https://doi.org/10.1038/s44303-025-00125-8

Reflect: reporting guidelines for preclinical, translational and clinical fluorescence molecular imaging studies

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Fluorescence molecular imaging (FMI) is becoming a powerful tool to improve surgical precision and diagnostics. Although significant advances have been made in imaging technology and contrast agents, debate continues regarding suitable standards for FMI. Standardizing all FMI aspects is crucial for adoption in clinical procedures and routine practice. Currently, the lack of clear-cut guidelines on FMI study reporting complicates comparison between studies and impedes standardization. This work presents community-driven REFLECT guidelines for FMI study reporting for fluorescence-guided surgery and interventions, covering preclinical, translational, and clinical studies. It aims to improve quality of reporting, enable comparison between studies, and enhance interpretation of results. The resulting structured checklist encompasses all essential details for reporting on the contrast agent, imaging device, imaging protocol, and image processing and analysis methods. Adoption of this framework is encouraged to enhance reproducibility and establish alignment with standardization efforts in the field - thus fostering advancements in FMI.

Over the last three decades, fluorescence molecular imaging (FMI) in the field of biomedical optics has seen significant advancements, solidifying its role as a fundamental imaging modality capable of revealing anatomical, functional, cellular, and molecular information in living subjects and/or tissue samples. The relatively straightforward and cost-effective technology, the minimal infrastructure requirements and the absence of ionizing radiation exposure have contributed to the widespread adoption of optical fluorescence imaging. Furthermore, the availability of ready-to-use contrast agents and a variety of molecular reporters, along with the compatibility with microscopic and flow cytometry methods, has promoted utilization across numerous scientific disciplines. Hence, fluorescence imaging has become a powerful research tool for visualizing protein expression, investigating molecular interactions, tracking cells, and monitoring disease progression in a non- or minimally invasive manner[1–5]. In a clinical setting, fluorescence imaging is particularly valuable in the context of interventional guidance[5–8]. Given its high temporal resolution and sensitivity, fluorescence imaging can support real-time clinical decision-making. The near-infrared (NIR) fluorescent dye Indocyanine Green, for instance, is widely used for assessing tissue perfusion and detecting sentinel lymph nodes[9–11], whereas fluorescent 5-aminolevulinic acid metabolites can aid neurosurgeons in achieving maximal safe resections of gliomas[12,13]. Notably, the first FDA-approved molecular contrast agents, designed to highlight or respond to specific biomarkers, have recently emerged. These include CYTALUX® (pafolacianine), a folate receptor binding fluorescent imaging agent enabling surgeons to visualize ovarian and lung cancer lesions during surgery[14], and LUMISIGHT™ (pegulicianine), a cathepsin and matrix metalloprotease activatable agent used to detect residual cancer during lumpectomy procedures[15]. A growing number of novel fluorescent molecular agents (also known as imaging tracers) are currently under preclinical development and clinical translation for diverse applications[16–18].

Broader adoption of FMI in clinical procedures and routine preclinical imaging requires standardization of all aspects related to the process, including contrast agents, imaging equipment, imaging procedures, and image processing and analysis. Such standardization ensures that the interpretation of fluorescence images is not only reproducible and comparable to alternate methods, but also accurate, precise, reliable, and meaningful. Numerous high-quality papers and consensus reports have already been published with guidelines, recommendations, and frameworks for the evaluation and validation of novel fluorescent molecular agents in clinical trials[19–25]. Furthermore, significant efforts have been made to develop standards and calibration methods that can assess the performance characteristics of fluorescence imaging systems, to verify their safe operation and to compare different imaging systems, either in relation with a specific contrast agent used in preclinical or clinical studies, or not (e.g., by using quantum dots or other fluorescent dyes)[21,24,26–31]. Still, no widely accepted consensus has been reached. Consequently, research and debate as regards to suitable standards and calibration methods are ongoing. Moreover, owing

to the complex interactions among the imaging device, the fluorescent contrast agent (e.g., spectral properties and pharmacokinetics), the imaging protocol, the optical properties of tissue, and any image processing employed, the accurate quantification of fluorescent signals remains challenging[29]. Earlier, the field of light microscopy, although outside the scope of this paper, has faced similar challenges, leading to the launch of an international initiative QUAREP-LiMi, which aims to enhance data quality, reliability, and reproducibility through standardization, reporting protocols, and best practices[32]. Likewise, amidst the increasing diversity of fluorescence imaging systems, agents, protocols, implementations, and patient physiologies, preclinical, translational and clinical studies would greatly benefit from clear guidelines for reporting the performance of a complete FMI approach. Adhering to consensus guidelines should enhance quality, transparency and reproducibility of FMI results, enabling accurate interpretation, critical reviewing as well as direct and comparable quality assessments across drastically different setups used for similar implementations.

A survey conducted by the STANDARD study group of the European Society for Molecular Imaging (ESMI) revealed that most of the researchers involved in molecular imaging (including optical imaging), acknowledge the importance of reporting on acquisition parameters and image analysis methods in publications[33]. However, despite this recognition, a significant proportion of researchers were unfamiliar with international guidelines on preclinical imaging research practices[34,35]. The consequence of this lack of awareness and adherence to guidelines, is that the information provided in the methodology section of publications can be challenging to evaluate against any kind of agreed upon standard, introducing the risk of disseminating inconsistent or even inadequate approaches. Such concerns were substantiated in findings of the ESMI study group on INTRAOPERATIVE IMAGING that conducted a small-scale analysis of research papers in the domain of fluorescence-guided surgery, and highlighted the pressing need for improvement in the metrics used for reporting of research methodologies.

