence intensity in specific blood vessels, directly observing the endothelial cells and vascular lumen is an

effective hemodynamic imaging method. This technique has been used to study neurovascular coupling in live animal brains, however, it requires a cranial window and is limited to surface vessels due to depth constraints with traditional fluorophore labels.[110,111] A recent breakthrough in NIR-II fluorescence imaging uses fluorescence-amplified nanocrystals doped with NIR-II emitting Er^{3+} and Tm^{3+} ions to label cerebral vessel linings and lumens. The deep penetration of NIR-IIb photons enables dynamic monitoring of changes in the width of cerebral arteries, veins, and capillaries during neurovascular coupling through an intact mouse skull.[46]

[H3] Longitudinal NIR-II fluorescence imaging

Compared to cross-sectional studies, where images are collected from subjects at a single point in time to assess variations within a population, longitudinal studies involve time-dependent structural, molecular, and functional imaging of the same group of subjects over extended time periods. Longitudinal NIR-II fluorescence imaging data is typically analyzed using the same approach as static and dynamic NIR-II fluorescence imaging. During each imaging session, subjects may undergo static imaging, dynamic imaging, or both. The data from these images, which ranges from hours to months, is plotted over the sessions. For instance, the NIR-II fluorescence intensity in specific areas — such as the liver,[25,26] tumors[29,41,112,113] or lymph nodes[32,114] — is normalized to its peak value and charted at various intervals post-injection or treatment. Furthermore, chronic assessment of blood perfusion in the cerebrovasculature via dynamic NIR-II fluorescence imaging can indicate the recovery trajectory following a traumatic brain injury.[109,115]

Besides these analysis methods, emerging data mining and machine learning methods, such as principal component analysis (PCA) and deep learning, have also been used for NIR-II fluorescence imaging. A detailed theoretical discussion of PCA and deep learning can be found in **Supplementary Note 6**.

[H2] Statistical analysis and error calculations

In NIR-II fluorescence imaging, an accurate representation and understanding of the data uncertainty and variability is crucial.[116] When interpreting results, it is essential to note the number of independent experiments — for example, the number of animals administered with NIR-II fluorescent probes — typically denoted in figure legends. For consistency and reproducibility, experiments are often performed multiple times and in multiple biologically distinct samples, with the number of replicates indicated as n.[117] Power analysis during experiment design determines the minimum sample size required to detect statistical significance in pairwise comparisons.[118,119]

The uncertainty and variability of NIR-II imaging studies are usually reported with the standard deviation and standard error of the mean (**Supplementary Note 7**). Several statistical tests are commonly used to determine whether a comparison shows statistical significance, especially when comparing the NIR-II fluorescence intensity across different conditions. For example, when NIR-II fluorescent sensors are used to

detect concentrations of specific markers, such as pH, hydroxyl radical, or dopamine, it is essential to compare the NIR-II fluorescence intensity across different treatment groups.[6,15,18,120] Furthermore, when assessing fluorescent probes' tumor-targeting efficiency, metrics such as the tumor-to-background ratio or tumor-to-spleen ratio are often used.[24,29,114]

The t-test, analysis of variance (ANOVA) and certain non-parametric tests are the most frequently used statistical methods. The t-test compares the means of two groups to see if they are statistically different. The p-value from a t-test indicates the probability of observing the given data if the null hypothesis, typically positing no difference between the groups, were true. A smaller p-value suggests a stronger case against the null hypothesis.[121] When there are more than two groups to compare, ANOVA is employed. It evaluates the differences among group means in a sample. Similar to the t-test, a smaller p-value in ANOVA suggests that at least one of the group means is significantly different from the others.[122] However, when the data does not meet certain assumptions, such as normal distribution, non-parametric tests become preferable. These tests, such as the Mann-Whitney U test or the Kruskal-Wallis test, do not rely on the usual assumptions of parametric tests. As a result, they are more robust in certain situations.[123] Regardless of the test, a common threshold for significance is a p-value less than 0.05, implying that the observed result would be unlikely under the null hypothesis.[121]

[H1] Applications

NIR-II fluorescence imaging is emerging as a crucial tool in various biomedical research domains. It has been instrumental in the study of cardiovascular and cerebrovascular diseases, such as peripheral ischemia,[10,124] stroke,[5,31] and traumatic brain injury.[8,115] The technique has also been applied to study the lymphatic system by imaging lymphatic vessels and lymph nodes.[2,14,100,125] Additionally, in vivo NIR-II fluorescence imaging has demonstrated potential in early cancer detection and diagnosis, image-guided tumor surgery, and cancer immunotherapy.[7,14,16,19,22,24,29,74,108,114,126,127] Applying NIR-II fluorescence imaging requires optimization of NIR-II fluorophores, refining imaging systems and tailoring delivery methods while conducting thorough evaluations in preclinical models for potential clinical translation. Additionally, NIR-II fluorescence imaging applications are expanding into new areas, such as neural activity imaging,[46,128] genetically encoded NIR-II reporters,[25,26,39] and innovative instrumentation approaches such as light-sheet and structured illumination microscopy.[8,50]

[H2] Demonstrated applications

[H3] Hemodynamic imaging in cardiovascular and cerebrovascular diseases

By dynamically imaging femoral vessels immediately after intravenous administration of NIR-II fluorophores, the hemodynamics can be imaged in a mouse model of peripheral ischemia. This provides deeper anatomical penetration, distinguishes

between arterial and venous vessels based on their unique hemodynamics, and enables precise blood velocity quantifications in normal and ischemic femoral arteries.[10] NIR-II fluorophores with enhanced brightness enable precise imaging of cardiac cycles by measuring intensity changes in femoral arteries, which are distant from the heart, via ultrafast dynamic imaging.[36,47,48] Cardiac cycles can also be directly monitored by dynamic NIR-II imaging in a mouse heart.[22]

