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Essential parameters to consider for the characterization of optical imaging probes

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Abstract

The Molecular Imaging and Contrast Agents Database (MICAD) was launched in 2005 to promote the development and application of imaging and contrast agents (probes) to advance the field of molecular imaging. As of March 2012, there are approximately 1170 agents available in MICAD. Based on the modality used for imaging, the largest category of probes described in MICAD are those used for PET (41.6%), followed by agents used for single-photon emission computed tomography (30.3%), optical imaging (12.0%), MRI (9.3%), multimodality imaging (3.4%), ultrasound (2.4%) and x-ray/computed tomography (1.0%). This article is intended to be a guideline for new investigators and students who wish to characterize an optical imaging probe that will be used to perform *in vivo* molecular imaging studies. It is necessary, however, to ensure that these agents meet certain quality control parameters before they are used in various *in vitro* and *in vivo* applications.

Keywords

characterization; dyes; MICAD; molecular imaging; nanoparticles; near-infrared fluorescence; NIR; optical imaging; organic fluorophores; targeted optical probes

The Molecular Imaging and Contrast Agents Database (MICAD) [101] provides freely accessible online information regarding molecular imaging and contrast agents (probes) that are published in peer-reviewed journals available on the PubMed database [1,102]. The primary aim of MICAD is to promote the development and application of imaging and contrast agents to advance the field of molecular imaging. For inclusion in MICAD, a molecular imaging probe must have *in vivo* animal or human studies. A systemic and comprehensive search on PubMed showed that there were approximately 4800 imaging and contrast agents published as of October 2011, and hundreds of agents are added to the list every year. Molecular imaging as well as biological and chemical information about an agent is obtained from original publications in scientific journals and summarized by the MICAD editors in a chapter format for publication on the MICAD website. A single chapter is compiled for each agent. As of March 2012, there were approximately 1170 chapters

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(agents) in MICAD. To facilitate the expansion of the database, members of the imaging community are encouraged to write chapters as guest authors of the database [2].

MICAD includes chapters written on molecular imaging agents developed for PET, single-photon emission computed tomography (SPECT), MRI, x-ray/computed tomography (CT), ultrasound, optical imaging, planar radiography and planar γ -imaging. Any new imaging modality developed in the future will be added to MICAD. Based on the modality used for imaging, the largest category of probes described in MICAD are those used for PET (41.6%), followed by agents used for SPECT (30.3%), optical imaging (12.0%), MRI (9.3%), multimodality imaging (3.4%), ultrasound (2.4%) and x-ray/CT (1.0%). These percentages do not reflect the real distribution of these imaging modalities in PubMed, however, because inclusion in MICAD requires *in vivo* animal or human studies and because the MICAD editors and guest authors select agents according to their research interests and priorities. During the process of compiling the various chapters published in MICAD, the editors have observed that important basic chemical and biological information regarding the characterization of an agent is inconsistently reported in publications. Each imaging modality measures a specific signal that is used to generate imaging data, which then must be interpreted. Although there is a similar set of techniques that can be used to characterize the various imaging probes, some characterization parameters are specific for a particular modality (e.g., PET, SPECT, optical and MRI) and are subject to different considerations [3]. The characterization guidelines of PET and SPECT have been published [3], whereas those for MRI are in preparation for publication. This article is intended to be a guideline for new investigators and students who wish to characterize an optical imaging probe that will be used to perform *in vivo* molecular imaging studies.

