Enhancing fluorescence in vivo imaging using inorganic nanoprobes
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**Abstract**

Fluorescence imaging is a versatile tool for biological and preclinical studies with steady improvements in performance thanks to instrumentation and probe developments. The sensitive detection and imaging of deep targets in vivo is especially challenging due to the diffusion and absorption of light by the tissues and to the emission of autofluorescence from intrinsic chromophores. Fluorescent inorganic nanoparticles present interesting optical properties that may significantly differ from organic dyes. In this short review, we present recent developments in the design of these nanoprobes and their use for new in vivo fluorescence modalities which provide enhanced imaging capabilities.

**Introduction**

Fluorescence in vivo imaging is becoming a commonly used tool to study biological functions and pathologies in living model organisms, and is being increasingly developed for clinical diagnostics and treatment. [1] Fluorescence imaging typically offers access to tissues a few mm or cm below the surface. It also offers unique advantages that make it a precious, powerful and very popular technique. Besides its ease of use, low cost, noninvasiveness and absence of ionizing radiation, it possesses distinct performance characteristics. For example, it offers high sensitivity, down to the single molecular tracer, as well as straightforward multiplexing capabilities by selecting probes with different spectral properties. It can also reach unrivaled spatial and temporal resolutions. For these reasons, in vivo fluorescence imaging techniques and applications are being developed at an accelerating pace. Several organic chromophores are being used in small animal models and in clinical applications. [1-4] In general however, for these dyes, the maximum imaging depth, resolution and sensitivity are limited by the optical properties of the tissues.[5] More specifically, the major obstacles limiting imaging performance are the absorption and diffusion of excitation and emission photons, causing a loss of signal and resolution, and the emission of autofluorescence from intrinsic chromophores, giving rise to spurious background signal. Inorganic nanoparticles, on the other hand, present unique optical properties that help alleviate some of these limitations. In this short review, we do not aim to offer a comprehensive review on fluorescent nanoparticles (NPs), but instead we will focus on how their optical properties can be used to enhance the performance of fluorescence in vivo imaging.
**Near infrared emitting nanoprobes for in vivo imaging**

The near infrared window (700-1300 nm) enables deeper imaging thanks to reduced light absorption and scattering compared to the visible range, where intrinsic chromophores such as hemoglobin absorb, and to longer NIR wavelengths, where water and lipids strongly absorb. In addition, autofluorescence from intrinsic chromophores is strongly reduced when exciting and/or detecting in the NIR range. Two near infrared bands are usually distinguished, NIR I (700-950 nm) and NIR II (1000-1300 nm). Several classes of organic fluorophores emit in the NIR I band; indocyanine green, for example, is used for angiography. [6] Even though continuing progress is being made in the synthesis of brighter and more stable organic dyes, [2] nanoparticles offer interesting alternatives as NIR I-emitting probes. These include semiconductor quantum dots (QDs), metal nanoclusters, rare earth doped nanoparticles (RE-NPs) and nanodiamonds (NDs). After initial demonstrations of *in vivo* imaging based on CdTe and PbS materials, new generations of low toxicity NIR emitting QDs have emerged. [7] These are mainly composed of CuIn(S/Se), [8-11] Cu-doped InP, [12] InAs, [13-16] and AgS [17, 18] nanocrystals. These QDs offer a high brightness thanks to their high extinction coefficient and quantum yields (QY), and a higher photostability compared to organic chromophores. Their emission is tunable with their size and composition. Metal nanoclusters are a novel class of fluorescent nanomaterials. While the exact underlying photophysical mechanism is not completely elucidated yet, NIR emitting gold nanoclusters have been synthesized. [19-22] These clusters are not as bright in terms of extinction coefficient and quantum yield as QDs, but their small size and lack of toxicity make them an attracting alternative. Carbon-based materials also present interesting optical properties. In particular, N-V centers in nanodiamonds (NDs) fluoresce in the far red-NIR range. [23, 24] These NDs are inert, which leads to a superior photostability and low toxicity. Finally, the fluorescence properties of rare earth doped nanoparticles rely on 4f transitions of trivalent lanthanide ions inserted in a suitable host matrix. The optical properties of these RE-NPs are therefore tunable by choosing appropriate emissive and sensitizing dopants. The choice of the host materials is determinant for the size control during the synthesis, as well as for the optical properties and colloidal and chemical stability. [25]

