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Fluorescence imaging *in vivo*: recent advances

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In vivo fluorescence imaging uses a sensitive camera to detect fluorescence emission from fluorophores in whole-body living small animals. To overcome the photon attenuation in living tissue, fluorophores with long emission at the near-infrared (NIR) region are generally preferred, including widely used small indocarbocyanine dyes. The list of NIR probes continues to grow with the recent addition of fluorescent organic, inorganic and biological nanoparticles. Recent advances in imaging strategies and reporter techniques for *in vivo* fluorescence imaging include novel approaches to improve the specificity and affinity of the probes and to modulate and amplify the signal at target sites for enhanced sensitivity. Further emerging developments are aiming to achieve high-resolution, multimodality and lifetime-based *in vivo* fluorescence imaging.

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Introduction

In vivo fluorescence imaging resembles fluorescence microscopy in that both use a low-light camera and appropriate filters to collect fluorescence emission light from samples, but differs in that it works at a macroscopic level. The objects for imaging are whole-body small animals instead of cells in culture dishes or on slides. This extension into the *in vivo* setting allows visualization of biology in its intact and native physiological state, but it is a technically challenging process for at least two reasons. First, thick, opaque animal tissue absorbs and scatters photons and generates strong autofluorescence, all of which obscure signal collection and quantification [1]. Second, the complicated *in vivo* environment puts additional demands on the contrast agent or imaging probe: it has to be biologically stable once distributed in an organism, preferentially accumulate at the intended target site, and produce imaging contrast specific to the target.

Significant advances in mathematical models for describing photon propagation in tissues and in the available instrumentation for illumination and detection have been made in the past to improve the capacity of quantitative fluorescence imaging in tissue, and have recently been reviewed [2,3••]. This short review is not intended to be a comprehensive discussion of all aspects of *in vivo* fluorescence imaging, but instead focuses on the latest progress in imaging probe chemistry and reporter gene techniques.

Imaging probe chemistry

Molecules that absorb in the near-infrared (NIR) region, 700–1000 nm, can be efficiently used to visualize and investigate *in vivo* molecular targets because most tissues generate little NIR fluorescence [4,5]. The most common organic NIR fluorophores are polymethines (Figure 1a). Among them, pentamethine and heptamethine cyanines comprising benzoxazole, benzothaizole, indolyl, 2-quinoline or 4-quinoline have been found to be the most useful [6]. Their physical properties, biodistribution, pharmacokinetics and applications for *in vivo* fluorescence imaging have been summarized in two recent reviews [4,5].

