Journal of Nuclear Medicine, published on February 10, 2022 as doi:10.2967/jnumed.121.262975

A guideline for clinicians performing clinical studies with fluorescence imaging

Wido Heeman[1,2,3#], Jasper Vonk[4#], Vasilis Ntziachristos[5,6], Brian W. Pogue[7], Rudi A.J.O. Dierckx[8], Schelto Kruijff[2,8], Gooitzen M. van Dam*[8,9]

Running title: Clinical fluorescence imaging guideline

#Authors contributed equally and share first authorship.

1. University of Groningen, Faculty Campus Fryslân, Leeuwarden, the Netherlands
2. Department of Surgery, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands
3. LIMIS Development B.V., Leeuwarden, the Netherlands
4. Department of Oral & Maxillofacial Surgery, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands
5. Chair of Biological Imaging, Center for Translational Cancer Research (TranslaTUM), Technical University of Munich, Munich, Germany. v.ntziachristos@tum.de.
6. Institute of Biological and Medical Imaging, Helmholtz Zentrum München, Neuherberg, Germany. v.ntziachristos@tum.de.
7. Thayer School of Engineering & Department of Surgery, Dartmouth College, Hanover NH USA
8. Department of Nuclear Medicine and Molecular Imaging, Medical Imaging Center, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands
9. AxelaRx / TRACER B.V., Groningen, the Netherlands

*Corresponding author: Gooitzen M. van Dam, MD, PhD, Department of Nuclear Medicine and Molecular Imaging, Medical Imaging Center, University Medical Center Groningen, PO Box 30.001, 9700 RB Groningen, the Netherlands. AxelaRx / TRACER B.V., L.J. Zielstraweg 1, 9713 GX Groningen. E-mail: g.m.van.dam@umcg.nl / go@tracercro.com, phone: +31 50 361 10183, mobile: +31 6 22914614, Fax: +31 50 361 2283

Word count: 3560

ABSTRACT

Fluorescence imaging is an emerging imaging technique that has shown many benefits for clinical care. Currently, the field is in rapid clinical translation, and an unprecedented number of clinical trials are performed. Clinicians are inundated with numerous opportunities and combinations of different imaging modalities. To streamline this process, a multidisciplinary approach is needed with drug discovery, software and systems engineering, and translational medicine. Here, we discuss the main constituents of a uniform fluorescence imaging protocol to match the clinical need and ensure consistent study designs and reliable data collection in clinical trials. In an era in which the potential of fluorescence imaging has become evident, consistent conduct of studies, data analysis, and data interpretation are essential for implementation into standard of care.

Noteworthy

- A fluorescence imaging protocol results from multiple constituents, such as clinical indication, applied fluorescence imaging camera system, target moiety, signalling compound, standardized image acquisition, data processing and image interpretation. (Page 4)
- Benchmarking camera systems is required for inter-comparable data since results are greatly affected by characteristics such as camera detection sensitivity, depth sensitivity, field illumination homogeneity, exposure time, resolution and dynamic range. (Page 6)
- Imaging procedures must be standardized regarding tracer administration, working distance, incident angle and ambient light. (Page 11)
- Clinical acceptance of fluorescence imaging requires standardized and reproducible clinical data based on an imaging approach that relies on the cornerstones of science; standardization and reproducibility. (Page 12)
- The discriminatory power of a tracer for a certain indication should be reported using the contrast-to-noise ratio and images should be presented using perceptually uniform scientific-derived colour maps. (Page 13)

INTRODUCTION

Wide-field fluorescence imaging (FI) is a rapidly evolving imaging technique. By probing optical contrast, FI visualizes biochemical or (patho-)physiological processes that human vision cannot detect*(1)*. In medicine, and specifically in surgery, the potential of FI has been shown for non-targeted indications such as assessment of tissue perfusion, retinal vasculature and sentinel lymph node mapping*(2, 3, 4, 5)*. Efforts to improve specificity of the signal have led to the development of targeted FI for the detection of (pre)malignant lesions and locoregional metastases (e.g., lymph node or peritoneal metastases), delineation of tumour margins, evaluation or prediction of treatment response and more recently, the visualization of critical anatomical structures, such as nerves*(6, 7, 8, 9)*. Although the field has grown exponentially in FI camera system performance and fluorescent tracers, broad implementation into standard of care has not yet been established*(10, 11, 12)*

Currently, the first phase II and III trials are being reported – overviews of currently ongoing clinical trials have been presented recently *(11, 13)* and the first Food and Drug Administration breakthrough therapy designation has been assigned for use in breast cancer surgery*(14)*. As such, the number of clinicians having access to FI camera systems (e.g., surgical robot-assisted systems with incorporated FI) is also rapidly increasing. Choosing the appropriate imaging approach for a clinical problem is based on the strengths and weaknesses of the available FI imaging systems and fluorescent tracers. This requires a basic understanding of the underlying physics of FI and the chemistry of the fluorescent tracers used.