Hemodynamic NIR-II imaging facilitates detection of cerebrovascular abnormalities in a mouse model of stroke caused by MCAO. By dynamically monitoring the NIR-II fluorescence intensity within the mouse cerebrovasculature through an intact scalp and skull, areas of reduced cerebral blood flow can be identified (**Fig. 6a**).[5] A similar decrease in NIR-II fluorescence signal is observed in a mouse traumatic brain injury model, suggesting that dynamic cerebrovascular NIR-II imaging can effectively detect hypoperfusion.[115] The dynamics of NIR-II fluorescence in cerebral vessels also reveals cardiac cycles with sufficiently bright fluorophores for fast video-rate imaging.[29] The different absorption characteristics of oxygenated and deoxygenated hemoglobin at two distinct excitation wavelengths (650 nm and 980 nm) of NIR-II emitting RENPs, enables differentiation of cerebral arteries from veins based on their varying oxyhemoglobin saturation levels.[129]

Methods based on single particle tracking and vessel diameter changes are also used. Particle image velocimetry **[G]** can track NIR-II fluorescent particles in the blood, enabling high-resolution 3D flow maps of microvascular networks. From this, healthy brain tissue and the glioblastoma margin in a mouse brain can be differentiated.[20] Cerebrovascular hemodynamics can be monitored by observing changes in the diameter of the lumen, which is labeled by NIR-II fluorescent agents, such as thulium-based cubic-phase downshifting nanoparticles (α-TmNPs) with 1,632 nm fluorescence amplification. Using this method, changes in vessel diameters in response to drugs such as norepinephrine can be dynamically imaged.[46]

[H3] Lymphatic imaging in cancer monitoring and immunotherapy

The lymphatic system can be imaged in the NIR-II spectrum by labeling the lymphatic fluid or lymphatic cells with NIR-II fluorescent agents. For example, intradermal injection of NIR-II fluorescent agents at the base of a tumor-bearing mouse tail enables visualization of internodal collecting lymphatic vessels and inguinal lymph node (iLN).[14] By intradermally and intravenously injecting two unique NIR-II fluorophores with different excitation or emission wavelengths, lymphatic and vascular systems can be differentiated using two-color NIR-II imaging (**Fig. 6b**). Being able to distinguish and monitor both systems simultaneously enhances non-invasive diagnostics and advances fluorescence-guided surgical techniques.[100,125]

Noninvasive imaging of lymph nodes, such as iLNs, is possible with the deep tissue penetration of NIR-II fluorescence. Without the invasive installation of transparent

windows needed for conventional intravital microscopy, researchers can simultaneously map the peripheral node addressin **[G]** on high endothelial venules **[G]** and surrounding blood vessels in the same iLN region. In addition, by labeling macrophages and T cells with spectrally resolved NIR-II agents, two-color NIR-II fluorescence microscopy can reveal the distribution of different immune cells in iLNs in a noninvasive manner.[2]

[H3] Molecular imaging and immunotherapy in cancer

Several small-molecule NIR-II fluorophores and NIR-II emitting nanoparticles have been used for molecular imaging of cancer. Functional groups in small molecules can be attached to a peptide, antibody, or affibody **[G]** to achieve tumor-specific targeting.[130] Some representative examples include cetuximab-IRDye800CW, with tail emission in NIR-II; trastuzumab-IRDye800CW; and anti-EGFP affibody-CH1055 with peak emission in NIR-II, **Table 1** and **Fig. 3**.[14,22,131] Besides small molecules, NIR-II emitting nanoparticles, such as RENPs, can be conjugated with antibodies for molecular tumor imaging. For example, the anti-PD-L1 monoclonal antibody (aPDL1), atezolizumab, has been conjugated to cubic-phase (α-phase) erbium-based RENPs (ErNPs) yielding a ErNPs-aPDL1 complex. NIR-II molecular imaging of mice with tumors that had undergone intravenous administration of the nanoparticle complex showed a higher specificity to CD-26 tumors, which have high PD-L1 expression, compared to 4T1 tumors with lower PD-L1 expression.[29] Using lifetime-engineered RENPs, multiplexed images of tumor-bearing mice can resolve the composition of different biomarkers in distinct tumor types.[16]

Cancer immunotherapy benefits from NIR-II fluorescence imaging for noninvasive tracking and visualization of immune cells following treatment with monoclonal antibodies or cancer vaccines. For instance, labeling $CD8^+$ cytotoxic T lymphocytes with 1,600-nm emitting PbS QDs that have a short fluorescence lifetime, accumulation of T cells in the tumor periphery can be detected while simultaneously imaging tumor cells with ErNPs.[29] Additionally, pure $NaErF_4$ nanoparticles (pErNPs) were used to tag a cancer vaccine formulated from the ovalbumin antigen combined with the adjuvant **[G]** class-B cytosine-phosphate-guanine (CpG B). This nanovaccine compound has strong fluorescence emission in the NIR-IIb range, facilitating in vivo tracking of the vaccine's movement through the lymphatic system, from the subcutaneous injection site, through iLNs and axillary lymph nodes (aLNs), before finally reaching the tumor.[114] The efficacy of this trackable nanovaccine is confirmed by noninvasive three-color microscopy in an E.G7 mouse lymphoma tumor model. Using three distinct channels, the molecular characteristics of $CD8^+$ and ovalbumin-antigen-specific T cells, as well as the nanovaccine's distribution can be concurrently visualized with minimal crosstalk (**Fig. 6c**). The NIR-II emitting cancer nanovaccine can achieve in vivo tracking and imaging of the associated immune response with approximately 1 μm resolution and 1 mm penetration depth. To ensure that labeling does not compromise nanovaccine efficacy, the effectiveness and safety should be validated through preclinical studies in a variety of animal models and cancer types.[132]