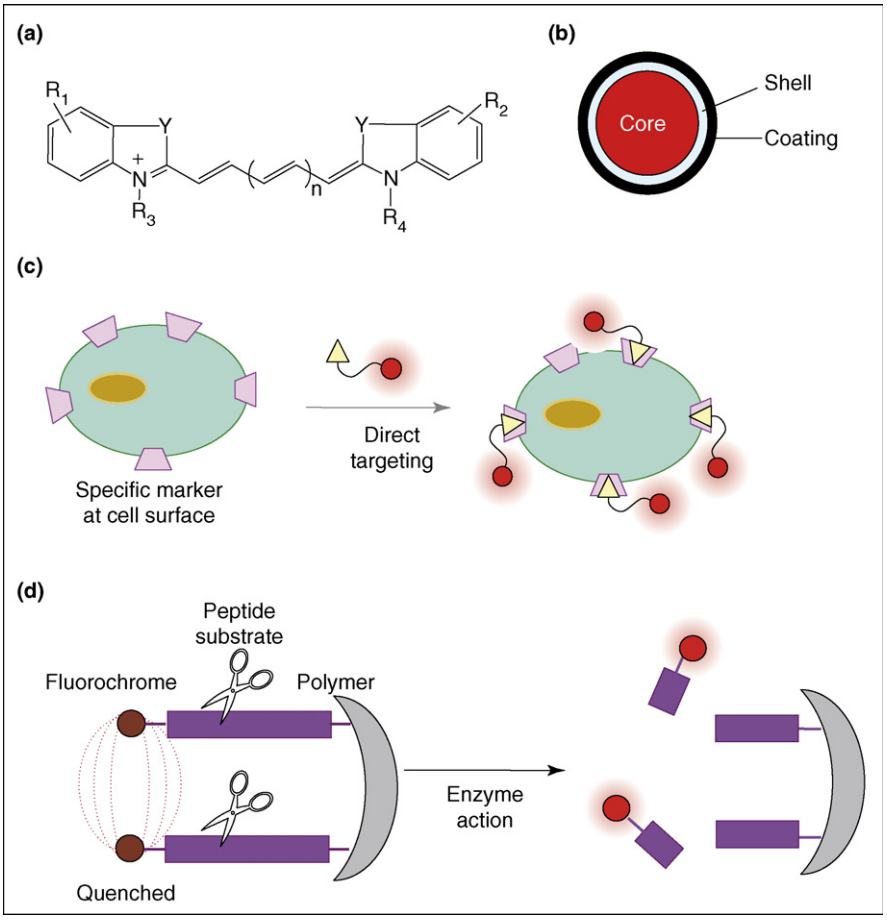
An emerging new class of probes for *in vivo* fluorescence imaging is semiconductor nanocrystals or quantum dots (Figure 1b). Quantum dots (QDs) typically have a core/shell structure of 2–8 nm in diameter with size-dependent fluorescence emission. The unique optical properties of QDs for *in vivo* optical imaging include high absorbency, high quantum yield, narrow emission bands, large Stokes shifts, and high resistance to photobleaching. QDs that emit at several different wavelengths can be excited with a single wavelength, and thus are suitable for multiplex detection of multiple targets in a single experiment. Several recent reviews summarize the synthesis, bioconjugation chemistry, optical features and applications of QDs for *in vivo* imaging [5,7••,8].

The general strategies for designing fluorescent imaging probes can be loosely grouped into non-targeting and targeting. Targeting probes can be further categorized as active or activatable (Figure 1c,d).

Non-targeting probes

Indocyanine green is a non-targeting NIR probe that is currently in clinical use for evaluating blood flow and clearance [9]. QDs have also been used as non-targeted probes and have been shown useful as contrast agent for imaging the vasculature of an adult rat [10,11]. Along similar lines, Kim *et al.* [12] prepared type II QDs with a novel core-shell (where electrons are mostly confined to

Figure 1



Common NIR fluorophores and imaging strategies. **(a)** General chemical structure of organic cyanine NIR fluorescent dyes. Y = C, O, S etc.; n = 1 for pentamethine; n = 2 for heptamethine; R1 and R2 can be identical or different from each other and represent alkyl, sulfoalkyl, cycloalkyl, alkoxyl or aryl groups; R3 and R4 can be sulfoalkyl, haloalkyl or hydroxycarbonylalkyl groups. **(b)** Schematic representation of a QD, comprising an inorganic core, an inorganic protective shell and a coating layer to which targeting molecules can be attached. **(c)** Active probes target cell-surface receptors. The specific cell marker on a target cell (purple) binds to its ligand (yellow), which is conjugated to a fluorochrome (red). **(d)** Scheme of action of enzyme-activatable NIR fluorogenic probes. In the absence of the target protease, the fluorochrome (brown) is quenched by the close proximity of a second fluorochrome molecule. In the presence of the protease, the peptide substrate is cleaved releasing the fluorochromes and resulting in fluorescence.

the shell and holes in the core) and fairly broad emission at 850 nm, which were successfully used to image sentinel lymph nodes in mice and pigs.

Active targeting probes

A general, and simple, approach to improving the accumulation of contrast agents at the target site is to conjugate the fluorochrome to a ligand that binds to a specific molecular target (active probes). The probes bind the targets and are retained at the target site, while non-bound probes are cleared from the circulation (Figure 1c). This approach is most useful for tumor imaging, as cancers often overexpress certain surface receptors.

