

# ***In vivo* near-infrared fluorescence imaging**

John V Frangioni

Photon penetration into living tissue is highly dependent on the absorption and scattering properties of tissue components. The near-infrared region of the spectrum offers certain advantages for photon penetration, and both organic and inorganic fluorescence contrast agents are now available for chemical conjugation to targeting molecules. This review focuses on those parameters that affect image signal and background during *in vivo* imaging with near-infrared light and exogenous contrast agents. Recent examples of *in vivo* near-infrared fluorescence imaging of animals and humans are presented, including imaging of normal and diseased vasculature, tissue perfusion, protease activity, hydroxyapatite and cancer.

## **Addresses**

SL-B05 Beth Israel Deaconess Medical Center, 330 Brookline Avenue,  
Boston, MA 02215, USA  
e-mail: jfrangio@bidmc.harvard.edu

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## **Abbreviations**

**HD** hydrodynamic diameter  
**ICG** indocyanine green  
**NIR** near-infrared  
**QD** quantum dot  
**QY** quantum yield  
**SBR** signal to background ratio

## **Introduction**

This review focuses on recently published examples of *in vivo* near-infrared (NIR) fluorescence imaging. To be successful, each example addressed a common set of parameters (outlined below and summarized in Box 1) and optimized the signal to background ratio (SBR; also called signal to noise ratio).

### **Selection of excitation and emission wavelengths**

The use of NIR (700–1000 nm) light for biomedical imaging is grounded in first principles, and is best understood in the context of photon propagation through living tissue and the SBR. An excitation photon typically travels through tissue to reach the fluorescent contrast agent, and has several possible fates depending on the tissue's scatter, anisotropy ( $g$ ), and refractive index(ices) [1]. The photon emitted by the fluorophore is subject to

the same fates. Generally, the photon absorbance of a particular tissue or organ is the sum of all absorbing components present. In living, non-pigmented tissue, the major NIR absorbers are water, lipids, oxyhemoglobin and deoxyhemoglobin, with the absolute value of  $\mu_A$  depending on the molar concentration of each component [2,3<sup>••</sup>]. In a 'typical' tissue, having an 8% blood volume and 29% lipid content, the dominant absorber is hemoglobin, accounting for 39–64% of total absorbance at NIR wavelengths [3<sup>••</sup>].

Whenever tissue absorbs light, there is a chance that fluorescent light will be emitted. In addition to absorption, tissue 'autofluorescence' can severely limit SBR. Figure 1 demonstrates the relationship of excitation and emission wavelengths to tissue autofluorescence. 'Green' autofluorescence of the skin and viscera, and especially the gallbladder, small intestine and bladder, is astoundingly high when excited with blue light. Even a small amount of urine leaking from the urethra (overlying the tail) is easily seen. Autofluorescence of the gallbladder and bladder are markedly reduced using a 'red' filter set (green light excitation), but intestinal autofluorescence remains significant. Use of a NIR filter set essentially eliminates autofluorescence. Hence, high tissue autofluorescence precludes the use of visible light for most *in vivo* imaging applications, and NIR light solves this problem by reducing fluorescence background.

For reflectance-type imaging (see below), scatter is as important as absorbance with respect to photon attenuation. Scatter describes the deviation of a photon from the parallel axis of its path, and can occur when the tissue inhomogeneity is small relative to wavelength (Rayleigh scattering), or roughly equal to wavelength (Mie scattering). Empirical measurements suggest that the wavelength dependence of the scatter coefficient is strongly dependent on tissue composition. In rat skin, the reduced scattering coefficient is proportional to  $\lambda^{-2.4}$  [4], suggesting Rayleigh dominance; however, in post-menopausal human breast it is proportional to  $\lambda^{-0.6}$  [5], suggesting Mie dominance. A detailed analysis of how tissue absorption and scatter affect the selection of excitation and emission wavelengths for reflectance *in vivo* NIR fluorescence imaging has recently been published [3<sup>••</sup>].

### **Exogenous contrast agents**

Because there is little NIR fluorescence contrast generated by most tissues, most *in vivo* studies administer exogenous contrast agents. Until recently, exogenous contrast was limited to organic fluorophores, the most

**Box 1 Optimization of parameters for *in vivo* NIR fluorescence imaging.**

|                                             |                                              |
|---------------------------------------------|----------------------------------------------|
| <b>Fluorophore properties</b>               | <b>Tissue and/or cell targeting</b>          |
| Aqueous solubility                          | Tissue/target refractive indices             |
| Excitation wavelength(s)                    | Rayleigh and Mie scatter of tissue           |
| Extinction coefficient ( $\epsilon$ )       | Tissue absorption of excitation light        |
| QY                                          | Tissue autofluorescence                      |
| Emission wavelength                         | Quenching of fluorophore                     |
| Local environment effects on above          | Cellular and sub-cellular diffusion barriers |
| <b>Excitation light delivery</b>            | <b>Signal amplification</b>                  |
| Fluence rate ( $\text{mW}/\text{cm}^2$ )    | Concentration in sub-cellular compartments   |
| Photobleaching threshold                    | Unquenching of fluorescence (stealth probes) |
| Homogeneity of light delivery               |                                              |
| <b>Biodistribution and pharmacokinetics</b> | <b>Collection of emitted fluorescence</b>    |
| Plasma protein binding                      | NIR transmission properties of optics        |
| Vascular permeability barriers              | Filter design                                |
| Blood clearance                             | Detector spectral sensitivity and response   |

