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Optical imaging in biomedical research: guidelines and practical insights

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Abstract

Preclinical imaging is crucial in biomedical research for non-invasive monitoring of various parameters in live animals over time, providing vital information while minimizing animal use. Optical imaging, the most commonly employed preclinical tool, offers ease of use, cost-effective instrumentation, and diverse applications. However, these advantages complicate the rational design, execution, and analysis of optical imaging experiments. This review highlights the specific conditions, strengths, and weaknesses of bioluminescence and fluorescence imaging. We discuss critical parameters essential for optimizing imaging settings to ensure accurate experimental outcomes. Additionally, we provide guidelines and address intrinsic limitations to assist scientists in making informed decisions and avoiding potential data shortcomings.

Keywords Optical imaging, Preclinical imaging, Bioluminescence, Fluorescence, Cancer, Fungi

Introduction

Molecular imaging is a rapidly evolving field in biomedical research that enables the observation, characterization, monitoring, and quantification of biomarkers and biological processes within living organisms. This discipline encompasses a wide range of imaging techniques, each offering anatomical, functional, or metabolic insights [1]. There has been a strong focus on the use of preclinical imaging in a bid to reduce the number of animals required for testing [2]. Preclinical imaging refers to a suite of techniques used to visualize biological processes in animal models before clinical trials in humans. These methodologies are essential for understanding disease mechanisms, evaluating drug efficacy, and developing new therapeutic strategies. Preclinical imaging encompasses various modalities, each with unique advantages and limitations, making them suitable for different applications. The most commonly used modalities include magnetic resonance imaging (MRI), positron emission tomography (PET), computed tomography (CT), single-photon emission computed tomography (SPECT), ultrasound, and optical imaging techniques such as bioluminescence and fluorescence imaging.

MRI is a non-invasive imaging technique that uses strong magnetic fields and radio waves to produce detailed images of internal structures. It excels in providing high-resolution images with excellent soft-tissue contrast, making it invaluable for anatomical and functional imaging of the brain, muscles, heart, and tumors [3]. However, MRI has limitations, including high cost and long scanning times and while excellent for anatomical details, it is not always ideally suited for extracting functional information (*e.g.* is the cell under investigation alive; has the cell differentiated etc.) [4].

PET is known for its high sensitivity and ability to quantify physiological processes in vivo, such as glucose metabolism, receptor binding, and enzyme activity. This makes PET highly valuable for oncology, cardiology, and

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neuroimaging studies [5]. SPECT, although similar to PET, has the advantage of being able to use a wider variety of radiotracers due to the longer half-life of its isotopes [6]. Both modalities, however, involve exposure to ionizing radiation, which limits their use in longitudinal studies. Additionally, the spatial resolution of PET and SPECT is lower compared to MRI, and the high cost of radiotracers and the need for specialized equipment are significant disadvantages [7].

CT uses X-rays to produce detailed cross-sectional images of the body. It is particularly useful for imaging bone structures, lung tissue, and for vascular studies when combined with contrast agents. However, the use of ionizing radiation in CT poses risks, especially for repeated scans in longitudinal studies [8]. Moreover, CT offers lower soft-tissue contrast compared to MRI, which can limit its application in certain types of biomedical research [9].

Ultrasound imaging is widely used due to its real-time imaging capability, portability, and relatively low cost [10]. Its major limitations include operator dependency, limited penetration depth, and lower resolution compared to MRI and CT. Additionally, ultrasound is less effective in imaging air-filled and bony structures [11].

Optical imaging is an economical technique that employs non-ionizing light to explore cellular and molecular processes in living organisms. This method captures photons within a spectrum from ultraviolet to near-infrared using specialized optical instruments [1, 12]. It provides spatial resolution at the nanometer scale, along with nanomolar-level sensitivity for detection. Compared to imaging techniques like MRI and PET, it is more affordable, simple to apply, and offers exceptional sensitivity. In a study, Zhao and colleagues [13] showcased the application of BLI in mice to track vascular alterations in luciferase-tagged human breast tumor xenografts following treatment with a vascular-disrupting agent, aimed at screening new cancer therapies. Ghost et al. [14] reported that fluorescence imaging enabled the removal of tumors as small as 1 mm in diameter, which would not have been possible without image-guided surgery. These findings highlight the potential of optical imaging for non-invasive cancer detection and treatment.

This review introduces the principles of optical imaging, focusing on bioluminescence and fluorescence imaging. It then focuses on the mechanisms underlying BLI and explores advancements in new substrates, along with their biological applications and developments. Potential challenges associated with BLI are also examined, along with proposed solutions. Following this, a detailed discussion of fluorescence imaging is presented. Finally, the review concludes with an exploration of the future prospects of optical imaging technologies, considering their potential impact on biomedical research and clinical practice.

Optical imaging

Optical imaging techniques, particularly bioluminescence and fluorescence imaging, have gained widespread use in preclinical research due to their unique advantages.

Bioluminescence Imaging (BLI)

BLI involves the use of genetically engineered cells or organisms that express luciferase enzymes, which emit light upon substrate oxidation. The primary advantage of BLI is its high sensitivity and ability to monitor biological processes in real-time in living animals. BLI is particularly useful for tracking tumor growth, metastasis, and gene expression patterns over time [15–17]. It allows for longitudinal studies with minimal invasiveness, and since it does not involve external light sources, background signal is minimal, providing high signal-to-noise ratios [18].

However, BLI has several limitations. The light emitted from bioluminescent sources can be attenuated by tissues, limiting the depth of imaging [18, 19]. Additionally, the quantification of light signals can be affected by the heterogeneous distribution of luciferase-expressing cells and substrate delivery. The spatial resolution of BLI is relatively low compared to other imaging modalities, which can limit its use in determining precise localization of the signal source [20].

Fluorescence Imaging (FLI)

FLI involves the use of fluorescent proteins or dyes to visualize cellular and molecular processes. This technique can be applied in a wide range of studies, including cancer research, cardiovascular disease, and neuroscience. FLI offers several advantages, such as high sensitivity, multiplexing capability (simultaneous imaging of multiple targets using different fluorophores), and the ability to image at the cellular and subcellular levels [21–23].

One of the significant strengths of FLI is its versatility. Various fluorophores can be engineered to target specific molecules or cellular structures, enabling highly specific imaging. Additionally, advancements in near-infrared (NIR) fluorescence imaging have improved tissue penetration and reduced background autofluorescence, enhancing image quality [21, 24].

Despite its advantages, FLI has limitations. Tissue autofluorescence and light scattering can reduce signal clarity,

particularly when using visible light fluorophores [25]. The depth of imaging is also restricted, although NIR fluorophores mitigate this issue to some extent. Moreover, the potential phototoxicity and photobleaching of fluorescent dyes can affect longitudinal studies. As with BLI, the spatial resolution of FLI is generally lower than that of modalities like MRI and CT [26].

The advantages of combining optical imaging and other tools

MRI and CT provide detailed anatomical information but are less sensitive for molecular imaging. Ultrasound is excellent for real-time imaging but has limited resolution and penetration. PET and SPECT offer high sensitivity for metabolic and functional studies but involve ionizing radiation. Optical imaging, particularly BLI and FLI, offers high sensitivity for tracking molecular and cellular processes with minimal invasiveness., making them invaluable for longitudinal studies [27]. Most important of all, due to the biocompatibility of luciferases and their substrates [28], along with the lack of ionizing radiation during bioluminescence imaging, animals can be imaged repeatedly without harm [29]. This significantly decreases the number of animals needed for long-term studies.

In recent years, the integration of multiple imaging modalities, known as multimodal imaging, has become increasingly popular in preclinical research. Combining modalities can provide complementary information, enhancing the overall understanding of biological processes [30]. For example, to advance the efficacy of glioblastoma (GBM) therapies, Molotkov et al. [31] developed a high-throughput and precise method to assess tumor growth and location in preclinical models. They established a multimodal 3D optical imaging (OI)/CT platform. Using Cy7 fluorescent dye-labeled albumin, the 3D OI/CT imaging was able to confirm the opening of the blood–brain barrier (BBB) for drug delivery, thereby enabling visualization of drug distribution. Similarly, the combination PET and FLI provides muti-scale and multi-parameter exploration of cellular system. Another study describes the successful synthesis and characterization of a NIRF/PET dual-modality imaging probe targeting cancer [32]. he probe showed specific targeting and accumulation in mice and rabbits with liver tumors, with clear tumor outlines visible in the NIRF/PET images after injection.

In short, preclinical imaging has been and still is a cornerstone of biomedical research, offering invaluable insights into disease mechanisms, drug efficacy, and therapeutic development. Each imaging modality has its own strengths and limitations, and the choice of modality depends on the specific requirements of the study. Optical imaging techniques, particularly bioluminescence and fluorescence imaging, are widely used due to their high sensitivity, minimal invasiveness, and suitability for longitudinal studies. While they have limitations in terms of depth and resolution, advances in technology and the integration of multiple imaging modalities continue to expand their utility and effectiveness in preclinical research.

Here we want to discuss the strengths and possible applications of preclinical optical imaging, but mainly focus on the practical aspects and need for careful design of experiments, optimization and analysis of results. As the widespread use of optical imaging is still vastly increasing and many researchers are being exposed to using this technology, we aim to provide some guidelines and how to maximally ensure robustness of the data. While optical imaging is indeed very easy to generate an image, the correct interpretation of the images and the conclusions that can be drawn will depend highly on the factors involved in the experiment. We will also highlight some cases where the data generated is difficult to analyze due to the wrong choice of imaging modality.

Principles of bioluminescence imaging

Bioluminescence imaging is a powerful and widely used technique in biomedical research that exploits the natural phenomenon of bioluminescence—the emission of light by living organisms. This imaging modality is non-invasive and allows real-time monitoring of various biological processes in live animals, such as gene expression, tumor growth, and microbial infections. The fundamental principles of BLI involve the enzymatic conversion of a substrate into light, which requires specific conditions including the presence of oxygen, adenosine triphosphate (ATP), and the saturation of the substrate.

Basic mechanism of bioluminescence

Bioluminescence results from a biochemical reaction catalyzed by luciferase enzymes, which oxidize a substrate known as luciferin. The most commonly used luciferase in BLI comes from the North American firefly (Photinus pyralis) [33]. In the firefly bioluminescence system, luciferase catalyzes the oxidation of D-luciferin in the presence of ATP, magnesium ions (Mg^{2+}), and oxygen, producing light, oxyluciferin, CO_2, and AMP as byproducts. The emitted light can then be captured by sensitive cameras (usually charge-coupled devices, or CCD cameras) and quantified.

The reaction can be summarized as follows:

$$\text{Luciferin} + \text{ATP} + \text{O}_2 \rightarrow \text{Oxyluciferin} + \text{Light}\ (\lambda\,\text{max} \approx 560\ \text{nm}) + \text{AMP} + \text{CO}_2 + \text{PPi}$$

This reaction illustrates the necessity of ATP and oxygen for light production.

Role of ATP

ATP is crucial for the bioluminescence reaction because it provides the energy required for the activation of luciferin. In the initial step of the reaction, luciferase binds to luciferin and ATP, forming luciferyl-adenylate and releasing pyrophosphate (PPi). This intermediate then reacts with molecular oxygen, producing a transient dioxetanone structure that decomposes to produce light.

The dependency on ATP means that bioluminescence is an excellent reporter for cellular viability and metabolic activity. Live, metabolically active cells produce ATP, which is necessary for the bioluminescent reaction. This property allows researchers to use BLI to monitor cell proliferation, viability, and energy status in vivo [34].

Role of oxygen

Oxygen is another critical component of the bioluminescent reaction. After the formation of the luciferyl-adenylate intermediate, molecular oxygen is required for the next step of the reaction, which involves the formation of a high-energy dioxetanone intermediate. The subsequent breakdown of this intermediate produces oxyluciferin in an excited state, which emits a photon of light as it returns to the ground state.

The requirement for oxygen implies that BLI can also be used to monitor oxygen levels and hypoxic conditions within tissues. Areas of low oxygen concentration, such as the hypoxic core of tumors, might exhibit reduced bioluminescent signals [35]. This characteristic can provide valuable insights into the tumor microenvironment and the effectiveness of therapies aimed at modifying oxygenation levels.

Substrate saturation

The availability and saturation of the luciferin substrate are essential for optimal bioluminescent signal generation. When luciferin is abundant, the reaction proceeds efficiently, producing a strong and detectable light signal. However, if luciferin is limited, the bioluminescent signal can become weak, leading to reduced sensitivity and inaccurate quantification [36].

In practical applications, researchers often administer luciferin exogenously to ensure that substrate levels are sufficient to saturate the luciferase enzyme fully. Zhou et al. developed a fibroblast activation protein (FAP)-monitoring probe [37]. This probe encapsulates d-luciferin within self-assembled micelles made from a peptide-linked amphiphilic block copolymer. Due to its nanoscale size, the probe can effectively enter living cells and prolongs the residence time of d-luciferin. In the presence of FAP, d-luciferin is gradually released from the micelle. Compared to free d-luciferin, the designed luciferin enabled continuous monitoring for up to five hours in vivo. The mode of administration (*e.g.*, intraperitoneal, intravenous, subcutaneous) and the dose of luciferin can significantly impact the bioluminescent signal. For example, by administering AkaLumine intraperitoneally, the growth or reduction of engrafted Antares-expressing MG63.3 osteosarcoma tumors, as well as the circulation and localization of CAR-T cells injected intravenously, could be sensitively visualized in the same mice [38]. Therefore, careful optimization and standardization of luciferin delivery are crucial for consistent and reproducible results.

Novel luciferase enzymes and substrates: enhancements to bioluminescence imaging techniques

New luciferase enzymes

NanoLuc Luciferase (Nluc) NanoLuc luciferase, derived from the deep-sea shrimp Oplophorus gracilirostris, is a highly compact enzyme with exceptional brightness and stability. Nluc utilizes a synthetic substrate, furimazine, to produce bioluminescence. One of the key advantages of Nluc is its smaller size (19 kDa compared to 61 kDa for Fluc), which allows for easier genetic engineering and fusion protein creation. Nluc's brightness is reported to be over 150-fold higher than that of Fluc, making it an excellent choice for offering the capability to elucidate protein dynamics in living cells without the necessity for repeated excitation of samples, such as single cell imaging and tracking of low abundance targets (Fig. 1) [39, 40].

Gaussia Luciferase (Gluc) Gaussia luciferase, derived from the marine copepod Gaussia princeps, is another alternative that offers several advantages. Gluc is naturally secreted, enabling the detection of bioluminescence in bodily fluids, which is particularly useful for monitoring systemic processes such as blood-borne metastasis and circulating biomarkers. Gluc uses coelenterazine as a substrate, emitting light at a peak wavelength of 480 nm. The enzyme is extremely bright and can be used for both in vitro and in vivo applications. Its secretory nature also facilitates non-invasive longitudinal studies [28].