Ligands can be small molecules, peptides, proteins and antibodies. For example, by taking advantage of expression of the folate receptor on activated but not resting synovial macrophages, Tung and colleagues [13] synthesized a folic acid and NIR fluorochrome conjugate (NIR2-folate) to image activated macrophages involved in inflammatory joint disease. An NIR fluorochrome (Cy5.5) was also employed for the *in vivo* imaging of murine tumors in intact animals [14]; in this case, endostatin, an endogenous inhibitor of angiogenesis with high specificity for proliferating endothelial cells, was labelled. In a further example, annexin V was tagged with a cyanine dye to image phosphotidylserine in apoptotic cells [15]. Fluorescence imaging of osteoblastic activity in living animals has also met with success using an active probe: a tetrasulfonated heptamethine indocyanine conjugated to the hydroxyapatite-binding ligand pamidronate [16]. Both monoclonal antibody and single-chain antibody fragments have been

conjugated with cyanine dyes and QDs for *in vivo* tumor imaging [17••,18].

Activatable targeting probes

Activatable probes are commonly used for functional imaging of enzyme activity [19,20]. They often contain more than two identical or different chromophores, which are joined in close proximity to each other by an enzyme-specific peptide linker (Figure 1d). The probes are essentially dark with little or no fluorescence emission, owing to the quenching effect induced by proximity (identical chromophores) or by resonance energy transfer (different chromophores). Cleavage of the peptide linker releases the fluorophores from each other, resulting in the restoration of the fluorescence emission. Thus, the background signal of activatable probes is generally low, but the contrast and detection sensitivity is higher than for active probes. Several activatable probes have been documented in the literature and been extensively reviewed elsewhere [21]. The enzyme target is mainly limited to proteases, including cathepsins, caspases, matrix metalloproteinases, thrombin, HIV and HSV proteases, and urokinase-type plasminogen activator.

Novel fluorescent probes

Small organic fluorophores

Chemists continue to develop new NIR fluorochromes with improved fluorescence quantum yield, high chemical and photostability, low aggregation tendencies and low cytotoxicity. For example, Dehaen and colleagues used palladium-catalyzed coupling reactions to synthesize several BODIPY derivatives with emission ranging from green to near-infrared [22]. Zhao and Carreria reported conformationally restricted Aza-BOBIPY dyes with improved physical properties and chemical and photo stability [23]. Tung and coworkers designed a series of Nile Blue analogs with excitation and emission near 640 nm and 680 nm, respectively [24]. These new small-molecule organic dyes are yet to be evaluated for *in vivo* fluorescence imaging. Gremlich and colleagues [25] described an NIR fluorescence oxazine dye AOI987 that emits maximally at 670 nm with a maximal excitation of 650 nm in serum. AOI987 can penetrate the intact blood–brain barrier and allowed *in vivo* NIR fluorescence imaging of amyloid plaques in transgenic mice.

Organic fluorescent nanoparticles

A large group of organic nanoparticles such as liposomes, dendrimers and polymersomes have been developed for drug delivery, but can also be applied to *in vivo* optical imaging. Therien and colleagues [26] reported the synthesis of NIR-emissive polymersomes (polymer vesicles with a diameter of 50 nm to 50 μm) through the cooperative self-assembly of amphiphilic diblock copolymers and conjugated multi(porphyrin)-based NIR fluorophores. Dendrimers were previously used as carriers for magnetic resonance imaging (MRI) contrast reagents and, recently, McIntyre *et al.* [27] designed a polyamidoamine dendrimer-based fluorogenic substrate to image tumor-associated matrix metalloproteinase-7 *in vivo*. A boronated dendrimer labeled with a vascular endothelial growth factor (VEGF) and an NIR dye Cy5 has been shown to selectively bind upregulated VEGF receptors in mouse breast carcinoma [28].