Swift implementation of FI into standard of care requires a multidisciplinary approach that is especially important when conducting a clinical study with FI. We strongly advise clinicians to partner with FI experts (e.g., engineers, physicists, chemists) in early phases of trial design. Choices of the fluorescent tracer and FI camera system must be made carefully. Perhaps most importantly; it requires the users to be cognizant of both the drug- and device- limitations for clinical use. The protocol should result from multiple constituents, such as clinical indication, applied FI

camera system, target moiety, signalling compound, standardized imaging acquisition, data processing and finally, image interpretation. An inadequate imaging approach leads to a flawed clinical trial or individual imaging procedure but, more importantly, comes with unnecessary patient risk and societal burden. These risks include elongated anaesthesia and operation time, unnecessary healthcare costs and the exposure to novel compounds without a fully elucidated pharmacological profile.

Clinical FI studies should be based on a scientifically substantiated imaging approach that relies on the cornerstones of science; standardization and reproducibility. This paper aims to provide a guideline for clinicians who want to perform wide-field FI trials that lead to clinical implementation or for translational research and development.

DEFINE THE CLINICAL INDICATION AND IDENTIFY THE APPROPRIATE TARGET

The driving motivator for a new trial is a clinician with a clinical challenge and the desire to test a new (optical) imaging approach, potentially leading to the birth of a new relevant application. In contrast to radiographic imaging techniques, FI can be seamlessly integrated into standard of care. It directly relates to the surgeon's vision and uses portable and relatively low-cost instrumentation, non-ionizing radiation and real-time feedback*(15)*. Yet, the clinician needs to think of the clinical value and practical issues. For example, an urgent surgical procedure requires fluorescent tracers that accumulate rapidly at the target site.

When such practical issues have been addressed, a more refined imaging approach can be developed (Fig. 1). FI imaging in the visible spectrum (e.g., fluorescein, methylene blue) is often not sufficient due to its low penetration depth resulting from strong photon absorption in this spectrum. Most clinical indications require the assessment of sub-surface structures (i.e., >1 mm) where the absorption and scattering of light are the main limiters of penetration depth. The user should be aware of the tissue of interest's optical properties (i.e., scattering and absorption) and its impact on light propagation*(16)*. Tissue types exhibit specific optical properties; for example,

more absorption occurs in a highly vascularized liver than in muscle tissue. Improved penetration depth can be obtained by imaging in the near-infrared (NIR) window (i.e., 750-1700 nm). This spectral region benefits from reduced scattering and lowest absorption by tissue chromophores (e.g., haemoglobin, water). A critical note here is that the signal is heavily surface-weighted due to light attenuation in tissue (i.e., absorption and scattering), and that the spatial resolution decreases with depth due to scattering (Fig. 2) *(17)*.

When the user is aware of the tissue of interest's optical properties, the biochemical phenomenon or (patho)physiological process should be concretized. All possible targets, including biomarkers and phenomena/processes, should be examined to determine which is most suitable for localization or evaluation of the target tissue. For example, one can image breast cancer through visualizing nonspecific intra tumoral phenomena (e.g., enhanced permeability and retention effect), a specific cell membrane-bound receptor, or a pathophysiological phenomenon in the tumour microenvironment. Methods for target selection have been reported previously*(18, 19)*. Briefly, the potential target should be prevailing in the target tissue compared to directly adjacent tissue, benefitting high binding sensitivity and specificity as well as improving the contrast. Target expression is commonly determined by immunohistochemistry. However, it is increasingly questioned whether this is representative of the complete tumour due to tumour heterogeneity and variations in target expression over time. Data-driven methods based on genomic alterations are studied to identify and prioritize relevant targets for clinical trials*(20)*. In addition, many targets (e.g., cell membrane receptors) are present in a microscopically heterogeneous pattern. For solid tumours that require wide local excision, the latter does not per se impede guiding the surgeon in tumour resection since the margin is of primary interest*(21, 22, 23)*. Contrary, in debulking surgery procedures (e.g., glioblastoma surgery) homogenous contrast is of clinical importance since microscopic residues should be identified in order to excise all tumour tissue*(24, 25)*.

SELECT THE APPROPRIATE IMAGING MODALITY

When selecting FI camera systems for a clinical trial, the systems' form factor must fit in the expected clinical setting. For instance, tumour visualization in oral cancer can be performed using an open system, but perfusion assessment during minimally invasive surgery requires a laparoscopic system. Next, the user should be aware of its performance characteristics to obtain the desired imaging data, as these parameters greatly affect results*(10)*. There are numerous parameters to consider, but one should focus on those that directly influence imaging data, such as the camera detection sensitivity to the desired tracer, depth sensitivity, field illumination homogeneity, spatial and temporal resolution, and dynamic range. These minimum requirements of these parameters should be finetuned for a specific imaging study, preferably in cooperation with an engineer and a physicist.

