Quo Vadis, Nanoparticle-Enabled In Vivo Fluorescence Imaging?

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ABSTRACT: The exciting advancements that we are currently witnessing in terms of novel materials and synthesis approaches are leading to the development of colloidal nanoparticles (NPs) with increasingly greater tunable properties. We have now reached a point where it is possible to synthesize colloidal NPs with functionalities tailored to specific societal demands. The impact of this new wave of colloidal NPs has been especially important in the field of biomedicine. In that vein, luminescent NPs with improved brightness and near-infrared working capabilities have turned out to be optimal optical probes that are capable of fast and high-resolution in vivo imaging. However, luminescent NPs have thus far only reached a limited portion of their potential. Although we believe that the best is yet to come, the future might not be as bright as some of us think (and have hoped!). In particular, translation of NP-based fluorescence imaging from preclinical studies to clinics is not straightforward. In this Perspective, we provide a critical assessment and highlight promising research avenues based on the latest advances in the fields of luminescent NPs and imaging technologies. The disillusioned outlook we proffer herein might sound pessimistic at first, but we consider it necessary to avoid pursuing “pipe dreams” and redirect the efforts toward achievable—or ambitious—goals.

Optical imaging techniques make use of light—matter interactions to extract information about biological systems. This family of diagnostic approaches generates contrast in images by capitalizing on different physical mechanisms involving light, such as scattering, absorption, conversion to heat, and photoluminescence. Although endogenous tissue components often already provide optical signals, exogenous materials (i.e., contrast agents) are frequently introduced in the biological system to increase contrast. The search for suitable contrast agents is a major driver in nanomedicine, along with the development of new approaches capable of localized therapy. These two subfields seamlessly merge in the research area of theranostics, wherein nanoparticles (NPs) that combine therapeutic and diagnostic properties are targeted.

Whether executed with a theranostic or a contrast agent, the initial step of disease imaging is pivotal to direct the therapeutic efforts and to minimize side effects on healthy tissues. Therefore, the development of NPs meeting the requirements of an ideal contrast agent is key. One question arises though: What are the requirements of an ideal contrast agent? A clear answer to that question provides an approach to the issue of preparing contrast agents with real potential to be implemented in the clinic. In this Perspective, we attempt to answer that question, drawing inspiration from recent publications on the use of inorganic NP-based contrast agents for in vivo fluorescence imaging. In doing so, we highlight the latest trends in the field while also pinpointing exciting challenges that lie ahead. Although our discussion focuses on a specific category of contrast agents (inorganic luminescent NPs), many general considerations concerning toxicity and selectivity can be extended to any NP to be used in the biomedical realm. Our hope is that the critical assessment of the state-of-the-art presented in this Perspective will support the evermore thought-out design of NP-based contrast agents. This process entails moving away from some common misconceptions as well as implementing methodologies that will enable a smoother transition toward use by physicians. Finally, we identify the fields where NP-supported fluorescence imaging applications can have a real impact (both at clinical and preclinical stages)—a necessary and disillusioned appeal to
reason, mainly enforced by the intrinsic physical constraints of the technique.

**FLUORESCENCE IMAGING: FUNDAMENTALS, ADVANTAGES, AND DRAWBACKS**

Fluorescence imaging is based on the detection of the electromagnetic radiation within the ultraviolet-visible-near-infrared (UV-vis-NIR) range that is emitted by either tissues or exogenous elements. It is used to build up images of cells, tissues, and animals. Although the autofluorescence of tissues can be used itself for bioimaging, it lacks specificity as different tissues usually show very similar emission spectra, which are located for the most part within the visible range. In addition, the fast attenuation of autofluorescence, caused by the large optical extinction of tissues in the visible range, hampers its use for deep-tissue and whole-body anatomical imaging. Alternatively, fluorescence imaging is made possible by introducing optical probes (contrast agents) that can be excited by light into the specimen to be visualized. The use of this low-energy excitation radiation avoids the intrinsic risks associated with ionizing radiation (mainly alterations of DNA structure). The use of suitable contrast agents improves the performance of the different fluorescence imaging modalities, which can be distinguished depending on their specific biomedical aim: anatomical, functional/metabolic, or molecular imaging.

If opting to use an exogenous contrast agent, the properties of this optical probe should be tailored to the needs of the specific application. In addition to the use of non-ionizing radiation, fluorescence imaging offers relevant advantages over other imaging technologies such as magnetic resonance imaging.

**Figure 1.** The three biological windows and fluorescence anatomical imaging with near-infrared-emitting nanoparticles. (A) The three biological windows (BW) all fall in the near-infrared (NIR) wavelength range and are delimited by the optical properties of several tissue components. The absorption of water and hemoglobin (oxygenated HbO2 and deoxygenated Hb) is shown along with the scattering from skin. The overall extinction that results from the combination of light absorption and scattering from these components (and others) frames three wavelength ranges known as biological (or optical transparency) windows: BW-I, 750–950 nm; BW-II, 1000–1350 nm; and BW-III, 1500–1800 nm. Note that the limits of the BWs are not strictly defined, and often different numbers are reported. The best performance for luminescence imaging contrast agents have been obtained so far with nanoparticles (NPs) that are excited in BW-I and whose emission falls in BW-II. In (B–H), different anatomical fluorescence images acquired using NIR-emitting NPs are presented. (B) Cerebral vascular image in the BW-III region with corresponding principal component analysis (PCA) overlay showing arterial (red) and venous (blue) vessels of a C57Bl/6 mouse with Er-doped rare-earth-based NPs. Reprinted with permission from ref 34. Copyright 2017 Springer Nature. (C) Dynamic contrast-enhanced imaging with CNTs through PCA. Major features observed belong to the lungs, liver, kidneys, and spleen. Reprinted with permission from ref 28. Copyright 2011 PNAS. (D) BW-II fluorescence images (background-corrected) of the left hind limbs of two mice immediately after intravenous injection of Ag2S dots (left) or Ag2S superdots (right). Reprinted with permission from ref 35. Copyright 2020 Springer Nature. (E) BW-II/BW-III fluorescence image of blood flow in the nude mouse after injection of PEGylated Ag2S quantum dots (QDs) and (F) the corresponding PCA. Reprinted with permission from ref 36. Copyright 2014 Elsevier. (G,H) High-resolution, high-speed multicolor angiography images of a glioblastoma tumor through a cranial window using QDs composite NPs. Reprinted with permission from ref 37. Copyright 2017 Springer Nature.
The excitation/emission wavelengths of these NPs were tuned to best results have been obtained when the operating ranges such as carbon dots (CDs) and carbon nanotubes (CNTs). The most relevant results have been obtained by using rare-earth-based NPs (RENPs), semiconductor nanocrystals (among them, quantum dots; QDs), and carbon-based nanostructures (carbon dots (CDs) and carbon nanotubes (CNTs)).

The extinction of light in a medium as an exponential function, which is even more accentuated through optically dense biological tissues (in both the excitation and emission paths). This extinction entails scattering and absorption of photons. As a result, the penetration depth achievable with fluorescence imaging inside biological tissues is greatly limited, both in comparison with previously mentioned imaging modalities (i.e., MRI, PET, CT) and with optical imaging methods relying on the synergy between diffuse light (scattered) and advanced processing techniques. Therefore, even for small animals, infrared fluorescence imaging-based tomography for the acquisition of three-dimensional (3D) images of sufficient resolution appears to be a distant dream, although first steps are being taken.