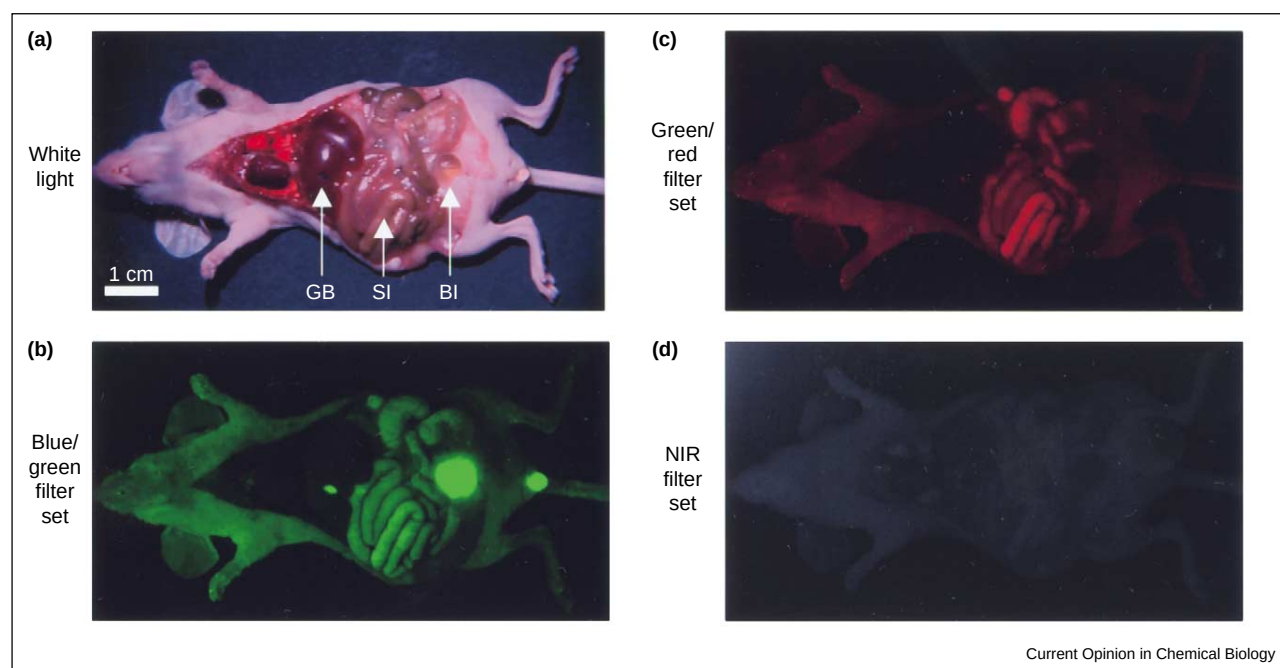
indolyl, 2-quinoline and 4-quinoline subclasses. Of these, the indocyanines are the most widely used as they do not have the aggregation problems associated with the other subclasses [6]. Peak excitation of this class is at 760–800 nm, and peak emission at 790–830 nm.

In 1958, indocyanine green (ICG; Cardio-Green; Figure 2b) was submitted for approval to the FDA for use in indicator-dilution studies in humans. It is one of the least toxic agents ever administered to humans, with the only known adverse reaction being rare anaphylaxis. Recently, several improved heptamethine indocyanines have become available. For example, Figure 2c shows the carboxylic acid form of IRDye78 (LI-COR, Lincoln, NE). This molecule is tetra- rather than di-sulfonated, which increases aqueous solubility and aqueous quantum yield (QY), and is available as an *N*-hydroxysuccinimide ester derivative for covalent conjugation to targeting molecules. Dozens of other heptamethine fluorophores have been reported [7,8,9]. Conjugation to the indocyanines requires careful handling [10], and a set of robust methods has recently been published [11].

common of which are polymethines. One important class of these molecules is the heptamethine cyanines (Figure 2a), comprising benzoxazole, benzothiazole,

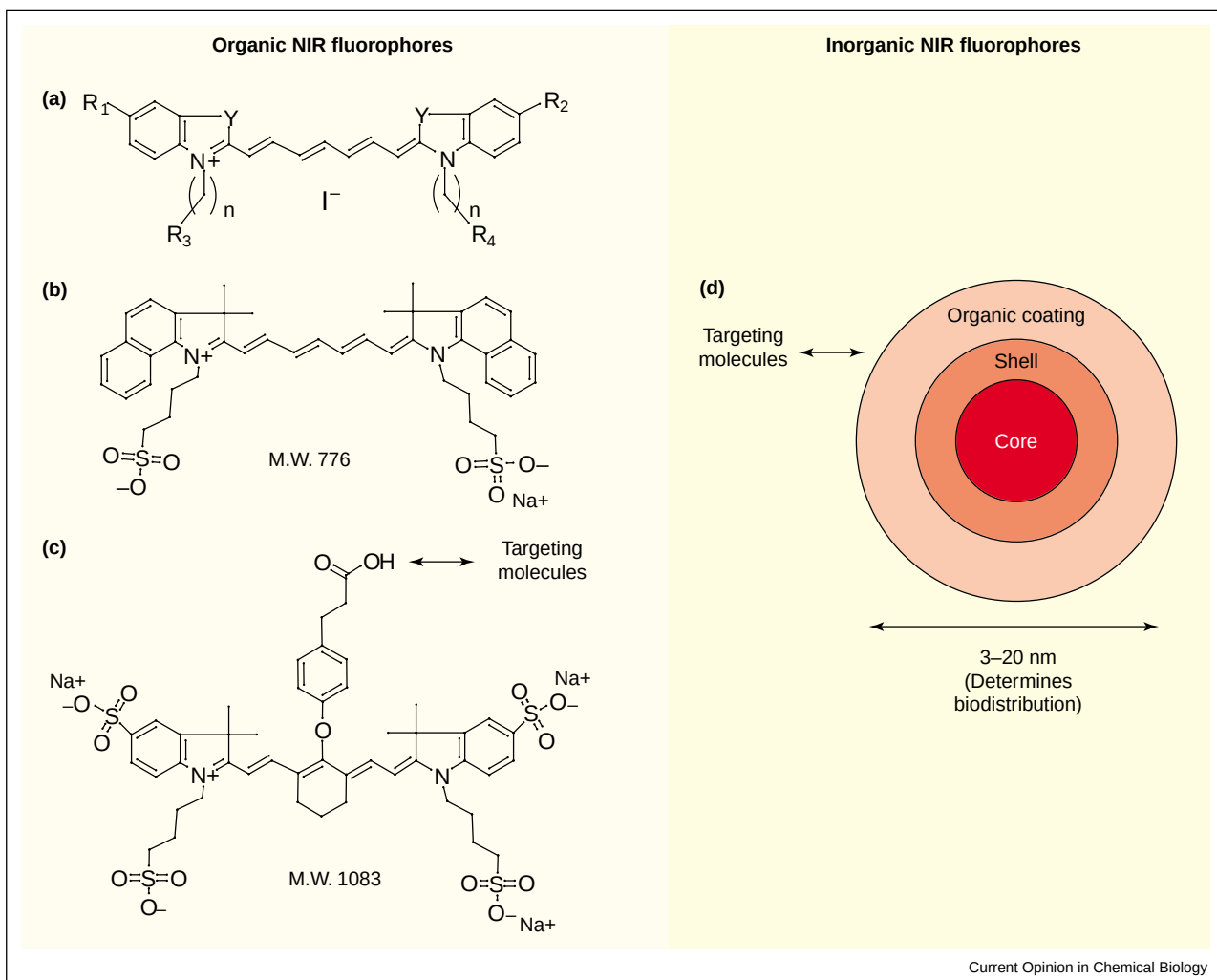
Conventional organic fluorophores suffer from significant limitations. First, and foremost, it is difficult to control excitation and emission wavelengths. These wavelengths