The camera detection sensitivity describes the ability of a FI camera system to detect a certain concentration of a specific contrast (i.e., fluorescent dye and corresponding emission wavelength). This should be determined for every combination of a FI camera system and fluorescent tracer since the systems' foremost influential characteristic is the sensitivity to the fluorescent tracer's emission peak. Commercially available FI camera systems are equipped with very specific narrow band optical filters. A mismatch between the optical filters and the fluorescent tracer results in a low fluorescence intensity and could lead to an erroneous conclusion that a fluorescent tracer (micro)dose does not accumulate in the region of interest since the contrast-to-noise ratio (CNR) is low (Fig. 3, panel B).

Depth sensitivity is the ability to measure fluorescence signal at a certain depth. This is largely dependent on the type of light (i.e., coherent or non-coherent) and the wavelength-specific penetration depth of the excitation light. Ideally, devices should evolve to account for this automatically, yet, the user should be aware for each clinical application of interest*(26)*. For margin assessment the imaging depth may vary among different tumours, since the definition of an adequate margin is different. Head and neck cancer requires a tumour-free margin of at least 5

mm, whereas for breast cancer this is at least 1 mm. Although the penetration depth of light increases with longer wavelengths (i.e., NIR versus visible spectrum), this does automatically translate to increased measurement depth. When deeper tissues are imaged due to increased scattering, the discrimination between target and surrounding tissue is impaired due to decreasing CNR with imaging depth (i.e., low depth sensitivity) (Fig 2).

Field homogeneity describes how uniform the region of interest is illuminated. Inhomogeneous field illumination can lead to over- or underestimation of the fluorescent signal throughout the field of view. Perfect field homogeneity is rarely achieved in practice, and only a few FI camera systems have implemented algorithms to improve field homogeneity. Most systems, especially endoscopic ones, have highly inhomogeneous light fields that lead to steep intensity fall-off towards the edge of the field. The user should validate the field homogeneity prior to every imaging procedure using a calibration phantom. An inhomogeneous field illumination is not an insurmountable problem, as long as the user is aware and knows how to interpret and correct for it*(27)*.

Resolution of a FI camera system is characterized by spatial and temporal resolution. The spatial resolution dictates the modalities' ability to differentiate between the smallest fluorescent sources. The spatial resolution should at least be half of the smallest feature that has to be detected, as described by the Nyquist theorem. The temporal resolution dictates the modalities' ability to detect changes in signal over time. This is of importance when a dynamic phenomenon is of interest, such as organ perfusion (e.g., semi-quantitative indocyanine green)*(28)*.

The dynamic range greatly influences the ability to measure fluorescence signal. The dynamic range (i.e., the detector's quantum efficacy) is the measure for the highest and lowest amount of measurable light for a set exposure time. A camera system with a low dynamic range can either measure very high or very low signals depending on exposure time. However, the camera cannot do so both at the same time. Hence, a camera with a high dynamic range can measure both very bright (i.e., high quantum yield) and very dim (i.e., low quantum yield) fluorescence signals (Fig. 3, panel A).

BENCHMARKING OF FLUORESCENCE IMAIGNG CAMERA SYSTEMS

To compare different FI camera systems, universal standards are required for benchmarking their performance, as is common in the other medical imaging modalities*(29)*. As such, solid tissue-mimicking phantoms have been developed to characterize the different FI imaging systems quantitatively. Wells filled with different concentrations of nanoparticles (i.e., quantum-dots) are used to measure i) camera detection sensitivity versus optical properties, ii) depth sensitivity, iii) dynamic range, iv) field homogeneity, and v) spatial resolution*(27)*. We advise that users acquire a FI camera system with high camera detection sensitivity in combination with a high dynamic range. Also, as described above, the camera wavelength specificity and emission light sources should match the excitation and emission spectra of the fluorescent tracer (Fig. 3, panel B)*(26, 30, 31)*.

Performing phantom measurements before each imaging procedure inform on system stability over time and provides users better insight into the performance capabilities. A standardized image of FI phantom should be taken under strict imaging acquisition parameters (i.e., camera distance, incidence angle, ambient light) and processed according to a strict protocol. *(27, 30, 31)*. Automated log files should be constructed according to a standardized format and recorded for review purposes, safeguarding a quality management system for FI in clinical use. Ideally, these log files are archived with the patient data and imaging results, allowing for calibration in later analysis of batch data, similar to the metadata archived in DICOM images taken with radiologic imaging systems. We propose a quality management system to enable comparative multicentre clinical trials and implementation in general practice, enabling uniformity.