In this community-led paper, we have established a comprehensive checklist—summarized in Table 1 and illustrated in Fig. 1—that encompasses a set of guidelines for reporting on the methodology used to obtain preclinical, translational and clinical FMI data in the context of fluorescence-guided surgery and interventions. The proposed parameters cover various aspects, including the characteristics of the fluorescent contrast agent, image acquisition parameters, and image analysis methods. Importantly, the aim of this paper is not to engage in the debate on best practices for '*how to perform FMI*'. Instead, the goal is to contribute to the standardization of FMI studies performed at preclinical, translational, and clinical levels by enhancing the transparency and quality of reporting, to facilitate comparison between studies, promote better interpretation of results, and foster advancements in the field.

Reporting guidelines

Fluorescent molecular imaging agent. FMI applications with clinical intent rely on the administration of a specific fluorescent imaging agent that may be a molecular targeted vector (e.g., antibody or small molecule) labelled with a fluorescent dye (or fluorophore)—or a molecule with a quenched fluorophore, where the fluorophore is activated upon a trigger such as cleavage of a bond or local pH—to generate fluorescent contrast[5,16,17]. When a new fluorescent molecular imaging agent is reported for the first time, a detailed description of the targeting moiety/backbone and selected fluorescent dye, as well as the synthesis or conjugation process used to link both, should be provided. Preferably, the chemical structure and a synthetic schematic representation should be included to illustrate the molecular design. If the fluorescent dye is obtained from a commercial source, the exact name, the supplier, and batch number should be reported if available. For in-house synthesized dyes, reporting full characterization is required. This characterization should include the chemical structure of the dye, along with excitation and emission spectra, molecular extinction coefficient at maximum excitation wavelength and quantum yield, all measured in a relevant buffer while considering and reporting potential homo-FRET quenching effects. The influence of physiological fluids such as blood or plasma should also be evaluated, as interactions with serum proteins can enhance fluorescence. Even if one can refer to relevant literature in which the fluorophore or dye has been previously described, minimal quality assessments should be conducted to verify chemical identity, homogeneity, and purity of the current batch of the dye (cf. Reporting guidelines from chemistry societies[36]). After preparing a fluorescent conjugate, a similar level of chemical characterization is required to ensure the purity and identity of the final product that should minimally include nuclear magnetic resonance (NMR)/mass spectrometry or high performance liquid chromatography (HPLC). Additionally, the spectral properties should be verified, as the conjugation process can lead to alterations in the absorption and fluorescence emission characteristics of the dye[37]. The degree of labeling (DoL), which refers to the average number of dye molecules attached to each targeting molecule, should be calculated and reported for each study as it may vary from batch to batch. The DoL can in fact impact the brightness of the imaging agent and may influence its pharmacokinetic profile, thereby influencing performance and detectability[38–40]. For instance, very high DoLs have been shown to lead to undesirable effects regarding biodistribution and clearance. Stability assessment should also be performed to confirm that the conjugate maintains its structural integrity and optical performance under relevant storage and experimental conditions. Finally, to ensure the effectiveness of the conjugated agent, demonstrating that the targeting specificity and affinity or its mechanism of action is not significantly diminished owing to the conjugation process, is essential. These properties of the imaging agent can be assessed via in vitro protein- or cell-based assays.

For clinical studies, fluorescent imaging agents must be manufactured following current Good Manufacturing Practices (cGMP) guidelines. These stringent regulations ensure per definition that the agents are produced under standardized, validated and reproducible conditions, guaranteeing their quality and safety. Thus, providing full manufacturing and quality control details in ensuing papers is not necessary. Still, it remains crucial to include essential general information about the agent, such as its molecular structure, batch number, DoL and spectral characteristics, either as supplementary data or in a reference, to ensure complete reporting to the audience.

Interactions between an imaging agent and biological tissue can lead to adverse effects in patients and study subjects (human or animal)[41,42]. Thus, information on toxicity and phototoxicity testing of an agent is also recommended for reporting[30,43,44], where the latter property is a function of both agent chemistry as well as procedure-specific optical exposure. This information may be based on directly relevant published studies or new results from in vitro or in vivo experiments.