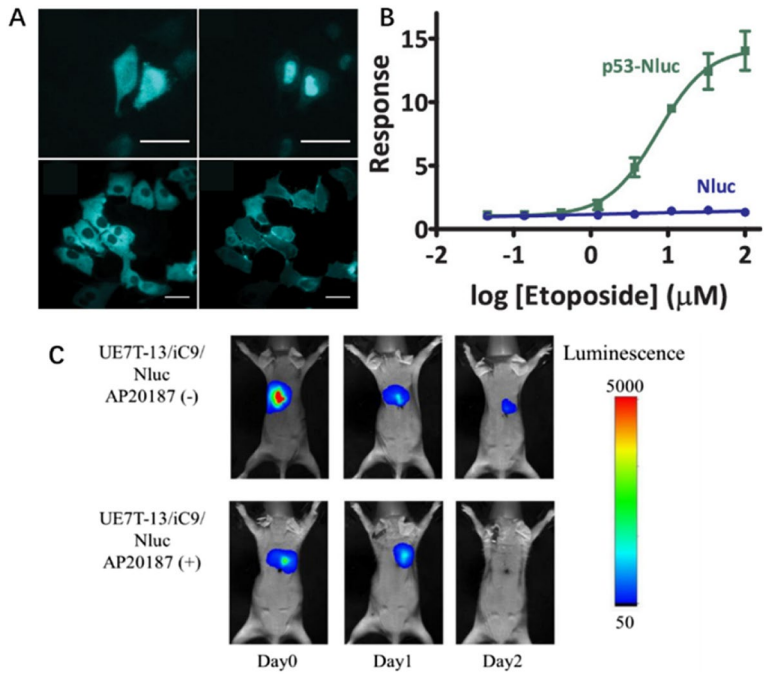

Fig. 1 Monitoring cells and proteins using BLI. a, Hela cells transiently expressing Nluc-GR fusions show cytosolic localization and nuclear accumulation after 15 min and 20 min of dexamethasone treatment. b, Nluc is used as a fusion tag for monitoring intracellular protein in p53 stability. Subpanels a and b have been reproduced with permission from Hall et al. (ref [39])© American Chemical Society, 2012, Open Access. c, nLuc was utilized to investigate the effects of AP20187 treatment on the rapid regulation of proliferation and function in cells expressing the iC9 gene following transplantation into mice. Subpanel c has been reused with permission (ref [40]) © MDPI, 2019

Renilla Luciferase (Rluc) Renilla luciferase, from the sea pansy Renilla reniformis, also utilizes coelenterazine as its substrate. Rluc is often used in bioluminescence resonance energy transfer (BRET) assays due to its emission spectrum and the ability to serve as an energy donor. Rluc emits blue light with a peak around 480 nm and is useful for studying protein–protein interactions, gene expression, and cellular signaling pathways. Its stability and compatibility with coelenterazine derivatives enhance its versatility in different experimental settings [41].

Akaluc Luciferase Akaluc luciferase is a red-shifted luciferase engineered for improved performance in deep tissue imaging. Developed to work with the near-infrared substrate AkaLumine, Akaluc produces bioluminescence at wavelengths that penetrate tissues more effectively than traditional luciferases. This enzyme is particularly beneficial for visualizing internal organs and tissues in live animals, thereby enhancing the study of complex biological processes such as cancer metastasis and cardiovascular diseases [42].

Antares and Antares2 Luciferases Antares luciferase is a synthetic enzyme created by fusing NanoLuc with a red fluorescent protein, leading to enhanced bioluminescence resonance energy transfer (BRET) and red-shifted emission. Antares provides significant improvements in signal intensity and tissue penetration, making it suitable for deep tissue imaging. Antares2 is an optimized version of Antares with further improved brightness and stability. Both Antares and Antares2 are valuable for multiplex imaging, enabling the simultaneous tracking of multiple targets in vivo with minimal spectral overlap [43, 44].

New Luciferase Substrates

Furimazine and furimamide Furimazine is the synthetic substrate for Nluc, providing a highly efficient bioluminescent reaction. Its high quantum yield and stability contribute to the exceptional brightness of Nluc. Additionally, furimamide, a modified form of furimazine, has been developed to further enhance the performance of Nluc in various experimental conditions. These substrates extend the dynamic range and sensitivity of BLI, particularly in challenging environments (Fig. 2) [38, 44].

AkaLumine AkaLumine, combined with the luciferase variant Akaluc, enables near-infrared bioluminescence

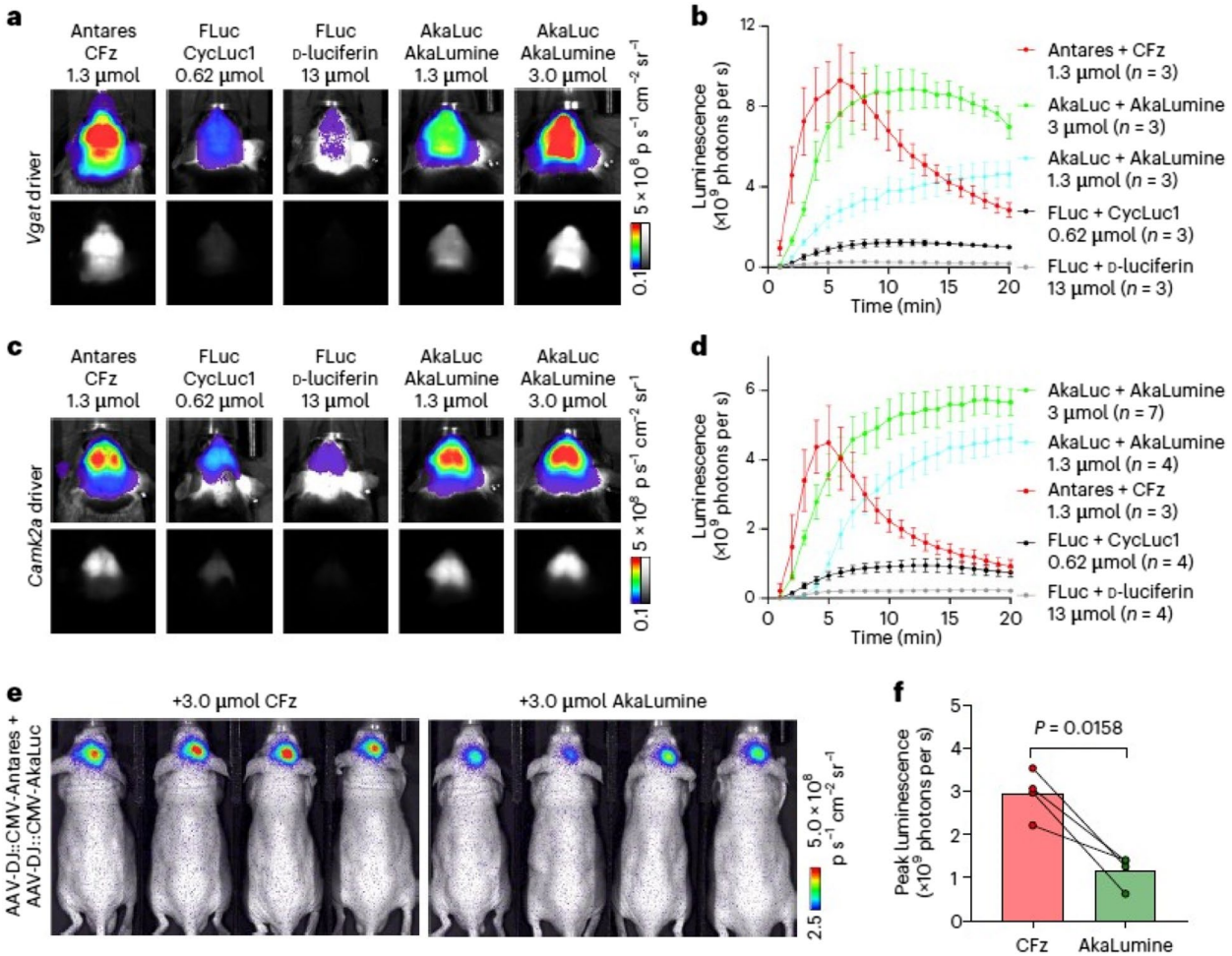

Fig. 2 **a**–**d**, Comparison between Antares–CFz and other luciferase reporters in transgenic mice. **a**, Representative images of peak bioluminescence of reporters expressed in VGAT+ neurons. **b**, Bioluminescence intensity over time. Data are presented as mean values ± s.e.m.; $n = 3$ independent animals in each condition. **c**, Representative images of peak bioluminescence of reporters expressed in CaMKIIα+ neurons. **d**, Bioluminescence intensity over time. Data are presented as mean values ± s.e.m.; $n = 7$, 4, 3, 4 and 4 animals for AkaLuc with 3 µmol of AkaLumine, AkaLuc with 1.3 µmol of AkaLumine, Antares with 1.3 µmol of CFz, FLuc with 0.62 µmol of CycLuc or FLuc with 13 µmol of D-luciferin, respectively. **e**, Representative bioluminescence images of Antares–CFz and AkaLuc–AkaLumine in the hippocampus of J:NU mice co-infected with Antares- and AkaLuc-encoded AAV. f, Comparison of peak signal intensities. Data are presented as mean values ± s.e.m.; $n = 4$ animals, each of which was measured for both Antares–CFz and AkaLuc–AkaLumine signals. *P* values were determined by two-tailed Student's paired t-test.Copyright ref [44], 2023 Nature Publishing Group

imaging. This system is particularly beneficial for deep tissue imaging due to the reduced light absorption and scattering at near-infrared wavelengths. AkaLumine allows for better penetration and clearer images of internal organs and tissues, enhancing the capabilities of BLI for in vivo studies. Using the Akaluc/Akalumine combination, single cell imaging could be performed of tumor cells migrating in the lung of mice [42].

Red-shifted luciferins Red-shifted luciferins, such as CycLuc1, have been developed to produce bioluminescence at longer wavelengths (600–650 nm). These substrates enhance tissue penetration and reduce background noise caused by tissue autofluorescence. Red-shifted luciferins are particularly useful for imaging in larger animal models and for applications requiring high signal-to-noise ratios [45].

The introduction of new luciferase enzymes and substrates addresses several limitations of the classical firefly luciferase system. Enhanced brightness and sensitivity are achieved with enzymes such as Nluc and substrates like furimazine, significantly increasing bioluminescent signal intensity. This enhancement facilitates the detection of lower-abundance targets, enabling applications

such as single-cell imaging and early tumor detection. The improved sensitivity also elevates the signal-to-noise ratio, resulting in clearer and more accurate images [39].

Novel luciferases and substrates offer greater stability, which is essential for long-term imaging studies. For instance, Nluc and furimazine maintain stable signals over extended periods, allowing continuous monitoring of biological processes. Their versatility permits use in various experimental conditions, including high-throughput screening and multiplex assays. The availability of luciferases with different emission spectra, such as red-shifted luciferins, supports multicolor imaging and multiplex experiments, enabling researchers to track multiple biological processes simultaneously within the same organism, thereby enhancing understanding of complex interactions and dynamics [46].

Near-infrared luciferases and substrates like Akaluc with AkaLumine significantly improve deep tissue imaging. These systems minimize light scattering and absorption, enhancing visualization of internal organs and tissues. This capability is crucial for studying complex biological processes and disease progression in vivo [42]. Antares and Antares2, which feature enhanced BRET efficiency and red-shifted emission, offer improved imaging capabilities for tracking cellular and molecular events deep within the body. Such advancements enable the investigation of disease progression and therapeutic responses in ways previously unattainable with traditional luciferases [43].

Luciferases such as Gluc, which are naturally secreted, facilitate non-invasive monitoring of systemic processes. This characteristic allows researchers to measure bioluminescent signals in bodily fluids, reducing the need for invasive procedures and enhancing animal welfare in longitudinal studies [28].

As mentioned above, the classical BLI system is based on the firefly luciferase (Fluc) enzyme and its substrate D-luciferin, which has been widely used due to its high sensitivity and relatively simple implementation. These innovations enhance the capabilities of BLI, addressing some limitations of the traditional firefly luciferase system and expanding its applications.

Current application and future directions in biomedical research

BLI is extensively used in various fields of biomedical research due to its high sensitivity, non-invasiveness, and ability to monitor dynamic biological processes in vivo. Some of the key applications include:

Oncology

BLI is widely used to study tumor growth, metastasis, and response to therapy. Cancer cells can be genetically engineered to express luciferase, allowing researchers to track tumor progression and monitor therapeutic efficacy in real-time [17, 47, 48]. The advantage over more anatomical imaging methods as CT or MRI is that any therapeutic response resulting in tumor cell death will result in a rapid decline in BLI signal, while the reduction of tumor size as observable by CT or MRI will take much longer. BLI therefore allows one to adjust treatment strategies more rapidly, depending on the initial therapeutic results measured, while for MRI/CT, this would be impeded by the slow functional readout.

Infectious diseases

Pathogens such as bacteria and viruses can be labeled with luciferase to monitor infection spread and treatment responses [49]. BLI can then be used to study the dynamics of bacterial, fungal or viral infections in vivo, providing insights into pathogen behavior and host response.

Gene expression and regulation

BLI can be employed to study gene expression patterns by placing luciferase under the control of specific promoters. This allows researchers to visualize the temporal and spatial expression of genes in living organisms. In a recent study, Malik *et al*. [50] created a BLI method for freely moving mice, allowing voluntary luciferin intake to reduce stress. This approach involved monitoring Per1 gene expression, using cooled electron-multiplying CCD cameras for accurate measurements without anesthesia. The luciferin itself was provided in the drinking water, enabling researcher to monitor gene expression in the animals longitudinally with minimal handling of the mice and hence, reduced animal stress.

Drug discovery and development

BLI is a valuable tool for high-throughput screening of drug candidates. The technique enables rapid and non-invasive assessment of drug efficacy and toxicity in animal models, accelerating the drug development process [51].

The field of BLI continues to evolve, with ongoing advancements aimed at improving sensitivity, specificity, and versatility. Some of the emerging trends and future directions include:

Engineered Luciferases

It refers to modified versions of natural luciferase enzymes that have been genetically altered to improve their performance. Researchers are developing engineered luciferases with enhanced brightness, stability,

and spectral properties. For example, engineered luciferases are designed to be more resistant to degradation, which helps maintain a consistent signal over extended periods. This is particularly important for monitoring dynamic biological processes in living organisms. These improvements can increase the sensitivity of BLI and enable multiplexed imaging of multiple biological targets simultaneously [52].