Yet, in the context of contrast-agent-based fluorescence imaging, working in parallel on the fronts of increasing the brightness of the contrast agent’s fluorescence signal and its spectral position, a sizable increase in the achievable penetration depth has been witnessed in recent years.
DEGRADABLE NANOPARTICLES: ADVANCES TOWARD ZERO TOXICITY

This Perspective was inspired by the paper by Liu, Hong, and co-workers in the December 2020 issue of ACS Nano, which reports RENPs with higher solubility in biological environments compared to canonical RENPs such as NaYF₄-based NPs (Figure 2). NaREF₄ NPs are known to undergo degradation upon exposure to aqueous media containing phosphate groups, particularly at low NP concentrations. The RENPs reported by Liu, Hong, and colleagues are composed of K₂/ZrF₄, a material whose “soft” crystal lattice imparts superior water solubility to the system. Indeed, the lattice features [ZrF₄]⁻ units and K⁺ ions as building blocks that can be easily solubilized by water. The distorted pentagonal-bipyramidal coordination environment with O₆ site-symmetry experienced by Zr⁴⁺ offers a favorable site for RE³⁺ substitution, given its high coordination number. In the structure, the 7 F⁻ anions occupy > 100 dynamically equivalent positions, a situation that possibly underscores a “fluidity” of the lattice and a high tolerance to defects. In fact, RE³⁺ ions can be doped at high concentration in the structure despite the charge and size mismatch with Zr⁴⁺, which is bound to introduce anion vacancies in the lattice upon substitution. Both NIR-emitting ions (Nd³⁺/Yb³⁺) and the iconic upconverting pair Yb³⁺/Er³⁺ can be doped in the structure, thus yielding downshifting or upconversion emission under NIR excitation within biological window (BW)-I and BW-II. Importantly, both high and low pH values prompt dissolution of the RENPs at a fast rate: A behavior observed also after the dispersion of the hydrophobic RENPs in peanut oil and subcutaneous injection in mice. The authors followed the dissolution within animals spectroscopically as well as via mass spectroscopy on collected excretions and harvested organs. They observed almost complete excretion of the solubilized material, which dramatically reduces the risk of toxicity due to accumulation of particles or ions (Zr⁴⁺ in this case) at specific organs, such as lungs and liver. At the same time, the upconversion emission was observed up to 2 h post-injection, a time span sufficient to perform imaging. Despite the relevance of the results published by Liu, Hong, and co-workers, the potential application of their visible emitting NPs for in vivo imaging is limited due to the strong tissue extinction in this spectral range (Figure 1A). This limitation could easily be overcome by an adequate choice of RE ions. For example, as already proposed by the authors of the study, one can move from the Er/Yb pair to Nd/Yb to enable shifting the emission wavelength from the visible to BW-II. This alteration yields a nanostructure capable of deep-tissue, in vivo imaging that is soluble in biological environments.

These RENPs, which diligently vanish after they have done their job as contrast agents, constitute a step in the right direction for future research efforts on fluorescence imaging for in vivo applications. Indeed, these RENPs address one main concern linked to the use of NPs in biomedicine generally speaking, namely their long-term toxicity owing to accumulation in organs. Achieving dissolution of the NPs is one way to approach the issue of accumulation. However, this strategy only works for NPs whose dissolution products exhibit negligible toxicity. Most of the NPs investigated for biomedical applications are far less soluble in biological media and/or are composed of materials that can be leached into potentially cytotoxic ions (e.g., Ag⁺ and Cu²⁺). Moreover, an extended circulation time is usually required to enable NP accumulation at the tissue of interest (to be imaged or treated). Strategies have been developed to design stealthier (i.e., biomimetic) NPs to avoid premature uptake from macrophages (vide infra). In those cases, it is beneficial to limit the NP dissolution further (via suitable surface coatings) and rely on their clearance. Intravenously administered NPs usually undergo elimination mainly via renal (smaller NPs, <5.5 nm) and hepatobiliary elimination (larger NPs). The former mechanism is better understood than the hepatobiliary pathway; thus, future efforts should be devoted to increase our understanding of the complex mechanisms underpinning the latter. To that end, Chan and co-workers recently investigated the barriers encountered by model Au NPs of different sizes during hepatobiliary elimination after intravenous injection (Figure 3). Combining their results with previous literature,

![Figure 3. Mechanism of hepatobiliary elimination for non-soluble nanoparticles (NPs) in the liver sinusoid proposed by Chan and co-workers. The authors suggest that, after intravenous injection, the NPs enter the liver and are subsequently taken up by Kupffer cells in the liver sinusoid (A). The larger circulating NPs are sequestered more easily, whereas smaller NPs cross the sinusoidal endothelium (B). Upon removal of the Kupffer cells, the larger NPs can also more easily extravasate. However, the fenestrae size limit of the liver sinusoidal endothelial cells can hinder this process (C). Lastly, the NPs accumulate at the space of Disse, where hepatocytes take them up, favoring the NP transport into the bile canaliculus (D). The final step is constituted by the transit of the NP outside the liver and into the intestine, from where they are eventually expelled from the body through the feces. Adapted from ref 48. Copyright 2019 American Chemical Society.](image-url)
CHARACTERISTICS OF A nanoparticle-based fluorescence imaging contrast agent

In a more holistic way, there are several points to consider when designing NPs for biomedical use, some of which are specific to the case of NP-based fluorescence imaging contrast agents. To that end, in this Perspective, we identify the following aspects as the most important ones to take into account for future experimental efforts in the field.

- Toxicity: This is a rather broad and multifaceted issue, and control over it requires attention to the composition of the constituent material, NP surface chemistry, and size/morphology.

- Stealth properties: The NPs should elude the lines of defense of the organism, residing within the body long enough to accumulate at the site of interest rather than being prematurely cleared.

- Specificity: The NPs should bind as specifically as possible to the targeted tissue, aiming for a "magic bullet"-like type of properties and overcoming possible physical barriers within the body.

- Range of excitability and emission: Light of specific wavelengths interact less with the constituents of tissues, thus affording deeper penetration within the human body as well as less spectral distortion.

- Brightness: The more photons that are emitted per photons shined on the NP, the more effective the contrast agent will be because it can afford higher penetration depth, contrast, and resolution (both spatial and temporal).

The next sections will specifically deal with these aspects, reviewing those we believe have been highlights in past years and suggesting future directions of investigation.

Minimizing Toxicity and Immune Response while Enhancing Specific Targeting. Human beings did not invent NPs. In nature, they can be found in dust, sand, volcanic ash, and even in some biological entities such as magnetotactic bacteria or viruses (which are nanosized themselves). However, anthropogenic activity has led to an increased presence of NPs in the environment, which translates to heavier exposure to a greater variety of materials. Before the era of nanotechnology, NPs were grouped with larger submicrometric materials in the broader category of ultrafine particles (UFPs), a term still used in Toxicology to refer to this class of tiny materials. Therefore, although the term nanotoxicology appears in the literature between the years 2003/2004, concerns and studies about the effect of NPs (or UFPs) on health predate the coinage of this word.

Exposure to NPs in nature mainly occurs via inhalation, and from the tissue of the upper and lower respiratory tract, the NPs can enter the bloodstream and/or translocate to other systems. NPs can also enter the body via skin contact (e.g., sunscreen) and oral ingestion, and the advent of nanomedicine will result more consistently in the direct injection of NPs into the body. The latter exposure route is the one most likely to be of interest in the framework of fluorescence imaging for in vivo applications and has been shown to pose higher risks connected to NP accumulation. Importantly, once the NPs are in the bloodstream and/or accumulated at the site of interest, they can elicit an immune response. This side effect is to be avoided, but what are the features of NPs that play a role in this context? In the past couple of decades, several studies have highlighted the effects of NP composition, size, morphology, and surface chemistry/charge in the human body. Before delving into these aspects, it is important to mention that it is not always easy to investigate their effects separately, owing to the difficulty in carrying out such investigations ceteribus paribus (i.e., all other conditions fixed).

Controlling particular variables is somewhat possible within the framework of a single study; however, it is almost impossible to compare the results obtained in different investigations carried out around the world due to the lack of standardization in nanomaterial toxicity assessments and the inherent difficulty in controlling the properties of nanomaterials on a batch-to-batch scale in a fully reproducible way. Setting standards in terms of procedures and parameters reported in each study is of utmost importance to advance the knowledge in the field of nanobiotechnology. To that end, Caruso, Crampin, and co-workers have already proposed guidelines for minimum information reporting in bio–nano experimental literature, openly encouraging the bio–nano science community to adopt a "reporting standard" to enhance the quality and reuse of published research. Following such guidelines should become the praxis for a more impactful and relevant advancement of our knowledge. We argue that efforts to screen for...
compliance to this good practice at the editorial level will also be most beneficial in this regard.

Before discussing NP toxicity, stealth properties, and targeting ability in more detail, we should point out how the use of artificial intelligence (AI) is emerging as a powerful tool to predict the toxicity and fate of NPs possessing different compositions, sizes, morphologies, and surface chemistries (Figure 5). The use of these neural networks requires large amounts of data as a training set to then be able to predict the behavior of a NP reliably. Therefore, the identification of a minimum set of parameters to be provided in studies focusing on the biomedical application of NPs (and that can be used to feed the learning algorithm) is key to support the advancement of this predictive tool. As we detail further toward the end of this Perspective, we believe that the contribution of AI to the future of luminescence imaging does not end here.