For the methodological details of imaging agent administration, the administration route (and infusion rate if relevant), the composition and volume of the injection formulation used to solubilize and deliver the imaging agent (in addition to details of any post-injection flush), and the dose should be specified. While clinical studies typically

Table 1 | Checklist for reporting on fluorescence molecular imaging studies

Fluorescent molecular imaging agent	Image acquisition	Processing and analyzing
Commercially available fluorophore/agent □ Name and supplier of the fluorophore/agent **In-house developed fluorophores** □ If previously described, reference □ If novel, chemical structure, excitation- and emission spectra, molar extinction coefficient, and quantum yield in relevant solvent □ Minimal quality assessments confirming chemical identity, homogeneity, and purity □ Information/assessment on (photo)toxicity **In-house-developed fluorescent molecular agents** □ If previously described, reference □ If novel, synthesis details, conjugation chemistry, and provide a chemical structure □ Minimal quality assessments confirming chemical identity, homogeneity, purity, and stability. Report on the DoL, spectral properties, and affinity characteristics □ For newly cGMP produced agents, provide the molecular structure, DoL, affinity, and spectral characteristics □ Information/assessment on (photo)toxicity **Administration** □ Route of administration (and injection formulation/buffer) □ Dose of administered agent (molar dose) □ Time between administration and imaging □ Excretion route (renal or hepatic) and known metabolites □ For preclinical animal studies, check ARRIVE 2.0 guidelines. In addition, specify the food provided to the animals and if the imaged region of interest has been shaved/epilated	**Fluorescence imaging system** □ For standard/commercial fluorescence imaging systems, specify name and version □ For custom-build systems, either provide a reference to a relevant publication or include details on the light source (e.g., wavelength (range), power at imaging field), optical filter characteristics (transmission bandwidth, rejection OD), specifics of objective lens, and detector specifications. Include system performance (sensitivity, dynamic range, linearity, cross-talk, ambient light leakage, illumination/collection spatial uniformity, field of view, depth of field spatial resolution, distortion)[28] □ Provide all system-specific user settings (e.g., illumination power, gain, exposure time/frame rate, magnification, binning, resolution) □ Describe how the system is regularly maintained and recalibrated and how its performance is assessed **Acquisition parameters** □ If possible, report on the exact positioning of the camera with respect to the subject being examined (working distance, viewing, and illumination angles) □ Report whether and how any undesired signals in the field of view were suppressed. □ Report the presence or absence of ambient illumination, and its source	**Image processing** □ Report on the image processing software utilized □ Report on any image adjustments, enhancements, alterations, and use of ratiometric or kinetic modeling approaches □ Report how multiple fluorescence spectral windows/agents are handled/acquired/processed □ Raw unprocessed images should be included as supplementary data □ Assign a specific intensity window for each (group of) image(s), specifying minimum and maximum values along with the corresponding units □ Include a spatial scale bar in each image □ Present clinical FMI data in a three-panel figure format (White light, White light + Fluorescence overlay, Fluorescence alone) □ Report on the impact of tissue optical properties or other contrast sources (i.e. reflectance, autofluorescence, etc.) and describe steps implemented to mitigate their effects **Image analysis** □ Report image analysis method and quantification protocol (incl. formulas) □ Specify the definition of target and background regions of interest (ROIs) □ Indicate ROIs/line profiles for target and background signals on the images

express doses in grams (or grams per body weight), reporting the dose in moles (or moles per body weight) is also relevant, as it is the molar concentration of the fluorescent dye that will be detected by the camera during imaging. This approach enables straightforward comparison between different fluorescent agents, irrespective of their molecular weight or DoL (since larger molecules contain fewer molecules per gram compared to smaller molecules). The dose in terms of molar mass of fluorescent dye can be easily converted to grams of fluorescent agent, and vice versa, given that the molecular weight and DoL are provided. (*Note*: the same principle is common practice in the field of nuclear imaging, where the molar activity (Bq/mol) or specific activity (Bq/g) should be reported, along with the amount of activity of the administered agent[45]). Adjuvant or possible side medication should be reported, for example a pre-dose of an unconjugated agent ('cold-dosing') or medication given as prevention of allergic reactions. Finally, careful reporting of the time between injection and imaging is essential, as the manifestation of the fluorescent agent in a particular organ or tissue is a dynamic process, influenced by accumulation and clearance of the agent. Those studies performing pre-injection autofluorescence photobleaching should also report details on how this was carried out[46].

Two key aspects of preclinical studies that are essential to report in the context of FMI are whether a specialized low-fluorescent diet is fed to the laboratory animals, including the duration they have been on this diet, as diets can potentially introduce significant autofluorescence background signals[47,48], and the removal of fur or application of hair removal agents, as fur will attenuate fluorescent signals. Moreover, skin pigmentation can also influence fluorescence signal detection, which is especially problematic when pigmentation is mottled or uneven. For all other aspects of animal experiments, we refer to the ARRIVE2.0 guidelines[49].

Fluorescence imaging systems and image acquisition. To visualize fluorescent imaging agents effectively and consistently, a dedicated imaging setup must be employed, capable of both exciting the fluorescent agent and detecting/capturing the emitted signal. Sensor-based acquisition enables the visualization of the emitted fluorescence, which otherwise may be invisible to the human eye (i.e., in the near infrared spectral region), drowned out by scattered excitation light, filtered by the laser safety eyewear (i.e., when laser sources are employed for excitation), or simply not exposed (i.e., during endoscopic or laparoscopic procedures to access internal tissue structures).