Surface chemistry of these different nanoprobes is of course crucial to guarantee their colloidal stability, preserve their optical properties *in vivo*, reduce non specific adsorption and provide specific targeting and functionality. The development of optimized chemistries is an ongoing research effort, which has already allowed most of these NPs to be used in small animals for different imaging applications. These include vasculature [26, 27] and lymph node [10] imaging, tumor detection [14], cell tracking [28, 29] and cell lineage. [30] In addition to providing higher detection sensitivity compared to standard NIR I organic fluorophores, these nanoprobes share several unique features. Their large hydrodynamic size, while restricting diffusion into tissues and preventing renal excretion above 5 nm, [31] also extends the circulation time in the bloodstream and limit leaching from the blood vessels into surrounding tissues, leading to more specific targeting. In addition, tumor detection using nanoprobes benefits from the enhanced permeation and retention (EPR) effect: [32] nanoparticles in the 50-150 nm size range can penetrate loose epithelial barriers and remain much longer in the tumor than smaller organic dyes which get washed away much faster. [33] Similarly, endocytosed NPs tend to remain much longer inside targeted cells compared to smaller organic dyes, facilitating long term cell tracking. [34] In contrast to monofunctional organic dyes, the large surface of NPs can be used to assemble different components on a single nanoplatorm. This potentially
enhances the targeting efficiency, thanks to the multiplicity of targeting moieties, or provides multifunctional NPs carrying different targeting probes, drugs, contrast agents for complementary imaging modalities, and/or environment reporters. Interestingly, these nanoprobes also offer optical properties that can be exploited to develop new improved imaging modalities in terms of accessible depth, resolution and sensitivity. In the following sections, we will more specifically review recent developments of NPs that emit in the NIR II range, undergo efficient nonlinear excitation, present slow fluorescence decays for time-gated detection or can be efficiently coupled to other imaging modalities.

**NIR II emitting nanoprobes**

*In vivo* imaging in the NIR II band has long remained difficult due to the absence of fluorophores and sensitive detectors in this region. In principle however, the NIR II band offer several advantages compared to NIR I, including better detection sensitivity, thanks to a much lower autofluorescence background, and better spatial resolution deep in tissues, thanks to reduced light scattering. [35] In addition, NIR II probes can usually be excited in the NIR I band, which reduces photodamage and improves the excitation penetration. Water soluble NIR II organic dyes are not available so far, and the QY of their hydrophobic counterparts is limited to a few percents in organic solvents. [36] For these reasons, three main classes of NIR II nanoprobes are being actively developed (see Table 1).

Single wall carbon nanotubes (SWNT) present a broad series of emission peaks between 1,000 and 1,400 nm. [37-40] RE-NPs can be designed to emit at different wavelengths in the NIR II band, from 1,200 nm (Er³⁺) to 1,525 nm (Pr³⁺). [41] Ag₂S and Ag₂Se QDs have been synthesized with tunable spectra up to 1,300 nm and good quantum yields. [18, 42-45] In addition, the high chemical stability of Ag₂S reduces significantly the potential toxicity of these QDs, compared to other QD materials. These nanoprobes have been used for blood vessel imaging. The Dai group in particular, demonstrated that carbon nanotubes could provide images of cerebral vasculature through the skull and scalp, while maintaining an impressive sub-10 µm resolution at a 2 mm depth. [37] Another example is *in vivo* tracking of Ag₂S QD-labeled cells. The absence of autofluorescence led to a low detection limit (< 1000 cells) and allowed tracking these cells for up to 30 days. [46]
### Table 1. Water soluble near infrared emitting nanoprobes, their respective size, QY and emission wavelength. (For RE-NPs, QY is defined for standard one photon excitation, down-conversion fluorescence).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Size (nm)</th>
<th>QY (%)</th>
<th>Emission</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NIR I nanoprobes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantum dots (CuIn(S/Se)$_2$, InP, InAs, Ag$_2$S, Si)</td>
<td>2-10</td>
<td>10-40</td>
<td>Tunable from 700 to 1000 nm FWHM 80-150 nm</td>
<td>[8-18]</td>
</tr>
<tr>
<td>Gold clusters</td>
<td>≈1.5</td>
<td>≈3</td>
<td>700-800 nm FWHM 100-150 nm</td>
<td>[19-22]</td>
</tr>
<tr>
<td>Nanodiamonds</td>
<td>20-100</td>
<td>≈100</td>
<td>700 nm FWHM 100-150 nm</td>
<td>[23, 24]</td>
</tr>
<tr>
<td>Rare earth doped</td>
<td>5…200</td>
<td>5-15</td>
<td></td>
<td>[25, 26, 47]</td>
</tr>
<tr>
<td><strong>NIR II nanoprobes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbon nanotubes</td>
<td>Ø ≈ 1 L = 100-1000</td>
<td>&lt; 1</td>
<td>Multiple broad peaks 1000-1350 nm</td>
<td>[37-40]</td>
</tr>
<tr>
<td>Quantum dots (Ag$_2$S, Ag$_2$Se)</td>
<td>2-7</td>
<td>10-15</td>
<td>Tunable from 1000 to 1350 nm FWHM 30-200 nm</td>
<td>[18, 42-45]</td>
</tr>
<tr>
<td>Rare earth doped</td>
<td>10-12</td>
<td>≤ 1</td>
<td>≈1200nm; ≈1300nm; ≈1500 nm FWHM 50-100 nm</td>
<td>[41]</td>
</tr>
</tbody>
</table>
Figure 1. *In vivo* through-skull fluorescence imaging of the brain vasculature using SWNT injected in the tail vein, in the NIR I (left) and NIR II (right) range. (Reproduced from ref. [37••] with permission from Macmillan Publishers Ltd.).