Additionally, FI camera systems should have the option to export raw data without interference of (undesired) image post-processing to obtain (semi-)quantitative data rather than qualitative images. However, some commercial intraoperative imaging devices often opt for an

underlay for the surgeon's orientation purposes, which impedes the possibility of quantification*(10)*.

FLUORESCENCE CONTRAST

Fluorescence contrast can be either endogenous (i.e., autofluorescence of intrinsic tissue compounds) or exogenous (i.e., administered fluorescent tracer)*(32)*. Although the use of endogenous contrast has some advantages, such as inherent non-toxicity and absence of regulatory issues, we focus on the use of exogenous contrast as this has been shown to increase specificity and detection sensitivity*(33)*. The main criteria for selecting a fluorescent tracer include efficient fluorescence light output (i.e., quantum yield), biodistribution and pharmacokinetic characteristics, signal enhancement strategies (i.e., "always-on" versus "activatable" or "smart") and regulatory approval*(11)*. Lastly, the clinician must be aware of regulatory issues that can result in tremendous costs when designing and using new fluorescent tracers, such as intellectual property, animal tox studies, availability of compounds in a good-manufacturing practice facility and regulatory approval*(34, 35)*.

Generally, exogenous fluorescent tracers can be divided into targeted and non-targeted tracers. Non-targeted tracers do not bind to biomarkers for disease-specificity but accumulate passively into the tissue through metabolism or nonspecific uptake (e.g., enhanced permeability and retention effect in tumours). A well-known non-targeted fluorescent tracer is indocyanine green, which has Food and Drug Administration approval for tissue perfusion assessment, sentinel lymph node mapping and biliary duct visualization. As fluorescent dyes itself are not tumour-specific, efforts to improve specificity have led to the development of targeted fluorescent tracers that bind to receptors or biomarkers*(36)*. Particularly in interventional oncology (e.g., surgery, gastroenterology), phase I studies have shown its potential for margin assessment and characterization of lesions. Recently, breakthrough therapy designations have been assigned by

the Food and Drug Administration (i.e., Pegloprastide, a ratiometric fluorescent probe for breast-conserving surgery)*(14)*.

The clinical indication should be leading when deciding between a targeted or non-targeted approach. The targeted approach is generally more complex and thus not always preferred. A non-targeted tracer could suffice for sentinel lymph node mapping as this generates contrast between the lymph nodes and the adjacent tissue. Contrarily, tumour delineation requires a targeted tracer with higher tumour-specificity. Even though targeted tracers are used, one should realize that the signal is not proportional to the concentration of the target, but is confounded by nonspecific sources of contrast. This nonspecific accumulation of fluorescent tracer is intrinsically determined by its receptor affinity but is also affected by physiological phenomena, such as vascularity, vascular permeability, interstitial pressure and lymphatic drainage*(37)*. Paired-imaging methods are currently studied to correct for the nonspecific tracer accumulation by co-administering an untargeted control agent with similar pharmacokinetics*(38, 39)*. A wide range of fluorescent tracers is currently studied in clinical trials, including small molecules, peptides, proteins and nanoparticles, as described elsewhere*(36)*.

Current developments to improve fluorescence contrast include the use of “activatable” or “smart” fluorescent tracers that only fluoresce after interaction with or binding to the target*(40, 41)*. Rather than visualizing one fluorescent tracer in a single lesion, multispectral imaging (i.e., imaging fluorescent probes at different or multiple wavelengths) could simultaneously visualize multiple fluorescent tracers that report on different targets within the same patient. The advantages include the delivery of a more homogeneous signal, increased sensitivity, and the ability to obtain anatomical-molecular information*(42)*. For example, one might strive to both perform molecular imaging of the tumour and identify critical structures (e.g., nerves), both contributing to an optimal surgical outcome, both requiring a specific tracer with different fluorescent excitation and emission wavelengths. Technical challenges include accurately separating signals and correcting for differences in fluorescent dyes (i.e., efficiency of fluorescence signal generation, wavelength-

dependent tissue optical properties), as described elsewhere*(43)*. The clinical introduction, safety and applicability of multispectral FI remains to be investigated in clinical trials.

IMAGE ACQUISITION: REPRODUCIBILITY AND STANDARDIZATION

Reproducibility and standardization should be central within the two primary components of a FI study protocol; tracer administration and image acquisition. Similar to PET, the tracer administration must be dosed and timed consistently throughout the entire study population*(44)*. The exact dose is commonly determined using dose-escalation schemes, with pharmacokinetics, biodistribution and toxicology studies in animals, healthy volunteers or subjects belonging to the target population. Whether timing between tracer administration and image acquisition is crucial, depends on the biodistribution and pharmacokinetic profile of the tracer. When studying a dynamic perfusion assessment (i.e., semi-quantitative use of indocyanine green) the timing comes down to seconds. In such a setting, the administration can be standardized by using a syringe pump with a pre-programmed infusion rate. On the other hand, many targeted fluorescent tracers need substantial amounts of time (i.e., days) to bind to the target moiety and ensure clearance of unbound tracer from the blood.