[H3] Image-guided tumor surgery

Image-guided tumor surgery benefits from early tumor detection. This early detection relies on the ability to distinguish small tumor tissue from normal healthy tissue with high sensitivity.[133] One promising method for early detection is NIR-II fluorescence imaging. Advantages include the low autofluorescence background of biological tissue in the NIR-II spectrum, providing greater contrast; high SNR due to reduced tissue scattering, creating sharp images with clearly defined tumor boundaries; minimal interference from ambient lighting, making the technique reliable in various imaging settings[7] ; and, because NIR-II imaging is an optical imaging method, it has a high spatiotemporal resolution to detect and eliminate small lesions at primary and metastatic locations (**Fig. 6d**). Additional examples of NIR-II image-guided tumor surgery, as well as those of NIR-II fluorescence imaging of inflammation and the gastrointestinal system can be found in **Supplementary Note 8**.

[H2] Considerations when applying NIR-II imaging

[H3] Selection of appropriate NIR-II fluorophores

Selecting the right fluorophore with optimal excitation and emission wavelengths within the NIR-II window is crucial. The goal is to achieve high SNR, sufficient spatial resolution, and deep penetration while minimizing interference from the inherent autofluorescence of tissues. Scattering and autofluorescence considerations typically favor fluorophores with longer emission wavelengths. Various biological tissues display an inverse relationship between scattering and wavelength.[1] Additionally, tissue autofluorescence rapidly diminishes with increasing wavelength, becoming negligible beyond 1,300 nm.[134] Using NIR-II fluorophores that emit in the NIR-IIb and NIR-IIc regions can substantially reduce scattering and autofluorescence. However, when considering tissue absorption in the context of NIR-II imaging and the requirement for diffraction-limited resolution in NIR-II microscopy, it is essential not to select excessively long wavelengths. There are overtone absorption bands of water at 970 nm, 1,200 nm, 1,450 nm, 1,900 nm, and beyond 2,300 nm.[60] Effective imaging requires the peak emission wavelength of the chosen NIR-II fluorophores not to overlap with these bands. Since the diffraction-limited spatial resolution is roughly equivalent to the wavelength of imaged photons, the resolution may deteriorate to ~2 μm in the lateral plane and considerably above 2 μm in the axial direction when performing confocal microscopy in the NIR-IIc spectrum.[2]

[H3] Biocompatibility of NIR-II fluorophores

Ensuring the biocompatibility and non-toxicity of NIR-II fluorophores and their conjugated targeting agents is crucial, as they interact with biological tissues and cells. It is imperative that NIR-II agents undergo rigorous evaluations to confirm their non-toxicity at the desired concentrations, both in cell cultures and within living organisms. Additionally, thorough assessments of the pharmacokinetics of NIR-II agents are essential. These assessments include understanding their behavior in the bloodstream, how they accumulate in tumors or other desired locations, their

distribution in various organs, if and how they are metabolized into subsequent compounds, and their excretion pathway. When using NIR-II agents to label and monitor immune cells, care must be taken. It is vital to ensure that the labels do not alter the natural behavior of the cells, in particular that they don't block membrane receptors, which would affect cell functionality. An emerging strategy to improve biocompatibility is to use the biological system to naturally produce NIR-II fluorophores through genetic engineering. This approach could reduce the potential toxicity associated with externally synthesized NIR-II agents.

[H3] Delivery routes of NIR-II fluorophores

Delivery routes of NIR-II fluorophores should be meticulously chosen based on their intended application. For instance, hemodynamic NIR-II imaging requires intravenous or retro-orbital injection. By contrast, imaging the lymphatic system typically requires fluorophores to be introduced via intradermal or subcutaneous injections. However, it is vital to acknowledge that the chosen delivery method introduces a degree of invasiveness to the NIR-II imaging process. This means that both the efficacy and potential toxicity of the NIR-II agents needs to be evaluated within the entire trajectory towards the target. For all delivery routes, there is a limited time frame for imaging post-delivery due to the wash-out effect. There is growing interest in prompting biological tissues to intrinsically produce NIR-II agents through genetic engineering. This approach holds promise in reducing toxicity, enhancing tissue and cell specificity, and negating the wash-out effect, presenting a more streamlined method.

[H3] Optimization of NIR-II imaging systems

Optimization of NIR-II imaging systems is at the forefront of advancing medical imaging techniques. Enhancing the sensitivity and speed of detectors is crucial. This can substantially increase the frame rate, enabling faster capture of dynamic biological processes. Extending the response wavelength of existing InGaAs detectors can broaden imaging capabilities into longer wavelength regions, such as the NIR-IIc and NIR-IId windows. A notable development is the incorporation of superconducting nanowire single photon detectors, which were used for NIR-IIc confocal imaging.[2] Advances in excitation methods offers the potential to transition from conventional one-photon excitation to two- and three-photon excitation for NIR-II fluorescence with deep-tissue optical sectioning. This shift necessitates longer-wavelength, pulsed IR light sources. To push the boundaries of imaging resolution, super-resolution methodologies, such as stimulated emission depletion, photoactivated localization microscopy, and superresolution imaging with minimal photon fluxes may be integrated to enhance the resolution beyond the diffraction limit of NIR-II photons.[135–137]

[H1] Reproducibility and data deposition

[H2] Reproducibility of NIR-II fluorophores

Reproducibility in NIR-II fluorescence imaging largely depends on the consistency of the NIR-II fluorescent agents. Besides validating their structures and compositions (**Supplementary Note 9**), it is crucial to ensure that the spectral properties and targeting specificity of NIR-II fluorescent probes are reproducible between batches and across different laboratories.