Since the recorded fluorescence signals depend strongly on illumination and detection parameters, having a thorough understanding of these key device factors is imperative. Several fluorescence imaging systems are commercially available for clinical or preclinical purposes[24,50–52], and the landscape continues to evolve. These systems come equipped with distinct hardware components and inherent imaging capabilities that are not always adjustable or known to the users. In such cases, specifying the system model, the version (including software version), and the values of any user defined operational parameters are sufficient for referencing the hardware specifications. However, in the case of custom-built systems, a more comprehensive description of the system is required. This should encompass specifications of the light source (central wavelength(s) and bandwidth(s), power, optical filters characteristics (excitation and emission), the objective lens (f-number) and resulting illumination power at the sample surface),

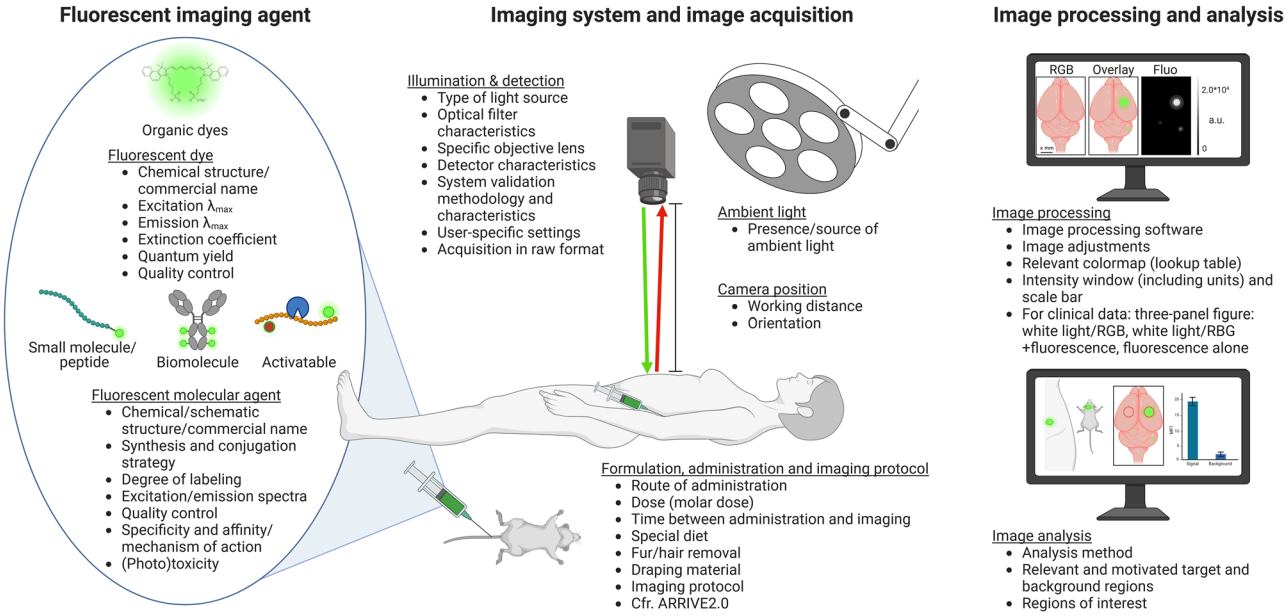

Fig. 1 | Graphical summary of the checklist of Table 1. Graphical summary of the checklist for performing and reporting preclinical, translational and clinical in-vivo fluorescence imaging studies.

and detectors (pixel size, bit-depth/dynamic range, spectral quantum efficiency, dark and read noise levels). Table 2 provides, based on current literature cited in the table, a few examples of the specifications to be reported, including units to use for reporting, for an intraoperative, endoscopic and preclinical fluorescence imaging system. Validation methodology and performance characteristics of fluorescence imaging systems obtained through, for instance, phantom studies should also be included in such cases[26,27,31,53]. Key characteristics include: field of view, image uniformity, spatial resolution, depth of field, linearity, penetration depth and distortion (esp. for endoscopic/laparoscopic devices)[28] (see Table 2). Reporting these detailed parameters will enable readers to assess how well the imaging system aligns with the chosen fluorophore's excitation and emission properties and application, and the trade-offs in performance that may exist with other systems. As part of a standardization protocol, one should also ensure the system is maintained and characterized (evaluated/recalibrated) at appropriate intervals. This can be performed with commercially available phantoms or calibrated fresh solutions[21,22,26–29,54]. Since materials used for adding absorption and scattering can vary significantly from batch to batch and through the shelf life of a single batch, preference to commercial phantoms should be given. Although unfortunately rarely done in the existing literature, the method of characterization should be reported. Solid phantoms provide an opportunity to collect and report these data using standardized analysis methods, where openly available protocols further enhance reproducibility[53,55,56].

Most systems also provide end-users with the ability to adjust various acquisition settings through dedicated acquisition software. These user-adjustable parameters typically encompass illumination power, detector gain, lens aperture, focus, exposure time/frame rate, magnification, and binning/resolution. Even when a series of different settings is employed as part of the experimental design (e.g., the same view is acquired at different frame rates), specification of the exact acquisition settings used for the generation of a displayed image/video is crucial as images of the same object can show different output (see Fig. 2). Of utmost importance is that the images are acquired in a raw format, avoiding any mode that automatically optimizes the acquisition settings, since auto-scaling might compromise comparison of images. Imaging settings should be kept constant as much as possible to enable comparison and data referencing, even within the same experiment/patient. Unfortunately, automatic optimization is sometimes the only option for certain commercial systems. In such cases, this limitation should be explicitly reported as such, and the researchers should exercise extreme caution when analyzing such data, particularly in terms of quantification and comparison with control conditions. The best practice in such cases is to have an unchanging reference standard (preferable with multiple dye concentrations) in all images that can be used to at least correct for uncontrollable automated changes in detection efficiency (e.g., gain, light power, exposure time, and aperture)[57], though even this may not account for all effects.