Optical imaging is increasingly being used to monitor biological functions of specific targets *in vitro* and *in vivo* to provide real-time imaging [4–6]. Optical imaging is becoming a noninvasive complement to PET, SPECT and MRI imaging in small animals with the ultimate goal of clinical use and drug development in humans [4,7,8]. Optical imaging uses light at various wavelengths for image generation. However, the intrinsic fluorescence of biomolecules in tissues interferes with the fluorescence signal from fluorophores that absorb visible light (350–650 nm) [9]. Near-infrared fluorescence (NIR) fluorescence (700–900 nm) detection exhibits a reduction of the natural background fluorescence interference of biomolecules (e.g., chlorophyll, cytokeratins, folate derivatives, coenzymes and vitamins), providing a high contrast between target and background tissues in small animals [10,11]. Diets high in vitamins, folate and chlorophyll are thought to contribute to background NIR fluorescence [12–15]. NIR fluorophores have a wider dynamic range and minimal background fluorescence as a result of reduced tissue scattering and absorption of light compared with visible light fluorescence detection. NIR fluorophores also have a high sensitivity, attributable to low background fluorescence, and high extinction coefficients, which provide high quantum yields. The advantages and disadvantages of various noninvasive imaging modalities as well as some of their applications have been reviewed [16,17]. In brief, nuclear imaging involves the use and special handling of radioactive materials; this poses a radiation burden to the patient but provides a high sensitivity for molecular target detection [17]. MRI provides a high spatial resolution but low sensitivity for molecular target detection. One main disadvantage of optical imaging is the limited tissue depth penetration [7]. Photoacoustic imaging is an emerging hybrid (optical/ultrasound) biomedical imaging modality based on the photoacoustic effect to improve the depth penetration [18]. Optical fluorophores have been linked to nuclear and MRI probes to form multimodality imaging probes that provide synergistic advantages of both imaging modalities [19]. Various optical imaging techniques and instrumentation have been reviewed [10,11,20,21] and will not be discussed here.

There are two major classes of optical imaging probes: organic fluorophores (dyes) conjugated to small molecules, peptides or antibodies; and optical nanoparticles conjugated or coated with small-molecule ligands, peptides or antibodies for specific targeting [20–22]. Organic dyes and unmodified optical nanoparticles by themselves are often used for nontargeted studies [8,20,23]. It is necessary, however, to ensure that these probes meet certain quality control parameters before they are used in various *in vitro* and *in vivo* applications. Some of the parameters commonly studied to characterize optical imaging probes are shown in Tables 1–4. Among the various optical imaging agents, only indocyanine green, with NIR fluorescence absorption at 780 nm and emission at 820 nm, is approved by the US FDA for clinical applications in angiography [24,25], blood flow evaluation [26,27] and liver-function assessment [28,29]. It is also under evaluation in several clinical trials for other applications, such as optical imaging, mapping of the lymphatic vessels and lymph nodes in cancer patients for surgical dissection of tumor cells, and endoscopic imaging of the colon [25,30,31].

Physical & chemical characterization of optical imaging probes

Characterization of an optical probe (organic fluorophore) is an important initial step to evaluate its use in biomedical imaging [32]. All determinants should be reported as an average with a statistical analysis (e.g., correlation coefficient and standard deviation), and the total number of experiments used to derive these parameters should be reported. HPLC or column chromatography with fluorescence spectroscopy is the standard method to determine the chemical purity, conjugation efficiency and yield of a dye-conjugated small molecule probe (Table 1). The structure of an optical probe is usually confirmed with nuclear magnetic resonance spectroscopy and mass spectroscopy. Conjugation of a dye to peptides or antibodies usually requires the use of column chromatography to separate the conjugated probe from the free dye. The number or percentage/concentration of dye molecules linked to the probe must be determined so that the probe is not under or over conjugated. An under conjugated probe may not provide a strong enough signal for detection and may provide false-negative results. Conversely, excess conjugation may decrease the binding capacity of the probe due to steric hindrance; self-quenching of the signal may also result as the dye molecules are in very close proximity [33], and the concentration for self-quenching should be reported. Optical characterization of the probe (e.g., excitation/emission spectra, photostability, quantum yield and excitation efficiency, among others) is important to determine optimal imaging parameters to obtain and accurately interpret results.