The detected fluorescence is dependent on different specifications of the FI camera system (e.g., exposure time, gain) in combination with the contrast, as well as variable imaging parameters of the experiment itself (e.g., working distance, incident angle and ambient light). Imaging with varying working distances substantially impacts the data consistency since the intensity measured is distance-dependent (Fig. 3, panel C). Consequently, higher fluorescence intensity is detected when the distance of the tissue of interest to the detector decreases, even when the fluorescent light emitted is the same. The camera should be perpendicular to the tissue to maximize the effective surface area of the detector (Fig. 3, panel D. When all variable imaging parameters are standardized in every FI measurement, the imaging data allows for reproduction

and represents the tracer distribution more realistically*(26)*. Ideally, all imaging parameters should also be registered to allow for post hoc correction.

Although the impact of ambient light in FI has never been underestimated*(45)*, it is rarely standardized or corrected for. The most common solution is to keep the ambient light to a constant minimum as relatively few systems can deal with high ambient intensity. The choice of lighting in the operating room can be optimized, typically by minimizing NIR light. This is specifically emitted from commonly used tungsten bulbs that could simply be replaced by light-emitting diodes. Needless to say, this only reduces the problem for NIR-based emission probes such as indocyanine green.

REPORTING ON FLUORESCENCE IMAGING DATA

Apart from a standardized imaging protocol, standardized data processing, representation and reporting are necessary for the implementation of FI in standard of care. Contrary to some other imaging techniques (e.g., CT), wide-field FI does not provide quantitative data. Even when imaging parameters are standardized, variations in tissue optical properties affect the fluorescence signal. Additionally, the signal is heavily surface-weighted, meaning that anything closer to the surface will generate more fluorescence signal. These factors need to be taken to account when analysing FI data. The most used semi-quantitative unit is mean fluorescence intensity (MFI), defined as the average pixel intensity within a region of interest. Yet, reporting the MFI as an absolute and quantitative measure without a thoroughly standardized protocol can lead to incorrect conclusions.

Since FI is a detection or discrimination method, relative measures (i.e. ratios) are more appropriate for FI as these demonstrate the ratio between the target and the background. Commonly used ratios in clinical FI include tumour-to-background ratio, signal-to-background ratio and CNR*(46)*. We advocate the use of CNR, defined as the target's MFI subtracted by the background's MFI, divided by the standard deviation of the background. Using a CNR is favourable since this is more informative on the detectability of the contrast (i.e. target) of

interest*(47)*. A high CNR indicates good discrimination between the target and background tissue. Still, the CNR is influenced by the FI camera systems dynamic range and quantum efficiency. For example, using a fluorescent tracer with a relatively high quantum yield together with two different FI camera systems with a low- and high dynamic range may result in two very different CNRs. In other words, a FI camera system with a low dynamic range may underestimate the CNR as the signal of the tumour is limited (Fig. 3, panel A). Also, despite the seemingly straightforward definition, these quantities are prone to bias due to the strong dependency on the definition of the surrounding tissue. Ideally, the target and the background are based on the gold standard (i.e., histopathology). The appropriate background must be adjacent tissue as it mimics the clinical scenario.

Clinical use of FI relies on the interpretation of data that is typically shown as an image or video, even though the ratios are most important in clinical trials. Fluorescence images should be uniformly reported across the field to avoid difference in image interpretation. This list includes the choice of colour map, functions for the lookup table and image compression. Perceptually uniform scientific-derived colour maps represent actual data variations, reduce complexity, and are accessible for colour-deficient people*(48)*. Yet, even when data is uniformly reported, the interpretation of FI signal without correction for tissue optical properties may lead to inaccurate conclusions. This may, for example, lead to erroneous tumour delineation due to scattering in margin assessment when interpreted by different clinicians Lastly, as mentioned earlier, the used FI camera system settings must be described in detail. Reporting these settings is essential for the reproducibility of study results as the FI camera system settings severely influence the obtained FI data.

CONCLUSION

The rapidly increasing interest in FI has led to serious improvements in FI camera systems and fluorescent tracers available. Although FI has shown enormous potential for a variety of

indications, the field has not yet established clinical implementation. Here, we have provided a guideline for clinicians to perform FI clinical trials (Fig. 1). The same conceptual thinking applies to other optical imaging modalities, such as laser speckle contrast imaging or spectroscopy-based techniques. Similar to the classical medical imaging field, the FI field should focus on training clinicians and supportive staff in a multidisciplinary way to better understand the underlying physics and chemistry. Still, we advise clinicians to collaborate with researchers that have experience with FI camera systems and fluorescent tracers in order to correctly acquire, analyse and interpret the imaging data in an accurate and reproducible manner. To establish the clinical implementation of FI, phase II and III trials need to commence based on a consistent study design, imaging protocol and data analysis. By emphasizing standardization and reproducibility, the full potential of FI can be realized, and its clinical value can be proven.