Measures of spectral reproducibility include the absorption, excitation, and emission spectra, sometimes including the 2D photoluminescence vs. excitation (PLE) spectrum.[14] Lifetime measurements also play a vital role in characterizing NIR-II fluorescent agents used in time-multiplexed imaging. A critical parameter, the quantum yield (QY), often defines the brightness of specific NIR-II fluorophores. However, measuring QY in NIR-II fluorescence imaging is debated, especially regarding discrepancies in the reported QY of the reference fluorophore, IR-26, which ranges from ~0.05 to 0.5%.[138,139] Consequently, absolute QY measurements using integrating spheres rather than relative measurements is now advocated.[89] To establish the QY measurement standard, it is recommended to use an integrating sphere connected to a sensitive spectrophotometer in the NIR-II spectrum, following the methodologies outlined in previous reports.[138] The photostability of NIR-II fluorophores can differ considerably. Certain NIR-II fluorophores with extended light exposure might exhibit compromised photostability, leading to potential data discrepancies.[38] As a result, it is crucial to report the duration and power density a fluorophore was illuminated under for accurate image interpretation.

Reproducibility of specificity in targeted NIR-II fluorescence imaging is also essential for an NIR-II agent. In vitro cell targeting experiments, complemented by negative controls — where the same NIR-II probe is used but without the targeting ligand or cells lacking specific receptors — provide an assessment of specificity. For in vivo experiments, incorporating a control group is essential. This control group should be injected with a solution of the same NIR-II agent, at the same concentration, but without the targeting ligand.[114] Another method of establishing a control group is to simultaneously administer a blocking dose of the anti-receptor affibody or antibody alongside the bioconjugate, which contains the NIR-II agent and targeting ligand.[14]

[H2] Reproducibility of NIR-II imaging systems

One of the primary challenges in NIR-II fluorescence imaging reproducibility is the use of relative fluorescence intensity scales. Many research papers normalize their fluorescence intensity to the maximum intensity in their images, masking the original intensities in the raw data. This normalization process complicates direct comparison between different studies. To address this lack of transparency, it is crucial that researchers provide detailed specifics about the experimental conditions. Details such as the concentration of NIR-II probe, exposure time for image acquisition, type and wavelength of excitation light source, incident power density, emission filters, and camera make and model are crucial for standardization. The incident power density,

not the output power density of the light source, should be reported. This distinction is critical because the output power density can be attenuated by optical components, such as filters and diffusers in the excitation path.

To drive uniformity, it is strongly recommended that p s^{-1} cm^{-2} sr^{-1} be used as an absolute unit for direct, standardized comparison of brightness in NIR-II fluorescence imaging. This standard has been widely adopted in rodent bioluminescence imaging.[140] Adopting this practice would supplant the current trend of reporting relative fluorescence intensities, enabling more direct comparisons across studies.

Another complicating factor is emission filters. Researchers often use filters to obtain images in different subregions of the NIR-II spectrum, such as NIR-IIa, NIR-IIb, NIR-IIc, and NIR-IId. However, stating that a filter for a particular subregion, such as the NIR-IIb window, was used is insufficient to ensure reproducibility. Filters, even with identical nominal cutoff wavelengths, can differ substantially in their optical density both in their pass bands and stop bands. Furthermore, the edge steepness between passband and stopband can vary between filters. This variation means that even if two imaging studies claim to be in the same NIR-II subregion, the actual photon detection efficiency varies depending on the exact filters used, leading to discrepancies in results. A laser's excitation power, for instance, can seep through the stop band of an emission filter if the optical density is not sufficiently high. A specific filter might not perform strictly to its nominal specification. A filter labeled as 1000LP might have some level of attenuation at wavelengths much longer than its cutoff, such as around 1500 nm. Similarly, it might show transparency at wavelengths much shorter than its nominal cutoff, around 600 nm. This often requires a combination of filters to achieve the desired filtration effect.

[H2] Reporting of image processing and analysis

Other reproducibility issues relate to a lack of clarity in image processing and analysis. An absence of standardized protocols for data processing, analysis, and quantification can lead to inconsistent interpretation of results. To overcome this, standardized guidelines and best practices for data analysis and dissemination need to be adopted. A unified approach would ensure that findings are reliable within individual studies and comparable across different laboratories.

Equations and methods for analysis should be clearly described. For instance, use of background subtraction and flatfield correction in widefield NIR-II imaging; details on theoretically-calculated versus experimentally-measured point spread functions in confocal NIR-II microscopy; the functions peaks are fitted to; and algorithms for machine learning-enhanced NIR-II imaging, should all be transparently reported.