For a nanoparticle-based optical probe, some additional parameters of characterization must be considered (Table 2). The hydrodynamic diameter (HD) or length of the nanoparticle should be determined, before and after modifications, with dynamic light scattering or transmission electron microscopy techniques [32,34]. Nanoparticles with HD <6 nm are usually eliminated from the body through the kidneys, whereas nanoparticles with HD >30 nm accumulate rapidly in the reticuloendothelial system [34,35]. Hence, the size of a nanoparticle probe will affect the blood half-life *in vivo*. Other factors, such as composition, dispersity (polydispersity index), shape and surface charge (ζ -potential), are also important because they influence the route of elimination from the body. On the other hand, addition of serum proteins to a nanoparticle probe increases the likelihood that it is taken up by the macrophages in the reticuloendothelial system [36] and may affect its target availability *in vivo*. For a specific targeted probe, the number of ligand, peptide or antibody moieties per nanoparticle should be reported because they affect the binding characteristics of the probe. The number of dye molecules per probe can be estimated with nuclear magnetic resonance spectroscopy, mass spectroscopy, UV-visible spectroscopy, fluorescence spectroscopy or SDS-PAGE depending on the nature of the probe (small molecule, peptide or antibody).

This information should be reported as ‘nmol/mg nanoparticle’ or ‘nmol/nmol optical active element’.

Most publications report the HD of the nanoparticles before conjugation/coating; however, this information is sometimes not available for the finished probe. In addition, the number or percentage/concentration of dye molecules, antibody molecules or fragments, and ligands per agent are sometimes not reported.

***In vitro* studies**

In vitro studies are necessary to provide important information regarding the stability and biochemical characteristics of an imaging agent (Table 3). The targeting specificity needs to be validated and can help in the design of preclinical studies *in vivo*.

The chemical stability of a probe should be determined by storing it in the formulation buffer (pH ~7.4) for *in vitro* and *in vivo* studies. The probe should be soluble and not aggregated in the formulation buffer. Its stability should also be assessed by incubation with mouse, rat or human serum at 37°C for certain periods of time. HPLC analysis of non-nanoparticle agents demonstrates how much of the probe can be expected to remain intact under these conditions. Spectroscopic analysis should be performed for qualitative and quantitative determination of the ligand and serum content on the surface of a nanoparticle-based probe before and after incubation. Loss of ligand content will decrease the binding of the probe to its target. Optical stability under these conditions should also be evaluated to confirm that the fluorescence signal can be measured. The MICAD editors have observed that only some optical probes have been studied for stability.

The partition coefficient or lipophilicity index ($\log P$) is not measured for most protein- and antibody-based optical agents, probably due to their large size (>25 kDa) and complex structure. The partition coefficient is usually measured by the partition of molecules between organic solvent (octanol) and water. More lipophilic molecules (high $\log P$ values) will reside in the organic layer than in the aqueous layer. Lipophilic molecules can integrate into the biolipid layer of cell membranes and cross the blood–brain barrier [37]. On the other hand, hydrophilic molecules (low $\log P$ values) are most likely not to nonspecifically accumulate in cells. Hydrophilic agents are more likely to be cleared through the kidneys, whereas lipophilic agents tend to be eliminated through the hepatobiliary system [38,39]. For nanoparticle-based optical probes, measurement of ζ -potential (electrical potential) is important because it is a good indicator of the stability of colloidal dispersions in solution [40]. Nanoparticles with a high ζ -potential (negative or positive) are electrically stabilized, whereas nanoparticles with a low ζ -potential tend to aggregate. Aggregation hinders the delivery of nanoparticles to the target from the blood circulation. ζ -potential should be measured before and after modification of the nanoparticles. For polymer-type probes, the addition of optical dye molecules and ligands may affect the probe’s polydispersity index (a measure of the distribution of molecular mass in a given polymer sample) and alter its biodistribution characteristics [41]. ζ -potential is not usually reported for most optical probes.

The biological characterization of a probe is initially performed in an *in vitro* system for target validation. The binding affinity (K_d) and target density ($B_{\max}$) are usually determined with the use of isolated cell membranes, recombinant receptors, enzymes, and primary or cultured cells. The cellular expression levels (e.g., mRNA and protein) of the targeted molecules should be quantified. However, an optical probe does not usually undergo such binding characterization due to an inability to quantify fluorescence signals. Instead, the binding or cellular uptake of an optical probe to targeted cells is generally analyzed with fluorescence microscopy/spectroscopy, fluorescence flow cytometry or confocal laser