Fluorescent biological nanoparticles

When multiple fluorescent dyes are attached to the same molecule, such as an antibody, the fluorescent intensity can decrease instead of increase owing to dye–dye quenching. However, when a viral capsid is used as the scaffold for labeling, more than 40 Cy5 dyes can be loaded onto a single virus particle via specific chemical coupling and no fluorescence quenching is observed due to the large intermolecular distances [29]. This approach has resulted in the synthesis of highly fluorescent viral nanoparticles with a defined structure and a size of ~30 nm in diameter. The local dye concentration was reported to be as high as 1.8 mM without significant quenching [30]. Cowpea mosaic virus nanoparticles labeled with Alexa dyes have been used successfully to visualize the vasculature and blood flow and for imaging human fibrosarcoma-mediated tumor angiogenesis in living mouse and chick embryos [31].

Self-illuminating inorganic nanoparticles

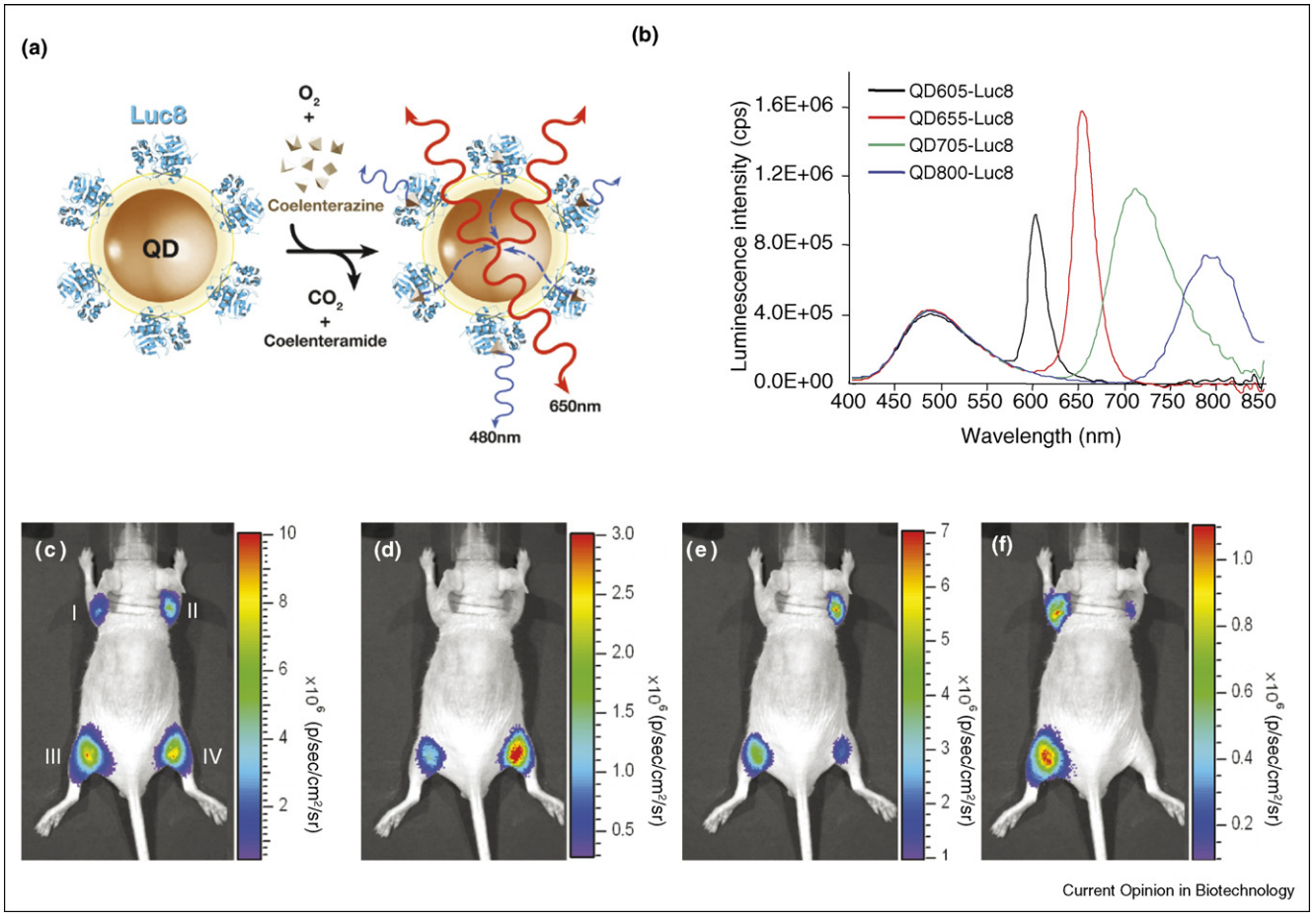
Fluorescent QDs require excitation from external illumination sources to fluoresce. This necessity limits their application for imaging living opaque subjects, owing to the resultant strong autofluorescence background and a paucity of excitation light at non-superficial locations. To overcome this challenge, we designed a new type of QD conjugate that can luminesce without the need for external excitation [32••]. These self-illuminating QD conjugates were prepared by coupling carboxylate-presenting QDs to a mutant of the bioluminescent protein *Renilla* luciferase (Figure 2a). The energy released by luciferase-catalyzed substrate catabolism can be transferred to QDs via resonance energy transfer, leading to QD light emission. These self-illuminating QD conjugates can emit long-wavelength (from red to near-infrared) bioluminescent light in living cells and in living animals, even in deep tissues, and can be applied for multiplex *in vivo* imaging (Figure 2). We have also demonstrated that similar self-illuminating QD conjugates can be obtained when the luciferase is fused to another protein; this will enable many new possibilities for imaging biological events in living subjects [33].