Hybrid imaging systems

Combining BLI with other imaging modalities, such as CT, MRI or PET, can provide complementary information and enhance the overall imaging capabilities. Hybrid imaging systems facilitate the real-time observation of biological processes over time. For example, BLI can monitor gene expression or tumor growth, while CT or PET offers insights into metabolic activity and structural alterations, enabling dynamic evaluations of disease progression and treatment effectiveness [53].

In Vivo Biosensors

Development of in vivo biosensors that couple bioluminescence with specific biological activities, such as enzyme activity or protein-protein interactions, can expand the utility of BLI. These biosensors can provide real-time readouts of cellular events and molecular interactions within living organisms [54]. For example, tumor-specific enzymes, offer valuable insights for cancer diagnosis, treatment, and prognosis. Numerous bioluminescent probes that can be identified and activated by these enzymes have been effectively utilized for cancer detection and therapy [55].

Therapeutic monitoring

BLI can be integrated into therapeutic monitoring protocols to assess the efficacy and safety of new treatments. New and alternative luciferase and substrates offer improvements in brightness, stability, wavelength emission, and versatility. For example, advancements of BLI can led to the extensive use of luciferins as bioluminescent probes for assessing the activity of tumor-specific enzymes and detecting bioactive small molecules for drug evaluation [56], due to their high biocompatibility and effectiveness in vivo. The use of non-invasive longitudinal imaging would allow researchers to monitor the effectiveness of any therapy given closely and to adjust the therapeutic strategy selected in case of lack of treatment success. By using multiple markers (*e.g.* tumor growth, levels of inflammation or T cell influx), the researchers can make more informed decisions on what they should change, while allowing them to monitor the impact of any change over time.

Potential problems and issues with BLI

Saturated substrate levels needed for proper quantification

As detailed above, the substrate that is provided will be converted by the engineered enzymes to create light. The amount of light generated therefore depends on the substrate availability for the enzymes, where for proper analysis, all luciferase enzymes should be able to convert the substrate at maximal level. If this is not the case, then the light output will not correlate any longer with the number of luciferases present, and therefore, the photon flux that is measured will not provide any real information on potential growth or reduced cell numbers. Instead, any differences in signal can then either be ascribed to variations in substrate availability and thus, luciferase activity level, as much as differences in cell, and therefore luciferase enzyme, numbers. For animal studies, the amount of d-luciferin, the substrate commonly used for firefly luciferase, is provided to the animals at 150 mg/kg body weight. This amount has carefully optimized to ensure saturating levels of the substrate for the luciferase enzymes, but this will still depend on the exact conditions.

A study by Yeh et al. found that different luciferase systems have expression levels that vary among different tissues and that substrates all have intrinsic variations in their biodistribution level and will therefore not be uniformly present throughout the body at saturation level. This means that if the cells or pathogens of choice are widely distributed throughout the body, and that biodistribution can vary widely between different animals, BLI may not provide accurate results to compare relative cell numbers [57]. However, if the cells or pathogens that express luciferase are generally in the same region or tissue, then this effect will not be a major cause of concern. Furthermore, d-luciferin is known to have a rather poor efficacy at crossing the blood brain barrier (BBB). This means that the number of cells or pathogens in the brain can typically not be studied properly using BLI. For glioblastoma (GBM) research, the outgrowth of GBM may cause ruptures in the BBB, through which the d-luciferin can pass and therefore lead to higher substrate levels in the brain. For different animals, or at different time points in the same animal, the photon flux measured is therefore not only dependent on the number of cells, but also on the extent of BBB permeability. Other substrates, such as cephalofurimazine, have been designed that are better suited at crossing the BBB [44].

BLI issues in fungal imaging

For infectious diseases models, such as fungi, BLI faces significant challenges, particularly regarding substrate availability and penetration, and biofilm formation. One major difficulty lies in the delivery and availability of the luciferin substrate within the infected host. Luciferin must reach the fungal cells to elicit a luminescent response, yet its diffusion can be hindered by host tissue barriers and the microenvironment of the infection site [58]. Additionally, fungal cell walls are complex and can limit substrate uptake, leading to inconsistent or weak luminescent signals. Furthermore, fungi can be rather complex structures, having varying levels of more hyphenated and more conidial structures, each of which have different abilities for allowing substrate penetration. Additionally, many fungi can form fungal biofilms [59]. Biofilms, which are structured communities of fungal cells encased in an extracellular matrix, are notoriously difficult to penetrate for the substrate itself, and can even influence signal readout by absorbing and scattering light.

To address these challenges, several strategies have been proposed. Enhancing substrate delivery by using more permeable luciferin analogs or nanoparticles that facilitate luciferin transport across biological barriers has shown promise. Additionally, genetic engineering of fungi to express more efficient luciferases or to produce luciferin autonomously can improve signal strength and reliability [60]. One example also involves the engineering of surface-located luciferases in order to remove the need for substrate penetration into the fungal cells [58]. Another approach involves the use of NIR bioluminescence, which penetrates tissues more effectively than visible light, thereby improving the detection of signals from deeper or more obscured infection sites [58]. These advancements aim to optimize BLI for more accurate and robust monitoring of fungal diseases, ultimately improving the understanding and treatment of these infections.

These findings suggest that BLI using the classical firefly luciferase and d-luciferin substrate is not really suited for imaging infectious diseases such as fungal models. While advances such as the ones above can be explored, other modifications are more questionable. One example includes increasing the concentration of the substrate in order to enhance sensitivity [61]. Any increase in photon flux generated therefore confirms that the signal generated is not optimal and that the luciferase enzyme is not converting the substrate at maximal capacity due to poor substrate penetration in the fungi. Furthermore, increasing the dose of d-luciferin more than threefold may also affect the overall pharmacokinetics and biodistribution of the substrate, and may in itself also cause potential toxicity. While d-luciferin is non-toxic at the recommended concentration, it is not advisable to increase the concentration without evaluating for potential adverse effects. Additionally, while increasing the dose may help generate more signal, it is impossible to really know if saturation levels have been reached for all animals, and hence, quantification of the signal cannot be accurately linked to fungal cell numbers. If d-luciferin cannot cross a thick biofilm or fungal cell walls and hence cannot reach the fungi themselves, increasing the substrate concentration may not overcome this problem. Recently, the same group also utilized BLI for screening of fungal pathogens in Galleria Melonella larvae, where, due to low signal, the dose of d-luc used was increased up to 4000 µg/g body weight [62], which is more than 25-fold higher than the recommended dose (126 µg/g). This excessive amount resulted in clear levels of toxicity, after which the level was reduced. However, it was unclear whether the substrate distribution in the larvae was uniform and could reach saturating levels at the dose provided. In view of longitudinal imaging, both examples clearly illustrate that the photon flux obtained cannot be correlated with proper fungal counts without additional controls and studies.

Substrate interactions with other compounds

In line with the section above, which emphasizes the need to have maximal luciferase activity and thus, saturating substrate levels needed for proper BLI analysis, this may also be in part impeded due to potential difficulties in using pharmaceutical agents for treatment of cancer or infectious diseases. Many therapeutic agents are relatively poorly soluble, and in order to increase their solubility for intravenous administration, the agents are mixed with b-cyclodextrin. β-Cyclodextrin (β-CD) is a cyclic oligosaccharide consisting of seven d-glucopyranoside units connected by α−1,4-glucosidic bonds. In view of its shape, it is conal and exists of a outer hydrophilic surface and inner hydrophobic cavity. This renders β-CD highly soluble in water, and it can help to encapsulate poorly soluble molecules in its inner cavity. Due to this, CDs have attracted great interest in recent years as a specific nanocarrier for the delivery of a wide range of chemotherapeutic agents and act as a promising anti-tumor nanomedicine [63]. For fungal diseases, common therapeutic agents such as voriconazole (used for treatment against Aspergillus fumigatus) also consist mainly of b-CDs. Recent work by Kumar et al. revealed that b-CDs have a high affinity for d-luciferin and that the presence of b-CDs can reduce the signal generated by luciferase enzymes [64]. To verify this finding, we evaluated the possible effect that voriconazole could have on luciferase signals. Using E0771 triple negative breast cancer (TNBC) cells expressing firefly luciferase as well as a NIR fluorescent protein (iRFP713), however, the addition of

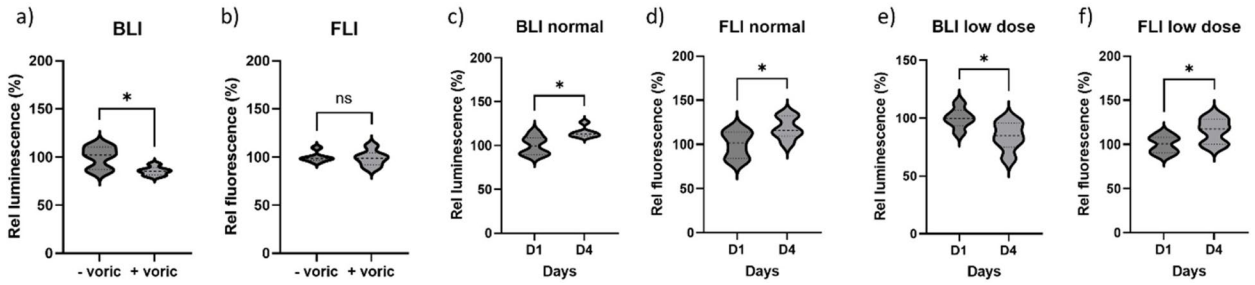

Fig. 3 The effect of b-cyclodextrin-containing pharmaceutical agents on firefly luminescence output. **a** luminescence and **b**) fluorescence levels of cultured E0771 cells expressing both firefly luciferase and niRFP713 reporters. Cells were cultured and BLI/FLI measurements were performed in the presence or absence of voriconazole given 30 min prior to the measurement. **C-f** Relative 3D luminescence (**c**,**e**) or 3D fluorescence (**d**,**f**) of C57Bl6 mice having received E0771 cells expressing both firefly luciferase and niRFP713 reporters 5 days previously through hemodynamic perfusion, resulting in high levels of cells in the liver (day 1). Luminescence and niRFP713 signal was measured when d-luciferin was provided at 150 mg/kg body weight (normal dose, c) or under restrictive (low dose, e) conditions at 30 mg/kg body weight. The animals were treated with voriconazole (40 mg/kg via tail vein injection) 30 min prior to the second measurement (day 4). Data are expressed as violin plots ($n = 6$). Significant differences between (**a**,**b**) the voriconazole and non-voriconazole conditions or (**c**-**f**) D1 and D4 were analysed using GraphPad Prism 10 using a standard t-test with the degree of significance indicated when appropriate (*: $p < 0.05$)

voriconazole significantly reduced luminescence photon flux of the cultured cells, whereas no effect was observed on fluorescence-based recordings (Fig. 3a,b). This was also confirmed using mouse models bearing E0771 tumor models provided by hemodynamic transfusion in the liver. Here, no effect of voriconazole was observed when d-luciferin was provided at the 150 µg/g concentration, as this is the saturating concentration and the substrate does not have difficulties passing the cellular membranes. Therefore, reducing the d-luciferin concentration a little would have no clear effect. However, as explained above, with fungi the substrate does not cross the fungal wall properly, resulting in insufficient d-luciferin levels at the level of the luciferase enzyme. To mimic these restrictive conditions, animals were given only 30 µg/g d-luciferin, in which case the saturating level is not reached. Under this condition, the addition of voriconazole reduced luminescence output compared to 4 days prior, and would indicate a potential anti-tumor effect, but this was countered by iRFP713 fluorescence signal, where the tumor had slightly grown (Fig. 3c-f). These data therefore clearly indicate that the possible interaction of d-luciferin with other pharmaceutical agents must be carefully considered. As the chapter above already indicated that classical firefly luciferase-based BLI with d-luciferin as a substrate is intrinsically ill-suited for monitoring fungal diseases, any studies that focus on the effects of agents such as azole-based antifungals obtained using longitudinal BLI, [61, 62], must be interpreted with caution.

Host parameters that may influence BLI efficacy

As detailed in the description of BLI above, the enzymatic reaction requires not only sufficient substrate, but also oxygen and ATP to perform well. Therefore, the accuracy of BLI can be impacted by several physiological and microenvironmental parameters. Key factors include hypoxia, low perfusion, and extracellular matrix (ECM) density, among others. Understanding these influences is crucial for interpreting BLI data correctly and improving the reliability of this imaging modality.

Hypoxia, or low oxygen levels, can significantly affect the accuracy of BLI. The enzymatic reaction that produces bioluminescence, typically involving luciferase and its substrate luciferin, is oxygen-dependent. In hypoxic conditions, the reduced availability of oxygen can lead to a decrease in the efficiency of the bioluminescent reaction, thus lowering the signal intensity. Studies have shown that regions of tumors, which are often hypoxic, exhibit diminished bioluminescent signals compared to well-oxygenated tissues [65]. This oxygen dependency implies that variations in tissue oxygenation must be considered when quantifying bioluminescent signals, as hypoxia could lead to underestimation of cell viability or activity in these regions [66].

Low perfusion, or reduced blood flow, can also impact BLI accuracy. Perfusion affects the delivery of luciferin to the cells expressing luciferase. In areas of low perfusion, such as in certain tumor microenvironments or ischemic tissues, the delivery of luciferin is compromised, leading to a lower bioluminescent signal. This phenomenon is compounded by the fact that low perfusion often correlates with hypoxic conditions, further exacerbating the reduction in signal intensity. Studies have documented that improving perfusion can enhance bioluminescent signal by ensuring adequate delivery of luciferin to the target cells [67].

The density of the extracellular matrix (ECM) is another critical parameter influencing BLI accuracy. The

ECM provides structural support to tissues and can vary significantly in density and composition, affecting the diffusion of luciferin and oxygen to luciferase-expressing cells. In dense ECM environments, such as in certain fibrotic tissues or desmoplastic tumors, the diffusion of these molecules is hindered, leading to lower bioluminescent signals. Research indicates that ECM remodeling or degradation can enhance the diffusion of luciferin and improve signal intensity in such tissues. Furthermore, the heterogeneous nature of the ECM across different tissue types adds an additional layer of complexity to BLI interpretation, necessitating tissue-specific considerations [68].

Other parameters influencing BLI accuracy include tissue depth and optical properties. Bioluminescent photons must traverse various tissue layers to reach the detector, and their intensity can be significantly attenuated by absorption and scattering within the tissue. Hemoglobin, water, and lipids are major absorbers, while cellular structures cause scattering, both of which reduce the detected signal as tissue depth increases (Fig. 4) [69]. Advanced computational models and correction algorithms are being developed to account for these optical properties and improve quantitative accuracy.