**Chemical Composition.** Since 2003, Europe has banned heavy metals from most vehicle components, and an extension of this ban to electronic devices has been on the table for a long time. Along with mercury and hexavalent chromium, cadmium and lead are on the blacklist; coincidentally, they are among the most used metals for producing highly efficient QDs. Cd-based QDs are the benchmark in terms of luminescence intensity in the visible part of the spectrum, with photoluminescence quantum yields (PLQYs, number of emitted photons per number of absorbed photons) approaching unity. PbS QDs, particularly in their PbS/CdS core/shell form, are uniquely interesting for in vivo biomedical applications owing to their brightness and NIR working capabilities, which ensure better penetration depth (vide infra). Admittedly, exceptions to the ban have been introduced, due to technological limitations that prevent obtaining the same standards achievable with materials and processing techniques involving heavy metals. However, it is natural to think that if heavy-metal-containing QDs have an uncertain future in several technological fields, it is highly unlikely that similar NPs will work their way into clinical practice. One of the main issues is in the possibility of metal ion leaching within the body. One might argue that the total amount used is generally small (see Table 1), hence, the benefit of accurate detection leading to a reliable diagnosis should greatly outweigh the risks associated with the toxicity of the contrast agent. However, at times it is necessary to repeat the imaging procedure more than once to assess the development of the disease or the efficacy of the treatment. Cumulative effects can therefore come into play. Moreover, the disposal of heavy-metal-contaminated material is a problem, both at the level of NP production and after (possible) use at the clinical level, posing a threat mainly to aquatic life and soil. Therefore, heavy-metal-free NPs are preferable over classical, Cd- and Pb-based QDs. (Ag,Cu)In(S,Se,Te)2 (even though Ag+ and Cu+/2+ are also cytotoxic), InAs, InP, and Ag,S are possible candidates as far as QDs (or semiconductor nanocrystals more broadly speaking) are concerned. Regarding the other class of candidates for luminescence imaging, RENPs, with a real potential to be used in biomedical applications, are usually based on fluorides or oxides and often contain alkaline metals. Whereas alkaline metal ions can be considered safe, the toxicity of RE ions is debated; however, they are generally considered less toxic than heavy metals. Toxicity of RE ions is a matter of concern particularly regarding exposure in the workplace via inhalation. Nonetheless, further studies have shown that continuous exposure via ingestion or injection also has negative effects, including damage to various organs (liver, kidneys, heart, brain; see Table 3 in ref 78 for more details). This negative repercussion is far from unexpected, because RE ions are used as catalysts in many reactions, are known to interact with biological molecules, and are thus likely to act as substrate for unwanted reactions within the body.

To prevent metal ion leaching from NPs, a protective shell of inert material can be grown on the surface of both QDs and RENPs, a strategy that usually has the advantage of also increasing the brightness of the luminescent NP. ZnS and CaF2, both of which do not contain toxic elements, are two exemplary materials for shelling QDs and RENPs, respectively. In addition, Santos et al. recently demonstrated how ultrastar laser irradiation can be used to build a protective layer on Ag,S NPs, ultimately resulting in an increased luminescence bright-
Table 1. Selection of Studies Where Pb-Containing Quantum Dots (QDs) Were Used As Contrast Agents for Fluorescence Imaging

| material | PLQY (%) | λ_{em} (nm) | surface | material composition | mouse | injected (µL) | total mass injected (µg/kg) | ref |
|----------|----------|-------------|---------|---------------------|-------|--------------|----------------------------|-----|
| PbS/CdS  | 1270     | 75          | NA      | mercaptopropionic acid | female CDBL/6 | 100 | 0.004 (3 × 10^{-7}) | 75  |
| PbS/CdS  | 1650     | 2.2         | NA      | oleyamine-branched poly(acrylic acid) + PEG | female C57BL/6 | 200 | 0.65 (1.7 × 10^{-7}) | 81  |
| PbS/CdS  | 1060     | 16          | NA      | oleyamine-branched poly(acrylic acid) + PEG | female nude | 100 | 0.6 (1.2 × 10^{-7})  | 82  |
| PbS      | 1300     | 20.3        | NA      | oleyamine-branched poly(acrylic acid) + PEG | male nude | 30 | 0.04 (1.3 × 10^{-7}) | 83  |
| PbS      | 1630     | 16          | NA      | oleyamine-branched poly(acrylic acid) + PEG | Balb/C | 100 | 0.001 (3 × 10^{-7})  | 84  |
| PbS      | 903      | 200         | NA      | silic | female nude | 0.25 | 0.05 (1.7 × 10^{-7}) | 85  |
| PbS      | 900      | 200         | NA      | dihydrolipoic acid | female nude | 6 | 0.6 (1.2 × 10^{-7})  | 86  |

Because quantum dots and rare-earth nanoparticles have operation capabilities in both the visible range and the near-infrared, they are suitable for in vitro as well as in vivo use.

All this being said, we argue that in the case of a single exposure to inorganic NPs for diagnostic purposes, the drawbacks and risks for the patient associated with the intrinsic toxicity of elements composing the NPs could (in general) be overlooked. However, problems might arise in the study of chronic or slowly developing diseases that would require the acquisition of multiple images (i.e., the repeated administration of inorganic NPs). In this case, the concentration of NPs in organs retaining them could overcome the damage limit. In preclinical units, the use of NPs containing hazardous elements could be justified, particularly for ex vivo or in vitro experiments aimed at investigating biological phenomena. A gradual farewell, particularly from NPs containing heavy metals, is both far-sighted and judicious. It is foreseeable that restrictions on the use of those hazardous materials might be put into place in the (more or less distant) future. In addition, the toxicological impact of materials containing heavy metals should be considered at different stages of the NP life cycle (production, handling, storage, and disposal). And from this point of view, the risks increase, due to cumulative toxic effects and the possibility of these elements entering the food chain.

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Size and Morphology. One of the important characteristics of NPs is their large surface-to-volume ratio. This geometric consideration is pivotal because it translates to a higher surface available for interaction with the environment. The surface of a material—particularly at the nanoscale, where exceedingly small curvature radii are found—is substantially different from the bulk, featuring vacancies, dangling bonds, and strained lattice.33,34 Thus, overall, the surface of NPs is disseminated with high-energy sites that are highly reactive. Moreover, the surfaces of QDs and RENPs are inherently positively charged due to the presence of exposed metal ions with unsaturated valency.92 These two last considerations become more relevant when designing surface functionalization strategies. Given the above considerations, it is not surprising that often a trend has
been observed correlating smaller NP size with greater toxicity,97,98 although this is not always the case and case-by-case distinctions should be made (Figure 7).99

The size of NPs also affects their penetration capability through specific physical barriers. For example, smaller (20 nm) insulin-coated NPs were found to more efficiently cross the blood–brain barrier (BBB) than larger ones (50 and 70 nm).100 Similarly, in another study, polysorbate-coated polybutylcyanoacrylate NPs with a size smaller than 100 nm were shown to cross the BBB more effectively.101

At a cellular level, Guo et al. showed that hydrophobic lipid-covered Au NPs feature different kinetics in the translocation through a lipid bilayer.102 In particular, NPs with a diameter below 5 nm remain trapped in the bilayer, while larger particles can translocate through it. However, as discussed below, the crossing efficiency of NPs into specific tissues and cellular membranes is greatly influenced by the surface chemistry of the NPs, with the probabilities of...
accumulating in different brain regions being governed by the type of functionalization.103,104

We should observe at this point that NPs in dispersion might form aggregates. Due to geometric factors, these aggregates can interact differently with cells compared to single NPs of equivalent overall volume.105 Ideally, one would want a dispersion to be administered where single NPs are individually dispersed and do not aggregate over time.106,107 In this situation, possible enhanced toxicity due to aggregates would be avoided, although there are contrasting reports regarding this potential increased toxicity.105,106 Preventing the aggregation-induced broadening of the size distribution also reduces the variability in the behavior featured by the NP ensemble (see Figure 3). The colloidal stability and lack of unwanted aggregates in dispersion is, hence, another relevant aspect. To that end, the strategies developed to impart biomimetic/biocompatible features to a NP generally also afford long-term stability to the dispersion. When a NP dispersion is injected intravenously, the flow of the bloodstream is expected to prevent aggregation between the NPs. Thus, aggregation is mainly investigated when the NPs reach a specific tissue. Indeed, sometimes controlled aggregation is harnessed to increase the effectiveness of the delivery at the desired site. For example, pH108,109 and microRNA-induced110 aggregation of Au NPs has been exploited as a means to promote accumulation at solid tumors and retention within cancer cells.

Size is not the only geometric factor at play when gauging the toxicity of NPs. It is a well-established fact now that CNTs, despite their biocompatible chemical composition, have inflammatory and carcinogenic potential on par with the geometrically similar asbestos fibers (Figure 7C).113 This behavior is a result of the extreme aspect ratio (length/diameter) of CNTs, as also shown by the fact that their toxicity scales with their length (from hundreds of nanometers to a few microns).112,114 The toxicity of CNTs mainly arises from their inhalation followed by their nesting within the tissues of the lower respiratory tract.115 As a result, concerns for their use in fluorescence imaging can partially be allayed because dispersions are employed to limit the presence of airborne CNTs. Aside from this specific case, the NPs used in fluorescence imaging are generally much less anisotropic with sphere-like morphologies and low aspect ratios. Therefore, save for CNTs, this aspect is one of the least worrying as far as toxicity is concerned.