Emerging new imaging strategies

Polyvalency to improve probe affinity

Tight and specific binding of the probe to the target is always the key to successful imaging. As many imaging targets are located outside of the cell surface, the principle of polyvalency can be applied to improve the binding affinity of the probe [34]. Two recent studies

Figure 2



Self-illuminating QDs for *in vivo* imaging. **(a)** Schematic representation of the QD conjugated with six bioluminescent protein molecules (Luc8). The bioluminescence energy released during the Luc8-catalyzed oxidation of coelenterazine is transferred to the QD, resulting in QD emission. **(b)** Bioluminescence spectra of conjugates QD605-Luc8, QD655-Luc8, QD705-Luc8 and QD800-Luc8. All four QD conjugates emit at 480 nm upon the addition of coelenterazine, but also have their own distinct QD emissions (e.g. at 605, 655, 705 and 800 nm), which make them suitable for *in vivo* multiplex imaging. **(c–f)** Multiplexed *in vivo* imaging of self-illuminating QD conjugates in (b) intramuscularly injected at the indicated sites: (I) QD800-Luc8, (II) QD705-Luc8, (III) a mixture of QD665-Luc8, QD705-Luc8 and QD800-Luc8, and (IV) QD655-Luc8. Images were collected with the following emission filters: (c) without any filter, (d) with 575–650 nm filter, (e) with x-Cy5.5 filter (680–720 nm) and (f) with ICG filter (810–875 nm).

have reported the design of oligomeric RGD peptides for imaging the $\alpha_v\beta_3$ integrin receptor in xenografted tumors *in vivo*. Cheng *et al.* [35] synthesized monomeric, dimeric and tetrameric cyclic RGD units and conjugated them to Cy5.5 for tumor imaging in living mice [35]. The Cy5.5–RGD tetramer displayed the highest tumor uptake and tumor-to-background fluorescence ratio. In another study, Ye *et al.* [36] monitored the internalization and localization of multimeric linear RGD–cypate conjugates in intact mice, and found $\alpha_v\beta_3$ integrin receptor binding affinity and tumor uptake to depend on both the number and spatial alignment of the multivalent RGD units.