In addition to the camera system specifications and user settings, the precise positioning of the camera with respect to the subject being examined can also significantly influence the measured fluorescence signals, for instance changes in the viewing angle can result in light reflection[23]. Therefore, it is advisable to adjust the camera position to minimize these artifacts. As such, information on working distance, as well as viewing and illumination angles (ideally close to normal incidence) and any use of polarization to further reduce specular reflections, should be provided, whenever possible. However, it is important to acknowledge the inherent challenges of recording such details, particularly for imaging sessions in vivo, where the region of interest (ROI) is not a flat surface and often embedded in a cavity. Moreover, blood and sutures present additional variables that can impact imaging results. It is also crucial to document and justify the use of draping materials, which are employed to hide high-intensity signals originating from injection sites or excretory organs, and reveal the visualization of lower-intensity signals. Lastly, addressing the presence of ambient white light, whether natural or artificial, emanating from sources such as room light, surgical lights or even the imaging system

Table 2 | Various parameters to report on fluorescence imaging system and acquisition parameters used

Reporting parameter	Description		Exemplary systems		
			Intraoperative system[54]	Endoscopic system[53]	Preclinical system[70]
Illumination					
Excitation wavelength (nm)	The wavelength or spectral band of the excitation source.	Influences overall sensitivity through match with imaging agent spectrum (absorption).	760	750	620
Illumination power (or irradiance) (mW/cm²)	Power of the illumination source at the imaging plane per unit area.	Influences overall performance and saturation, photobleaching of imaging agent and scattering and thereby overall sensitivity plus safety consideration.	20	35	N/A
Detection					
Sensor type	The technology of the sensor.	Defines sensitivity and spectral response.	CMOS	EMCCD for fluorescence CCD for color	CCD
Pixel size (µm)	The length /width of each individual pixel.	Determines resolution and sensitivity through number of photons detected per area.	85	16 for fluorescence 5.5 for color	13
Binning / Resolution (pixel×pixel)	The imaging sensor array dimensions (pixel numbers) and the binning value used.	Determines resolution and sensitivity.	N/A	1 / 512×512	1 / 1024×1024
Gain	The level of electronic amplification used in the imaging sensor.	May influence SNR and/or introduce nonlinear response distorting the image.	Automated	300	N/A
Exposure time / FPS	The value of the exposure time or frame rate of the imaging sensor.	Relates to real time performance and sensitivity.	Automated	50 ms for fluorescence 40 ms for color	4.65 ± 1.65 s
Sensor temperature (°C)	The operating temperature of the imaging sensor and the type of cooling module.	Influences the noise characteristics of the sensor (lower temperature lower noise).	N/A	-90	-90
Detection wavelength range (nm)	Spectral band used for fluorescence detection (and filter type).	Influences overall sensitivity through match with the dye emission and detector spectral response.	800-840 (band pass)	800-840 (band pass)	660-680 (band pass)
Reporting parameters related to the experimental procedure					
Working distance (cm)	Distance between the objective and imaging plane.	Influences resolution and sensitivity esp. if imaging distance is not at the focal distance.	20	1.1	19-23
Field of view (cm²)	System's field of view and magnification range.	Influences resolution, illumination power and applicability.	14×14	85° forward looking	12.5×12.5

itself, in addition to any methods employed to suppress the effect of these light sources, is essential[58]. This ambient light can markedly contribute to background signals and is therefore imperative to report.

Fluorescence image processing and analysis. Post-processing of the images and the strategies employed for image analysis constitute a third critical set of parameters that can significantly influence data interpretation, comparison and quantification. Unfortunately, these aspects in particular suffer from underreporting, potentially introducing biases both within and between studies. Moreover, there is a risk of inadvertently or deliberately misrepresenting original data, making it essential to adhere to established best practices for image representation[59–61]. Although these best practices were predominantly developed for microscopy data, the same principles apply for FMI, encompassing various facets related to color coding, contrast and brightness adjustments, background and noise correction methods, filtering, motion correction, cropping and resizing. To assure transparency and maintain image integrity, meticulously documenting all image adjustments is imperative. Enhancements and alternations made during the image processing stage and any modification should be clearly denoted for each figure. The methods section should also explicitly mention the image processing software utilized. Furthermore, we refer to the perspective paper authored by Crameri et al., reflecting on the correct application of color gradients (also known as lookup tables (LUT)) to prevent visual distortions of data and to accommodate individuals with color vision deficiencies[62,63]. In this context, assigning a specific intensity window to each image (or group of images acquired and processed identically) is essential, while specifying the minimum and maximum values. Without an intensity window, images can be misinterpreted easily as shown in Fig. 3. In this figure, without the proper scaling, one might mistake the basal side to be tumor positive (Fig. 3B), whereas with the proper scaling, corresponding with mucosal tumor intensities, the image (Fig. 3C) shows the tumor margin to be negative. It should be noted that the corresponding units used for reporting fluorescence intensity images must also be provided, either in the text or within the intensity windows. Currently, relative measures such as arbitrary units and counts are widely used. However, as fluorescence imaging