Author contributions

WH, JV, SK and GMvD designed the manuscript. WH and JV identified literature, drafted the manuscript and conceptualized the figures. RAJOD, BWP and VN critically revised the manuscript.

Disclosure

VN is an equity owner and consultant of iThera Medical GmbH, an owner of Spear UG and a member of the Scientific Advisory Board of SurgVision B.V. / Bracco Sp.A. GMvD is CEO, founder and shareholder of TRACER Europe B.V. / AxelaRx. The other authors declare no conflict of interest regarding this work.

Figures

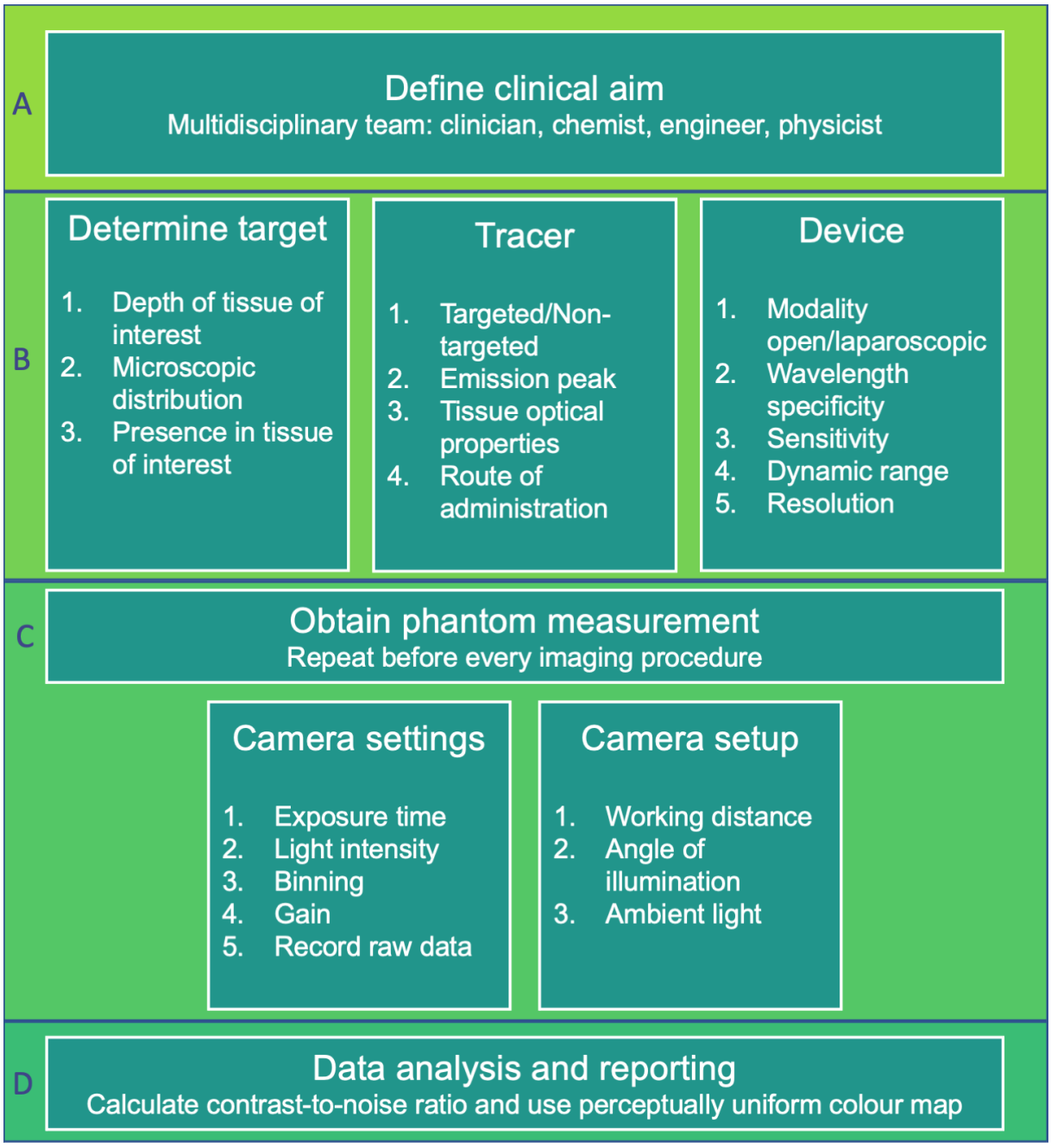

Figure 1: Checklist for performing *in vivo* fluorescence imaging studies.