scanning microscopy. The immunoreactivity of an antibody-based probe and the binding characteristics of a nanoparticle-based probe should be analyzed. The immunoreactivity of an antibody-based probe should be compared to that of the native antibody used to generate the probe to confirm that the modified antibody maintains binding characteristics similar to those of the parent antibody [42]. Blocking studies with excess non-labeled ligand or antibody should be performed to confirm the target specificity of the probe. Control studies with a nanoparticle probe involve the use of a non-conjugated nanoparticle for comparison with the probe using cells expressing the target and cells with little or no expression of the target with the probe. Some investigators do not report any binding characteristics, such as binding affinity, immunoreactivity and binding sites per cell.

The potential toxicity of optical dyes and nanoparticles is a major concern for any diagnostic agent for application in humans [43]. The effects of a probe on cell proliferation and viability should be tested with the targeted cells and in normal control cells, with a range of agent concentrations at several incubation time points. Intracellular accumulation and retention should be assessed and compared with the corresponding ligand without dye conjugation or with the corresponding nonfunctionalized nanoparticle without surface modification. Most investigators report the binding of probes to the surface of the target cell; however, only a small number of investigators report cell internalization and cell viability data, and few perform *in vitro* blocking of the probe to the target.

Animal studies

It is necessary to perform *in vivo* target validation, target specificity, biodistribution, pharmacodynamics and pharmacokinetics studies of optical probes in small animals before human studies (Table 4). These studies (*ex vivo* and *in vivo*) are generally performed in normal, disease-modeled or tumor-bearing mice. Doses of the probe administered to the animals should be reported as the number of particles, mass (e.g., mg) or molar unit (e.g., nmol). A reasonable blood half-life is essential for the probe to reach its target to generate a signal/noise ratio that is sufficient for target detection. However, the blood circulation half-life is infrequently reported. In most published studies, three animals per group are studied; however, to obtain statistically significant results, the use of five or more animals per group is recommended [44]. Data obtained from *ex vivo* biodistribution studies with excised tissues to assess the accumulation of the probe in different tissues should be quantitated with statistical analysis. A high kidney fluorescence signal will indicate that the probe is eliminated by the renal route. On the other hand, a high liver fluorescence signal will indicate the probe's elimination via the hepatobiliary route. The target tissue should have a reasonably high accumulation of the probe. Noninvasive *in vivo* optical imaging is performed either with tissue reflectance imaging or tomographic optical imaging systems [16,17]. Both *ex vivo* and *in vivo* imaging studies should be performed to provide complementary data for tissue biodistribution and target specificity.

For a targeted optical probe, it is important to confirm its binding specificity in a well-defined animal model for the target. Two major blocking studies are usually utilized to assess the specificity of the probe. First, the non-conjugated ligand or antibody (100-fold excess) is coinjected with the optical probe. Second, the animals are pretreated with the non-conjugated ligand or antibody, and the optical probe is injected 10–60 min later. The degree of fluorescence signal inhibition in the target tissues indicates the probe's specificity in binding to the target. Other controls are necessary depending on the nature of the study. Non-conjugated nanoparticles are administered in the same animal model to determine the degree of accumulation in the target that is due to enhanced permeability and retention effects. Another method of control is to compare the accumulation levels and patterns of the optical probe in targeted and nontargeted xenografts or in diseased versus nondiseased

models to assess the specificity of the probe. Immunohistochemical analysis of the target tissue sections can provide information regarding the specificity of the probe with colocalization of the probe to the target. Most investigators do not perform *in vivo* blocking studies, which involve the administration of a blocking agent to determine the target specificity of a probe. Although other methods are used to demonstrate target specificity, the *in vivo* blocking study is the ultimate proof of specificity. Some investigators report the short-term toxicity effects of optical probes in animals, but few report long-term toxicity effects.

Conclusion

The parameters discussed here are essential for the systematic evaluation of an optical probe in *in vitro* and *in vivo* imaging studies. Understanding the physical and chemical properties of a probe is necessary to determine optimal imaging parameters to obtain and accurately interpret results. The *in vitro* characterization studies using cells help in the design of preclinical studies *in vivo* with the probe. The *in vivo* target validation, target specificity, biodistribution, pharmacodynamics and pharmacokinetics studies of an optical probe are usually performed in small animals before human studies to generate safety and tissue distribution profiles as well as to confirm target specificity.