[H2] Data deposition and sharing

To enhance collaboration, verify data, and improve reproducibility, data from NIR-II fluorescent imaging studies should be deposited in universally accessible repositories. It is suggested that the repositories listed in **Table 2** be used for in vivo NIR-II imaging data. Specifically, the Image Data Resource (IDR) is advised for general reference image datasets from scientific publications.[141] Clinical NIR-II images should go to The Cancer Imaging Archive (TCIA), while NIR-II brain images are best suited for the Brain Image Library (BIL) or Distributed Archives for Neurophysiology Data Integration (DANDI). In these repositories, authors should submit datasets that meet the highest standards for reproducibility and comply with the FAIR (Findable, Accessible, Interoperable, and Reusable) principles. Submissions usually require the inclusion of comprehensive metadata that details experiments, samples, imaging techniques, and processing methods. Depositing raw data, processed images, and analytical methods is also recommended for thorough assessment and results interpretation. By following these data submission standards, an increasing collection of NIR-II fluorescence images can be produced and shared, encouraging progress and novel applications.

[H1] Limitations and optimizations

Current NIR-II fluorescent imaging techniques excel in capturing structural, hemodynamic, and molecular information, such as vascular and lymphatic imaging, blood flow dynamics, and specific targeting to tumor and immune cells. However, vital functional and molecular data remains beyond the reach of NIR-II imaging. For instance, dynamic intracellular calcium concentrations, membrane potential changes, neurotransmitter levels, neuropeptide concentrations, and the presence of signaling molecules are not readily accessible with this approach (**Supplementary Note 10**).

[H2] Equipment constraints

The equipment required for NIR-II fluorescence imaging has some limitations, particularly in terms of accessibility and cost-effectiveness. A key factor contributing to these limitations is the high price of InGaAs cameras, which are essential for capturing NIR-II signals. These cameras can be prohibitively expensive, making it challenging for researchers and institutions with limited budgets to access. NIR-II fluorescent imaging often uses additional advanced optics in, for example, confocal microscopy and light-sheet microscopy. This limits NIR-II fluorescent imaging to well-funded universities and laboratories, creating a barrier to enter the NIR-II imaging research community.

While cost is highly prohibitive, technical limitations also exist. InGaAs cameras require deep cooling to reduce thermal noise, which can be expensive and technically challenging to maintain at optimal operating conditions. InGaAs cameras need to be placed in a humidity-regulated room to prevent condensation on the sensors. Additionally, the thermal background noise generated by living organisms can interfere with detection in the NIR-II window. This would be particularly impactful for future applications in the long wavelength, NIR-IId region. Using Planck's radiation law, the

blackbody radiation of an organism at 310 K is over 10^5 times more intense at 2,200 nm in the NIR-IId subregion than at 1,300 nm in the NIR-Iia subregion. Consequently, long-wavelength fluorophores need to be made exponentially brighter to overcome thermal background before the benefits of reduced scattering can be realized. Lastly, the Abbe limit, which defines the maximum spatial resolution attainable, is more restrictive for fluorescence microscopy performed in the NIR-II than in the visible spectrum. While NIR-II fluorescence imaging produces sharper features and higher resolution at greater depths, it may not surpass visible spectrum microscopy in terms of resolution for superficial features.

In clinical applications, manufacturing NIR-II imaging instruments faces several challenges. The cost of InGaAs cameras, along with their requirement for deep cooling and humidity control, limits their accessibility. Clinical imaging of human subjects demands large-area, high-quality focal plane arrays (FPAs). The commonly used 640 × 512 pixel FPA restricts the potential for high-resolution and large-area imaging in humans. The readout integrated circuit, which is essential for high-quality NIR-II images, presents challenges, especially when the FPA size is large. While current systems primarily use high-power lasers as excitation sources, achieving uniform illumination across the large size of a human subject, while remaining within the maximum permissible exposure, poses a technical challenge. Sourcing large and high-quality cutoff filters and focusing lenses in the NIR-II spectrum further complicates instrument manufactured for clinical applications.[142]

[H2] NIR-II fluorescent agent constraints

Alongside equipment limitations, NIR-II fluorophores have limitations, including low quantum yields and poor aqueous solubility. Improving quantum yields has been an area of investigation, but there is still room for development. For inorganic NIR-II fluorophores, adding a shell around the fluorescent core can prevent Förster energy transfer to ligands and solvent molecules, improving the quantum yield.[143] For organic NIR-II fluorophores, engineering the donor and acceptor moieties and introducing shielding units to prevent intermolecular interactions can create brighter NIR-II fluorophores.[37,38,81]

Poor water solubility and serum stability are also issues when developing NIR-II fluorophores. These problems can lead to aggregation of fluorophores in physiological environments, which is often associated with fluorescence quenching. Strategies to improve water solubility include covalent and non-covalent functionalization with hydrophilic groups — such as PEG and sulfonate[14,20,74,82] — and incorporation in proteins and amphiphilic polymers.[29,39,48,114] Despite this aggregation challenge, AIE and J-aggregates can be used to enhance fluorescence and red-shift the emission of potential fluorophore candidates.[42,88] Emerging genetic engineering approaches hold promise for addressing limitations with the stability, biocompatibility, pharmacokinetics, and excretion of NIR-II fluorophores.[25,26,39]