**Nonlinear excitation/Upconversion**

Most inorganic nanoparticles present higher nonlinear absorption cross sections compared to organic fluorophores. Nonlinear excitation in the NIR enables deep penetration and reduces autofluorescence, thus leading to improved signal-to-background ratio. W. Webb and co-workers demonstrated that 2-photon excitation (2PE) is particularly suited to CdSe quantum dots (QDs) because they have 2-photon absorption cross sections two to three orders of magnitude higher than organic fluorophores, enabling dynamic observation of QDs through the skin of living mice, in capillaries hundred of micrometers deep. [27•]. However, 2PE requires expensive equipment and is better suited for cellular scale than for whole animal imaging where efficient focusing is hard to achieve deep in the tissues. For the latter application, rare-earth doped nanoparticles present the great advantage of being easily and efficiently excited in the near infrared with a continuous laser source by up conversion. [25] The large blue spectral shift between excitation and emission thus obtained and their high resistance to photobleaching contributes to enhance the detection sensitivity of up-conversion nanoparticles (UCNP). Lim *et al.* demonstrated first *in vivo* imaging using 50 to 200 nm lanthanide doped NPs in *Caenorhabditis elegans* (see figure 2a). [35] Since then, lanthanide doped NPs have been efficiently detected when injected deep in a rat and have allowed tracking cells in live animals. [28] Vinogradov and co-workers reported the use of UCNP doped with both Yb$^{3+}$ and Er$^{3+}$ to acquire vascular images with high contrast and resolution up to 340 microns deep into a mouse brain (see figure 2B). [48] To enhance the depth of *in vivo* imaging, further works focused on the synthesis of biocompatible NIR-to-NIR up conversion nanoparticles. [49] Within lanthanides, Tm$^{3+}$ ions are thus especially well suited because they provide a main up conversion emission band around 800nm. Prasad *et al.* recently reported the development of core/shell NIR-to-NIR UCNP with enhanced up conversion efficiency, which enable whole body mice imaging with high signal to background ratio (see figure 2C). [26]
Figure 2: Up-conversion nanoparticles imaged in live organisms: A) Fluorescent and bright field images of *C. elegans* NaYF₄ : Yb,Er@PEI nanoparticles; B) Maximal intensity projection image of a 200-μm-thick image stack showing vasculature in the mouse brain. Scale bar is 100 μm (Reproduced from [48••] with permission from the National Academy of Sciences); C) Whole-body imaging of a nude mouse injected via tail vein with NIR-to-NIR core−shell nanoparticles. (Reproduced from ref. [26•] with permission from the American Chemical Society).