Future perspective

Optical probes hold a great deal of promise as molecular imaging agents. Inconsistent characterization of optical probes, however, inhibits the preclinical development required to ensure the safety and success of these agents for humans in clinical applications.

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Executive summary

- The Molecular Imaging and Contrast Agents Database (MICAD) provides freely accessible online information regarding molecular imaging and contrast agents (probes) that are published in peer-reviewed journals available on the PubMed database.
- Physical and chemical characterizations of optical probes are important to evaluate their use in biomedical imaging.
- The biological characterization of optical probes is initially performed in an *in vitro* system for target validation.
- Parameters for characterization of optical imaging probes for performing *in vivo* molecular imaging studies are essential to validate the probes.
- It is necessary to perform *in vivo* target validation, target specificity, biodistribution, pharmacodynamics and pharmacokinetics studies of optical probes in small animals before human studies.
- Optical probes hold a great deal of promise as molecular imaging agents complementary to other imaging modalities, such as PET, single-photon emission computed tomography, MRI, ultrasound and x-ray/computed tomography.

Essential parameters for the characterization of optical imaging probes: conjugated small molecule/peptide/antibody probe.

Table 1

Criteria	Quality control parameters
Conjugation yield	Percentage of product formed from the primary chemical, peptide, protein and antibody
Chemical purity	Determined with HPLC, NMR, MS or composition analysis
Chemical properties	Solubility, acid ionization constant (reported as pK _a)
Structure	NMR or spectroscopic analysis
Number of dye moieties per probe	Optical dye molecules conjugated to each probe entity (e.g., small molecule, peptide and protein)
Optical characteristics	Excitation/emission wavelength, quantum yield, photostability, extinction coefficient

MS: Mass spectroscopy; NMR: Nuclear magnetic resonance.

Table 2
Essential parameters for the characterization of optical imaging probes: nanoparticles.

Criteria	Quality control parameters
Composition	Elements (e.g., gold, iron, silver, indium and cadmium, among others), nontargeted coating (e.g., polyethylene glycol and dextran), number of ligand/antibody moieties per nanoparticle (nmol/ng nanoparticles), percentage of optical element per ng nanoparticles
Shape	Dot, sphere, nanotube, nanorod, nanoshell, nanocage, nanowire
Size	Hydrodynamic diameter, length, width
Polydispersity index	Distribution of molecular mass per polymer/nanoparticle
ζ-potential	Net charge of nanoparticles, stability of colloidal dispersions
Optical characteristics	Excitation/emission wavelength, quantum yield, photostability, extinction coefficient

Table 3
Essential parameters for the characterization of optical imaging probes; *in vitro* studies.

Criteria	Quality control parameters
Stability of probe	In buffer, animal serum and human serum at different temperatures and pH levels
Cell line(s)	Generation or source and oncological origin
Characterization of cell line(s)	Expression of receptor, antigen or binding site (determined with immunochemical staining, western blot analysis, ELISA or biosensor techniques)
Binding characteristics	B_{max} , K_d , immunoreactivity
Cell-labeling efficiency	Percentage of labeled cells and accumulation and internalization by cells
Target specificity	Blocking studies

B_{max} : Maximal binding capacity; K_d : Dissociation constant.

Table 4

Essential parameters for the characterization of optical imaging probes; animal studies.

Criteria	Quality control parameters
Animal description	Strain(s) (e.g., for mice and rats); details of disease model; origin, generation, location and size of tumors
Number of animals	In a group or per data point
Quantitative biodistribution	Presented as percentage injected dose/g tissue or percentage injected dose/organ, contrast enhancement ratio
Instrumentation	Description and specifications of optical imaging equipment
Imaging protocol	Quantity and route of injection; anesthesia used; time and duration of scan at each time point
Optical tomography	Qualitative and/or quantitative
Target specificity	Blocking studies
Histological confirmation	Colocalization of the probe to target