Temperature is another factor that can affect BLI. The luciferase-luciferin reaction is temperature-sensitive, with reaction rates typically increasing with temperature up to an optimal point beyond which enzyme denaturation occurs. Variations in body temperature, therefore, can lead to fluctuations in bioluminescent signal intensity. Maintaining consistent and physiologically relevant temperatures during BLI experiments is crucial to minimize this source of variability [67].

Cellular metabolic state and pH can also modulate bioluminescent signal. The production of ATP, which is required for the luciferase reaction, can vary with the metabolic state of the cell. In stressed or dying cells, ATP levels may be lower, leading to reduced bioluminescent output. Similarly, extreme pH conditions can affect the enzyme activity of luciferase, further impacting signal intensity. Thus, monitoring and potentially correcting for these metabolic parameters can enhance the reliability of BLI [70].

Efforts to mitigate these influences and improve BLI accuracy include the development of luciferase variants with altered substrate affinities or oxygen dependencies, the use of alternative substrates that might be less affected by hypoxic conditions, and the incorporation of complementary imaging modalities such as magnetic resonance imaging (MRI) or positron emission tomography (PET) to provide more comprehensive data [71]. Additionally, advances in image processing and analysis techniques are enabling more precise quantification and localization of bioluminescent signals, accounting for the aforementioned physiological and microenvironmental factors. Hardware components are also changing, with more efforts being made towards more precise and fully quantitative 3D BLI systems to overcome these issues. Choosing the right system and settings, and ensuring that adequate controls are included (hypoxia measurements, CT or MRI for appropriate tumor location…) to define what exactly is causing any change or a lack of expected change in BLI signal.

Influence of genetic transformation on the cell population of interest

Bioluminescence imaging (BLI) is a critical tool in biomedical research, enabling non-invasive visualization of cellular and molecular processes in living organisms. Central to BLI is the transformation of cells to express luciferase enzymes, which catalyze the bioluminescent reaction upon exposure to a suitable substrate such as luciferin. While this technique has provided significant insights, the process of transforming cells to express luciferase enzymes can introduce several variables that impact the accuracy and interpretation of BLI data. These include the necessity for high expression levels of luciferase, the influence on cell metabolism and functionality, and the potential immunogenicity of the foreign luciferase enzyme.

High expression levels

Achieving sufficiently high expression levels of luciferase is crucial for generating detectable and quantifiable bioluminescent signals. Low expression can result in weak signals that are difficult to distinguish from background noise, thereby reducing the sensitivity and reliability of BLI. To ensure high expression levels, strong promoters are typically used in the genetic constructs for luciferase expression. However, high expression can also pose challenges. Overexpression of luciferase can lead to metabolic burden on the host cell, potentially affecting cellular physiology and viability. This metabolic burden arises from the increased demand for transcriptional and translational machinery, as well as the potential toxicity of high levels of protein synthesis. For example, research has shown that overexpression of exogenous proteins can trigger the unfolded protein response (UPR) and other stress pathways, which may alter cellular behavior and function [72].

Impact on cell metabolism and functionality

The introduction and expression of luciferase enzymes can influence cellular metabolism and overall functionality. The luciferase reaction itself consumes ATP, oxygen, and luciferin. Depending on the level of expression and

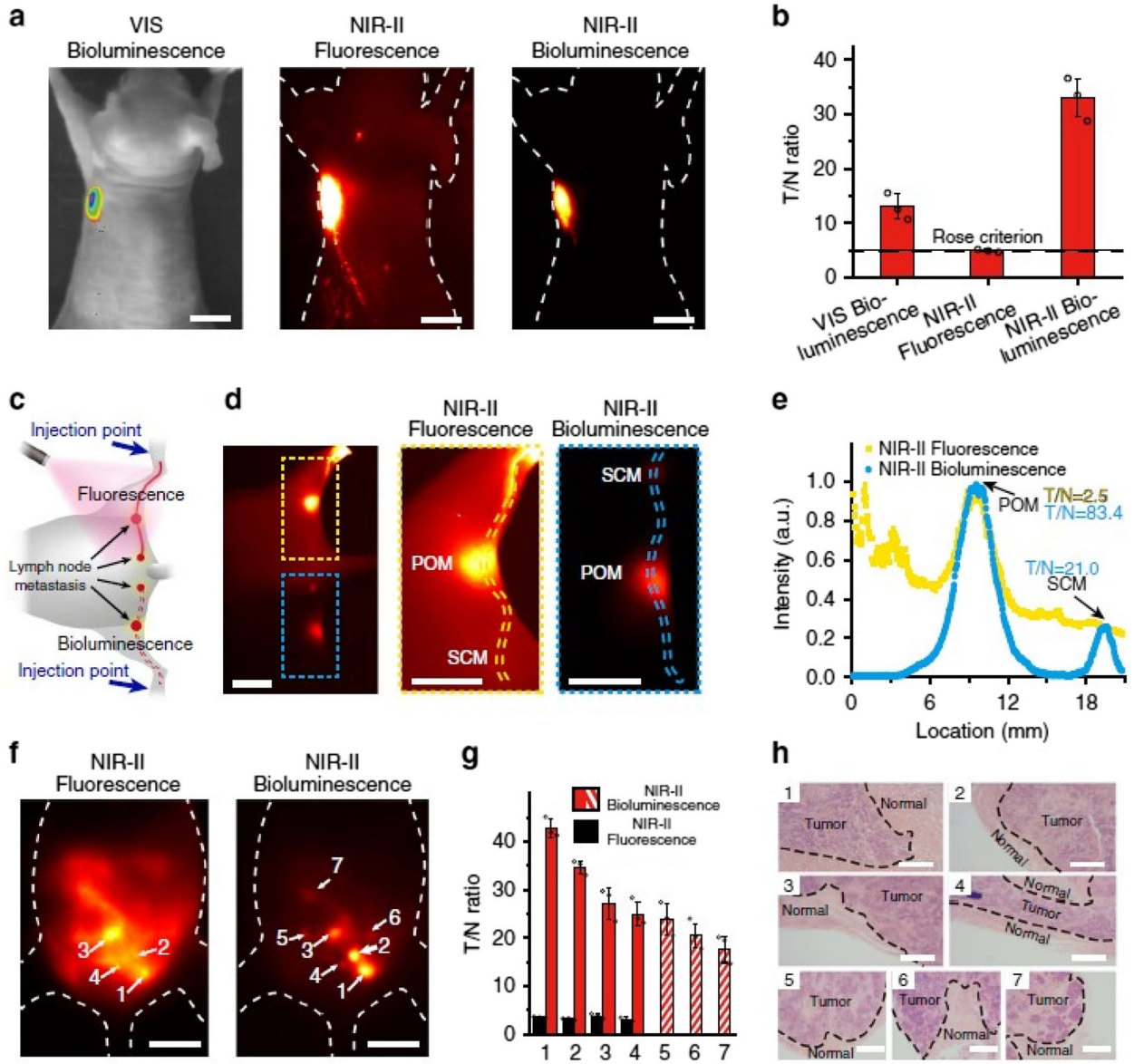

Fig. 4 **a** VIS bioluminescence imaging, NIR-II fluorescence imaging and NIR-II bioluminescence imaging of the same tumor in a nude mouse. Scale bar, 1 cm. **b** T/N ratios of different optical imaging methods in a, black dashed line indicates the Rose criterion. Bars represent mean ± s.d. derived from n = 3 independent measurements. **c** Schematic illustration of executing fluorescence imaging and bioluminescence imaging simultaneously on one nude mouse carrying lymph node metastases. Blue arrows indicate the subcutaneous injection location. **d** Fluorescence imaging (top) and bioluminescence imaging (bottom) of lymph node metastasis and the corresponding high-magnification imaging (right). Scale bar, 5 mm. **e** Intensity profiles along the lymphatic vessels in d. **f** NIR-II fluorescence imaging and NIR-II bioluminescence imaging of peritoneal metastases (No. 1–7) Scale bar, 1 cm. g T/N ratios of corresponding serial number tumors in f. Black dashed line indicates the Rose criterion. Bars represent mean ± s.d. derived from *n* = 3 independent measurements. h H&E staining results of metastatic tumors (No. 1–7) margin in **f**. All borderlines between early metastatic lesions and normal tissues can be observed. All the metastases were confirmed to be malignant and repeated for three times in independent experiments. Scale bars, 0.2 mm. Source data underlying b, e and g are provided as a Source Data file. Copyright ref [69], Nature Publishing Group 2020

the frequency of bioluminescent measurements, the ATP consumption can be significant, potentially affecting cellular energy balance. In high-demand tissues or cells with limited ATP reserves, this additional consumption could impact normal cellular processes. For instance, studies have indicated that the ATP-dependent nature of the luciferase reaction can lead to lower intracellular ATP levels, which may compromise cell viability and function under certain conditions [67].

Furthermore, the luciferase reaction generates light, but also heat and reactive oxygen species (ROS) as by-products. While the heat generated is typically negligible, the production of ROS can contribute to oxidative stress, which can damage cellular components and affect cell viability and function. For example, elevated ROS levels can lead to oxidative damage to DNA, proteins, and lipids, potentially triggering apoptotic pathways and altering cell fate [73].

Immunogenicity of luciferase enzymes

Since luciferase enzymes are typically derived from non-mammalian organisms, such as fireflies (Photinus pyralis) or sea pansies (Renilla reniformis), they are recognized as foreign entities by the mammalian immune system. The immunogenicity of these enzymes can lead to an immune response, particularly in immunocompetent animals. This response can manifest as the production of antibodies against luciferase, which can neutralize the enzyme and reduce the bioluminescent signal over time. Additionally, immune responses can cause inflammation and other immunopathological effects, potentially complicating the interpretation of BLI results.

The immunogenicity of luciferase enzymes has been well-documented. For instance, studies have shown that repeated administration of luciferase-expressing cells or luciferin substrate can lead to the generation of anti-luciferase antibodies, which can significantly attenuate the bioluminescent signal [74–76]. This immune response can vary depending on the host species, the site of luciferase expression, and the duration of the study. To mitigate this issue, strategies such as using immunodeficient animal models, transient expression systems, or engineering less immunogenic luciferase variants have been explored [77].

Cellular transformation and tumorigenicity

The process of transforming cells to express luciferase typically involves the introduction of genetic material using viral vectors, plasmids, or transposons. While these methods are generally efficient, they carry the risk of insertional mutagenesis, which can disrupt endogenous genes and potentially lead to tumorigenic transformations. This risk is particularly pertinent when using integrating viral vectors, which can integrate into the host genome at multiple sites.

Insertional mutagenesis can activate oncogenes or inactivate tumor suppressor genes, leading to uncontrolled cell proliferation and tumorigenesis. For example, retroviral vectors have been associated with the development of leukemia in clinical gene therapy trials due to insertional activation of proto-oncogenes [78]. Although non-integrating vectors and site-specific integration systems reduce this risk, they are not completely free from insertional effects. It is therefore imperative that cells expressing the luciferase reporter are carefully evaluated compared to untransformed wild-type cells. For tumor cells, their in vitro homeostasis and the in vivo growth or immunogenicity should not be altered. Alternatively, for fungal cells, the growth rate and dissemination rate as well as the response against antifungal therapeutics should be the same. These factors should be carefully evaluated to ensure the reliability of the data generated.

Expression stability

The stability of luciferase expression over time is another critical factor that affects BLI accuracy. Stable expression ensures consistent bioluminescent signals, which is crucial for longitudinal studies. However, silencing of transgenes can occur through epigenetic mechanisms such as DNA methylation and histone modifications, leading to reduced or variable expression levels over time [79]. This silencing effect can be more pronounced in certain cell types or under specific physiological conditions, complicating the interpretation of bioluminescent signals in long-term studies.

To address this, researchers have employed strategies to enhance the stability of luciferase expression, such as using insulator sequences, optimizing vector design, and employing inducible expression systems. These approaches aim to minimize transgene silencing and ensure reliable bioluminescent signals throughout the study period [80].

Bioluminescence kinetics and imaging protocols

The kinetics of the luciferase-luciferin reaction also influence BLI accuracy. The reaction kinetics can be affected by factors such as substrate availability, enzyme concentration, and cellular environment. Understanding these kinetics is essential for optimizing imaging protocols and accurately interpreting the bioluminescent signals.

For instance, substrate availability can be influenced by tissue perfusion and diffusion. In poorly perfused or densely packed tissues, substrate delivery to luciferase-expressing cells may be limited, leading to lower bioluminescent signals. Furthermore, the timing of substrate administration relative to imaging is critical, as the bioluminescent signal typically peaks shortly after substrate injection and then declines. Optimizing the timing and dosage of substrate administration can enhance signal intensity and reproducibility [81].

A) Signal background

Accurate quantification of BLI signals requires proper handling of background luminescence, which can originate from sources such as autofluorescence, nonspecific binding of substrates, or intrinsic tissue bioluminescence. When expressing bioluminescence signals on a logarithmic scale, normalizing data by dividing by background levels rather than subtracting them offers significant advantages for ensuring accurate and reliable measurements.

B) Logarithmic scale and signal normalization

Bioluminescence signals often span several orders of magnitude, making logarithmic scaling a suitable method for data representation. Logarithmic scaling compresses large data ranges, facilitating the visualization and comparison of signals with substantial variability. However, the mathematical properties of logarithmic functions necessitate careful consideration when dealing with background subtraction versus normalization.

C) Background subtraction

Subtracting background levels from bioluminescence signals on a logarithmic scale can lead to significant inaccuracies, particularly with low-intensity signals. Subtraction of a constant background value from low-intensity signals can result in negative or near-zero values, which are problematic in logarithmic transformation since the logarithm of zero or negative numbers is undefined. For example, a low-intensity bioluminescence signal of 10 photons with a background of 8 photons would result in a net signal of 2 photons after subtraction. On a logarithmic scale, this would be log(2), a small positive value. However, if the actual signal were slightly lower, such as 7 photons, the net signal after subtraction would be negative (7—8 = −1), leading to an undefined logarithmic value.

D) Background division

Normalizing by dividing bioluminescence signals by background levels avoids these issues by maintaining the proportionality of the signals regardless of their intensity. This approach ensures that low-intensity signals remain positive and interpretable on a logarithmic scale. Using the previous example, if the signal is 10 photons and the background is 8 photons, dividing gives a normalized value of 1.25. Similarly, if the signal is 7 photons, dividing by the background yields a normalized value of 0.875. Both values are positive and can be easily transformed logarithmically, resulting in log(1.25) and log(0.875), respectively. This method preserves the relative differences in signal intensities without introducing undefined values or disproportionate scaling issues. The need for signal division by background levels rather than subtraction is further exemplified for those cases where low signal levels are measured. When the 'real' signal is multifold higher than the background, then the subtracting or dividing will not interfere too much with the analysis. However, when the 'true' signal is close to the background signal, background subtraction can give rise to false positive results. Please see Table 1 as an exemplified data table showing the differences between background subtraction and background division.