Surface Chemistry. This parameter is by far the most important for determining the fate of a nanoparticle and its interactions with biological tissues and, thus, both its toxicity and its ability to target specific tissues.103 The chief impact of surface chemistry over size and morphology has been showcased several times for different NPs (e.g., Au nanorods, RENPs, and QDs).79,116 For example, heavy-metal-free InP QDs have been shown to exhibit cytotoxicity when featuring amine groups on their surface, whereas hydroxyl- and carboxyl-bearing QDs are not toxic.117 This cytotoxicity is a direct consequence of the presence of positively charged cationic species (−\(\text{NH}_2^+\)) as compared to negatively charged anionic ones (−\(\text{COO}^-\)). In general, a surface featuring positive charge is associated with higher cytotoxicity, due to its stronger interaction with the cellular membrane.118 Surface charge is also a key parameter for cellular internalization of NPs, along with size and morphology.119 The step of internalization is fundamental to guarantee the residence of NPs at the site of interest, and it usually proceeds via two types of pathways: endocytosis-based uptake and direct cellular entry. Induced aggregation can also be exploited to avoid exocytosis once the particles have been internalized via endocytosis.110 The pathway by which internalization occurs depends not only on the NP parameters but also on the cell type. For a comprehensive summary of the state-of-the-art of cell uptake and trafficking, the reader is encouraged to read the recent review by Donahue et al.119

Surface chemistry is by far the most important parameter for determining the fate of a nanoparticle and its interactions with biological tissues and, thus, both its toxicity and its ability to target specific tissues.

The subject of surface functionalization of NPs is incredibly broad, and its discussion is a consistent element in many review articles dedicated to the use of NPs for biomedical applications.118–123 Simply put, a successful surface functionalization boils down to finding the right balance between minimizing interactions with biological components that elicit an immune response (and, hence, premature clearance of the NPs), while prompting selective recognition of and interaction with cells that form the tissue of interest. Silica coating77 as well as the use of zwitterionic molecules127,128 or polymers129 are among the most explored strategies to enhance biocompatibility and extend NP circulation time. Decoration with polyethylene glycol (PEG), in particular, is often regarded as the golden standard for imparting "stealth" properties to NPs.106,131 This approach builds on the success obtained with PEGylation of proteins in terms of improving circulation time by preventing uptake from macrophages.132 However, studies have shown that PEG antibodies are produced upon repeated injections of PEGylated species.133,134 In terms of fluorescence imaging, this finding translates to a possible premature uptake of the PEGylated contrast agent, leading to a shorter circulation time and less effective accumulation at the desired site.

A successful surface functionalization boils down to finding the right balance between minimizing interactions with biological components that elicit an immune response (and hence premature clearance of the nanoparticles), while prompting selective recognition of and interaction with cells that form the tissue of interest.

But, even considering perfectly stealthy NPs, how do we achieve their selective accumulation at the desired site? The literature contains countless examples of intratumoral injection of NP dispersions followed by imaging and/or treatment of the tumor.135–137 However, these approaches have limited appeal in clinical practice, and they are justified in proof-of-concept studies, where emphasis is put on the side of the material or an innovative treatment. The ideal strategy entails the engineering of a NP so that it can be injected intravenously in the proximity of the tissue of concern and then autonomously identifies and accumulates in correspondence with the targeted cells.138 This scenario can be achieved by taking advantage of two approaches:
The formation of a so-called protein corona (PC) atop the NPs foreseen use of the NPs. This approach might also avoid or limit capabilities. Any cell membrane can be used depending on the lipid insertion) can be performed to enhance selective targeting tumors after intravenous injection of NPs.144 Despite this trend, using xenografts from patients, one can prepare cancer-cell-cellular evolution. For example, researchers have shown that by relying on the naturally available products of millions of years of evolution, one can prepare cancer-cell membranes, as well,149,150 because they are recognized as endogenous components from the body, thus eliminating risks of NP opsonization and premature clearance.

The complementarity between the moieties attached to the NPs and cellular receptors is critical to successful accumulation and cellular internalization. In some cases, physical barriers must be overcome to reach a particular tissue. The most famous and difficult barrier to cross is arguably the BBB, whose effectiveness in preventing nonspecific crossing (and, thus, in protecting our brain) is also the reason for the difficulty in accessing the brain with NPs through the circulatory system.145 However, the brain is not the only organ/tissue that is difficult to reach. A great deal of effort is being invested in improving the targeting efficiency of tumors after intravenous injection of NPs.146 Despite this trend, the current targeting efficiency (percentage of injected NPs reaching tumor site) has been reported to be below 1%. Therefore, future investigations devoted to modifying the pharmacokinetics of injected NPs to increase the targeting efficiency are desirable.

We believe that significant future research efforts for NP surface functionalization should be directed toward the personalization of nanomedicine. One powerful approach is to use the cell membranes already present in the body,147 thus relying on the naturally available products of millions of years of cellular evolution. For example, researchers have shown that by using xenografts from patients, one can prepare cancer-cell-membrane-coated NPs for personalized cancer treatment or tumor imaging (Figure 8).148 This approach exploits the tendency of tumor cells to adhere one to another, which can be harnessed to promote accumulation of coated NPs at the tumor site. Red blood cells and platelets can be used as sources of biomimetic membranes, as well,149,150 because they are recognized as endogenous components from the body, thus eliminating risks of NP opsonization and premature clearance.

Further functionalization (either via surface conjugation or lipid insertion) can be performed to enhance selective targeting capabilities. Any cell membrane can be used depending on the foreseen use of the NPs. This approach might also avoid or limit the formation of a so-called protein corona (PC) atop the NPs when they enter a bodily fluid (Figure 9).151 This PC is composed of proteins found in the body fluid, and its composition determines the fate of the NP, governing the interaction with biological species.152 Indeed, even PEGylated nanoformulations approved for medical use become covered by the PC, which has the fingerprint of the environment in which the NPs are introduced.153 The use of cell membranes that are already present in the body to coat the NPs should minimize issues related with PC formation and changes in the NP surface chemistry induced by it. Customization of nanoformulations by encapsulation of NPs within the membranes of cells extracted from the patient and their further functionalization with...
molecules to enhance active targeting is expected to open the door to ever more effective diagnosis and treatment of a number of diseases. As we discuss in the next section, for the specific case of luminescence imaging, these opportunities are restricted by some physical considerations that are intrinsic of the imaging modality.

Brightness and Tissue-Induced Attenuation: Will We Ever Give It the Required Attention? In NP-based fluorescence imaging, the signal level is of paramount importance. An ever-increasing number of technologies aimed at improving the contrast between the signal level and the autofluorescence present in biological tissues have been proposed of late. Perhaps the most remarkable technology entails the incorporation of time-dependent analysis of fluorescence images obtained under excitation with a pulsed source.\textsuperscript{154–156} This strategy affords the removal of the background contribution of autofluorescence (characteristic of biological tissues) and significantly improves the quality of the image. Nevertheless, even after applying this general optimization procedure, some drawbacks remain difficult to circumvent under \textit{in vivo} conditions, namely tissue-induced attenuation of light and the low fluorescence efficiency of NPs. Thus, to expand the applicability of NP-based fluorescence imaging, a two-pronged approach is necessary: (i) increase the fluorescent efficiency (fluorescent brightness, defined below) and (ii) minimize the effects of the optical extinction caused by tissues.