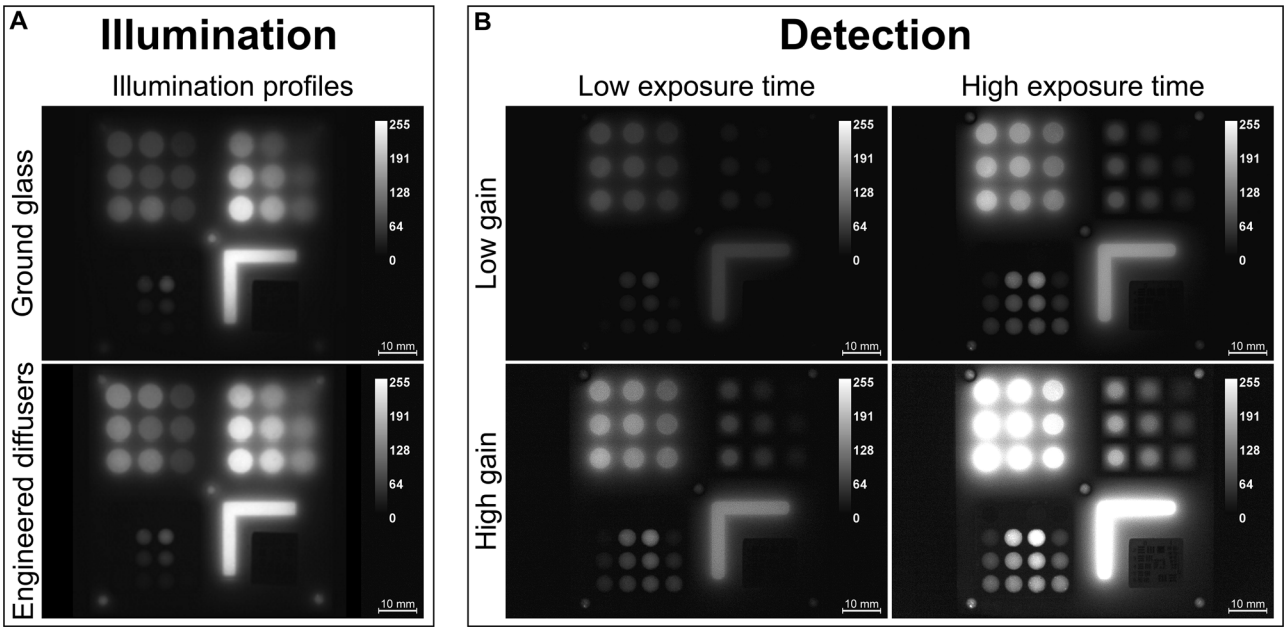

Fig. 2 | Examples displaying the importance of illumination and detection parameters on fluorescence image outcome. A The effect of varied illumination profiles. Illumination through ground glass results in a heterogeneous illumination profile, excessively exciting the center of the image and lacking intensity at the edge of the image. Illumination through engineered diffusers results in a more homogeneous illumination profile, shown by similar fluorescence signal intensity emitted by the center and four corner wells. In (**B**), the fluorescence images obtained of the same phantom using a variety of settings show the importance of reporting on user-settings as adjustments, in this case gain and exposure time, yield changes in the output. All color bars represent 8-bit pixel intensity, in arbitrary units (a.u.); All images per panel have been normalized to the same global pixel intensity.

enters clinical practice and multicenter studies become more common, absolute measures in SI units may enable more reliable comparison between centers using different fluorescence systems, and could accelerate advancements in upcoming technologies, such as short-wave infrared imaging[64,65]. Furthermore, incorporation of a scale bar into each image is highly advisable: "a must" and, as Fig. 3 has demonstrated, this scale bar should be identical across images for meaningful comparison. This practice is particularly valuable in clinical contexts, as it provides viewers with a tangible spatial reference within the image, facilitating an accurate assessment of the size and dimensions of image features.

To support FMI data with anatomical information, overlay images that merge fluorescent images with bright field or RGB images are invaluable. However, the overlay process can cause signal blending, color interference and contrast changes, which can alter the perceived fluorescent signals[66]. Therefore, for reporting on clinical images, a three-panel figure format is recommended. This figure should include a) the white light or RGB image presented on its own, serving as a reference for visual orientation, b) an overlay image that combines the fluorescence signals (displayed in monochromatic LUT) with the reference image, and c) an image displaying only the fluorescence signals (in gray-scale or color LUT). This format may not be necessary for preclinical data presentations, especially when a series of longitudinally acquired images or multiple control conditions need to be presented. The fluorescence images alone can always be provided as supplementary data. When providing the overlay images, care should be given to the assessment of the spatial co-registration between these images, whether that is automatically done by the camera or is based on suitable phantom tests.