Table 1 A simplified example of the influence of background subtraction on exaggerating fold differences in low signal measurements

Average Bck	Average Gr1	Average Gr2	Ratio Gr2/Gr1	
1000	1100	1200		
(Gr – Bck)	100 (1100–1000)	200 (1200–1000)	twofold 200/100	**subtraction**
(Gr/Bck)	1.1 (1100/1000)	1.2 (1200/1000)	1.09-fold 1.2/1.1	**division**

Bck Background

Gr Group

E) Proportional representation

Dividing by background levels also provides a more accurate representation of the signal-to-noise ratio (SNR), an essential metric in imaging. The SNR is the ratio of the true signal to the background noise, directly impacting the ability to distinguish between different signal levels. Normalizing by division inherently considers the proportional contribution of the background to the observed signal. This approach maintains the fidelity of the relative changes in bioluminescence intensities, especially in low-signal scenarios where background noise can constitute a significant fraction of the measured signal.

F) Statistical considerations

From a statistical perspective, dividing by the background is preferable because it reduces the impact of background variability on the normalized signal. When background levels fluctuate, subtracting these values can amplify the variability, leading to inconsistent and less reliable data. Conversely, division tends to stabilize the variability by proportionally adjusting the signal relative to the background. This normalization method

thus enhances the statistical robustness of the bioluminescence measurements, improving the accuracy of downstream analyses such as comparative studies and quantitative assessments. Please see Table 1 for a simplified example on how the choice for background subtraction can create the illusion of large differences when low level signals are generated. It is therefore vital to always apply proper statistical analysis and not simply put photon levels over an artificial background. It is also important for proper analysis that the background itself is measured correctly and for multiple animals to enable proper statistical analysis. For example, if the background is measured at every time point, one must make sure that the region where the background is located is not affected by light emitted by the primary source (bioluminescent tumor or pathogen). Additionally, one must be sure that the background region is free from the actual luminescent probes. For metastatic tumors or spreading pathogens, this can be problematic. As background values can differ at different time points, it is ideally measured at every time point as this can be affected by technical issues, but also due to differences in the efficacy of animal shaving or even temperature. To circumvent the problems above, for background levels, separate control mice can be used that do not express the bioluminescent reporter, but still receive the substrate of choice to account for possible background luminescence, as described for Akalumine in the liver-region [82]. When choosing this option, it is best to measure the background at a fixed wavelength corresponding to the optimal wavelength for the luciferase-luciferin pair. When control animals are mixed for luciferase-expressing animals, and the latter generate only low level signal, as commonly observed in fungi, then the background at low wavelengths may be higher than the signal of the test animals. In this case, the signal will be measured at a wrong wavelength, resulting in higher background levels and potentially missing the actual signal. In longitudinal studies, this would change once the luminescence signal becomes stronger and then the background value will also change. This would harden any quantitative analysis later on.

G) Improved data interpretation

Normalization by division also simplifies the interpretation of bioluminescence data in comparative studies. Researchers often compare signals from different regions, time points, or experimental conditions. Dividing by background levels standardizes the data, allowing for direct comparison of normalized signals across different contexts. This method ensures that any observed differences in bioluminescence intensities are due to true biological variations rather than artifacts of background subtraction.

H) Non-specific substrate-based luminescence

Apart from incorrect analysis of BLI data, which can give rise to either false positive as well as false negative results, false signals in BLI can also arise from the background luminescence generated by the substrate itself. In some instances, the substrate can exhibit auto-luminescence or be partially converted by host enzymes, leading to spurious signals that are not indicative of the biological process being studied. This issue is particularly problematic when substrates are administered systemically, as they can be distributed throughout the body and interact with various tissues and enzymes, generating non-specific signals [83].

A notable example of substrate-induced background signal is observed when using Akalumine, a substrate often employed in BLI due to its superior properties, such as high sensitivity and ability to penetrate deeper tissues [42]. Akalumine, like other luciferase substrates, is subject to metabolism and conversion by endogenous enzymes present in the host organism. This enzymatic activity can lead to the generation of background signals that are unrelated to the target biological process.

The liver is a key site of metabolic activity and is rich in enzymes capable of modifying a wide range of substrates, including Akalumine. Studies have shown that when Akalumine is used in BLI, there is a significant liver-specific background signal. This phenomenon occurs because the liver enzymes partially convert Akalumine into luminescent compounds, even in the absence of the target luciferase [82]. The high metabolic activity in the liver exacerbates this issue, as the substrate undergoes rapid and extensive enzymatic transformation.

The liver-specific background signal presents a substantial challenge in BLI, particularly in experiments where liver function or liver-based processes are under investigation. For example, in studies aimed at visualizing tumor growth or therapeutic responses in hepatic tissues, the background luminescence from Akalumine metabolism can obscure true signals, leading to potential misinterpretation of the data. This non-specific luminescence can mask the bioluminescent signal from the luciferase-expressing cells or tissues, thus complicating the distinction between genuine biological activity and background noise [82].

To mitigate the impact of substrate-induced background signals, several strategies have been proposed. One approach involves the use of alternative substrates with reduced susceptibility to host enzyme conversion. Substrates designed to be more selective for the target

luciferase and less prone to metabolic transformation can help reduce non-specific signals. Additionally, pretreatment protocols that block or saturate endogenous enzyme activity in the liver before substrate administration have been explored. Such methods can decrease the extent of substrate conversion and, consequently, reduce background luminescence [84].

Moreover, advances in imaging techniques and data analysis can also aid in differentiating between true bioluminescent signals and background noise. Enhanced imaging systems with higher sensitivity and resolution can better capture the spatial and temporal dynamics of bioluminescence, allowing for more accurate discrimination of specific signals. Computational approaches, such as background subtraction algorithms and signal deconvolution, are also valuable tools in refining the analysis and interpretation of BLI data [85].

Preclinical whole-body fluorescence imaging

Preclinical whole-body fluorescence imaging in small animals has emerged as a powerful tool in biomedical research, offering a non-invasive method to visualize and quantify biological processes in vivo. This technique relies on the administration of fluorescent probes or proteins that emit light upon excitation by a specific wavelength, enabling the study of molecular and cellular events in real-time. The versatility and high sensitivity of fluorescence imaging make it particularly suitable for various applications, ranging from tumor imaging to tracking gene expression and monitoring therapeutic interventions.

Mechanism of fluorescence imaging in mice

Fluorescence imaging in mice involves the use of fluorescent molecules, such as fluorophores, fluorescent proteins, or nanoparticles, that are introduced into the organism either systemically or targeted to specific tissues or cells. Upon excitation by an external light source, typically in the visible or NIR spectrum, these molecules absorb photons and re-emit them at a longer wavelength. The emitted light is then captured by sensitive detectors, such as charge-coupled device (CCD) cameras, enabling the visualization of the fluorescence signal.

The process begins with the selection of an appropriate fluorescent probe. The choice of probe depends on various factors, including the target tissue, the depth of imaging, and the potential for background autofluorescence. NIR fluorophores are often preferred for whole-body imaging in mice due to their deeper tissue penetration and reduced background interference from tissue autofluorescence [86].

Once the probe is administered, the mouse is placed in an imaging chamber, and the excitation light is directed at the animal. The emitted fluorescence is filtered to remove the excitation light and captured by the imaging system. The resulting images can be analyzed to provide quantitative information about the distribution and intensity of the fluorescence signal, offering insights into the underlying biological processes.

Sensitivity and spatial resolution

Fluorescence imaging is renowned for its high sensitivity, capable of detecting signals from very low concentrations of fluorescent probes. This sensitivity is primarily attributed to the high quantum yield of fluorescent molecules and the efficiency of modern detection systems. Fluorescence imaging can achieve detection limits in the picomolar to nanomolar range, making it suitable for detecting low-abundance targets [87].

Spatial resolution in fluorescence imaging depends on several factors, including the wavelength of the emitted light, the optical properties of the tissues, and the imaging system's specifications. Typically, fluorescence imaging offers a spatial resolution of around 1–2 mm for whole-body imaging in mice. However, resolution decreases with increasing tissue depth due to scattering and absorption of light. Techniques such as fluorescence molecular tomography (FMT) can improve spatial resolution by reconstructing three-dimensional images from multiple two-dimensional projections [88, 89].

Comparison with bioluminescence imaging

Bioluminescence imaging (BLI) and fluorescence imaging are both valuable tools in preclinical research, each with its advantages and limitations. BLI relies on the emission of light produced by enzymatic reactions, typically involving luciferases and their substrates, whereas fluorescence imaging requires external excitation of fluorescent probes.

One of the main advantages of BLI is its low background signal, as there is no need for external light sources that can induce autofluorescence. This results in a high signal-to-noise ratio (SNR), making BLI highly sensitive, often detecting signals from single cells or small clusters of cells [90]. BLI also offers deeper tissue penetration compared to fluorescence imaging, as the emitted bioluminescent light can travel through tissues with less scattering and absorption.

However, fluorescence imaging has distinct advantages over BLI in certain applications. The primary advantage of fluorescence imaging is the availability of a wide range of fluorescent probes that can be tailored for specific applications, including probes that are activated by

specific biological events, such as enzyme activity or pH changes. This allows for the visualization of a broader array of molecular and cellular processes [91, 92].

Moreover, fluorescence imaging can be used to simultaneously monitor multiple targets using probes with different emission spectra, enabling multiplexed imaging. This capability is particularly useful in studies that require the concurrent tracking of several biological markers or processes [93].

Applications where fluorescence imaging is beneficial

Fluorescence imaging is superior to BLI in applications where the ability to use targeted probes or visualize multiple targets simultaneously is crucial. For instance, in cancer research, fluorescence imaging can be used to track the distribution of tumor-targeted probes, monitor drug delivery, and visualize tumor microenvironment interactions. Fluorescent probes can be conjugated to antibodies or ligands that specifically bind to tumor-associated antigens, providing high specificity and enabling the detection of small or early-stage tumors [94].

Another area where fluorescence imaging excels is in the study of gene expression and regulation. Fluorescent proteins, such as green fluorescent protein (GFP) and its variants, can be genetically encoded and expressed in specific tissues or under the control of particular promoters. This allows researchers to visualize and quantify gene expression in living animals over time, providing insights into developmental processes, disease progression, and responses to therapy [95].

Fluorescence imaging is also advantageous in applications requiring high temporal resolution. The rapid acquisition of fluorescence images enables the monitoring of dynamic processes, such as calcium signaling, neuronal activity, and blood flow, in real-time. This temporal resolution is critical in studies where understanding the kinetics of biological events is essential [96].

Applications where bioluminescence imaging is beneficial

Bioluminescence imaging is particularly well-suited for applications where high sensitivity and low background noise are paramount. For example, in infectious disease research, BLI can be used to monitor the spread of pathogens, such as bacteria and viruses, in living animals. The low background signal of BLI allows for the detection of small numbers of pathogens, facilitating the study of infection dynamics and the evaluation of antimicrobial therapies [49].

BLI is also advantageous in studies involving deep tissue imaging. The bioluminescent light produced by luciferase reactions can penetrate tissues more effectively than the externally excited fluorescence, making BLI suitable for imaging deep-seated tumors or monitoring metastatic spread in orthotopic cancer models. This deeper tissue penetration is crucial for accurately assessing disease burden and therapeutic efficacy in preclinical models [97].

Additionally, BLI is often preferred in longitudinal studies where repeated imaging of the same animal is required. The non-invasive nature and low phototoxicity of BLI make it ideal for monitoring disease progression and treatment responses over extended periods without adversely affecting the animal's health [98].

Interference with whole body fluorescence imaging

Despite the strong potential of fluorescence imaging, several factors can interfere with the accuracy and clarity of the imaging results, leading to potential misinterpretations and limitations in data reliability.

One of the primary sources of signal interference in fluorescence imaging is tissue autofluorescence. Biological tissues can emit their own fluorescent signals when excited by light, a phenomenon known as autofluorescence. This intrinsic emission originates from endogenous fluorophores, such as collagen, elastin, flavins, and porphyrins, present within the tissues. For example, collagen exhibits strong fluorescence when excited with ultraviolet or blue light, significantly contributing to background noise in imaging studies [99]. The overlap between the emission spectra of these endogenous fluorophores and the exogenous fluorescent probes can complicate the interpretation of the specific signals of interest. Strategies to mitigate this issue include the use of NIR fluorophores, which have longer excitation and emission wavelengths that are less prone to overlap with tissue autofluorescence.

Food intake is another factor that can significantly affect fluorescence imaging outcomes. Certain food components, particularly those containing fluorescent compounds like chlorophyll, can contribute to increased autofluorescence in the gastrointestinal tract. Ingestion of such compounds can lead to elevated background signals, especially in the abdominal region, complicating the interpretation of fluorescence data [25]. To address this issue, animals are often fasted prior to imaging to reduce the presence of fluorescent compounds in the digestive system, thereby minimizing background fluorescence.

Furthermore, the biodistribution and clearance of fluorescent probes can introduce variability in imaging results. The pharmacokinetics of the fluorescent agents, including their absorption, distribution, metabolism, and excretion, can affect the intensity and localization of the fluorescence signal. Factors such as the size, charge, and hydrophobicity of the fluorophore, as well as the animal's physiological state, can influence these processes. For instance, hydrophilic probes may be rapidly cleared from

the bloodstream, reducing their availability for imaging, while lipophilic probes might accumulate in fatty tissues, leading to non-specific background signals [100]. Tailoring the physicochemical properties of the probes and optimizing their administration routes can help mitigate these issues.

Additionally, optical properties of the tissues, such as scattering and absorption of light, can impact the quality of fluorescence imaging. Biological tissues can scatter and absorb the excitation and emission light, causing attenuation and distortion of the fluorescence signals. The extent of light scattering and absorption is wavelength-dependent, with shorter wavelengths being more affected. This effect is particularly pronounced in thicker tissues, where the penetration depth of light is limited, resulting in reduced signal intensity and resolution [86]. Utilizing advanced imaging techniques, such as multispectral imaging and fluorescence lifetime imaging, can help distinguish specific signals from background noise and improve signal-to-noise ratios.

Moreover, the presence of blood can also interfere with fluorescence imaging. Hemoglobin, a major component of blood, exhibits strong absorption in the visible and NIR regions, which can quench the fluorescence signal. This absorption can vary depending on the oxygenation state of the blood, introducing additional complexity in the interpretation of imaging data [101]. Techniques such as spectral unmixing and the use of fluorescence probes that emit at wavelengths less affected by hemoglobin absorption can help address this issue.