Looking for Brighter Nanoparticles. The pursuit of evermore bright NPs is an ongoing process. In fluorescence imaging, one of the main drivers for this search is the fact that if brighter NPs are used, fewer of them are required for acquiring an image. The ability to use fewer NPs alleviates concerns related to the injection of a large number of exogenous species in the body and the possible adverse effects they might have. One of the most important parameters for improving the fluorescence of a given NP is the PLQY.\textsuperscript{157} PLQY (\(\Phi\)) is defined as the ratio between the number of photons emitted and the number of photons absorbed.\textsuperscript{158} Although researchers looking for high-brightness NPs often focus on increasing the PLQY, years of experience have shown that this strategy is insufficient, as a high PLQY coupled with low absorption efficiency of the excitation light also yields a poorly luminescent material. To account for the interplay between these two quantities, the concept of brightness (PLQY times the absorption coefficient) is introduced, which is especially useful when comparing different luminescent materials or probes.\textsuperscript{158}

Two strategies can be used simultaneously to increase the NP brightness: the development of highly absorptive NPs and the modification of the NP structure to enhance the PLQY. The second half of this two-pronged approach implies the enhancement of radiative transition probabilities. In NPs, this enhancement is achieved by reducing surface defects that act as luminescence quenchers and isolating the NP from environmental molecules whose vibrations promote nonradiative decays.\textsuperscript{159,160} The preparation of core/shell structures is an optimal approach in this context. The presence of a protective shell has been widely demonstrated to produce significant enhancements in the PLQY of luminescent NPs.\textsuperscript{74,161} Unfortunately, one-size-fits-all solutions do not exist. One example of this is PbS QDs, whose PLQY cannot be effectively increased \textit{via} ZnS shelling\textsuperscript{162} even though this strategy has proven successful with some semiconductor NPs.\textsuperscript{89,163} Some of the authors of this Perspective recently demonstrated how combining ultrafast photochemistry with traditional wet chemistry could lead to core/shell Ag$_2$S NPs with PLQY close to 10\% (as illustrated in Figure 6).\textsuperscript{85} This is a remarkable result, considering the usually low values of PLQY featured by Ag$_2$S NPs.\textsuperscript{164} Following this path, we believe that more unconventional yet effective strategies might and should be developed to push the boundaries of achievable PLQY.

One can also focus on increasing the excitation light absorption capability of the NP. In this context, QDs have an edge on RENPs, with a light absorption cross-section that is \(3−10\) orders of magnitude larger (\(\sigma_{\text{QD}}=10^{-17}−10^{-11}\) \text{cm}^2\) vs \(\sigma_{\text{RE}}=10^{-21}−10^{-20}\) \text{cm}^2). CNTs also have a larger absorption cross-section compared to single RE$^{3+}$ ions (\(\sigma_{\text{CNT}}=10^{-18}−10^{-17}\) \text{cm}^2). No reliable data could be found for single-photon excitation of CDs; multiple-photon excitation processes are instead much better quantified in these NPs.\textsuperscript{165} Given the poor absorption capabilities of RE$^{3+}$ (owing to the forbidden 4f–4f nature of most of the electronic transitions involved in the photoluminescence of these ions),\textsuperscript{166} RENPs are contrast agents that would substantially benefit from a boost of their light-harvesting prowess.\textsuperscript{167,168} To that end, researchers have coupled semiconductor NPs and RE$^{3+}$ both decorating RENPs with Ag$_2$S\textsuperscript{169} or Ag$_2$Se\textsuperscript{170} NPs and doping RE$^{3+}$ ions in QDs.\textsuperscript{171} These strategies are incredibly advantageous on paper, but their efficiency strongly depends on (and is often limited by) how effectively the energy is transferred from the absorbing moiety (semiconductor NP) to the emitting RE$^{3+}$ ion. This consideration is equally relevant when organic dyes (antennas) are employed as light harvesting species for the enhancement of RE$^{3+}$ brightness.\textsuperscript{168} The use of plasmonic moieties coupled to luminescent NPs is another highly coveted strategy, which has been used on several occasions to enhance the absorption and/or emission efficiency of RENPs\textsuperscript{172} and QDs.\textsuperscript{173} Harnessing the plasmonic effect is also challenging because quenching can be prompted, rather than photoluminescence enhancement, if the relative position between plasmonic moiety and fluorescent NP is not precisely controlled.\textsuperscript{174}

All in all, we believe that a combination of strategies to increase PLQY (\textit{e.g.}, \textit{ad hoc} developed shelling procedure) and absorption (\textit{e.g.}, plasmon-induced improvement of absorption and/or emission probability) would lead to brighter contrast agents whose use could improve the performance of fluorescence imaging.

A combination of strategies to increase photoluminescence quantum yield and absorption could lead to brighter contrast agents whose use would improve the performance of fluorescence imaging.

Tissue Attenuation. Even with the brightest possible NP as the contrast agent, the problem of low-contrast images still poses a serious impediment. Further limitations need to be considered, some of which could be overcome and others would have to be embraced by the scientific community. The latter is the case, for example, of the conditions imposed on the irradiation dose.\textsuperscript{175–178} The issue of light attenuation in a biological system is difficult to tackle, as it is entirely determined by the optical properties of the tissues (Figure 10).\textsuperscript{179–181} Although the exploitation of BWs in the electromagnetic spectrum minimizes these problems,\textsuperscript{1,182,183} the strategy does not completely
resolve them. For the sake of illustrating the implications of these restrictions, let us consider an idealized luminescent NP with a quantum energy efficiency (defined as the ratio between the energy output and the energy input)\textsuperscript{158} of 100%. Even in the situation where this material is found underneath a layer of one of the most transparent human tissues in the BWs (gray matter, for example),\textsuperscript{184} both the excitation and emission light are still absorbed and scattered. For our purposes, let us also consider the use of a BW-I excitation source, as it has been shown to minimize undesired heating effects,\textsuperscript{30,31,185} and that the emission falls within BW-II. Considering that the more external part of the gray matter has a usual thickness of 10 mm and average attenuation coefficients of 0.07 mm\(^{-1}\) and 0.06 mm\(^{-1}\) in BW-I and BW-II, respectively, this means that 50% of the subsequently generated emission is not transmitted to the detection system. Compared to the case in which the sample is directly illuminated by the laser, in this example 73% of the intended emission is lost. Such a value might still be acceptable for biomedical applications. However, had another tissue been chosen, the total loss would be even higher. The skin, for example, has average attenuation coefficients of 16.0 mm\(^{-1}\) and 16.6 mm\(^{-1}\) in BW-I and BW-II and would provide a total loss of more than 90%\textsuperscript{18,186} The situation is worsened by real-life scenarios where several tissues with different optical properties are simultaneously affecting photon propagation. In addition, NPs used as contrast agents have quantum energy efficiencies below 100%.

Pathways to Address the “Tissue Attenuation” Issue. To alleviate the negative repercussions of these large attenuations, one could try either of these two solutions: (i) increase the excitation power so that more excitation photons reach the contrast agent, causing more photons to be emitted and, therefore, detected or (ii) increase the exposure time so that more signal is collected. Although the first approach may prove useful in fields such as fluidics and electronics, it is not viable when dealing with biological tissues. Because certain safety guidelines need to be followed, the irradiation dose must never surpass a well-established maximum value.\textsuperscript{175-178} Though there are minor disagreements in the literature, a maximum excitation power density of 330 mW/cm\(^2\) is generally accepted for the study of tissues under normal conditions.\textsuperscript{175,187} If a photothermal treatment is being induced, however, this maximum value could reach up to 2 W/cm\(^2\).\textsuperscript{188} Be that as it may, increasing the excitation power is neither a safe nor a universal approach. Contrastingly, increasing the exposure time does not necessarily compromise the health status of the tissue if the irradiation dose is within the safety limits. Increased exposure time generates other problems, however, including loss in temporal resolution and accumulative heating during illumination. Furthermore, the effectiveness of the approach might also be compromised by the technological limits of the detection systems. For example, even after increasing the exposure time, if the transmitted intensity is only slightly larger than the noise-equivalent irradiance (NEI; \textit{i.e.}, the flux density that results in a signal-to-noise ratio of 1) of
the camera, the detected signal will be too poor for an accurate analysis.

Because these problems are mainly imposed by the optical properties of the various biological tissues, their corresponding