**Figure 1**



Wavelength-dependent autofluorescence of vital organs and bodily fluids. (a) Immediately after sacrifice, the viscera of a hairless, athymic *nu/nu* mouse were exposed. Tissue autofluorescence was then imaged using three different excitation/emission filter sets: (b) blue/green (460–500 nm/505–560 nm); (c) green/red (525–555 nm/590–650 nm); and (d) NIR (725–775 nm/790–830 nm). The fluence rate provided by each filter set was adjusted to 2 mW/cm<sup>2</sup>. To compensate for differences in emission filter wavelength width and camera sensitivity, exposure times were adjusted accordingly. Fluorescence images have identical normalization. For orientation, the white light color image of the animal is shown in (a). Arrows mark the location of the gallbladder (GB), small intestine (SI) and bladder (BI).

Figure 2



Organic and Inorganic NIR fluorescent contrast agents. (a) General structure of the heptamethine cyanine class of NIR fluorophores, where Y determines subclass. (b) ICG, a di-sulfonated indocyanine in clinical use. (c) The carboxylic acid form of IRDye78 (IRDye78-CA), a tetra-sulfonated indocyanine that is available as an *N*-hydroxysuccinimide ester for conjugation to targeting molecules. (d) General structure of QDs, comprising an inorganic core, an inorganic shell, and an aqueous solubilizing organic coating to which targeting molecules can be conjugated.

are dependent on chemical structure, and tuning a conventional fluorophore to precise wavelengths requires sophisticated chemistry. Moreover, changes in wavelength will usually be 'discrete'. For polymethines, the addition of each double bond results in an emission wavelength increase of 80–100 nm, but comes at the cost of a more hydrophobic and potentially unstable molecule. Second, QY is usually less than 15% in aqueous environments. Third, for conjugation of tumor targeting, or other ligands to fluorophores, there is usually one ligand per fluorophore, so detectability is a strong function of target concentration. Finally, conventional fluorophores are highly susceptible to photobleaching, which limits the fluence rate that can be applied to a sample, and hence the sensitivity of detection (see below).

Inorganic fluorescent semiconductor nanocrystals (also called quantum dots; QDs) have the potential to solve many of these problems. QDs are synthesized in organic solvents such as hexane, and typically comprise an inorganic core and inorganic shell of metal, the properties of which tune peak fluorescence emission. For *in vivo* imaging, QDs must be additionally coated with an aqueous-compatible organic layer (Figure 2d). QD fluorescence emission can be tuned to virtually any discrete wavelength and absorption is broadband. More importantly, QDs are remarkably resistant to photobleaching, permit dozens of targeting molecules to be conjugated to a single QD, and, at least in organic solvents, have very high QYs (discussed below). A detailed discussion of QD properties for *in vivo* imaging has been published [3\*\*].

### Solubility, extinction coefficient, QY and photobleaching threshold

Solubility is a significant issue for both organic and inorganic fluorophores. Non-sulfonated heptamethines, such as IR-786, have aqueous solubilities  $\leq 10 \mu\text{M}$ , and require carriers such as Cremophor EL and/or ethanol for *in vivo* use [12]. Solubility increases dramatically for every charged group, typically sulfonates, substituted on the fluorophore. For the heptamethine indocyanines, tetra-sulfonation typically results in aqueous solubilities of  $\geq 10 \text{ mM}$ . Of course, the cost of adding too much charge is that some targeting molecules will lose binding potency because of charge or steric effects. As a general rule, the degree of sulfonation should be chosen to render the NIR fluorophore/targeting molecule conjugate soluble in plasma, but not so charged as to interfere with target binding. For QDs, solubility depends entirely on the organic coating applied to the inorganic core/shell. Although this field is in its infancy, several organic coatings have been described [3<sup>•</sup>,13–17].

Heptamethine indocyanines typically have extinction coefficients in aqueous buffers of  $100\,000\text{--}200\,000 \text{ M}^{-1}\text{cm}^{-1}$ , which, when coupled with low tissue absorption in the NIR, results in reasonably good photon absorption by the fluorophore. QDs possess the interesting property of broadband absorption; they absorb all wavelengths below their emission wavelength, and their absorption coefficient actually increases as excitation wavelength becomes bluer. This property permits multiple QDs with different fluorescence emissions to be excited by a single wavelength, or even broadband light, although the absorption and scattering properties of tissue can negate much of this advantage [3<sup>•</sup>].

Absolute QY can be difficult to measure, and most reports of QY are given for methanol or other non-aqueous solvents. In general, the QY of heptamethine indocyanines in aqueous environments increases with their solubility, but even for tetra-sulfonated molecules is often less than 15% [12]. A recent report of novel heptamethine indocyanines with a 28% aqueous QY suggests that improvements are possible [9]. QD QYs, too, are typically cited for organic solvents, where QY can approach 90%. However, some of the newer aqueous-solubilizing organic coatings for QDs have been reported to produce QYs of 50–60%.

A major difference favoring QDs over organic fluorophores is their resistance to photobleaching. In our experience, heptamethine indocyanines rapidly photobleach in serum when fluence rate exceeds  $50 \text{ mW/cm}^2$  [12]. This effect precludes the use of higher fluence rates, which would otherwise improve the SBR. QDs can withstand fluence rates one to two orders of magnitude higher. Because  $50 \text{ mW/cm}^2$  is already a sizable fluence rate, and tissue damage can occur at high fluence rates, it

is unclear how much of an issue this will become for *in vivo* imaging.