A step-by-step approach to ensure a standardized and reproducible FI clinical trial, including trial design, imaging acquisition, data analysis and reporting results. First, the clinician involved should define a clear and specific clinical aim in close cooperation with a chemist, engineer and physicist. The team then defines a biological target with the microscopic distribution and required penetration

depth in mind. The tracer must match the target and should be selected based on the targeted/non-targeted approach, the tracers' emission peak, the tissue optical properties and the administration route. Simultaneously, the device emission and excitation filters must match the tracers' wavelength. Also, the form factor should be determined along with the desired resolution, sensitivity to light and dynamic range. Prior to every imaging procedure, phantom measurements should be obtained to evaluate performance characteristics over time. The user should set the camera settings such as exposure time, binning, gain, emission light intensity, and the data should be recorded without any pre-processing. Moreover, the camera setup should be identical in every procedure, with respect to the working distance, angle of illumination and ambient light levels, to compare results across patients. After data analysis, the performance of fluorescent tracer and imaging device combination should be reviewed based on the contrast-to-noise ratio. Images should be processed using perceptually uniform colour maps.

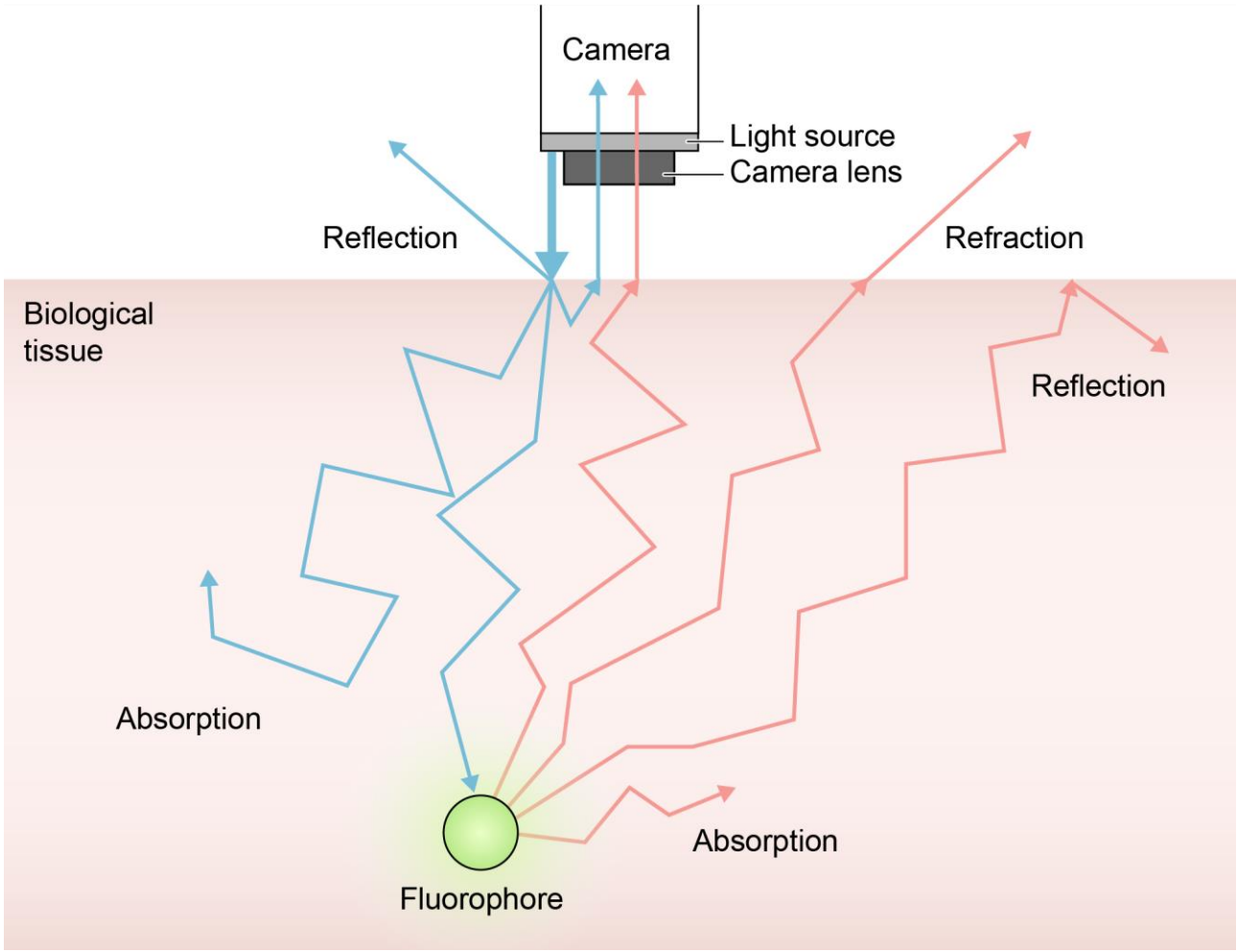

Figure 2: Basic principles of fluorescence and tissue optical properties. Fluorescent contrast generation starts with illuminating tissue at the appropriate wavelength for excitation of the fluorophore (i.e., endogenous or exogenous contrast). The fluorophore is excited from a ground state to an excited state by short-lived light absorption. Immediately after excitation, the fluorophore relaxes to a lower energy state and emits light of lower energy and longer wavelength