| Tissue | $\mu'_{\text{om}}$ (cm$^{-1}$) | $\mu_{\text{abs}}$ (cm$^{-1}$) | Surrounding Tissues | $I_{\text{em}}/I_{\text{exc}}$ (%) | $I_{\text{em}}^{\text{opt}}/I_{\text{exc}}$ (%) | $\beta_{\text{EM}}^{\text{opt}}$ (W/cm$^2$) |
|--------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Skin$^{17,185}$ (1.5 mm) | BW-I: 15.6 | BW-II: 0.38 | -- | 8.2 | 9.0 | $2.4\times10^{-3}$ |
| BW-II: 16.3 | BW-II: 0.34 | Skin (1.5 mm) | 2.8$\times10^{-3}$ | 1.3$\times10^{-3}$ | $1.2\times10^{-10}$ |
| Adipose tissue$^{165}$ (7 mm) | BW-I: 11.2 | BW-II: 8.68 | BW-I: 1.08 | BW-II: 1.00 | Skin (1.5 mm) | 8.1$\times10^{-7}$ - 8.3$\times10^{-4}$ | 1.1$\times10^{-7}$ - 8.8$\times10^{-4}$ | $2.9\times10^{-18}$ - 2.4$\times10^{-11}$ |
| BW-II: 5.95 | BW-II: 5.73 | BW-II: 0.51 | Adipose tissue (7 mm) | 5.0$\times10^{-6}$ - 6.0$\times10^{-2}$ | $1.5\times10^{-6}$ - 2.2$\times10^{-2}$ | $2.4\times10^{-16}$ - 4.3$\times10^{-8}$ |
| Muscles$^{165}$ (10-30 mm) | BW-I: 20 | BW-II: 16 | BW-I: 0.1 | BW-II: 0.5 | Skin (1.5 mm), possibly facial muscles (15 mm) | 6.3$\times10^{-12}$ | 6.6$\times10^{-15}$ | 1.37$\times10^{-3}$ |
| BW-II: 46.1 | BW-II: 1.29 | Skin (1.5 mm), adipose tissue (7 mm) | 3.1$\times10^{-14}$ - 1.3$\times10^{-4}$ | $6.8\times10^{-21}$ - 2.2$\times10^{-2}$ | 6.9$\times10^{-39}$ - 9.4$\times10^{-8}$ |
| Bone$^{16,217,218}$ (up to 25 mm) | BW-I: 16 | BW-II: 10.5 | BW-II: 0.02 | BW-II: 0.07 | Skin (1.5 mm), adipose tissue (7 mm), possibly muscle (up to 30 mm) | 1.8$\times10^{-4}$ | 1.2$\times10^{-4}$ | 7.2$\times10^{-14}$ |
| Dura mater$^{219}$ (2.5 mm) | BW-I: 8.27 | BW-II: 9.52 | BW-II: 0.5 | BW-II: -- | Skin (1.5 mm), Skull (6 mm) | 2.1$\times10^{-8}$ - 3.5$\times10^{-7}$ | $2.1\times10^{-11}$ - 9.0$\times10^{-7}$ | $1.4\times10^{-24}$ - 1.0$\times10^{-17}$ |
| Breast$^{220}$ (up to 55 mm) | BW-I: 12.0 | BW-II: 10.0 | BW-I: 0.13 | BW-II: 0.20 | Skin (1.5 mm), adipose tissue (7 mm) | 6.7$\times10^{-5}$ | 1.8$\times10^{-5}$ | 4.0$\times10^{-14}$ |
| Parietal peritoneum$^{221}$ (14 mm) | BW-I: 11.0 | BW-II: 12.0 | BW-II: 10.0 | Skin (1.5 mm), adipose tissue (7 mm), muscle (5.6 mm) | Skin (1.5 mm), adipose tissue (7 mm), muscle (5.6 cm), parietal peritoneum (1.4 mm) | 9.6$\times10^{-14}$ - 2.9$\times10^{-12}$ | $6.6\times10^{-13}$ - 1.5$\times10^{-13}$ | $2.1\times10^{-39}$ - 1.4$\times10^{-29}$ |
| Stomach$^{222}$ (46 mm) | BW-I: 7.59 | BW-II: 7.62 | BW-I: 1.0 | BW-II: 2.5 | Skin (1.5 mm), adipose tissue (15 mm), muscle (5.6 cm), parietal peritoneum (1.4 mm) | 4.6$\times10^{-20}$ - 2.7$\times10^{-14}$ | $1.6\times10^{-24}$ - 4.5$\times10^{-17}$ | $2.4\times10^{-48}$ - 4.0$\times10^{-33}$ |
| Liver$^{223}$ (69.6 mm) | BW-I: 9.0 | BW-II: 7.68 | BW-I: 1.0 | BW-II: 0.5 | Skin (1.5 mm), adipose tissue (15 mm), muscle (5.6 mm), parietal peritoneum (1.4 mm) | 9.9$\times10^{-14}$ - 6.5$\times10^{-9}$ | 1.1$\times10^{-11}$ - 9.9$\times10^{-11}$ | $3.6\times10^{-26}$ - 2.1$\times10^{-23}$ |
| Lung$^{224}$ (102 mm) | BW-I: 20 | BW-II: 8.56 | BW-I: 0.01 | BW-II: -- | Skin (1.5 mm), adipose tissue (7 mm), chest wall (14 mm) | 2.4$\times10^{-54}$ - 7.9$\times10^{-16}$ | 9.1$\times10^{-38}$ - 7.0$\times10^{-21}$ | $7.2\times10^{-334}$ - 1.8$\times10^{-40}$ |
| Intestine$^{219}$ (73.3 mm) | BW-I: 9.66 | BW-II: 8.56 | BW-I: 1.5 | BW-II: 2.3 | Skin (1.5 mm), adipose tissue (15 mm), muscle (3 mm), parietal peritoneum (1.4 mm) | 2.6$\times10^{-5}$ - 3.6$\times10^{-5}$ | $4.1\times10^{-6}$ - 5.8$\times10^{-6}$ | $3.5\times10^{-15}$ - 6.9$\times10^{-15}$ |
| Brain (grey matter)$^{219}$ (10 mm) | BW-I: 0.7 | BW-II: 0.6 | BW-I: 0.02 | BW-II: 0.04 | Skin (15 mm), skull (6 mm), dura Mater (2.5 mm) | 1.8$\times10^{-7}$ - 3.7$\times10^{-7}$ | $1.1\times10^{-8}$ - 5.5$\times10^{-8}$ | $8.3\times10^{-20}$ - 6.7$\times10^{-19}$ |
| Brain (white matter)$^{219}$ (15 mm) | BW-I: 5.2 | BW-II: 3.0 | BW-I: 0.08 | BW-II: 1.00 | Skin (15 mm), skull (6 mm), dura mater (2.5 mm), grey matter (up to 29 mm) | 5.4$\times10^{-6}$ - 7.2$\times10^{-5}$ | $3.7\times10^{-7}$ - 6.3$\times10^{-6}$ | $6.6\times10^{-17}$ - 1.5$\times10^{-14}$ |

*Estimation of the emitted light that is detected (column 4), the excitation light that reaches the inner part of the tissue (column 5), the detected power density after considering an excitation irradiance of 330 mW/cm$^2$ and luminescent nanoparticles (NPs) with perfect quantum energy efficiency (column 6). In the more internal organs, the NPs were considered to be located between the center of the organ and the proximities of its surface. The rightmost column contains the values of maximum detected irradiance and classifies the different organs according to the possibility of detecting the emission coming from nanoparticles found within them. Four categories are found: optimal (green), circumstantially positive (blue), potentially positive (orange), and negative (red). Dimensions were estimated with cryosections of the human body as provided by The Visible Human Project.$^{214,215}$

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The Visible Human Project.214,215

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solutions can only be resolved if one tries first to determine the best possible outcomes when dealing with different organs. Only then can expectations become realistic. To illustrate this concept, we evaluated the potential of narrowband NIR-emitting NPs for applications in various human organs. For the sake of brevity, we chose a model NP that can be excited in BW-I and that emits in BW-II, which is a common situation.27,189 For each case studied, the NPs were considered to have preferential accumulation in a specific organ. The optical properties of this organ, in turn, were registered (according to literature) alongside those of the surrounding tissues. By taking into account their average thicknesses in an adult, it was possible to determine, in a first approximation, (i) the fraction of excitation light that gets into the organ \( \frac{I_{\text{det}}}{I_{\text{exc}}} \) and (ii) the fraction of emitted light that gets detected, \( \frac{I_{\text{det}}}{I_{\text{em}}} \). The main results are included in Table 2 and reveal that, granted that luminescent NPs could accumulate in any desired human organ, most resulting measurements would still be problematic. Many organs provide values of \( \frac{I_{\text{det}}}{I_{\text{em}}} \) and \( \frac{I_{\text{det}}}{I_{\text{exc}}} \) below \( 10^{-86} \). Thus, depending on the excitation energy initially delivered, the emitted light might not be detected at all. To verify which cases could result in viable applications, we considered, without loss of generality, the excitation irradiance \( I_{\text{exc}} \) and the noise-equivalent irradiance \( (1 \text{ s of exposure time}) \) of InGaAs cameras currently available in the market is on the order of \( 10^{-12} \text{ W/cm}^2 \) (i.e., the allowed maximum).175 Under such conditions, the maximum detected irradiance, \( I_{\text{det}}^{\text{max}} \), is obtained through:

\[
I_{\text{det}}^{\text{max}} = \kappa_{\text{exc}} \left( \frac{I_{\text{det}}}{I_{\text{em}}} \right) \left( \frac{I_{\text{det}}}{I_{\text{exc}}} \right)
\]

where \( \eta \) is the quantum energy efficiency of the NPs. If we, once again, consider the best-case scenario where \( \eta \) is equal to 100% (maximum imposed by energy conservation), we then find the data included in the rightmost column of Table 2. By taking into account that the noise-equivalent irradiance (at 1 s of exposure time) of InGaAs cameras currently available in the market is on the order of \( 10^{-12} \text{ W/cm}^2 \),192 then the straightforward conclusion is that NP luminescence could only be detected in a small set of human organs. Broadly speaking, Table 2 presents at least four different situations:

- **Optimal:** \( I_{\text{det}}^{\text{max}} \) always exceeds the order of \( 10^{-12} \text{ W/cm}^2 \). The organs that satisfy this condition are highlighted in green in the last column. If luminescent NPs are located within these organs, their light is very likely to be detected.