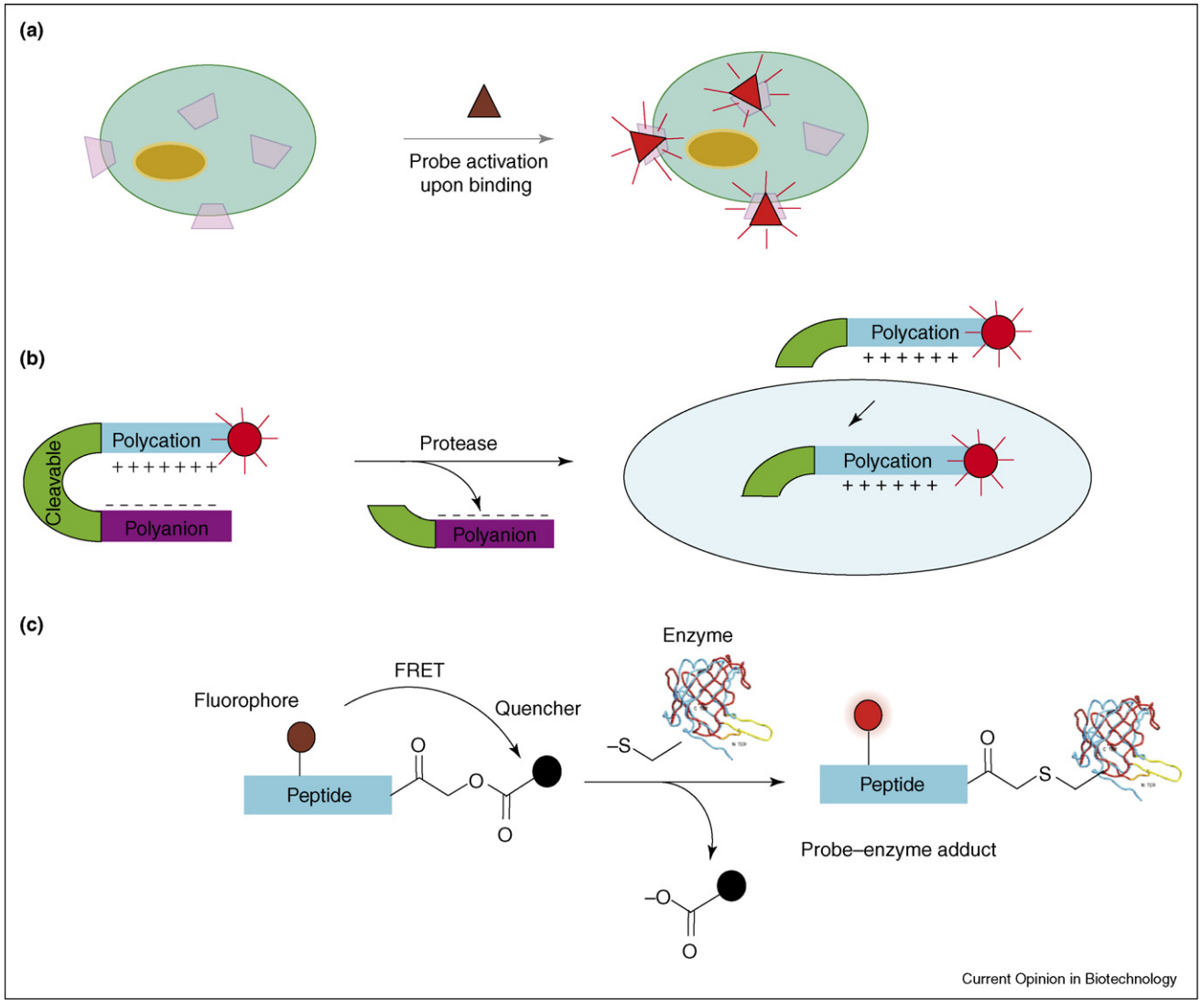
Library screening to discover novel probes

Systematic library screening strategies have long been developed to identify target-specific inhibitors and drug leads; recent studies have expanded them into molecular imaging. Newton *et al.* [37] performed an *in vivo* phage display and micropanning assay to select for phages that extravasate and bind human PC-3 prostate carcinoma xenografts in mice. The identified phage clone was labeled with the near-infrared AlexFluor 680 for *in vivo* imaging, showing a high tumor-to-muscle fluorescence ratio. Combinatorial chemistry has also been used to screen for high-affinity peptidomimetic probes. Peng *et al.* [38••] described a one-bead-one-compound combinatorial library method for live-cell screening and successfully identified a novel peptidomimetic ligand (LLP2A) that binds to $\alpha_4\beta_1$ integrin with high affinity and specificity and can be used for *in vivo* tumor NIR imaging of $\alpha_4\beta_1$ integrin.