than the excitation light. The emitted light propagates out of the tissue and is detected by the fluorescence detector that converts the recorded light into an image demonstrating the number of photons detected. Light propagation and imaging depth are limited by the tissue optical properties. Absorption causes light energy to be transferred to the tissue, decreasing the light intensity. Scattering is a process of short-lived absorption of a photon (typically) without energy loss, but

with a change of initial direction. Also, scattering decreases the ability to distinguish details. If there is no correction for tissue optical properties, the signal registered is rather qualitative than quantitative.

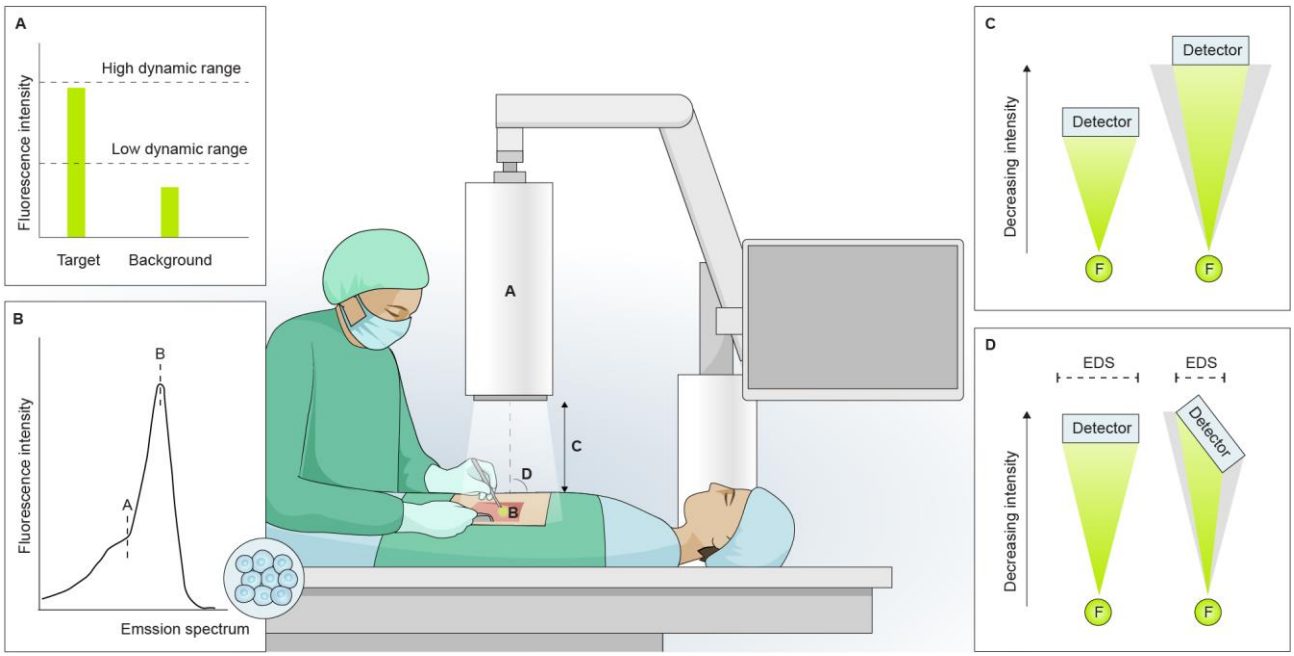

Figure 3: Potential pitfalls in fluorescence imaging studies.

A. The contrast-to-noise ratio is strongly dependent on the dynamic range of the fluorescence imaging camera system concerning the fluorescent tracer. When imaging tissue using a fluorescent tracer with a high quantum yield, the system with the high dynamic range would result in a higher contrast-to-noise ratio compared to the low dynamic range system. **B.** The fluorescence intensity detected by the fluorescence imaging camera system is dependent on the match between the systems' optical filter and the emission peak of the fluorescent tracer used. A mismatch between the emission peak and optical filter will result in suboptimal fluorescence intensity detected (wavelength A) compared to the most optimal (wavelength B). **C.** The fluorescence intensity exponentially decreases with increased working distance due to the diverging nature of light. **D.** When the detector is not placed perpendicular to the tissue of interest, the effective detection surface (EDS) that can detect emitted photons is smaller. As such, fluorescence intensity is falsely reduced, possibly leading to erroneous conclusions.

Abbreviations: EDS, effective detection surface.

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