- **Circumstantially positive:** \( I_{\text{det}}^{\text{max}} \) is circumstantially higher than \( 10^{-12} \text{ W/cm}^2 \) (highlighted in blue). This is the case, for example, of bones. Depending on which bone is selected, there might be ample muscle tissue around it and, consequently, more attenuation. Other bones (such as those in the hands and feet) might be less affected by attenuation.

- **Potentially positive:** \( I_{\text{det}}^{\text{max}} \) between \( 10^{-18} \text{ W/cm}^2 \) and \( 10^{-12} \text{ W/cm}^2 \) (i.e., lower than NEI but only by a few orders of magnitude). Although below the detection limits, the possibility of detecting light coming from these organs could have been negatively affected by the first-order nature of the approximation. This issue is important to consider because a simple Beer–Lambert law analysis cannot account for the role of the geometry of the tissue and, for example, the non-additivity of the scattering effect. Furthermore, there is no consensus on their optical coefficients (differences of up to 40% in the mean value have been reported in different works in the literature).193–199 These facts, together with the possibility of increasing the exposure time or the excitation irradiance (photothermal therapy), could actually result in light being detected. Therefore, by recognizing the limits of our approximations and the accuracy of the data provided in literature, we were led to classify these as potential candidates to be imaged via NP-enabled fluorescence imaging. They are highlighted in orange.

- **Negative:** Organs that had a \( I_{\text{det}}^{\text{max}} \) below \( 10^{-18} \text{ W/cm}^2 \). For these organs, the application of luminescent NPs is not viable even after considering the discrepancies in literature over their optical properties and the simplified nature of the approximations. To justify such a value, one only needs to think about the worst-case scenario where (i) the Beer–Lambert Law, (ii) the discrepancy on the optical coefficients, and (iii) the low integration time (or low excitation irradiance) is each reducing the value of \( I_{\text{det}}^{\text{max}} \) by 2 orders of magnitude. If that were the case, \( I_{\text{det}}^{\text{max}} \) would still fall behind \( 10^{-12} \text{ W/cm}^2 \). Such organs are highlighted in red.

Careful examination of Table 2 suggests that, with the optical coefficients currently reported, luminescent NPs and fluorescence imaging can only be feasibly applied in five main human tissues: skin, bone, muscle, breast, and the outer part of the brain (Figure 11). This conclusion is certainly more modest than what most reports on in vivo applications of luminescent NPs in animal models may suggest. The reason why these studies tend to overestimate the potential for human applications is primarily related to the reduced dimensions of the animal under investigation. If one inserts NPs into the livers of mice, an organ which for humans is outside the detection range, the maximum detected irradiance is on the order of \( 10^{-4} \text{ W/cm}^2 \) (assuming similar values for the attenuation coefficients and the dimensions of tissues provided in literature120–122) under 330 mW/cm² excitation. Such a value is far above the lower detection limit of currently available cameras. The community needs to be careful not to extrapolate their goals from animal models to humans when interpreting research results.

Figure 11. Feasible noninvasive applications of fluorescence imaging through luminescent nanoparticles (NPs). Main human tissues identified as optimal (or at least possible) for investigation with NP-based fluorescence imaging after considering the limitations imposed by the scattering and absorption of near-infrared light in biological tissues. The identification was based on the data inserted in Table 2.
Nevertheless, even if expectations are lowered, we should not disregard the effort put into luminescence-based nanotechnology in the past decades. Luminescent NPs can still play a role in the noninvasive detection and treatment of several malfunctions of the human body, including diseases such as osteoporosis, vitiligo, multiple sclerosis, stroke, breast cancer, and many others. Furthermore, the already established applications at the small-animal level are also beneficial for better understanding the biological processes that take place during malfunctions of the body. The future of the field, therefore, remains promising. Interestingly, the five human tissues that were selected for Figure 11 form the same set of tissues that have been successfully studied in a well-established technique known as diffuse optical tomography (DOT). DOT is an ongoing imaging modality in which a tissue is illuminated by NIR light from an array of sources. Under such conditions, the scattered light that emerges from the tissue is observed with an array of detectors, and then a model of light propagation is used to infer the optical properties of the illuminated tissue. Fluorescence imaging and DOT have similar applicability in that they are both limited by the level of tissue-induced attenuation. Researchers should be attentive to a overlaps between the two techniques as well as their relative advantages, disadvantages, and possible synergies. Albeit the noninvasive use of luminescent NPs in adult humans is limited to specific tissues, we are more optimistic for uses involving infants and children. Due to the reduced dimensions of their tissues, the number of organs considered viable as indicated in Table 2 would increase, potentially giving rise to more possibilities for the detection and treatment of diseases. Such a concept, in fact, has already been explored in DOT for deep-brain studies in newborn babies.

Considering all the extra quality checks that will need to be fulfilled for use in newborns, if luminescence-based nanotechnology follows a similar path, then the development of the materials should start focusing on these quality checks at the level of material design. If, however, the intention is still to keep the applicability of luminescent NPs as broad as possible (including adults), then the use of endoscopic techniques will have to be considered. If an endoscope is placed in the proximity of the tissue of interest, the light coming from there can still be collected. Under this circumstance, however, noninvasiveness could no longer be considered as one of the most attractive features of luminescent NPs.

**Raising the Stakes: Fluorescence Imaging and Machine Learning.** After laying out the problems related to the intrinsic limitations of fluorescence imaging imposed by the properties of luminescent NPs, tissue properties, and measurement setups, let us now delve into how AI can provide a helping hand in this framework. If we consider the use of fluorescence imaging in preclinical units, in addition to the problem of strong tissue-induced attenuation described in the previous section, the acquisition of fluorescence images requires complete immobilization of the animal under study. Typical integration times used for the acquisition of high-contrast in vivo fluorescence images are in the 0.1–1 s range. During this acquisition time, the displacement of a free mouse could be as large as 1 cm. This displacement causes blurring in the acquired image. Traditionally, this challenge has been solved by immobilizing the animal under study, usually by administering anesthesia. This approach not only avoids
blurring, it also enables the use of longer integration times, thus improving the signal-to-background ratio in the fluorescence images. However, depending on the goal of the imaging procedure, using anesthesia or physical constraints can be far from ideal. Anesthesia is known to alter vital functions, and several works have reported a general decrease of metabolic activity and whole-body temperature during anesthesia procedures. Thus, readouts regarding vital parameters extracted from the analysis of fluorescence images taken on an anesthetized animal could be misleading or erroneous, because the system under investigation is altered by the measurement procedure. This factor could be especially relevant when imaging organs that are more heavily affected by anesthesia, such as the brain. To overcome this limitation, imaging of freely moving animals becomes imperative. Indeed, this need has motivated the community to upgrade traditional techniques. For example, Kyme and co-workers developed an experimental setup capable of acquiring PET images in “freely” moving, awake rats. The experimental system (Figure 12) consisted of an unmodified small animal PET system, a robot-controlled animal enclosure, and an optical motion-tracking device.

Using this system, the authors were able to image the impact of drug (amphetamine) administration on brain activity without the need for immobilization. However, the system still imposed severe motion restrictions to the animal owing to the reduced space. Space restrictions limit the potential of this approach to one-animal studies and puts an end to the possibility of studying the impact that social interactions have on vital function. Nonetheless, this study demonstrates that the community is becoming aware of the importance of moving away from anesthesia to obtain results that paint a more truthful picture. The acquisition of fluorescence images of freely moving animals is a challenging task. Because of the use of excitation laser sources and fluorescence cameras, two main technical limitations need to be overcome. First, the technology required to localize the animal within the experimentation cage should be incorporated into the imaging system. Second, a new procedure for imaging acquisition and analysis to correct blurring caused by the rapid movements of the animal becomes essential. This latter point requires knowledge of the animal’s position as well as prediction of its motion. These two requirements can be satisfied simultaneously by using AI. To that end, deep learning has been recently applied for real-time tracking and motion prediction of freely moving animals.