### Contrast agent biodistribution and pharmacokinetics

*In vivo* imaging requires that the contrast agent is delivered to the target, has adequate contact time with the target for binding to occur, and is retained by the target while non-bound material is cleared from the circulation. Unconjugated organic NIR fluorophores are typically  $\leq 1200 \text{ Da}$ , but have widely different biodistributions and pharmacokinetics depending on their charge and the properties of their conjugated targeting molecule. In the absence of any solubilizing groups, heptamethine indocyanines are lipophilic cations that distribute freely and partition intracellularly. This property can be exploited to create a perfusion tracer [12,18<sup>•</sup>]. As charge (e.g. sulfonation) is increased, fluorophores remain extracellular and plasma half-life increases proportionally [8<sup>•</sup>]. Even so, the plasma half-life of unconjugated tetra-sulfonated heptamethine indocyanines is measured in minutes at best. Clearance is typically accomplished from a combination of renal filtration and excretion into bile.

QDs have the opposite problem. Because of their multi-layered structure, and especially given the thickness of the organic coatings, they are typically large structures of 3–20 nm hydrodynamic diameter (HD). Because there is a sharp decrease in renal filtration above a 3.5 nm HD, most QDs will be difficult to clear from the circulation, resulting in high background. This problem has yet to be addressed and solved systematically.

### In vivo toxicity

Without charged groups, heptamethine indocyanines can be quite toxic due to intracellular accumulation [12]. However, di-sulfonated ICG has been used routinely in humans for over 40 years and has an excellent safety profile. Less is known about other heptamethine indocyanines, although studies in mice suggest a dramatic increase in the  $\text{LD}_{50}$  with increasing sulfonation [8<sup>•</sup>].

The *in vivo* toxicity, if any, of QDs is unknown. They typically comprise metals that, in their elemental form, are toxic (e.g. cadmium, arsenic and lead). However, in the nanocrystalline form, these metals are already bound as a less reactive salt, and might be relatively inert in the body. This critical issue remains to be addressed.

### Sub-cellular localization, stealth probes and signal amplification

The Weissleder group has pioneered the concept of ‘stealth’ NIR fluorescent probes [19<sup>•</sup>]. These contrast agents are ‘silent’ in their native form by virtue of a quenching molecule connected via a short linker. After proteolysis or chemical scission of the linker, the contrast agent becomes unquenched and highly fluorescent. This system results in extremely low background because the

majority of the injected dose is non-fluorescent. Because the linker is typically hydrolyzed by an enzyme, the system also produces high signal via ‘amplification’. Examples of *in vivo* imaging with this system are given below. Amplification can also occur via endocytosis of a contrast agent bound to the cell surface, although the stability, over time, of NIR fluorophores in the endosomal compartment is unknown. It may also be possible to apply stealth technology to QDs given their relatively long fluorescent lifetime.

#### Imaging systems, delivery of excitation light and collection of emission light

There are two fundamental configurations for *in vivo* NIR fluorescence imaging: reflectance and tomographic. In reflectance imaging, continuous wave excitation light is delivered to the surface of the subject, photons traverse the boundary layer and reach the target, and emitted light travels back to the surface where it is collected directly. Although imaging is instant, there is exponential decay of intensity with tissue depth, and scatter limits the technique to several millimeters, at best. In tomographic imaging, signal intensity falls more linearly with target depth in tissue, permitting measurements to be made up to 30–40 cm into tissue [20,21]. However, the image must be reconstructed from multiple measurements made in the time or frequency domains using variants of the diffusion equation. Two excellent recent reviews describe the nuances of reflectance versus tomographic imaging [22<sup>•</sup>,23<sup>•</sup>].

Regardless of the imaging system employed, one must be extremely careful in selecting filters, lenses, cameras and other optics for *in vivo* NIR fluorescence imaging. Most optics are optimized only for visible light transmission, and many have a heat coating that will drastically reduce NIR transmission. For imaging, silicon CCD cameras are preferred because of their cost/performance ratio; however, sensitivity is fair to poor above 800 nm, and few silicon cameras have quantum efficiencies higher than 25% at 800 nm. The quantum efficiency of gallium arsenide is excellent up to 900 nm; however, cost is higher and availability limited. Indium gallium arsenide cameras are not useful for NIR imaging because of their poor quantum efficiency below 1000 nm, and higher noise.

#### Recent advances in *in vivo* near-infrared fluorescence imaging

Over the past several years, there has been an explosion of reports describing successful *in vivo* NIR fluorescence imaging. Many studies are qualitative; however, quantitative methods are beginning to emerge where dictated by necessity. The following summary describes those studies in the context of the principles discussed above.

##### Vascular mapping and tissue perfusion

Given its long FDA approval, it is not surprising that ICG is the most commonly used NIR fluorophore for vascular

studies, and the only one used in humans. As a disulfonated indocyanine it is only moderately soluble in aqueous buffer, is heavily protein-bound, and has a low QY in plasma. As a small molecule (775 Da), it exhibits a short plasma half-life (1–2 min), and diffuses readily from vessels into the interstitial space. As detailed below, more recent studies have utilized tetra-sulfonated indocyanines with higher QYs for angiography, and non-sulfonated indocyanines for measuring tissue perfusion.

##### Angiograms of the eye

ICG angiography is a routine test in the ophthalmology clinic for detection of posterior uveitis [24] and central serous chorioretinopathy [25]. It is supplanting fluorescein angiography for these diagnoses, given the deeper photon penetration in the NIR. Still, measurement of pre-injection autofluorescence can improve results [26], and care must be taken to avoid photobleaching when xenon flash lamps are utilized for excitation. Typically, 25–50 mg of ICG in saline is injected intravenously to achieve an initial blood concentration of 6–12  $\mu$ M. Angiography can be performed in three phases, with the early ( $\leq 2$  min) being most sensitive for vascular pattern, and the intermediate ( $\approx 12$  min) and late phases ( $\approx 40$  min) being more sensitive for permeability changes between vessels and interstitium [24]. A recent prospective study suggests that confocal ICG angiography can predict the time to growth of small choroidal melanomas by revealing characteristic patterns within their microcirculation [27].