Another consideration is the impact of skin pigmentation. Melanin, the pigment responsible for skin color, has strong absorption properties, particularly in the UV and visible light regions. In animal models with darker skin, the presence of melanin can significantly reduce the intensity of the detected fluorescence signal [102]. Adjusting the imaging settings, such as increasing the excitation light intensity or extending the acquisition time, can help compensate for the reduced signal in pigmented animals. Apart from skin pigmentation, the presence of fur can have a major impact on fluorescence imaging. Here, black fur will reduce fluorescence intensity more than white fur, but a lot of the excitation or emission light will be scattered by the animal hair. To overcome this problem, many researchers tend to shave the animals at the site of interest. This in itself has to be done carefully however, as bare skin will have less scattering and hence higher signal than the rest of the body. In case background regions are selected, it is important that these are also derived from shaven areas. Furthermore, for comparative or longitudinal studies, it is important that the quality of hair removal is reproducible and consistent between animals and at all time points. For this, proper shaving coupled with the use of depilation cream is recommended. To get a uniform background and to ensure a proper detection of specific fluorescence signal, it is also recommended to shave bigger parts of the body (as only shaving the region of interest will make it seem that there is signal in that area if sensitivity is higher enough) or in case of stronger signal, not shave the mice at all.

Technical artifacts related to the imaging equipment can also contribute to signal interference. Instrumental noise, uneven illumination, and detector sensitivity variations can introduce artifacts that may be mistaken for genuine biological signals. Regular calibration of the imaging system, along with appropriate image processing techniques, can minimize these artifacts and enhance the accuracy of the fluorescence measurements [103].

Multiplexing capabilities of fluorescence imaging

Multiplexing in whole body fluorescence imaging represents a significant advancement in preclinical research, enabling the simultaneous detection and quantification of multiple biological processes within a single living organism. This technique leverages the use of various fluorescent reporter proteins with distinct excitation and emission spectra, allowing researchers to monitor several molecular targets concurrently. Among the broad range of fluorescent reporter proteins utilized, near-infrared fluorescent proteins (NIRFPs) like iRFP713, iRFP720, and others are particularly advantageous due to their deep tissue penetration and minimal background interference from tissue autofluorescence. For instance, using different NIRFPs to label distinct cell populations allows for real-time observation of cell–cell interactions, tumor microenvironment changes, and immune responses, in the same animals, drastically reducing the numbers of animals needed while providing crucial information on cancer biology [104].

The use of NIRFPs such as iRFP713 and iRFP720 offers distinct benefits for multiplexed imaging. These proteins have excitation and emission maxima in the near-infrared range (iRFP713: 690/713 nm, iRFP720: 702/720 nm), which minimizes the overlap with the autofluorescence of biological tissues that predominantly emit in the visible range. This spectral separation enhances signal-to-noise ratios and improves the clarity of imaging results. Additionally, NIRFPs exhibit better tissue penetration compared to visible spectrum fluorophores, allowing for the visualization of deeper structures within the body (Fig. 5) [105].

Despite these advantages, multiplexing in fluorescence imaging presents several challenges. One of the key difficulties is ensuring that the different fluorescent signals can be perfectly separated and independently quantified.

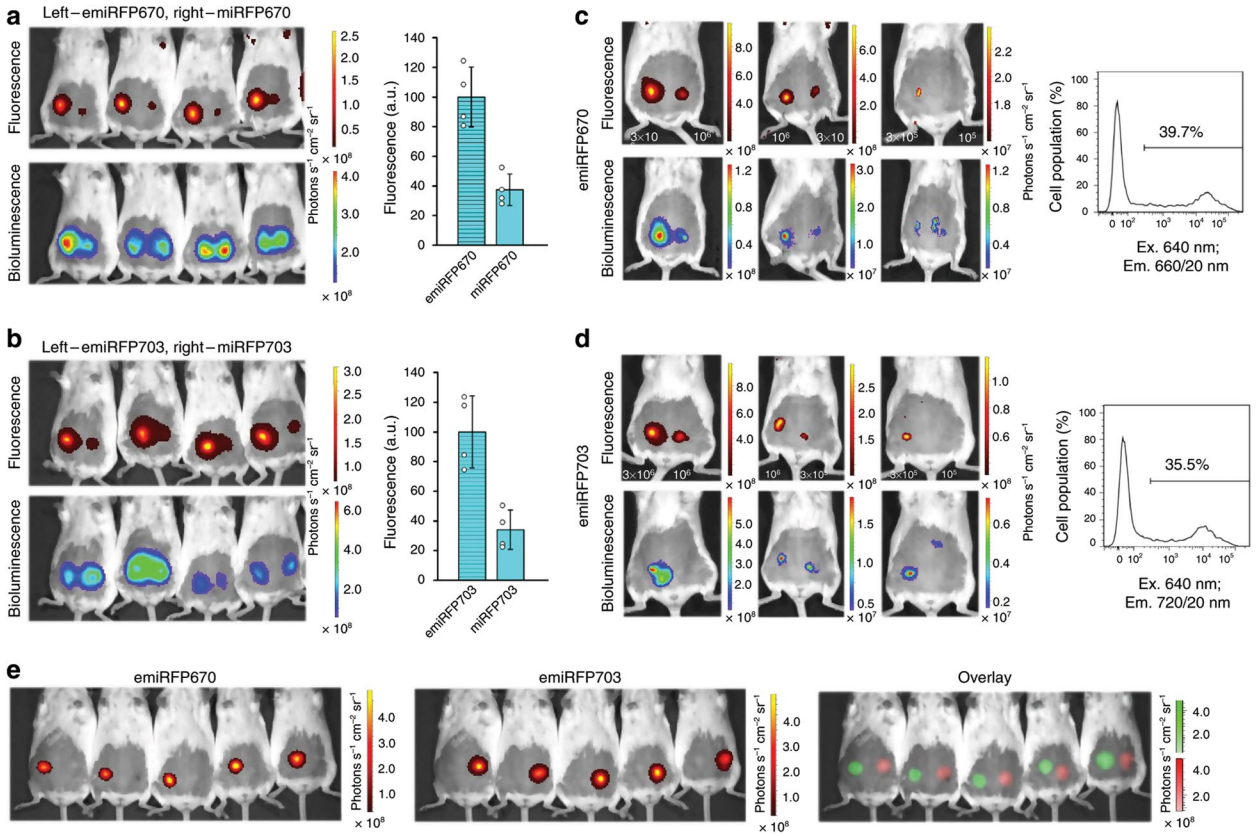

Fig. 5 **a**, **b** Comparison of emiRFP670 with parental miRFP670 (**a**) and emiRFP703 with parental miRFP703 (**b**). Fluorescence (top row) and bioluminescence (bottom row) images of living mice injected with 3×10^6 COS-1 cells expressing emiRFP670 or emiRFP703 (left) and miRFP670 or miRFP703 (right). Cells were co-transfected with Rluc8. The filter sets were 640/20 nm excitation and 680/30 nm emission (**a**) or 675/20 nm excitation and 720/30 nm emission (**b**). Bar plots at the right show quantified mean fluorescence intensities (normalized to bioluminescence) that correspond to **a** and **b**. Error bars, double s.e.m. ($n = 3$ experiments). **c**, **d** Minimal number of detectable fluorescent cells. Fluorescence (top row) and bioluminescence (bottom row) images of living mice injected with various quantities of COS-1 cells expressing emiRFP670 (**c**) or emiRFP703 (**d**) and co-transfected with Rluc8. The filter sets were 640/20 nm excitation and 680/30 nm emission (**c**) or 675/20 nm excitation and 720/30 nm emission (**d**). Transfection efficiency of injected COS-1 cells obtained by FACS analysis shown on the right of each panel. **e** Two-color imaging of emiRFP670 and emiRFP703. Fluorescence images of living mice injected with 3×10^6 COS-1 cells expressing emiRFP670 (left row) and emiRFP703 (middle row) and its unmixed overlay (bottom row) are shown. Images were acquired in 19 spectral channels using IVIS Spectrum instrument and spectrally unmixed. Copyright ref [106], Nature Publishing Group 2020.

This requires careful selection of fluorophores with minimal spectral overlap and the use of advanced imaging techniques to distinguish between the signals. Spectral unmixing algorithms are often employed to deconvolute the overlapping emission spectra, allowing for accurate quantification of each fluorescent signal. However, these algorithms must be precisely calibrated and validated to avoid cross-talk and signal bleed-through, which can lead to erroneous interpretations.

Another challenge is the differential expression and stability of fluorescent reporter proteins. The efficiency of fluorophore expression can vary depending on the genetic construct, promoter strength, and the cellular environment, leading to variability in signal intensity. Additionally, some fluorescent proteins may be prone to photobleaching or quenching, affecting their long-term stability and the accuracy of longitudinal studies. Strategies to mitigate these issues include the use of robust genetic constructs, optimizing expression systems, and employing photostable fluorophores [106].

Moreover, the pharmacokinetics of the fluorescent probes, including their biodistribution and clearance rates, can affect the imaging results. Different fluorophores may exhibit varying rates of uptake, distribution, and elimination in the body, leading to temporal and spatial variations in signal intensity. Understanding the pharmacokinetic profiles of the probes and optimizing their administration protocols are crucial for accurate multiplexed imaging. This might involve selecting fluorophores with similar pharmacokinetic

behaviors or timing the imaging sessions to capture the peak signals of each probe [100].

Instrumental factors, such as the spectral characteristics of the excitation light sources and the sensitivity of the detectors, also influence the success of multiplexed imaging. The imaging system must be capable of providing adequate excitation for all the fluorophores used while efficiently capturing their emission signals. This often requires multi-spectral imaging systems equipped with tunable light sources and advanced detectors capable of distinguishing between closely spaced emission wavelengths.

To further enhance the capabilities of multiplexed fluorescence imaging, researchers are exploring the development of novel fluorophores and imaging techniques. For instance, the creation of new NIRFPs with distinct spectral properties and improved photostability can expand the palette of fluorophores available for multiplexing. Additionally, advancements in computational imaging and machine learning algorithms hold promise for more accurate spectral unmixing and signal quantification, reducing the risk of cross-talk and improving the reliability of multiplexed data [107].

The combination of BLI and FLI

The combination of BLI and FI has the potential to enable researchers to monitor both gene expression and protein localization within the same animal, providing a more holistic understanding of cellular and molecular dynamics [4].

Several studies have demonstrated the utility of combining BLI and FI in mice. For instance, a study by Laxman et al. [108] utilized a dual-reporter system to monitor tumor growth and metastasis in vivo. They engineered tumor cells to express both firefly luciferase (for BLI) and green fluorescent protein (GFP, for FI). This dual-reporter approach allowed the researchers to use BLI for the sensitive detection of primary tumor growth and metastasis, while FI provided spatial information about the localization of tumor cells within tissues. The study revealed that BLI could detect metastatic lesions earlier than FI, highlighting the complementary nature of these imaging modalities [108].

Another example is the work by Ventura et al. [109], who developed a bioluminescent and fluorescent dual-imaging system to study the dynamics of viral infection and immune response in mice. They used a recombinant virus expressing both Nanoluc and green fluorescent protein (RFP). BLI was used to track the spread and replication of the virus over time, while FI enabled the visualization of viral localization in tissues. This dual-imaging approach provided valuable insights into the temporal and spatial dynamics of viral infection and the subsequent immune response, facilitating the evaluation of antiviral therapies and vaccine efficacy [109].

A recent study by Naatz et al. [110] reported on the use of luciferase-expressing tumor cells that were used to monitor tumor growth, while fluorescent probes were used to monitor neutrophil influx and caspase activity in the tumor region upon treatment with Fe-doped CuO nanoparticles.

While the combination of BLI and FI offers significant advantages, several potential limitations and challenges must be addressed to ensure accurate and reliable imaging results. One primary concern is signal interference between the two modalities. The use of external light sources for FI can potentially excite endogenous fluorophores or other light-emitting compounds within the animal, leading to background noise that may interfere with the bioluminescent signal. To mitigate this issue, researchers should carefully select fluorophores with minimal overlap in their excitation and emission spectra with the bioluminescent signal. For example, using near-infrared fluorescent proteins such as iRFP713 and iRFP720, which have excitation and emission spectra distinct from those of luciferase, can reduce spectral overlap and improve signal separation [104]. Interference can for sure also work the other way. Once the luciferase substrate is provided and luminescent signal is generated, great care must be taken that it does not interfere with the fluorescence imaging itself. As most bioluminescence probes give broad emission peaks that will continually generate light over a long period of time, even in the absence of excitation light, this may interfere with the emission light collected during fluorescence imaging. From a practical perspective, this is most easily dealt with by performing the fluorescence imaging prior to any BLI. In case repeated measurements are required, great care should be taken to investigate whether the BLI signal has sufficiently been reduced to not interfere with the fluorescence readout. This can be done by performing a new bioluminescence scan, where the imaging data will be collected at the wavelength where the fluorescent probes will typically emit. While the substrate is typically cleared quite quickly, depending on the substrate, remnant signal can still be observed sometimes hours after substrate administration.

Another potential limitation is the difference in the pharmacokinetics of the imaging agents. Bioluminescence imaging relies on the administration of luciferin, which must reach the luciferase-expressing cells to produce a signal. The distribution and clearance of luciferin can vary, potentially leading to temporal variations in the bioluminescent signal. Similarly, the pharmacokinetics of fluorescent probes, including their uptake, distribution, and clearance, can affect the fluorescence signal. These

differences must be carefully considered when designing experiments and interpreting results, as temporal discrepancies between the bioluminescent and fluorescent signals can complicate data analysis [111].

Moreover, the choice of luciferase and fluorescent proteins can influence the effectiveness of dual imaging. Different luciferases, such as firefly luciferase and Renilla luciferase, have distinct spectral properties and substrate requirements, which can be exploited to minimize spectral overlap with fluorescent proteins. For example, Renilla luciferase emits blue light, which can be used in conjunction with red or near-infrared fluorescent proteins to reduce spectral interference. Similarly, the use of different fluorescent proteins with non-overlapping spectra allows for multiplexing and the study of multiple biological processes simultaneously [112]. The choice of luminescent and fluorescent proteins are also important in order to rule out possible energy transfer between both systems. The emission light of luminescence can in itself lead to excitation of the fluorescent probes, in particular when these are in close proximity, through bioluminescence resonance energy transfer (BRET), which may affect the bioluminescence readout, as part of the energy can be absorbed by the fluorescent reporters.