**Time gated detection**

In order to improve detection sensitivity, the signal of interest can be discriminated from the autofluorescence background spectrally (as mentioned above by selecting NIR excited and/or emitting NPs), but also temporally. This can be easily achieved using inorganic NPs that present slow fluorescence decay (from 10 ns to several μs) compared to both extrinsic and intrinsic organic chromophores (a few ns). [5]

In the last decade, two techniques have emerged to perform time-gated detection using pulsed excitation. Time Correlated Single Photon Counting (TCSPC) provides a high temporal resolution with a pixel scanning acquisition. Fluorescence decay maps are then reconstructed from the raw data. This technique thus provides information on the distribution of fluorescence lifetimes, from which the slow NP decay can be deconvoluted from the fast autofluorescence. However, it can detect at most one fluorescence photon per pulse which limits the acquisition speed. It is therefore not well suited for dynamic sample imaging. In contrast, intensified gated cameras (iCCD) directly provide an integrated image for different controllable delays between the excitation and the detection window. Selecting a detection delay longer than the autofluorescence lifetime ensures its rejection, while efficiently detecting the signal from slower decaying NPs. Time-gated detection is particularly interesting to increase NP detection sensitivity in vivo, and several applications have been demonstrated for tumor targeting or cell tracking as illustrated in Table 2 and Figure 3. Finally, in order to completely overcome autofluorescence, le Masne de Chermont *et al.* have developed persistent luminescence nanoparticles that can be excited before the in vivo injection and followed for up to one hour without the need of an external illumination source. [50]
<table>
<thead>
<tr>
<th>Probe type</th>
<th>Probe lifetime component</th>
<th>Imaging system</th>
<th>Sample</th>
<th>Detection enhancement</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silanized CdSe QDs</td>
<td>2 (11%) 8 (49%) 24 (40%)</td>
<td>Confocal microscopy</td>
<td><em>In vitro</em> 3T3 mouse fixed fibroblasts</td>
<td>X 15</td>
<td>[51]</td>
</tr>
<tr>
<td><em>λ</em>&lt;sub&gt;em&lt;/sub&gt;=575 nm</td>
<td></td>
<td>5 MHz Ti:sapphire laser TCSPC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIR QDs</td>
<td>&gt; 20 ns</td>
<td>Wide-field imaging</td>
<td><em>In vivo</em>. Subcutaneous QD injection</td>
<td>X 10</td>
<td>[52]</td>
</tr>
<tr>
<td><em>λ</em>&lt;sub&gt;em&lt;/sub&gt;=705 nm</td>
<td></td>
<td>2 MHz 630-nm-LED iCCD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIR QDs</td>
<td>150 ns</td>
<td>Wide field microscopy</td>
<td><em>Ex vivo</em>. QD-labeled cell on an autofluorescent tissue</td>
<td>X 25</td>
<td>[53]</td>
</tr>
<tr>
<td><em>λ</em>&lt;sub&gt;em&lt;/sub&gt;=800 nm</td>
<td></td>
<td>5 MHz 659-nm-LED iCCD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silicon QDs</td>
<td>5-13 μs</td>
<td>Fluorescence tomography</td>
<td><em>In vivo</em>. QD-labeled cancer cells injected in a mouse</td>
<td>X 20</td>
<td>[54]</td>
</tr>
<tr>
<td><em>λ</em>&lt;sub&gt;em&lt;/sub&gt;=800 nm</td>
<td></td>
<td>40MHz 470-nm laser TCSPC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Applications of time-gated detection for biological imaging. Detection enhancement stands for the gain in sensitivity or signal-to-background ratio between a time-gated and an ungated detection.
Another way to extract a signal of interest from the autofluorescence background is to use a specific modulation of the fluorescence intensity. NDs fluorescence, for example, depends on the spin properties of their ground state, and can therefore be modulated by an external magnetic field or a microwave irradiation. Since autofluorescence is insensitive to these modulations, the signal from NDs can be extracted with an excellent signal to noise ratio. [55, 56]

**Multimodal imaging**

The interest of fluorescence in vivo imaging resides in its high sensitivity and spatial and temporal resolution. However, it is still limited to surface tissues; it is not quantitative and often requires additional information on anatomic environment. These limitations can be circumvented by combining fluorescence with other imaging modalities. For example, magnetic resonance imaging (MRI) and computed X-ray tomography (CT) provide whole body images and can be used for preoperative detection while fluorescence can be used intra-operatively for guided surgery. Positron emission tomography (PET) and single-photon emission computed tomography (SPECT) are very sensitive methods and can provide quantitative information on metabolic activity but suffer from a poor spatial resolution whereas fluorescence possesses a high spatial resolution but is not a quantitative technique. The combination of these modalities with fluorescence imaging can be achieved using nanoparticle-based multimodal probes.