##### Brain

ICG has been used to image the vasculature [28,29] and cerebrospinal fluid [30] of both rats and humans. In both rat models and human gliomas, ICG angiography improved tumor localization and permitted assessment of post-resection margins with high sensitivity and specificity [28,31]. In a study of 14 patients with known vascular abnormalities such as aneurysms and fistulae, ICG video angiography, performed after injection of 25 mg ICG intravenously, was shown to provide real-time information regarding the patency and malformation of vessels [29].

##### Breast

In proof-of-principle experiments with dog models of adenocarcinoma of the breast, frequency-domain photon migration techniques were used in conjunction with ICG to detect lesions as deep as 1.5 cm from the skin surface [32]. This same group of investigators created a carotene-derivatized NIR fluorophore/photosensitizer with peak emission at 715 nm and demonstrated preferential uptake into mammary tumors [33]. Another group developed a combined magnetic resonance imaging/diffuse optical tomography system and demonstrated, in human breast, that intravenously injected ICG could identify suspicious lesions [34].

### Brown adipose tissue

Di-sulfonated ICG remains extracellular after intravenous injection. Conversely, non-sulfonated heptamethine indocyanines, like IR-786, will partition intracellularly after intravenous injection, and can therefore be used as perfusion tracers. This technique has been demonstrated to permit quantitative measurement of brown adipose tissue perfusion in transgenic animals [12]. Such a technique permits optically based, high-throughput screening of modulators of adaptive thermogenesis and obesity, without sacrifice of the animal. The photobleaching threshold of IR-786 was determined to be approximately 50 mW/cm<sup>2</sup>, and experiments were designed to maintain the fluence rate at or below this level. Because of IR-786's hydrophobicity, intravenous injection required the carriers Cremophor EL and ethanol.

### Gastrointestinal tract

Using a rat laparotomy model, Borotto and co-workers demonstrated NIR fluorescence video angiography of exposed gastrointestinal vessels after intravenous injection of 1 mg/kg ICG [35]. They also adapted an endoscope with NIR optics to perform similar imaging from within the lumen of the small intestine. NIR endoscopy has the potential to demonstrate vascular abnormalities with high sensitivity and specificity, and given the number of endoscopic procedures performed annually worldwide, it is expected that such imaging may someday become routine.

### Heart

Several studies have utilized heptamethine indocyanines to image the vasculature of the heart and myocardial perfusion. Detter *et al.* performed fluorescence coronary angiography of human-sized pigs using ICG injected intravenously at 0.03 mg/kg [36•]. This amount would be expected to achieve an initial blood concentration of approximately 400 nM. Excitation was via a 785 nm laser diode (the fluence rate was not reported), and emission centered at 835 nm was imaged with a NIR-sensitive CCD camera. The technique could be used to image normal vasculature, to detect the location of significant stenoses, and to verify vessel patency after coronary artery bypass grafting (CABG; [36•]). The authors rightly noted that in reflectance geometry, imaging is limited to approximately 4 mm from the surface of the heart due to the combined effects of scatter and absorbance.

Nakayama *et al.* described a real-time intraoperative imaging system that displays both anatomy (color video image) and function (NIR fluorescence image) simultaneously for the surgeon [18•]. Using rat models of cardiovascular disease, this imaging system was utilized with a tetra-sulfonated indocyanine (IRDye78-CA) to image heart vasculature, and a non-sulfonated indocyanine (IR-786) to image myocardial perfusion.

A recent study utilized a commercial NIR fluorescence imaging system on 84 patients undergoing off-pump and on-pump CABG [37•]. The amount of ICG injected was approximately 0.04 mg/kg. Excitation was via a 806 nm laser diode at a fluence rate of 39 mW/cm<sup>2</sup>. The details of the emission optics and light collection system were not specified. The study was significant in that in 5% of patients, NIR fluorescence analysis suggested graft failure, which in turn necessitated surgical revision.

In the only study using QDs to image the vasculature *in vivo*, Lim *et al.* synthesized an aqueous soluble QD with peak emission at 752 nm, and demonstrated imaging of the coronary vasculature of an adult rat [3••]. This study exploited the broadband absorption of QDs, and their high absorption cross-section, and required only 2 mW/cm<sup>2</sup> of white light (400–700 nm) excitation to perform successful imaging.

### Skin and burn depth

Still *et al.* performed a very interesting study that utilized ICG NIR fluorescence to categorize the severity of patient burns [38]. ICG was injected intravenously at 0.1 mg/kg, and excitation was via a pulsed laser diode centered at 795 nm. Fluence was applied to a very large field of view (40 cm by 30 cm), and emission at 835 nm was collected over time with a non-intensified video camera. Fifteen burns on nine patients were analyzed, in an apparently non-blinded fashion, using this protocol. Correlation with the gold standard, biopsy, was excellent, although the authors note that the technique is qualitative, and cannot quantitate burn depth.

### Inflammation, atherosclerosis, protease activity and cell death

#### Inflammation and atherosclerosis

A very important recent study [39••] synthesizes virtually all of the principles reviewed herein. The Weissleder group had previously developed a 'stealth' NIR probe that is specifically activated by the protease cathepsin B [19•] and a small animal tomographic imaging system [40]. By combining these tools, they demonstrated that cathepsin B activity is high in inflamed atherosclerotic lesions of transgenic mice, and that these 'vulnerable plaques' could be identified in the living animal using optical tomography [39••]. Contrast agent was injected intravenously at 0.2 µmol/kg, tomographic fluorescence excitation was 2 mW at 675 nm, and light was collected through a 710 ± 10 nm emission filter. Twenty four effective sources and 36 effective detectors were utilized, and tomographic reconstruction was via a novel algorithm [40].

#### Protease activity

Over a dozen other studies, most from the Weissleder group, utilize 'stealth' macromolecular NIR fluorescent probes to assess protease activity *in vivo*. All studies

exploit the low background of quenched fluorophores, and the high SBR achieved after unquenching. To summarize a large body of data, stealth NIR fluorescent probes have been used to assess, quantitatively and *in vivo*, the potency of metalloproteinase inhibitors [41], thrombin activation in blood [42] and thrombi [43], dysplastic intestinal adenomas [44], and differential protease expression in breast cancer xenografts [45]. An optical/magnetic resonance imaging dual-modality stealth probe has also recently been reported [46].