Advances in fluorescence imaging hardware

The first systems that enabled fluorescence whole body imaging provided standard 2D images which suffered from drawbacks such as the depth of the source signal which impedes straightforward analysis, results in higher variability and makes absolute quantification impossible. Several systems have tried to overcome these problems upon using fluorescence tomography, which provides a 3D scan and can therefore more accurately determine the source of the signal. The efficacy of this 3D imaging however depends strongly on the system used. To date, many optical imaging systems employ so-called pseudo-3D, where images are acquired at different excitation spots to gain a better 3D understanding, but overall, all signals are collected by a single detector without any detailed knowledge of the source animal anatomy. Recently, newer technologies have been put on the market which enable more accurate 3D fluorescence imaging. The LagoX platform (Spectral Instruments Imaging; Bruker) uses a body-conforming mold that allows to generate an organ probability map. Using a mirror gantry to allow a 360° view of the images the organ probability map with the body conforming mold takes into account the tissue-specific optical properties and partial blood volume to allow for more accurate 3D localization and quantification. The optical imaging—μCT combination from MILabs, B.V. performs sequential scanning, where a μCT image is first acquired and analyzed on the spot prior to acquiring the optical images. This CT priors approach enhances the precise 3D localization of the signal and more robust quantification of signal [113].

Fluorescence Emission Computed Tomography (FLECT) combined with μCT is an advanced imaging modality that offers significant potential in preclinical research. This hybrid imaging technique integrates the high sensitivity of fluorescence imaging with the anatomical detail provided by CT, enabling comprehensive in vivo studies in small animal models. FLECT provides three-dimensional (3D) localization of fluorescent signals within the body, while CT offers precise anatomical context, facilitating the detailed mapping of molecular and cellular processes in relation to the structural features of the organism.

FLECT/CT imaging is particularly advantageous for studying the biodistribution and pharmacokinetics of fluorescently labeled compounds, as well as for tracking cellular and molecular events in disease models. The fluorescence component of FLECT relies on the detection of emitted light from fluorescent probes that are excited by an external light source. Unlike traditional planar fluorescence imaging, which provides only surface or superficial views, FLECT reconstructs 3D images by capturing multiple projection images from different angles around the subject. This capability allows for the precise localization of fluorescent signals deep within the tissues, overcoming the limitations of light scattering and absorption inherent in planar imaging techniques [114, 115].

CT imaging complements FLECT by providing high-resolution anatomical details. Using X-rays, CT generates cross-sectional images of the body, which can be reconstructed into 3D volumetric representations. These images reveal the structural context, including bone architecture, organ boundaries, and other tissue interfaces, essential for accurately correlating fluorescent signals with anatomical landmarks. The combination of FLECT and CT thus enables researchers to co-register functional and anatomical data, allowing for more accurate interpretation of biological processes and disease mechanisms (Fig. 6) [116, 117].

A major benefit of FLECT/CT is the ability to provide accurate quantification of fluorescent signal at the source location, and thus at the level of the cells or probes within the animal [115]. Classical planar fluorescence imaging detects light that is emitted at the surface of the animal and does not take into account the loss of signal due to tissue depth. So-called pseudo-3D imaging tries to gain more information on the depth of the signal source by looking at different wavelengths and use advanced computational processing to look at the loss of signal over different wavelengths to determine the 3D localization more correctly. However, this is still based on many assumptions, and for example, does not take into account

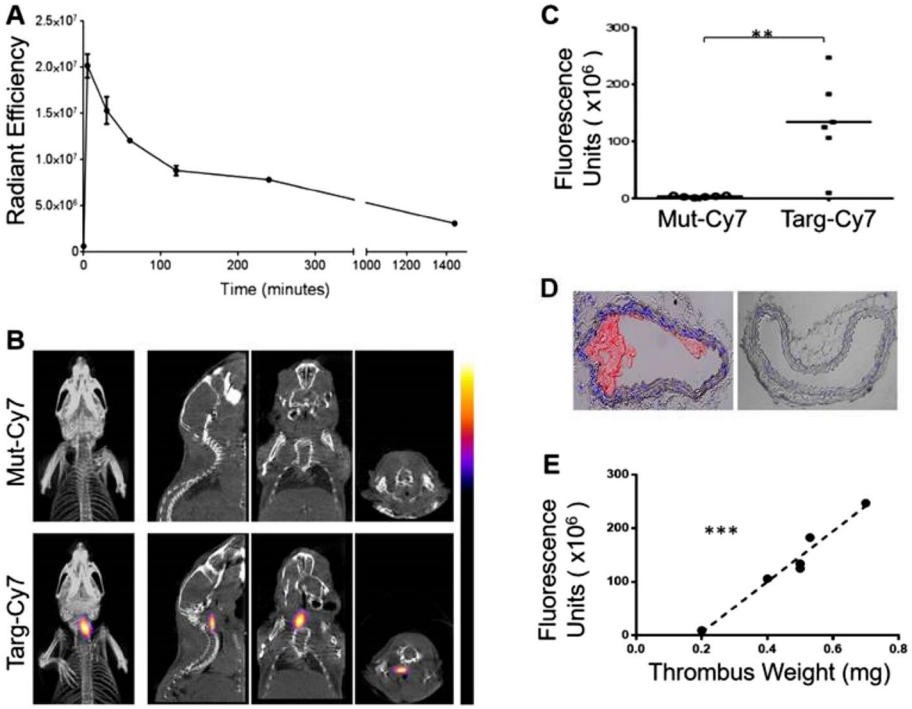

Fig. 6 Circulatory in vivo half-life of the FLECT fluoroprobe and its use in FLECT/CT imaging of mice with left carotid ferric chloride induced thrombus. **A** Mice (*n*=5) were i.v. injected with 1 µg/g of Targ-Cy7 and blood was collected at different time-points (0, 5, 30, 60 120, 240 and 1440 minutes). The NIR fluorescence signal in the collected samples was determined by the IVIS® Lumina imager and quantified as shown. **B** Upon arterial thrombus formation using the ferric chloride model, mice were i.v. injected with either mutated (Mut-Cy7; top panel) or targeting-fluoroprobe (Targ-Cy7; bottom panel) and allowed to circulate before they were scanned on the FLECT/CT imager. Following data reconstruction, coregistration and analysis, a representative comparison of maximum-intensity projection of FLECT/CT images of Mut-Cy7 (*n*=6) and Targ-Cy7 (*n*=6) mice is shown. The colour scale for each FLECT/CT image shows levels of detected NIR fluorescence with white corresponding to the highest intensity and blue the lowest. **C** Using Invivoscope software, the region of interest around the left carotid artery was determined, and detected fluorescence intensity was quantified between groups of mice (**: $p \leq 0.01$; Mann-Whitney nonparametric test, $p = 0.0022$). **D** A representative micrograph of the ferric chloride-injured carotid artery (top) and the contralateral uninjured carotid artery (bottom), where nuclear stain (DAPI) is blue, and platelet-specific (CD41- Allophycocyanin) is red. **E** Further analysis of the detected FLECT-signal in each mouse shows a strongly significant correlation to the weight of its *ex-vivo* thrombus (using Pearson's correlation analysis: r = 0.9807 and $p = 0.0006$, ***). Copyright ref [117], Ivy Spring 2017

the different tissue types and their effect on signal attenuation. FLECT/CT has a ring of 48 detectors and therefore acquires the signal at all these different angles to get a more precise location. FLECT/CT is also based on the initial CT scan, where the anatomical information is gathered, after which this is correlated with a surface scan to gain information on the specific tissue absorption at different locations. This information can then be used to make accurate measurements of the number of photons at the region of interest, irrespective of the depth of the signal. Due to this, standard concentration series can be generated and measured which allows the researcher to calculate the number of fluorescently labeled probes or cells based on fluorescence measurements. This is ideally suited to perform biodistribution studies and calculate the targeting efficacy of labeled compounds [115].

Despite its advantages, FLECT/CT imaging also faces several challenges. One primary concern is the use of photodiodes rather than CCD cameras which are less sensitive and thus require a strong signal.

Fluorescence imaging in the NIR-II region

Whole body fluorescence preclinical imaging in the near-infrared II (NIR-II) region, typically spanning 1000–1700 nm, represents a significant advancement in the field of biomedical imaging. This technique offers enhanced tissue penetration and reduced autofluorescence compared to traditional visible and NIR-I (650–900 nm) fluorescence imaging, providing more precise and deeper visualization of biological tissues. The transition to NIR-II wavelengths minimizes the scattering and absorption by biological molecules and water, thereby enabling higher resolution and contrast imaging deep within tissues [118].

Advantages of NIR-II Imaging

1. Enhanced Penetration Depth: One of the primary benefits of NIR-II fluorescence imaging is its ability to penetrate deeper into biological tissues. This is due to the lower scattering and absorption coefficients of tissues at these wavelengths. Studies have shown that NIR-II imaging can achieve tissue penetration depths of up to several centimeters, compared to millimeter-scale depths typical of visible and NIR-I imaging. This capability is crucial for visualizing deep-seated tumors and monitoring organ functions in small animal models [119].
2. Improved Signal-to-Noise Ratio (SNR): The reduction in autofluorescence in the NIR-II region significantly enhances the SNR, allowing for clearer and more accurate imaging. Autofluorescence from endogenous biomolecules such as collagen, elastin, and flavins is substantially lower in the NIR-II region, leading to a cleaner background and enabling the detection of weak fluorescent signals from the target molecules [120].
3. High Spatial Resolution: Due to reduced scattering, NIR-II imaging can achieve high spatial resolution even at greater depths. This is particularly beneficial for detailed anatomical studies and tracking the distribution and dynamics of fluorescent probes in vivo. For instance, high-resolution imaging of blood vessels, lymphatic systems, and tumors has been demonstrated using NIR-II fluorophores [121].
4. Minimal Photodamage: NIR-II imaging uses lower energy photons compared to visible light, resulting in reduced phototoxicity and photobleaching. This aspect is particularly important for longitudinal studies where repeated imaging sessions are necessary. The lower photodamage facilitates extended observation periods without adversely affecting the biological tissues under study [122].

Advances in NIR-II Fluorophores

The development of novel NIR-II fluorophores has been pivotal in advancing this imaging modality. Organic dyes, quantum dots, single-walled carbon nanotubes, and rare-earth doped nanoparticles are some of the fluorophores that have been optimized for NIR-II imaging. These fluorophores are designed to exhibit high brightness, stability, and biocompatibility. Recent innovations include the synthesis of water-soluble and biologically inert NIR-II probes that can be conjugated with targeting ligands for specific molecular imaging [123].

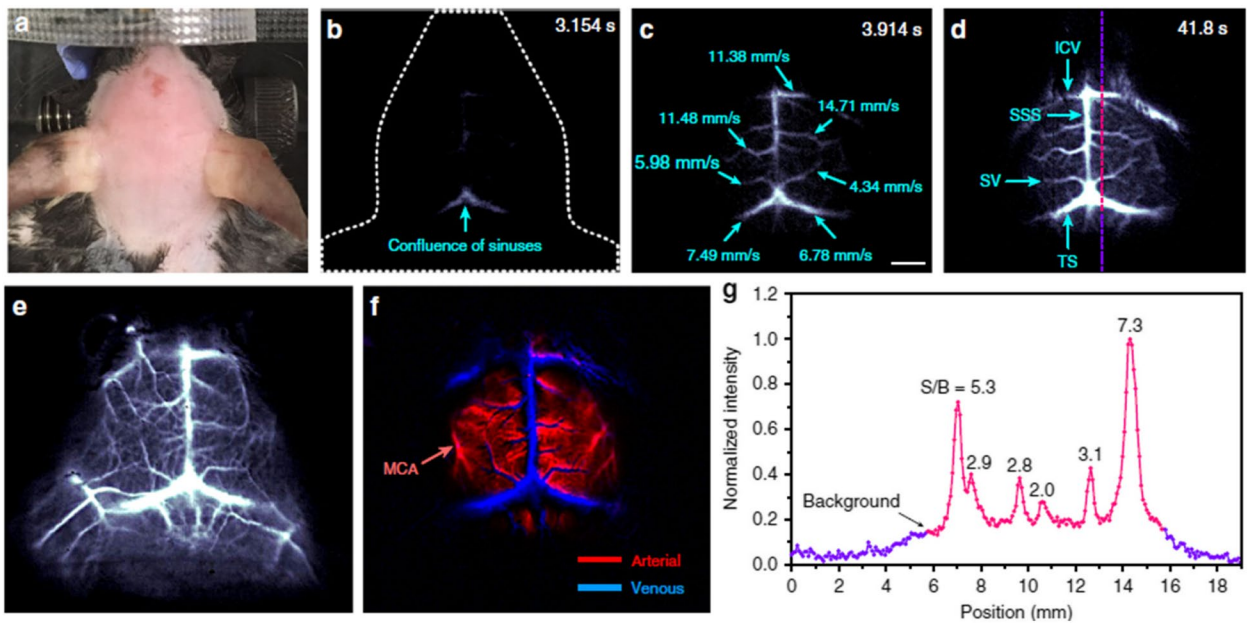

Fig. 7 Fast in vivo brain imaging with Er-RENPs@PMH-PEG in the NIR-IIb region. **a** Color photograph of a C57Bl/6 mouse (with hair shaved off) preceding NIR-IIb fluorescence imaging. **b**–**d** Time-course NIR-IIb brain fluorescence images (exposure time: 20 ms) showing the perfusion of RENPs into various cerebral vessels. The blood-flow velocities of cerebral vessels are given in **c** (scale bar corresponds to **b**–**d**: 2 mm). **e**, **f** Cerebral vascular image (exposure time: 20 ms) in NIR-IIb region with corresponding PCA overlaid image **f** showing arterial (red) and venous (blue) vessels. **g** SBR analysis of NIR-IIb cerebrovascular image **d** by plotting the cross-sectional intensity profiles. Copyright: ref [124], Nature Publishing Group 2017

Applications in Preclinical Research

NIR-II imaging has broad applications in preclinical research. It is particularly valuable in oncology for tumor detection, tracking metastasis, and monitoring treatment responses. In cardiovascular research, NIR-II imaging enables the visualization of vascular structures and blood flow dynamics in real-time, providing insights into cardiovascular diseases. Additionally, it has been used in neuroscience for imaging brain vasculature and neuronal activities in animal models (Fig. 7) [124].