The semi-quantitative character of FMI images remains one of the major limitations. As mentioned above, this limitation arises because absolute quantification of fluorescence intensities is challenging due to the various influencing factors that are difficult to control. To circumvent this, researchers often resort to calculating ratios, such as the target-to-background ratio (TBR) or contrast-to-noise ratio (CNR). Both metrics rely on subjective selection of ROIs within the images[21,67–69]. Consequently, they are susceptible to errors if ROIs are not chosen objectively (e.g. based on anatomical landmarks) or at least as objectively as practically achievable (Fig. 3B, C). Clearly indicating the target ROI, including its shape and boundaries, is crucial and should be provided together with rationale. While target regions (i.e. areas with high fluorescence intensity or regions affected by a certain disease) are often selected in a comparable manner between studies, this is not the case for background regions. Previous studies reported a wide variety in TBR, CNR, and signal-to-noise ratio (SNR) values resulting from differences in background selection[67,69]. For example, some may choose their background in tissue without illumination or from the contralateral site, resulting in higher TBR, CNR, and SNR values, whereas others may prefer a background directly adjacent to the target region, leading to lower values. Furthermore, automated ROI selection can be performed by image processing techniques such as thresholding[68]. Regardless of the methodology of background selection, specification of the background location, size and shape and the motivation for this selection, is essential. Ambiguous descriptions such as 'healthy tissue' are to be avoided to eliminate misinterpretations. Equally important is the need to quantify both the target and background signals from ROIs within the same image (taking illumination and detection uniformity of