#### Cell death

Although several studies have utilized annexin conjugated to visible wavelength fluorophores to assess cell death, a recent report utilized a Cy5.5-conjugated, NIR annexin molecule [47<sup>\*</sup>]. Substitution of more than one molecule of Cy5.5 per mole of annexin resulted in loss of bioactivity, reinforcing the importance of fluorophore interference with targeting molecule. NIR annexin was injected intravenously into nude mice at a dose of 3 mg/kg. The authors suggest that NIR annexin could be used to assess the cytotoxicity of cancer chemotherapy in animal model systems.

#### Hydroxyapatite

Zaheer and colleagues synthesized a NIR fluorescent version of the hydroxyapatite-binding molecule pamidronate [48<sup>\*\*</sup>]. Conjugation was performed to a tetra-sulfonated heptamethine indocyanine (IRDye78-NHS), and yielded a small molecule (Pam78) of 1388 Da. Contrast agent was injected intravenously at a concentration of 0.1  $\mu\text{mol/kg}$ . Excitation was via a 770 nm laser diode at a fluence rate of 18 mW/cm<sup>2</sup>, and emission from 790–830 nm was collected. Sites of osteoblastic activity could be imaged in the living animal using the NIR fluorescence signal from Pam78, although the reflectance geometry limited detection to  $\leq 4$  mm. Pam78 will probably be a useful reagent to investigators testing tomographic imaging systems with the potential for full-thickness imaging of small animals.

#### Tumor cell surface targeting

Both antibodies and small molecules have been conjugated to NIR fluorophores to create tumor-specific NIR fluorescence contrast agents. Ballou and colleagues conjugated Cy5.5 to IgG and IgM antibodies, at fluorophore to antibody ratios of 1–6, and demonstrated tumor-specific localization after intravenous injection of 0.4–4 mg/kg of conjugated antibody [49]. Such high substitutions were well tolerated, at least by the particular antibodies utilized in that study. As could be predicted by their MW and HD, optimal images were not acquired until at least 24–48 h after injection [49]. Similar results were obtained by Soukos and colleagues using Cy5.5 conjugated to an anti-EGF receptor antibody to detect oral cancers in hamsters, although an even longer washout time (4–8 days) was required for optimal SBR [50].

Neri and colleagues improved the pharmacokinetics of detection by employing affinity matured scFv antibody fragments ( $\approx 29\,000$  Da), in either monomeric or dimeric form, conjugated to Cy7 [51]. The fluorophore to protein ratio was controlled at 1:1 to maintain antibody bioactivity. Using this system, they demonstrated *in vivo* imaging of the angiogenesis-associated fibronectin isoform B-FN in teratocarcinoma xenografts.

Becker and colleagues labeled transferrin and albumin with NIR fluorophores, at a fluorophore to protein ratio of 2.4 and 2.1, respectively, and demonstrated preferential uptake of these macromolecules in xenograft tumors [52]. Excitation was via a pulsed laser at 740 nm and emission was collected from 780–900 nm onto an intensified CCD camera. Interestingly, mice were fed a low pheophorbide diet for 10–14 days before the experiment to reduce NIR autofluorescence.

Several studies have utilized small molecules conjugated to NIR fluorophores as tumor targeted contrast agents. Tung *et al.* created a folate-derivatized NIR fluorophore and demonstrated *in vivo* imaging of a metabolically active mouse xenograft [53]. Two groups have synthesized NIR-octreotide and demonstrated *in vivo* imaging of somatostatin-positive tumors [54,55]. These latter studies injected 0.012–0.02  $\mu\text{mol/kg}$  of NIR octreotide, and both achieved adequate SBR after only a few hours due to the rapid clearance of the low-molecular-weight contrast agent.

#### Conclusions

Organic and inorganic fluorophores that span the entire NIR portion of the spectrum are now available. When conjugated covalently to targeting molecules, sensitive contrast agents for NIR fluorescence imaging are created. However, to be used successfully *in vivo*, careful attention must be paid to those parameters that influence the SBR, including selection of excitation and emission wavelengths, fluorophore solubility, QY, photobleaching threshold, biodistribution, target binding affinity, pharmacokinetics, excitation light delivery and fluorescence light collection. NIR fluorescence imaging is already used daily in the ophthalmology clinic, and there is no doubt that other clinical uses will result from the ongoing advances in NIR fluorophore chemistry and optical engineering.