We anticipate that, in the near future, these algorithms could be implemented in fluorescence imaging systems already working in preclinical units. Knowing the exact location of the animal is necessary not only for determining the imaging field-of-view but also for selective and accurate excitation of contrast agents within the animal. This goal could be accomplished with the use of advanced optomechanics, which, guided by the output of deep-learning algorithms, could direct the excitation light inside the experimentation cage with high spatiotemporal precision. In this scheme, AI could also be used to develop a module capable of combining information about animal position and motion together with the acquired fluorescence images to correct for image blurring. We foresee that the implementation of AI-based technologies in this field could take time, but we are convinced that the future of fluorescence imaging in preclinical units will be experimentation of freely moving animals.

Lastly, a point must be made regarding spatial resolution, which is relevant both in preclinical and in foreseeable clinical applications. As noted in Table 2, spatial resolution is primarily dependent on the scattering of the light emitted by the contrast agent at a specific depth within the tissue. Due to difference in optical properties, not all the tissues would afford the same spatial resolution in fluorescence imaging. To mitigate this issue, the use of AI could prove advantageous because it is efficient not only in image processing but also in the solution of partial differential equation-related problems (such as the propagation of light). Indeed, convolutional neural networks have been successfully applied in CT, MRI, and, more recently, in DOT. With regards to fluorescence imaging, this technique could train neural networks to reverse the problem of nonlinear photon scattering and, as a consequence, reconstruct the original fluorescence signal coming from the NPs before being transmitted through the different sets of tissues. Such an application could extend the applicability of the field and even offer information on the possible anomalies present in organs and tissues.

### PERSPECTIVE AND OUTLOOK

The fate of NP-enabled in vivo fluorescence imaging is contingent upon how specific features of luminescent NPs are upgraded in the immediate future. We identify three lines of action:

- **Establishment of standards:** Standards in the assessment and report of the characteristics of different NPs, including brightness, specificity, and toxicity, should be developed, set, and accepted community-wide in order to afford a fair and reliable comparison between different systems. With these standards in place, the research community will be able to compare the new generation of luminescent NPs rigorously and quantitatively, ranking luminescent NPs not only based on their intrinsic characteristics, but also weighing the use of such contrast agents for each specific tissue.

- **Improvement of nanoparticle interbatch reproducibility:** This goal is closely related to the previous objective because the features of luminescent NPs of a given material should not differ from batch to batch. Their performance should not depend on who fabricated them, once the synthesis protocol is officially established. In pursuit of reproducibility, the use of robots, automation of the synthesis processes, and machine learning will prove fundamental.

- **Development of effective functionalization:** As far as contrast agents are concerned, nontoxic, high-brightness NPs that emit light within carefully chosen spectral ranges are ultimately worthless if they do not reach the desired organ. The NPs specifically have to reach the site of interest, accumulate in substantial number, and reside long enough at the site to have real applicable potential. This process requires advanced surface functionalization that should be accompanied by a thorough understanding of how a NP interacts with cells and tissues at the in vivo level. With in vitro experiments, researchers have often been too optimistic when evaluating the targeting efficiency of functionalized NPs.

For all of the steps above, great support is to be expected from AI. Although still relatively in its infancy in many of the subfields discussed so far, the use of deep learning based on neural networks is envisaged to reduce the time consumed with trial-and-error approaches drastically by supporting a more rational design of contrast agents.
After setting the boundaries above, we split our outlook of the realistic chances for a broader use of luminescent NPs for in vivo imaging between two different contexts: small-animal imaging for research purposes (preclinical) and applications in humans for disease detection (clinical), facilitating diagnosis or image-assisted therapies.

At the preclinical level, NP-enabled in vivo fluorescence imaging is already a reality in the imaging facilities of research institutions around the globe. However, we note that there are two steps yet to be taken to place institutions around the globe. However, we note that there are two steps yet to be taken to place fluorescence imaging at the level of PET, MRI, PA, and CT. Luminescent NPs as contrast agents for in vivo imaging are often considered to be one echelon below small organic (fluorescent) molecules. High brightness (which is ultimately conducive to low injected doses), well-known clearance paths, and minimal related toxicity, along with knowledge of ways to engineer the fluorophore chemically, are the prominent reasons for preferring small molecules in this context. However, we argue that this preference is also the fruit of habit (i.e., years of consolidated use of small molecules). For the forthcoming generation of luminescent NPs to become a viable option compared to such an established competitor, further progress in terms of brightness, certainty about the lack of toxicity, and clearance are key. On the positive side, luminescent NPs overpower small molecules in terms of emission tunability (easily reaching BW-II/BW-III emission, narrow emission, large Stokes shift) and long photoluminescence lifetimes. This latter characteristic affords the implementation of time-gated detection approaches, which alleviate the issues related with autofluorescence.

Irrespective of the chosen contrast agent, in vivo fluorescence imaging at the preclinical level has not yet been fully exploited. Specifically, the best of what fluorescence imaging has to offer in terms of real-time monitoring (e.g., of metabolic processes) is yet to come. Moreover, based on the continuous improvement of infrared-emitting NPs and of BW-II/BW-III detection systems, we expect that whole-body 3D fluorescence imaging of small animals (which we identified as the future of preclinical fluorescence imaging) will be achieved within the next decade.

The future of clinical fluorescence imaging deserves more elaborate analysis, as it is a more complex and multifaceted scenario. In the discussion below, we primarily cite works related to QDs/semiconductors nanocrystals as examples of the proven applicability of NP-enabled fluorescence imaging. We do not wish to imply that other luminescent NPs could not pass the bar for clinical applications anytime soon, but research in semiconductor-based nanomaterials is currently providing the most promising results.

According to what we presented in this Perspective, the number of organs that are accessible via NP-enabled fluorescence imaging is relatively limited. As a result, it is highly unlikely that this imaging method could ever replace techniques that are capable of seeing deeper within the human body. However, for the set of tissues that can be observed via fluorescence imaging (skin, breast tissue, muscles, joints, brain surface), this simple, fast, and inexpensive diagnostic tool is expected to make its way to the clinic. This result will be achieved by working along the lines highlighted for achieving whole-body 3D imaging at the preclinical level in terms of better NPs, detection systems, and image reconstruction via the use of algorithms.

As we mentioned, the physical constraints regarding the applicability of fluorescence imaging in humans are relaxed in infants, owing to the intrinsic reduced size of organs and tissues. However, this increased accessibility is countered by more stringent requirements to be met from a safety viewpoint.

If we are willing to yield in part the noninvasiveness promised by NP-enabled fluorescence imaging, however, the horizon broadens. Indeed, fluorescence imaging is approved for clinical use in endoscopic and surgery-related applications. For endoscopic fluorescence imaging, optical fibers or, better, bundles of them are incorporated in the endoscopic probe. This configuration enables clinicians to reach tissues at depths that prevent the employment of the less invasive approach of external illumination/collection of the fluorescence signal. Autofluorescence and/or emission of small dyes acting as markers can be leveraged to obtain information on the status of the tissue. Although this endoscopic approach can only be used for diagnostic purposes, it is a powerful weapon in real-time fluorescence imaging of tumoral areas during surgeries. The ability to visualize the tumoral mass clearly and in real time prevents removal of unaffected tissue and helps ensure that the entirety of the tumoral mass is removed, thus averting recurrence. In that context, fluorescence imaging provides both high sensitivity and remarkable resolution (1 μm), but is hampered by the non-ideal properties of fluorophores currently approved for oncologic indications. The three fluorescence dyes currently approved by the FDA for cancer imaging are 5-aminolevulinic acid (for fluorescence-guided surgery), methylene blue (for sentinel lymph node mapping), and indocyanine green (for intraoperative tumor identification). These molecules, to different extents, exhibit nonspecific tissue distribution, lack of stability, possible toxic side effects, and excitation/emission at wavelengths that are not transmitted efficiently within tissues. In addition, because all three are small-molecule dyes, they are cleared quickly by the lymphatic system.

Imaging-assisted surgery would ultimately benefit from preferring luminescent NPs over small organic dyes. Enlarging the picture, luminescent NPs can also be flexibly engineered to achieve longer retention times and more efficient tumor targeting, to provide multimodal imaging readouts and, arguably, to lead the way toward supplying nonmorphological data. We anticipate that luminescent NPs will rival dye-based probes in clinical applications of fluorescence imaging within the next decade.

We anticipate that luminescent nanoparticles will rival dye-based probes in clinical applications of fluorescence imaging within the next decade.

To conclude, NP-enabled fluorescence imaging has a bright future, and we foresee its increasingly widespread use for several preclinical and clinical purposes. With this Perspective, we attempted to provide guidelines for ensuring that the efforts in the field are not misplaced, thus enabling researchers to reach significant goals in a timely manner.

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Notes

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