[H2] Unexpected outcomes and alternatives

Unexpected technical issues — such as photobleaching, autofluorescence, signal interference, or photothermal effects — can influence data quality and interpretation. Recent advances have enhanced the photostability of NIR-II fluorophores, however, photobleaching remains a concern that can affect long-term performance and impact results (**Fig. 7a**). Inorganic nanoparticles, such as RENPs, typically demonstrate greater photostability than organic counterparts.[144] Anti-quenching NIR-II molecular fluorophores have been developed to address the challenges of organic NIR-II agents.[23] The reverse intersystem crossing strategy may help to reduce photobleaching in NIR-II emitting fluorescent proteins.[145] Autofluorescence can also impede data collection, particularly when imaging at the shorter end of the NIR-II spectrum (**Fig. 7b**).[1] A comprehensive examination of NIR-II autofluorescence showed that it is strongly influenced by mouse coat pigmentation and consumed food.[146,147] Signal interference is another potential pitfall in multiplexed NIR-II imaging, especially when using multiple probes with overlapping emission spectra and similar lifetimes, leading to signal crosstalk (**Fig. 7c**). If the excitation light source is powerful enough, its reflection can cause crosstalk in the emission channel, particularly if the optical density of the emission filter's stop band cannot adequately filter out the excitation photons. This issue becomes problematic when the fluorescence is substantially weaker than the excitation. When fluorescence emission wavelengths approach the far end of the NIR-II spectrum — closer to 2,300 nm — absorption by water and other organic molecules should be considered, along with the consequent heating (**Fig. 7d**). Such heating can alter the native physiological activity of the subject under study, potentially skewing results.

[H1] Outlook

It is expected that several challenges in NIR-II fluorescence imaging will be addressed in the coming years. Currently, most NIR-II imaging focuses on targeting and imaging extracellular structures, receptors, and events rather than intracellular molecules and processes. While there has been some development of intracellular NIR-II fluorescent imaging,[18] improving intracellular sensitivity would present opportunities to answer new biological questions. Potential advances include NIR-II sensors for monitoring dynamic calcium concentrations, membrane potential changes, and protein kinase activity. In addition, instrumentation limits are a critical challenge to solve. Developing 2D InGaAs arrays with sensitivity beyond 1,700 nm is vital for NIR-II fluorescence imaging in biomedical research, especially when offered at an affordable price with low thermal noise levels.

As the NIR-II fluorescent imaging field continues to develop, novel approaches and questions emerge. These potential directions include methods for imaging in the NIR-IIc and NIR-IId subregions, with further reduced scattering. Although different NIR-II subregions are intentionally defined to avoid water absorption, moderate absorption of photons by water can enhance resolution via absorption-induced image resolution enhancement in scattering media.[148,149] Based on this strategy, theories predict that

wavelengths up to 2,340 nm can provide best image quality through scattering tissues.[150] NIR-II imaging at these extremely long wavelengths requires high performance detectors such as SNSPDs and rationally designed nanoprobes [143,151] for deeper penetration and higher resolution.

Another emerging direction involves the use of machine learning and artificial intelligence to enhance NIR-II imaging. Deep learning techniques can be used to extract hidden information from images, providing insights that may not be apparent in the original NIR-II images. It is predicted that large language models will be combined with computer vision to enhance clinical NIR-II imaging by revealing information not obvious to the operator. In addition, deep learning-adaptive optics could be used for wavefront correction, improving resolution and correcting aberrations in the NIR-II fluorescent imaging process.[152] Integration of other imaging methods with NIR-II fluorescence imaging is another area of exploration. Integrating super-resolution microscopy with NIR-II imaging enables in vivo sub-diffraction imaging, compensating for the unfavorable long-wavelength-dependent resolution.

Over the next 5-10 years, several priorities should be addressed to advance NIR-II fluorescence imaging and increase its impact. A key priority is the development of compact and cost-effective, potentially portable NIR-II imagers to enable wider adoption by researchers and clinicians. In addition, cost-effective imaging will enable point-of-care NIR-II imagers to be distributed to under-resourced populations.[153] Another important focus is to create more specific NIR-II probes for imaging molecular and functional information with high resolution and deep penetration. This would expand the range of biological processes that can be studied. For example, activable NIR-II probes that respond to various biomarkers, may enable sensitive detection of neurodegenerative diseases.[154,155] Lastly, integrating deep-brain NIR-II imaging of neural activity with neuromodulation using widefield NIR-II illumination provides opportunities for an all-optical, bidirectional noninvasive brain-machine interfaces.[66,156–158]

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Fig. 1 | Schematic summary of NIR-II fluorescence imaging. Deep tissue penetration is highlighted as the main advantage, along with representative NIR-II fluorophores.

Fig. 2 | Representative in vivo NIR-II imaging systems. (**a**) Widefield NIR-II fluorescence imaging. (**b**) Raster-scan confocal NIR-II fluorescence imaging. (**c**) NIR-II light-sheet microscopy. (**d**) The image-formation lens system. WD: working distance; FOV: field of view; BFD: back focal distance; H: the horizontal dimension of the camera.

Fig. 3 | Different NIR-II fluorescent probes and their emission spectral ranges. Blue: inorganic nanoparticles; red: organic molecules; yellow: genetically engineered proteins.

Fig. 4 | Preparation and administration of NIR-II probes. (**a**) Evaluation of cytotoxicity. (**b**) Evaluation of systemic toxicity. (**c**) Evaluation of minimum excitation power. (**d**) Evaluation of photobleaching and photothermal effects. (**e**) Intravenous administration. (**f**) Retro-orbital administration.

Fig. 5 | Representative results of NIR-II fluorescence imaging. (**a**&**b**) Epifluorescence images showing the cerebrovasculature through the intact scalp and skull of a live mouse in the NIR-I window (<900 nm, **a**) and the NIR-IIb window (1,500-1,700 nm, **b**). (**c**&**d**) Principal component analysis (PCA) of dynamic NIR-II fluorescence images in the mouse cerebrovasculature, revealing arterial (red) and venous (blue) vessels in a healthy mouse (**c**) and in a mouse with surgically induced middle cerebral artery occlusion (MCAO) (**d**). (**e**&**f**) A representative fluorescence image of a mouse hindlimb taken in the NIR-IIa window (1,000-1,300 nm, **e**), alongside a contrast-enhanced image via deep learning (**f**). A corresponding ground truth image of the same region taken in the NIR-IIb window (1,500-1,700 nm) is shown as the inset of **f**. All scale bars represent 5 mm. Panels a,c,&d adapted with permission from ref. [5], Springer Nature. Panels e&f adapted with permission from ref. [106], National Academy of Sciences.