Challenges and disadvantages

Despite its advantages, NIR-II imaging faces several challenges and limitations:

1. Complexity and Cost: The equipment required for NIR-II imaging, including specialized lasers, detectors, and optical filters, is more complex and expensive compared to conventional fluorescence imaging systems. This high cost can be a barrier to widespread adoption in research laboratories [125].
2. Fluorophore Development and Safety: The synthesis of NIR-II fluorophores that are both highly efficient and biocompatible remains challenging. Ensuring the long-term safety and minimizing potential toxicity of these fluorophores are critical, particularly for clinical translation. Comprehensive toxicity studies and regulatory approvals are required before NIR-II fluorophores can be widely used in clinical settings [126]. Some of the dyes, more classically NIR I fluorophores have been found to have excellent SNR in NIR II settings, which is allowing the exploration of clinical studies using such dyes. Specifically, NIR II imaging has been successfully used to aid imaging-guided surgery in different pathologies, including liver carcinoma surgery [127].
3. Limited Availability of Commercial Probes: Currently, there is a limited selection of commercially available NIR-II probes. Most NIR-II fluorophores are still in the experimental stage, with ongoing research focused on improving their properties. This limited availability restricts the immediate application of NIR-II imaging in various research domains [128].
4. Quantitative Imaging and Standardization: Achieving quantitative imaging with NIR-II fluorophores is challenging due to variations in probe brightness, photostability, and interactions with biological environments. Standardization of imaging protocols and calibration methods is necessary to ensure reproducible and reliable results across different studies and research groups [122].

Future perspectives

The future of NIR-II fluorescence imaging holds promising prospects for both preclinical and clinical applications. Ongoing research is focused on developing more advanced NIR-II fluorophores with enhanced brightness, targeting specificity, and biocompatibility. The integration of NIR-II imaging with other imaging modalities, such as MRI, CT or PET, could provide comprehensive and multimodal imaging solutions for complex biological systems.

Additionally, advancements in image processing algorithms and machine learning could further enhance the interpretation and analysis of NIR-II imaging data. These technological improvements will facilitate more accurate disease diagnosis, monitoring, and personalized treatment planning.

General points of concern for fluorescence imaging

Fluorescence imaging relies on the use of fluorophores to label specific biomolecules or structures, allowing researchers to track and quantify dynamic biological events. However, several intrinsic challenges can affect the accuracy and reliability of fluorescence imaging, including phototoxicity, photobleaching, loss of the fluorophore, limited dynamic range, and others. Addressing these issues is crucial for optimizing imaging protocols and improving data interpretation.

Phototoxicity

Phototoxicity is a significant concern in fluorescence imaging, particularly in live animal studies. It arises when the excitation light required to induce fluorescence generates reactive oxygen species (ROS) within tissues, leading to cellular damage or death. This is especially problematic for prolonged or repeated imaging sessions. In preclinical studies, phototoxicity can distort physiological processes, leading to artifacts that affect the interpretation of results. To mitigate phototoxicity, researchers often use lower intensity light sources, minimize exposure times, and select fluorophores that require less energetic light for excitation (longer wavelengths) [129].

Photobleaching

Photobleaching refers to the irreversible loss of fluorescence from a fluorophore due to prolonged exposure to excitation light. This phenomenon results in a gradual decrease in signal intensity over time, which can compromise the accuracy of longitudinal studies and real-time imaging. Photobleaching is particularly problematic in preclinical imaging where prolonged observation is often

required. Strategies to reduce photobleaching include using more photostable fluorophores, such as quantum dots or certain organic dyes, and optimizing imaging parameters to balance signal strength and fluorophore longevity [130]. However, these approaches may not completely eliminate photobleaching, necessitating the use of computational techniques to correct for signal loss during data analysis.

Loss of the fluorophore

The stability of fluorophores in biological environments is critical for accurate imaging. Fluorophore loss can occur through various mechanisms, including photobleaching, chemical degradation, or biological processes such as cellular uptake and excretion. In preclinical studies, this can lead to erroneous conclusions about the distribution and dynamics of labeled molecules. To address this issue, researchers have developed more stable fluorophores and conjugation strategies that enhance the retention of the label. For example, covalent attachment of fluorophores to biomolecules can prevent dissociation, while encapsulation within nanoparticles can protect the fluorophore from the biological environment. Additionally, using genetically encoded fluorescent proteins, which are continuously produced by the cells, can provide a more consistent signal over time.

Limited dynamic range

The dynamic range of a fluorescence imaging system is its ability to accurately detect and quantify both low and high levels of fluorescence within the same sample. A limited dynamic range can result in the under-detection of low-intensity signals and saturation of high-intensity signals, thereby reducing the accuracy of quantitative analyses. This limitation is particularly relevant in preclinical imaging, where detecting subtle changes in fluorescence intensity can be crucial for understanding biological processes. Enhancing the dynamic range can be achieved by optimizing detector sensitivity, using fluorophores with different brightness levels, and employing advanced imaging techniques such as high dynamic range (HDR) imaging. Additionally, ratiometric imaging, which involves using two or more fluorophores that emit at different wavelengths, can help normalize the signal and improve quantification across a broader intensity spectrum [131].

Signal overlap and crosstalk

In multi-color fluorescence imaging, the overlap of emission spectra from different fluorophores can lead to signal crosstalk, where signals from one channel bleed into another. This spectral overlap complicates the interpretation of results and can lead to inaccurate conclusions about the co-localization and interaction of different biomolecules. In preclinical studies, where the accurate differentiation of multiple targets is often necessary, this can be a significant issue. To address signal overlap and crosstalk, researchers must carefully select fluorophores with minimal spectral overlap and employ advanced optical filters. Spectral unmixing algorithms and the development of fluorescent probes with narrow emission spectra also play crucial roles in minimizing crosstalk and enhancing the accuracy of multi-color imaging [124].

Autofluorescence

Autofluorescence is the natural emission of light by biological structures when excited by light of certain wavelengths. This background fluorescence can interfere with the detection of specific fluorescent signals, reducing the signal-to-noise ratio (SNR) and complicating data analysis. Autofluorescence is particularly problematic in preclinical imaging of tissues and organs, which inherently contain autofluorescent compounds such as flavins, NADH, and collagen. To minimize autofluorescence, imaging can be performed in spectral regions where background fluorescence is minimal, such as the near-infrared (NIR) region. Additionally, techniques like autofluorescence correction and fluorescence lifetime imaging microscopy (FLIM), which distinguishes between signals based on their fluorescence lifetimes, can improve the accuracy of fluorescence measurements in autofluorescent samples [93].

Photostability and brightness of fluorophores

The photostability and brightness of fluorophores are critical factors in preclinical fluorescence imaging. Highly photostable and bright fluorophores are essential for capturing high-resolution images and conducting long-term studies. However, many commonly used fluorophores exhibit limited photostability and brightness. Advances in fluorophore chemistry have led to the development of novel dyes and fluorescent proteins with enhanced properties. For example, near-infrared fluorophores and quantum dots offer superior photostability and brightness compared to traditional dyes. Encapsulation techniques, such as embedding fluorophores in protective matrices, can further improve their stability and performance, making them more suitable for demanding preclinical applications [132].

Environmental sensitivity of fluorophores

The fluorescence properties of many fluorophores are sensitive to environmental factors such as pH, ion concentration, and temperature. These sensitivities can lead

to variations in fluorescence intensity and complicate the interpretation of imaging data. In preclinical studies, this environmental sensitivity can affect the accuracy of measurements, particularly in heterogeneous biological environments. For example, pH-sensitive dyes can provide inaccurate readings in tissues with variable pH levels. To address this, researchers often use ratiometric fluorophores, which provide a built-in correction for environmental changes by emitting at two different wavelengths. This approach allows for more accurate and reliable measurements, even in fluctuating environmental conditions [132].

Future perspectives

Addressing optical imaging challenges

The field of preclinical optical imaging, particularly bioluminescence and fluorescence imaging, has become an indispensable tool in biomedical research due to its ability to non-invasively visualize and track biological processes in living organisms. As this field continues to evolve, several key trends and technological advancements are poised to address current challenges and expand its capabilities. The present review has mainly been addressing whole body fluorescence and bioluminescence imaging, but other optical imaging modalities are also rapidly gaining interest. Among those techniques is intravital imaging, where high resolution imaging can be performed of a small preselected region of the subject [133]. Another example includes photoacoustic imaging, where ultrasonic waves are generated by irradiating contrast agents with pulsed lasers and reconstructing the light energy absorption distribution throughout the tissue [134]. The ultrasound-mediated imaging enhances both the penetration depth as well as the spatial resolution, enabling efficient structural and molecular imaging in one system. Compared to more classical imaging methods like MRI, CT or PET/SPECT imaging and other optical imaging methods (BLI or optical tomography), the following comparison can be made in view of the sensitivity, spatial resolution and imaging depth of the different techniques (Fig. 8).

Figure 8 reveals the high spatial resolution of CT and MRI, but also their low sensitivity, making them particularly suitable for anatomical imaging, but les for molecular studies. PET and SPECT however suffer from poor spatial resolution, for high sensitivity and near unlimited imaging depth. Whole body optical imaging reaches similar sensitivities and spatial resolution, whereas photoacoustic imaging can reach higher resolution. All optical imaging techniques however suffer from limited penetration, making them highly suitable for small animal preclinical imaging, but less suited for clinical applications.

Genetic engineering approaches, such as the use of CRISPR/Cas9, could enable precise insertion of luciferase genes into specific genomic loci, ensuring consistent expression and minimizing variability between experiments. Furthermore, advancements in multiplexing capabilities, where multiple luciferase-substrate pairs with distinct emission spectra are used simultaneously, will allow for the monitoring of multiple biological processes

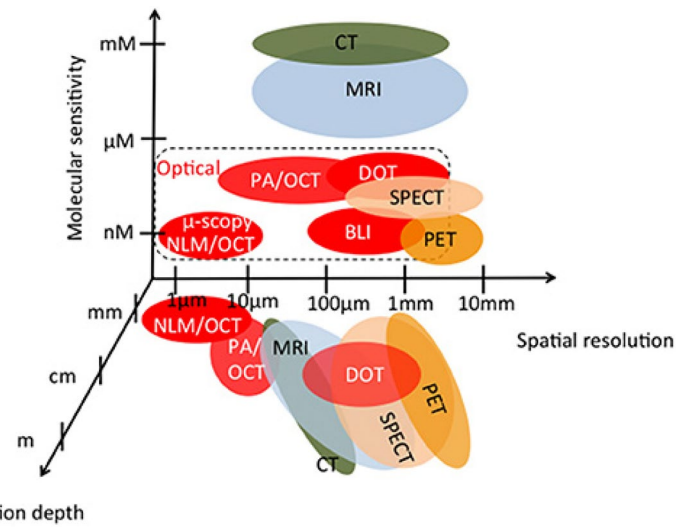

Fig. 8 Medical imaging technologies as function of penetration depth, molecular sensitivity, and spatial resolution; MRI, magnetic resonance imaging; CT, computed tomography: SPECT, spectroscopic CT; PET, positron emission tomography; PA/OCT, multimodal photoacoustics -OCT; NLM/OCT, multimodal non-linear microscopy-OCT; BLI, bioluminescence imaging; DOT, diffuse optical tomography. This Figure is reproduced with permission from Leitgeb and Baumann, © 2018, Frontiers [135].

in a single organism, providing a more comprehensive understanding of complex biological systems.

To overcome the challenges of FLI, related to autofluorescence, or photobleaching, the development of brighter and more photostable fluorophores is essential. The introduction of new classes of fluorescent proteins and synthetic dyes with enhanced quantum yields and resistance to photobleaching will prolong imaging sessions and improve signal-to-noise ratios. NIR fluorescent probes, similar to those being developed for BLI, will also play a critical role in improving tissue penetration and reducing background autofluorescence.

Another promising avenue is the integration of advanced imaging techniques such as fluorescence lifetime imaging microscopy (FLIM) and Förster resonance energy transfer (FRET). FLIM provides additional contrast by measuring the decay time of the fluorescence signal, which can distinguish between different molecular environments and interactions. FRET, on the other hand, allows for the detection of molecular interactions at nanometer scales by measuring energy transfer between closely situated fluorophores. Combining these techniques with conventional FLI can provide more detailed information about cellular and molecular dynamics.

The quantitative accuracy of optical imaging still remains a challenge. Variability in probe distribution, differences in tissue absorption and scattering, and photobleaching can all affect the accuracy of quantitative measurements. The development of robust computational models and algorithms for image reconstruction and analysis will be crucial in mitigating these effects. Machine learning and artificial intelligence (AI) approaches are particularly promising in this regard, as they can be trained to recognize and correct for these variabilities, providing more reliable quantitative data. With the increasing complexity and volume of imaging data, advancements in image processing and data analysis are imperative. AI and machine learning algorithms are expected to play a pivotal role in this domain. These technologies can automate the analysis of large datasets, identify patterns and correlations that may be missed by human observers, and even predict biological outcomes based on imaging data. AI-driven image enhancement techniques can also improve the quality of images obtained from deeper tissues, where signal-to-noise ratios are typically lower.

Moreover, the development of real-time imaging and analysis capabilities will be transformative for preclinical research. Real-time imaging can provide immediate feedback on biological processes, enabling dynamic studies of phenomena such as drug delivery, immune responses, and tumor progression. This will require the integration of fast, high-resolution imaging systems with powerful computational resources capable of processing and analyzing data on-the-fly.

Future directions

Extracellular vesicles are membrane-bound nanoparticles released by cells, containing various bioactive molecules. Stem cell-derived vesicles show immunomodulatory and trophic properties, positioning them as therapeutic candidates. For instance, vesicles from human liver stem cells enhance liver recovery after injury in rats [136]. To assess their anticancer effects, vesicles from luciferase-expressing mesenchymal stem cells were injected into lung tumors in mice. BLI confirmed their delivery to tumors and a subsequent reduction in tumor size. These findings highlight BLI's potential in evaluating the therapeutic effects of extracellular vesicles in vivo.

MicroRNAs (miRNAs) are short, non-coding RNAs that bind to messenger RNAs, affecting their translation. They play crucial roles in stem cell self-renewal and differentiation, exemplified by miRNA-145's influence on neural stem cells via the SOX2 transcription factor. Bioluminescence imaging (BLI) has been used to investigate miRNA functions in cancer, allowing real-time monitoring of miRNA biogenesis and regulation in P19 mouse embryonic carcinoma cells with luciferase reporter constructs [137]. This technique reveals the roles of primary and mature miRNAs in translation repression and extends to studying differentiation and regeneration in stem cells.

Nanotechnology also holds significant promise for the evolution of optical imaging. The development of nanoparticle-based probes that combine multiple imaging modalities (*e.g.*, optical and magnetic properties) can enable simultaneous tracking of biological processes with high sensitivity and resolution. Moreover, nanoparticles can be engineered to target specific cell types or molecular markers, enhancing the specificity of imaging.

Authors' contributions

All authors researched, wrote and amended the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests
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