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## References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
  - of outstanding interest
1. Alfano RR, Demos SG, Gayen SK: **Advances in optical imaging of biomedical media.** *Ann N Y Acad Sci* 1997, **820**:248-270.
  2. Chance B: **Near-infrared images using continuous, phase-modulated, and pulsed light with quantitation of blood and blood oxygenation.** *Ann N Y Acad Sci* 1998, **838**:29-45.
  3. Lim YT, Kim S, Nakayama A, Stott NE, Bawendi MG, Frangioni JV: **Selection of quantum dot wavelengths for biomedical assays and imaging.** *Mol Imaging* 2003, **2**:50-64.  
Detailed discussion of how tissue absorbance and scatter properties affect QD performance during *in vivo* imaging, and first demonstration of vascular imaging with NIR QDs.
  4. Cheong WF, Prael SA, Welch AJ: **A review of the optical properties of biological tissues.** *IEEE J Quantum Electron* 1990, **26**:2166-2195.
  5. Cerussi AE, Berger AJ, Bevilacqua F, Shah N, Jakubowski D, Butler J, Holcombe RF, Tromberg BJ: **Sources of absorption and scattering contrast for near-infrared optical mammography.** *Acad Radiol* 2001, **8**:211-218.
  6. Sima PD, Kanofsky JR: **Cyanine dyes as protectors of K562 cells from photosensitized cell damage.** *Photochem Photobiol* 2000, **71**:413-421.
  7. Flanagan JH Jr, Khan SH, Menchen S, Soper SA, Hammer RP: **Functionalized tricarbo-cyanine dyes as near-infrared fluorescent probes for biomolecules.** *Bioconjug Chem* 1997, **8**:751-756.
  8. Miwa N, Inagaki M, Eguchi H, Okumura M, Inagaki Y, Harada T: **Near-infrared fluorescent contrast agent and fluorescence imaging.** Patent Cooperation Treaty WO 00/16810, 2000.  
Patent application describing scores of different NIR fluorophores. Includes a correlation of the degree of sulfonation with blood half-life and *in vivo* toxicity.
  9. Lin Y, Weissleder R, Tung CH: **Novel near-infrared cyanine fluorochromes: synthesis, properties, and bioconjugation.** *Bioconjug Chem* 2002, **13**:605-610.
  10. Rajagopalan R, Uetrecht P, Bugaj JE, Achilefu SA, Dorshow RB: **Stabilization of the optical tracer agent indocyanine green using noncovalent interactions.** *Photochem Photobiol* 2000, **71**:347-350.
  11. Zaheer A, Wheat TE, Frangioni JV: **IRDye78 conjugates for near-infrared fluorescence imaging.** *Mol Imaging* 2002, **1**:354-364.  
Detailed discussion of the conjugation and purification of small molecules and peptides to heptamethine indocyanines, including proper handling.
  12. Nakayama A, Bianco AC, Zhang CY, Lowell BB, Frangioni JV: **Quantitation of brown adipose tissue perfusion in transgenic mice using near-infrared fluorescence imaging.** *Mol Imaging* 2003, **2**:37-49.
  13. Bruchez M Jr, Moronne M, Gin P, Weiss S, Alivisatos AP: **Semiconductor nanocrystals as fluorescent biological labels.** *Science* 1998, **281**:2013-2016.
  14. Chan WCW, Nie SM: **Quantum dot bioconjugates for ultrasensitive nonisotopic detection.** *Science* 1998, **281**:2016-2018.
  15. Mattoussi H, Mauro JM, Goldman ER, Anderson GP, Sundar VC, Mikulec FV, Bawendi MG: **Self-assembly of CdSe-ZnS quantum dot bioconjugates using an engineered recombinant protein.** *J Am Chem Soc* 2000, **122**:12142-12150.
  16. Wu X, Liu H, Liu J, Haley KN, Treadway JA, Larson JP, Ge N, Peale F, Bruchez MP: **Immunofluorescent labeling of cancer marker Her2 and other cellular targets with semiconductor quantum dots.** *Nat Biotechnol* 2003, **21**:41-46.
  17. Dubertret B, Skourides P, Norris DJ, Noireaux V, Brivanlou AH, Libchaber A: **In vivo imaging of quantum dots encapsulated in phospholipid micelles.** *Science* 2002, **298**:1759-1762.
  18. Nakayama A, del Monte F, Hajjar RJ, Frangioni JV: **Functional near-infrared fluorescence imaging for cardiac surgery and targeted gene therapy.** *Mol Imaging* 2002, **1**:365-377.  
Description of an intraoperative NIR fluorescence imaging system capable of displaying anatomy and function simultaneously.
  19. Weissleder R, Tung CH, Mahmood U, Bogdanov A: **In vivo imaging of tumors with protease-activated near-infrared fluorescent probes.** *Nat Biotechnol* 1999, **17**:375-378.  
Detailed description of 'stealth' NIR fluorescent probe technology and demonstration of *in vivo* imaging of protease activity.
  20. Ntziachristos V, Ripoll J, Weissleder R: **Would near-infrared fluorescence signals propagate through large human organs for clinical studies?** *Opt Lett* 2002, **27**:333-335.
  21. Ntziachristos V, Tung CH, Bremer C, Weissleder R: **Fluorescence molecular tomography resolves protease activity in vivo.** *Nat Med* 2002, **8**:757-760.
  22. Sevick-Muraca EM, Houston JP, Gurfinkel M: **Fluorescence-enhanced, near infrared diagnostic imaging with contrast agents.** *Curr Opin Chem Biol* 2002, **6**:642-650.  
Excellent review on NIR fluorescence imaging, including a well-described comparison of reflectance versus tomographic imaging.
  23. Ntziachristos V, Bremer C, Weissleder R: **Fluorescence imaging with near-infrared light: new technological advances that enable in vivo molecular imaging.** *Eur Radiol* 2003, **13**:195-208.  
Comprehensive, well-written review on reflectance versus tomographic imaging.
  24. Herbert CP, LeHoang P, Guex-Crosier Y: **Schematic interpretation of indocyanine green angiography in posterior uveitis using a standard angiographic protocol.** *Ophthalmology* 1998, **105**:432-440.
  25. Chen SJ, Lee AF, Lee FL, Liu JH: **Indocyanine green angiography of central serous chorioretinopathy.** *Zhonghua Yi Xue Za Zhi (Taipei)* 1999, **62**:605-613.
  26. Piccolino FC, Borgia L, Zinicola E, Iester M, Torrielli S: **Pre-injection fluorescence in indocyanine green angiography.** *Ophthalmology* 1996, **103**:1837-1845.
  27. Mueller AJ, Freeman WR, Schaller UC, Kampik A, Folberg R: **Complex microcirculation patterns detected by confocal indocyanine green angiography predict time to growth of small choroidal melanocytic tumors: MuSIC Report II.** *Ophthalmology* 2002, **109**:2207-2214.
  28. Haglund MM, Hochman DW, Spence AM, Berger MS: **Enhanced optical imaging of rat gliomas and tumor margins.** *Neurosurgery* 1994, **35**:930-940.
  29. Raabe A, Beck J, Gerlach R, Zimmermann M, Seifert V: **Near-infrared indocyanine green video angiography: a new method for intraoperative assessment of vascular flow.** *Neurosurgery* 2003, **52**:132-139.
  30. Sakatani K, Kashiwasake-Jibu M, Taka Y, Wang S, Zuo H, Yamamoto K, Shimizu K: **Noninvasive optical imaging of the subarachnoid space and cerebrospinal fluid pathways based on near-infrared fluorescence.** *J Neurosurg* 1997, **87**:738-745.
  31. Haglund MM, Berger MS, Hochman DW: **Enhanced optical imaging of human gliomas and tumor margins.** *Neurosurgery* 1996, **38**:308-317.
  32. Reynolds JS, Troy TL, Mayer RH, Thompson AB, Waters DJ, Cornell KK, Snyder PW, Sevick-Muraca EM: **Imaging of spontaneous canine mammary tumors using fluorescent contrast agents.** *Photochem Photobiol* 1999, **70**:87-94.
  33. Gurfinkel M, Thompson AB, Ralston W, Troy TL, Moore AL, Moore TA, Gust JD, Tatman D, Reynolds JS, Muggenburg B et al.: **Pharmacokinetics of ICG and HPPH-car for the detection of normal and tumor tissue using fluorescence, near-infrared reflectance imaging: a case study.** *Photochem Photobiol* 2000, **72**:94-102.
  34. Ntziachristos V, Yodanis AG, Schnall M, Chance B: **Concurrent MRI and diffuse optical tomography of breast after indocyanine green enhancement.** *Proc Natl Acad Sci USA* 2000, **97**:2767-2772.