A first strategy consists in coating the surface of fluorescent nanoparticles with active ligands. This method is quite universal as it only requires changing one part of the ligand. For example, the grafting of gadolinium complexes leads to dual MR-fluorescence imaging probes. [57] This method was also used to produce SPECT/fluorescence and PET/fluorescence imaging by grafting $^{111}$In- and $^{64}$Cu-complexes respectively. [58] Unfortunately, such probes lack stability due to a non-covalent grafting of such ligands.

Another strategy consists in incorporating different types of nanoparticles in a carrier nanoparticle. Such composite nanoparticles for fluorescence and MRI were obtained by incorporating QDs and superparamagnetic iron oxide nanoparticles (SPIONs) in silica or polymer matrices. [59] This method provides very stable probes and preserves the individual properties of each nanoparticle. However their use is limited by their generally larger size (typically a few hundreds of nanometers).

Alternatively, the doping of fluorescent nanoparticles with paramagnetic ions or radioactive nuclide is an effective method to obtain small and stable multimodal probes. The main drawback of this
method is the limited amount of doping ions successfully incorporated. In the case of RE-NPs, the incorporation (either as component of the host matrix or as codopant) of different ions can be used to implement new modalities: \(\text{Gd}^{3+}\) for MRI and CT and \(^{18}\text{F}\) for PET [25]. The same strategy has been used with quantum dots, mainly by incorporating \(\text{Mn}^{2+}\) ions to produce bimodal MR/fluorescent probes. For example, Sitbon et al have shown that Zn-Cu-In-Se/Zn\(_{0.9}\)Mn\(_{0.1}\)S QDs can be used to detect regional lymph nodes in both MRI and NIR fluorescence imaging (Fig. 4). [60] More recently, the incorporation of 64 Cu by cation exchange enable their use as PET/fluorescence contrast agents [61].

Another way to obtain multimodal probes is to grow directly a nanoparticle on top of another one. This method provides very stable probes but some charge transfer between the different materials may cause a diminution of QY. This system has been particularly studied for the synthesis of MR/fluorescent probes, as for example in the FePt/CdSe or Fe\(_3\)O\(_4\)/CdSe systems and more recently in the Si/Fe\(_3\)O\(_4\) system. [59, 62]

**Conclusions/Perspectives**

Inorganic nanoprobes present unique optical properties that can be harnessed to enhance the sensitivity, resolution and imaging depth of fluorescence *in vivo* imaging. They are therefore bound to play an increasingly central role in the development of new imaging techniques and applications. Continuing progress in the synthesis of these NPs and in the control and understanding of their optical properties, together with the development of adapted instrumentation, should further improve their imaging performance. To translate these advances into pertinent bio-applications, the development of their surface chemistry is also required, in order to better control their biodistribution and improve their targeting efficiency. This should in turn enable a better understanding and control of their potential toxicity. Ultimately, fast clearance of these NPs from the body will be required to bring these imaging modalities to the clinical field. We note some recent progress in this direction, with examples of fluorescent NPs that are either small enough [31] or degrade into molecular compounds [54] to undergo partial renal clearance. Future expected developments should also include smart nanoprobes that are able to report on their biochemical environment, or theranostics probes that use local heating, singlet oxygen production or controlled drug delivery as a therapeutic tool in addition to imaging.
References:


Detailed description and characterization of new bright NIR-to-NIR core—shell up conversion nanoparticles and demonstration of their use for whole body mouse imaging.


CdSe-based QDs were shown to possess 2PE cross -sections two to three orders of magnitude larger than organic dyes. QDs were used for 2PE imaging of the mouse brain vasculature.


NDs were used to label stem/progenitor stem cells and follow their biodistribution in vivo without impairing their regenerative capabilities for up to 7 days after intravenous transplantation.


SWNTs were used as NIR II emitters to demonstrate through-skull imaging of the mouse brain vasculature. A spatial resolution of less than 10 μm was preserved to a depth of more than 2 mm.


NIR II Ag2S QDs were synthesized with 15% QY and used for tumor detection and deep inner organ imaging in vivo.


This article demonstrates that dendrimer-bound up-conversion NPs are photostable and biocompatible, and enable in vivo mapping of the cortical vasculature down to 400 μm under the tissue surface with a continuous wave excitation.


This article demonstrates a significant in vivo detection enhancement of silicon nanoparticles using time-gated imaging. The authors also show that these nanoparticles present a low toxicity thanks to an effective renal clearance.