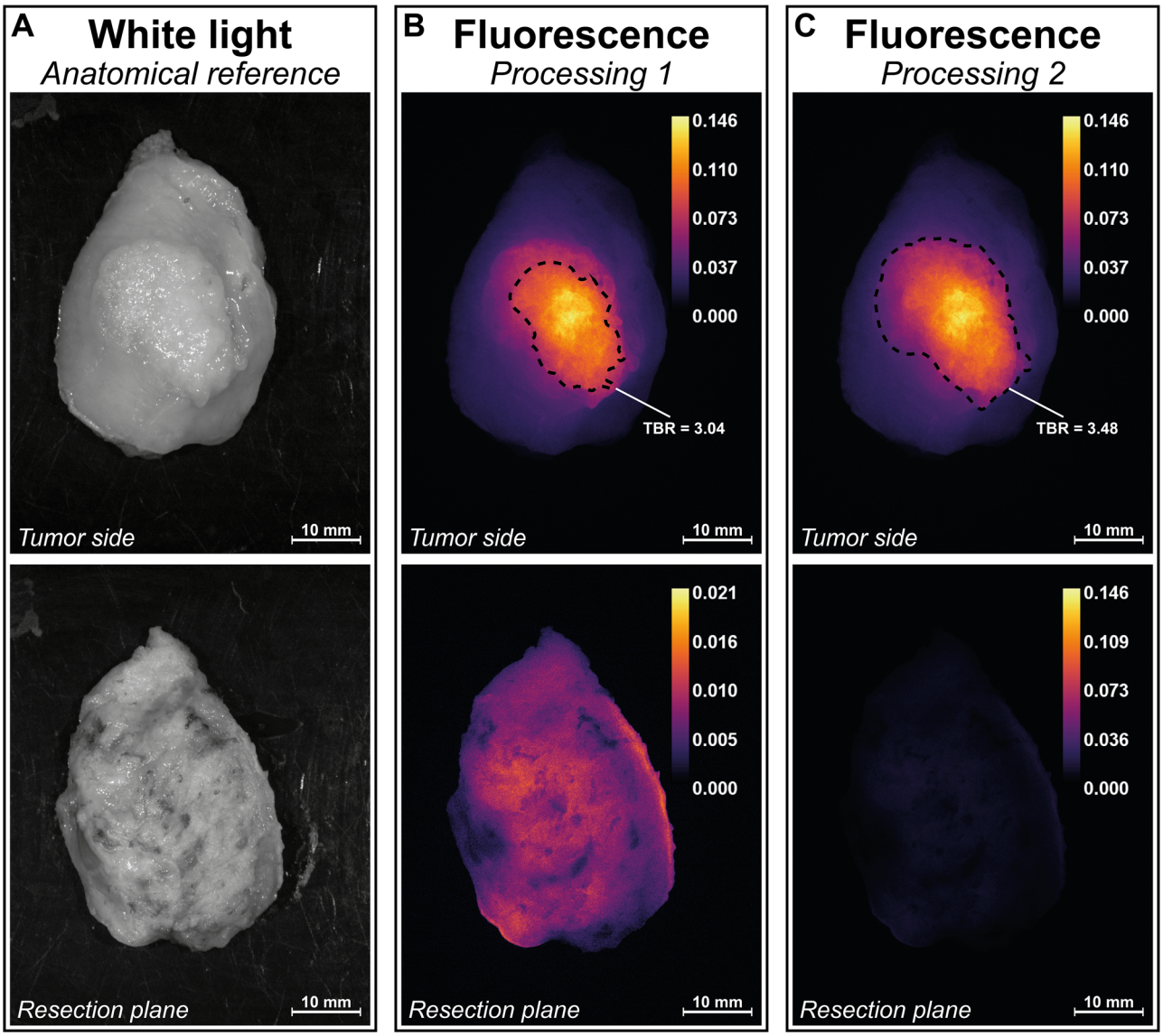

Fig. 3 | Importance of clear explanation of processing and analysis steps. A White light images of a tongue tumor resection specimen for anatomical reference. Panel B and C show various ways of analyzing and interpreting the fluorescence images. In (**B**), both the tumor and resection side images are scaled between the maximum and minimum values of the individual image, whereas (**C**) shows the images scaled to the scale bar of the tumor specimen. The calibration/intensity of the color bars are in arbitrary units (a.u.). The scale is based on automated, non-normalized system output. Note the differences in visualization of signal in the resection plane, where the way of processing images can alter conclusions drawn. Moreover, the importance of clearly defining and showing regions-of-interest (ROI) for target-to-background (TBR) calculation are shown. In (**B**), the dashed black line represents the 'target' ROI based on a fluorescence threshold, where the background is defined as 'all remaining tissue'. The TBR differs from the one in (**C**), where 'target' ROI is determined by anatomical reference (**A**).

the camera system into account), where both have been imaged at equal distances and orientations relative to the camera. Besides, it is highly recommended to provide at least one example in which the ROIs (both target and background) are visualized on an obtained fluorescence image to ensure transparency in reporting and enhance repeatability. Likewise, when employing line profiles as an alternative method to assess contrast, indication of the precise position at which the line profile was captured on the images is imperative. A recent study has demonstrated that the formulas used for the quantification of performance metrics, such as SNR and contrast, can similarly to ROIs influence the interpretation of the acquired data[69]. As this observation is also valid for metrics like TBR and CNR, reporting the specific formula applied is imperative to enable repeatability and transparency in data interpretation.

Moving forward with these guidelines. This work presents the REFLECT reporting guidelines, a comprehensive checklist for reporting on FMI studies in the context of fluorescence-guided surgery and interventions, spanning preclinical, translational and clinical studies. The checklist encompasses all essential details that should be

reported regarding the fluorescent contrast agent, the imaging device, and the methods used for fluorescence image processing and analysis. To promote adoption of these community-driven guidelines, we aim to collaborate closely with fellow scientists, scientific publishers, and journals and encourage journals to modify their submission guidelines for FMI studies to align with the REFLECT guidelines as we recognize that improving the reporting of fluorescence imaging studies requires community-wide standards supported by researchers, journals, reviewers, and publishers. It should be noted that this initial effort provides guidelines for reporting to support the transparency and standardization of FMI studies, but does not provide recommendations for study methodology. Increased transparency will, in the future, facilitate expert discussions allowing the development of further guidelines regarding recommendations for FMI studies, including template figures and data file standards to promote FAIR data practices. Such that the quality, and thereby clinical applicability, of FMI studies will be improved. We strongly encourage the adoption of this checklist when composing or evaluating scientific manuscripts involving FMI. The aim being to enhance the reproducibility of FMI research, align with ongoing standardization efforts in the field, and provide the foundation for advancements in the field of FMI. These reporting guidelines have received official endorsement from the European Society for Molecular Imaging (ESMI), the Dutch Fluorescence-Guided Surgery group (DFGS) and the World Molecular Imaging Society (WMIS).

Data availability

All data used is included in the manuscript. The tiff files of the figures of the current study are available upon reasonable request.

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Received: 19 June 2025; Accepted: 17 October 2025;
Published online: 25 November 2025

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Acknowledgements

We would like to thank the members of the ESMI Intraoperative Imaging (IOI) study group as well as T.J. Pfefer for stimulating discussions.In addition, DG and VN received funding from the European Union's Horizon Europe research and innovation program under grant agreement No 101046923 (msGUIDE) and from the DZHK (German Centre for Cardiovascular Research; FKZ 81X2600421). SH and HI received funding from the European Union's Horizon Europe research and innovation program under grant agreement No 101047263 (CodaFlight), Flander's Research Foundation - Strategic Basic Research program (grant number FWOSBO42), Strategic Research Program of the Vrije Universiteit Brussel (grant number SRP95), Innovation Research Fund of the Vrije Universiteit Brussel (grant number IOF3005). These reporting guidelines have received official endorsement from the European Society for Molecular Imaging (ESMI), the Dutch Fluorescence-Guided Surgery group (DFGS) and the World Molecular Imaging Society (WMIS).

Author contributions

B.K., M.S., M.A., S.F. and J.S. performed the initial analysis for this study, supervised by L.M., F.J.V., S.H., P.J.v.d.Z. The initial draft was written by B.K. and M.S., which was reviewed and edited by the other authors (M.A., L.M., E.J.D., S.L.G., S.t.G., S.G., H.I., S.K., V.N., E.L.R., S.R., E.L.R., K.S.S., K.M.T., A.L.V., M.J.H.W., F.J.V., D.G., S.H., P.J.v.d.Z.). D.G., S.H. and P.J.v.d.Z. coordinated this study.

Competing interests

SG is employed by Intuitive Surgical. EL is co-founder and share-holder of QUEL Imaging, a company marketing phantoms. VN is a founder and equity owner of Maurus OY, sThesis GmbH, iThera Medical GmbH, Spear UG, and I3 Inc. All other authors declare no competing financial or non-financial interests.

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