35. Borotto E, Engler J, Pourny JC, Naveau S, Chaput JC, Lecarpentier Y: **Detection of the fluorescence of GI vessels in rats using a CCD camera or a near-infrared video endoscope.** *Gastrointest Endosc* 1999, **50**:684-688.
36. Detter C, Russ D, Iffland A, Wipper S, Schurr MO,
  - Reichenspurner H, Buess G, Reichart B: **Near-infrared fluorescence coronary angiography: a new noninvasive technology for intraoperative graft patency control.** *Heart Surg Forum* 2002, **5**:364-369.
 First demonstration of NIR fluorescence coronary angiography in large animals including imaging of normal, stenotic and grafted arteries.
37. Taggart DP, Choudhary B, Anastasiadis K, Abu-Omar Y,
  - Balacumaraswami L, Pigott DW: **Preliminary experience with a novel intraoperative fluorescence imaging technique to evaluate the patency of bypass grafts in total arterial revascularization.** *Ann Thorac Surg* 2003, **75**:870-873.
 Largest human trial on intraoperative NIR fluorescence coronary angiography.
38. Still JM, Law EJ, Klavuhn KG, Island TC, Holtz JZ: **Diagnosis of burn depth using laser-induced indocyanine green fluorescence: a preliminary clinical trial.** *Burns* 2001, **27**:364-371.
39. Chen J, Tung CH, Mahmood U, Ntziachristos V, Gyrko R,
  - Fishman MC, Huang PL, Weissleder R: **In vivo imaging of proteolytic activity in atherosclerosis.** *Circulation* 2002, **105**:2766-2771.
 A stealth NIR fluorescent optical probe specific for cathepsin B was used in conjunction with a novel tomographic imaging system to distinguish vulnerable from stable atherosclerotic plaques in transgenic animals.
40. Ntziachristos V, Weissleder R: **Experimental three-dimensional fluorescence reconstruction of diffuse media by use of a normalized Born approximation.** *Opt Lett* 2001, **26**:893-895.
41. Bremer C, Tung CH, Weissleder R: **In vivo molecular target assessment of matrix metalloproteinase inhibition.** *Nat Med* 2001, **7**:743-748.
42. Tung CH, Gerszten RE, Jaffer FA, Weissleder R: **A novel near-infrared fluorescence sensor for detection of thrombin activation in blood.** *ChemBioChem* 2002, **3**:207-211.
43. Jaffer FA, Tung CH, Gerszten RE, Weissleder R: **In vivo imaging of thrombin activity in experimental thrombi with thrombin-sensitive near-infrared molecular probe.** *Arterioscler Thromb Vasc Biol* 2002, **22**:1929-1935.
44. Marten K, Bremer C, Khazaie K, Sameni M, Sloane B, Tung CH, Weissleder R: **Detection of dysplastic intestinal adenomas using enzyme-sensing molecular beacons in mice.** *Gastroenterology* 2002, **122**:406-414.
45. Bremer C, Tung CH, Bogdanov A Jr, Weissleder R: **Imaging of differential protease expression in breast cancers for detection of aggressive tumor phenotypes.** *Radiology* 2002, **222**:814-818.
46. Josephson L, Kircher MF, Mahmood U, Tang Y, Weissleder R: **Near-infrared fluorescent nanoparticles as combined MR/optical imaging probes.** *Bioconjug Chem* 2002, **13**:554-560.
47. Petrovsky A, Schellenberger E, Josephson L, Weissleder R,
  - Bogdanov A Jr: **Near-infrared fluorescent imaging of tumor apoptosis.** *Cancer Res* 2003, **63**:1936-1942.
 First demonstration of NIR fluorescence imaging with a probe specific for cell death.
48. Zaheer A, Lenkinski RE, Mahmood A, Jones AG, Cantley LC,
  - Frangioni JV: **In vivo near-infrared fluorescence imaging of osteoblastic activity.** *Nat Biotechnol* 2001, **19**:1148-1154.
 Development of a NIR fluorescent bisphosphonate derivative that targets sites of bone growth. Because this small molecule targets the entire skeleton, it is useful for testing the sensitivity and resolution of tomographic imaging systems.
49. Ballou B, Fisher GW, Hakala TR, Farkas DL: **Tumor detection and visualization using cyanine fluorochrome-labeled antibodies.** *Biotechnol Prog* 1997, **13**:649-658.
50. Soukos NS, Hamblin MR, Keel S, Fabian RL, Deutsch TF, Hasan T: **Epidermal growth factor receptor-targeted immunophotodiagnosis and photoimmunotherapy of oral precancer in vivo.** *Cancer Res* 2001, **61**:4490-4496.
51. Neri D, Carnemolla B, Nissim A, Leprini A, Querze G, Balza E, Pini A, Tarli L, Halin C, Neri P *et al.*: **Targeting by affinity-matured recombinant antibody fragments of an angiogenesis associated fibronectin isoform.** *Nat Biotechnol* 1997, **15**:1271-1275.
52. Becker A, Riefke B, Ebert B, Sukowski U, Rinneberg H, Semmler W, Licha K: **Macromolecular contrast agents for optical imaging of tumors: comparison of indotricarbocyanine-labeled human serum albumin and transferrin.** *Photochem Photobiol* 2000, **72**:234-241.
53. Tung CH, Lin Y, Moon WK, Weissleder R: **A receptor-targeted near-infrared fluorescence probe for in vivo tumor imaging.** *ChemBioChem* 2002, **3**:784-786.
54. Achilefu S, Dorshow RB, Bugaj JE, Rajagopalan R: **Novel receptor-targeted fluorescent contrast agents for in vivo tumor imaging.** *Invest Radiol* 2000, **35**:479-485.
55. Becker A, Hensenius C, Licha K, Ebert B, Sukowski U, Semmler W, Wiedenmann B, Grotzinger C: **Receptor-targeted optical imaging of tumors with near-infrared fluorescent ligands.** *Nat Biotechnol* 2001, **19**:327-331.