Fig. 6 | Applications of in vivo NIR-II fluorescence imaging. (**a**) Hemodynamic NIR-II imaging of a shaved healthy mouse head (top) and that with middle cerebral artery occlusion (MCAO) (bottom). (**b**) Dual-channel NIR-II fluorescence images of lymph structures (top left: EB766, an erbium(III)–bacteriochlorin complex) and blood vessels (top right: NaYF4:20% Yb, 2% Er@NaYF4 downconversion nanoparticles, DCNPs) in the same mouse (bottom: overlaid image). (**c**) 3D reconstructed NIR-II image of $CD8^+$ T cells (red), ovalbumin-antigen-specific T cells (green), and pErNP–OVA–CpG B

nanovaccine (blue) in the tumor. (**d)** Surgical removal of tumors with NIR-II fluorescence guidance. Representative whole-abdomen NIR-II images taken before NIR-II probe injection (top left), pre-surgery (top right), post-unguided surgery (bottom left), and after NIR-II-guided surgery (bottom right) are shown. A white arrow points to a nodule detected only in the NIR-II-guided surgery. Panel a adapted with permission from ref. [5], Springer Nature. Panel b adapted with permission from ref. [100], Springer Nature. Panel c adapted with permission from ref. [114], Springer Nature. Panel d adapted with permission from ref. [127], National Academy of Sciences.

Fig. 7 | Examples of unexpected outcomes in in vivo NIR-II imaging. (**a**) Photobleaching of NIR-II fluorophores. (**b**) Autofluorescence from illuminated biological tissues. (**c**) Fluorescence crosstalk between different emission channels, and between excitation and emission. (**d**) Photothermal effect of illuminated tissues.

1256 **Table 1 | NIR-II fluorophores.**

NIR-II fluorophores	Emission wavelengths (nm)	Quantum yield (%)	Fluorescence lifetime	Refs
Inorganic NIR-II nanoparticles				
CNTs	1,000 – 1,800	0.5	10 ps	1,71,159
CISe NTs	1,138	12.4	336.1 μs	74
Ag_2S QDs	1,050 – 1,200	5 – 15 (depending on surface coating)	ns – μs	19,107,160–163
PbS QDs	1,600 – 2,000	1.0 – 57 (depending on emission wavelengths)	46 μs	2,36,164
InAs QDs	1,000 – 1,400	30	100 ns	20,165
$NaGdF_4$: 5% Nd@$NaGdF_4$	1,060	NA	NA	166
$NaGdF_4$@$NaGdF_4$:Yb,Ln@$NaYF_4$:Yb@$NaNdF_4$:Yb nanoparticles	1,155 (Ln = Ho) 1,525 (Ln = Er)	0.009 – 0.24 (Ln = Er)	40 – 920 μs (Ln = Ho) 5.8 μs – 20.9 ms (Ln = Er)	16
$NaYbF_4$:2%Er,2%Ce,10%Zn@$NaYF_4$ nanoparticles	1,550	5	4.6 ms	29
Cubic phase $NaYF_4$:$Yb_{0.8}$/$Tm_{0.08}$@$NaYbF_4$@$NaYF_4$ (α-TmNPs)	1,632	14	1.5 – 3.7 ms	46
$NaErF_4$/$NaYF_4$ nanoparticles	1,550	NA	2.7 ms	114
AuNCs	1,000 – 1,350	0.1 – 4	ns – μs	30–33,167
Organic NIR-II molecules				
CH1055-PEG	1,055	0.3	NA	14
IR-26	1,130	0.05 – 0.5%	22 ps	138,139,168
IR-FTAP	1,048	5.3	NA	81
FNIR-1072	1,103	0.12	NA	24
ICG	820 (peak; tail extending to NIR-II)	0.9	0.166 ns	22,169

IRDye 800CW	800 (peak; tail extending to NIR-II)	3.3	0.5 ns	[22,170]
CH-4T@protein complex	1,000	11	NA	[48]
IR-783@BSA complex	800 (peak; tail extending to NIR-II)	21.2	NA	[87]
AIE nanoparticles of 2TT-*o*C26B	1,030	11.5	NA	[88]
J-aggregates of meso-[2.2]paracyclophanyl-BODIPY dye	1,010	6.4	NA	[41]
J-aggregates of FD-1080 cyanine dye	1,370	0.0545	172 ps	[97]
Erbium(III)–bacteriochlorin complex	1,530	0.01	1.73 μs	[100]
Genetically engineered proteins with off-resonance NIR-II emission				
miRFP718nano	718 (peak; tail extending to NIR-II)	5.6	NA	[25]
iRFP713	713 (peak; tail extending to NIR-II)	0.33	NA	[26]
IR783@DIII	810 (peak; tail extending to NIR-II)	0.97 – 9.73	NA	[39]

AIE: aggregation-induced emission; BSA: bovine serum albumin; CISe: copper indium selenium ($CuInSe_2$); CNT: carbon nanotube; DIII: domain III of human serum albumin; ICG: indocyanine green; NT: nanotube; PEG: polyethylene glycol.

Table 2 | Recommended repositories for depositing and sharing NIR-II imaging data.

Repository Name	Type of Data	Data Formats Accepted
Image Data Resource (IDR)	Image datasets	A study file, assay file including the images, and processed data files
The Cancer Imaging Archive (TCIA)	Cancer medical image datasets	De-identified images in Digital Imaging and Communications in Medicine (DICOM) international standard
Brain Image Library (BIL)	Brain image datasets	Both raw and processed data is accepted, preferred image format is tiff but for.swc format is acceptable for higher-level traced-neuron data
Distributed Archives for Neurophysiology Data Integration (DANDI)	Electrophysiology, optophysiology, and behavioral time-series, and images from immunostaining experiments	Neurodata Without Borders (NWB) format for electrophysiology and optophysiology data; Brain Imaging Data Structure (BIDS) format for neuroimaging data

Glossary

Scattering | The deviation of light rays from their original path, a phenomenon exacerbated in animal tissue by the inhomogeneity of refractive indices among components like water, lipid membranes, and subcellular organelles.

Autofluorescence | The natural emission of light upon excitation of biological tissues, largely contributed by endogenous chromophores such as NADH (emission ~460 nm) and flavins (500–600 nm), as well as pigmented cellular structures such as lipofuscin (450–650 nm) and reticulin (470–520 nm).

Epifluorescence | The fluorescence observed in an optical microscope or imaging system when the object is illuminated from the side that is being viewed.

Indium gallium arsenide | A compound semiconductor material that is sensitive to infrared light and commonly used in photodetectors for NIR-II fluorescence imaging.

Photodiode | A semiconductor device that converts light into an electrical current, the amplitude of which is directly proportional to the light intensity shining on the diode.

Photomultiplier tube | An electronic device that detects and greatly amplifies weak light signals by converting photons generated by a photocathode into an intensified electrical signal through a series of secondary electron multipliers.

Superconducting nanowire single-photon detector | An ultra-sensitive device that detects individual photons by measuring the disruption in the bias current, which arises when single photons absorbed by the superconducting nanowire break Cooper pairs.

Signal-to-noise ratio | The ratio of fluorescence signal to the background noise, the latter of which comprises the shot noise and dark noise of the photodetector, the readout noise from the camera electronics, as well as autofluorescence and scattering from biological tissues.

Reactive oxygen species | Chemically reactive molecules that contain oxygen, such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical (•OH), and singlet oxygen ($^1O_2$).

Overtone absorption | The absorption of light by a molecule at a frequency (or wavelength) that is a multiple of the fundamental frequency of a vibrational mode of that molecule.

Semiconductor diode laser | A type of laser with a semiconductor active medium, akin to an LED, but it produces coherent light through stimulated emission from the recombination of electrons and holes.

Optical density | A measure of how much a substance or an object attenuates the intensity of light that passes through it. Mathematically, optical density (OD) is defined as $OD = -\log_{10}(I/I_0)$ where I is the intensity of light transmitted through the substance, and I_0 is the intensity of the incident light.

Excitation filter | An optical filter, typically positioned in front of the excitation light source, selectively transmits wavelengths suitable for exciting a specific fluorophore, while blocking other undesired wavelengths.

Emission filter | An optical filter, typically positioned in front of the detector, selectively transmits wavelengths corresponding to the emission of a specific fluorophore, while blocking other undesired wavelengths, such as those from the excitation light source.

Optical diffuser | A device that scatters light in various directions to produce a uniform illumination.

Optomechanics | Elements including optical tables, breadboards, construction components such as mounts, and mechanically integrated optoelectronic devices.

Dichroic mirror | An optical filter that reflects light below (for shortpass) or above (for longpass) a specific cut-off or cut-on wavelength, respectively, while transmitting the rest.

Avalanche photodetector | A type of photodiode that is specifically designed to use the avalanche effect, which involves the multiplication of charge carriers (electrons and holes) due to high applied voltages, to amplify the electrical signals generated by the absorption of photons.

Infinity-corrected objective | An optical lens system designed to produce parallel rays between the objective and the eyepiece or camera, typically used in microscopy for clearer imaging and easier integration of additional optical components.

Aggregation-induced emission | a phenomenon where a material, often an organic compound, emits light more efficiently when it is aggregated or clustered together than when it is in an isolated, dissolved state.

Human embryonic kidney cells | A cell line derived from human embryonic kidney tissue, known for robust growth and ease of transfection, commonly used in the production of recombinant proteins, viral vectors, and in vitro drug toxicity assays.

Intralipid | A sterile fat emulsion commonly used in medical settings as a parenteral nutrition supplement and in research as a scattering medium to simulate biological tissues in optical imaging experiments.

Organic color centers | Synthetic defects in semiconducting single-walled carbon nanotubes created by covalently bonding organic molecules to the crystal lattice, resulting in quantum emitters that fluoresce in the NIR-II spectrum, emitting pure single photons at room temperature.

Particle image velocimetry | A visual measurement technique used to obtain instantaneous velocity fields by tracking the movement of small particles seeded in a fluid flow.

Peripheral node addressin | A carbohydrate ligand for L-selectin that plays a crucial role in the homing of white blood cells, specifically directing their migration to peripheral lymph nodes during the immune response.

High endothelial venules | Specialized post-capillary venous structures found in lymph nodes and Peyer's patches that facilitate the entry of lymphocytes from the bloodstream into lymphatic tissues.

Affibody | Small protein scaffolds derived from the Z domain of staphylococcal protein A, engineered to bind specific target proteins with high specificity and affinity.

Adjuvant | A substance added to vaccines to enhance the body's immune response to the vaccine's antigen.

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Competing interests

The authors declare no competing interests relevant to this work.

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