Activatable active probes

Active targeting probes generate contrast through retention of the probes at the target site and rapid clearance

Figure 3



New imaging strategies and reporter technologies. **(a)** Target-activated small-molecule fluorochromes. The fluorogenic probe (brown) binds to the target cell marker (purple) and becomes fluorescent (red). **(b)** Enzyme-mediated uptake of CPP-conjugated probes. The fluorophore (red) is linked to a polycationic (CPP) domain (blue) and further via a protease-sensitive peptide (green) to a polyanionic domain (purple), which inhibits the cellular uptake of the probe. In the presence of the protease, the substrate is cleaved causing the detachment of the polyanionic domain and the cellular uptake of the CPP-fluorophore conjugate. **(c)** Ligation-based imaging. The fluorophore (brown) is linked to a quencher (black) via an acyloxy group. The attack of the cysteine protease releases the quencher and forms an adduct with the activated fluorophore (red).

from the blood circulation and non-targeting sites. For probes with high molecular weights, the clearance can be very slow. A probe that will switch on to fluoresce only when it binds the target would overcome this problem (Figure 3a). Recently, Swager and colleagues [39] designed an NIR dye NIAD-4 that can cross the blood–brain barrier and specifically detect amyloid-β deposits in the brain of a living transgenic mouse. In contrast to previously mentioned AIO987 [25], NIAD-4 exhibited 400-fold enhancement in fluorescence emission at 610 nm when bound to aggregated amlyoid fibrils. In another report, Weissleder and coworkers [40] showed that a far-red indocyanine-based fluorophore significantly increases its fluorescence emission upon binding to albumin and can be readily used for *in vivo* specific visualization of tumoral tissues by means of fluorescence molecular tomography. This approach of using target-activated small-molecule fluorochromes is particularly useful for imaging intracellular targets, as the contrast mechanism does not rely on the fast clearance of the probe from the circulation and non-target sites.

Enzyme-mediated uptake of probes

Tsien and colleagues [41•] designed an enzyme-mediated 'gating' strategy to selectively deliver the

imaging probe exclusively to target cells (Figure 3b). The activatable probe consists of two fragments, one polycationic cell-penetrating peptide (CPP) labeled with an NIR dye and one polyanionic blocking peptide that hampers the CPP-induced cellular uptake. These two fragments are connected through a peptide linker sensitive to matrix metalloproteinase-2 (an enzyme highly expressed in tumors). Once the probe reaches the tumor site the linker undergoes proteolytic degradation, leading to the release of the labeled CPP fragment and its subsequent cellular translocation. The accumulation of the reporter probe in target cells can, in principle, lead to signal amplification and high sensitivity. This imaging strategy was successfully demonstrated *in vivo* by the visualization of fibrosarcoma cells in mice and *ex vivo* in human squamous cell carcinoma tissue. We have recently demonstrated that this strategy can also work with nanoparticles such as QDs [42].

Ligation-based probes

Activity-based proteomic profiling employs fluorophore-labeled protein substrates or inhibitors that can form covalent adducts with the target enzyme [43]. Blum *et al.* [44] recently extended this strategy to *in vivo* protease imaging (Figure 3c). Their design was based on the covalent inhibition of a cysteine protease by an acyloxymethyl ketone. The probe consisted of a fluorophore-labeled peptide connected to a quencher-acyloxy leaving group. In the presence of protease the probe becomes fluorescent and covalently attached to the enzyme. Although the efficiency of this design has been demonstrated in live cells, its *in vivo* utility remains to be tested.

New reporter technologies

Red and near-infrared fluorescent proteins

Reporter technologies employ reporter genes, and have been an indispensable tool to study gene expression and regulation. There are several reporter genes for *in vivo* imaging [45]. Among them, fluorescent proteins (FPs) remain highly popular because they are genetically encoded and easy to use with no need for systematic delivery of the imaging probe. FP-based *in vivo* imaging in cancer research allows direct visualization of primary tumor growth, invasion, metastatic seeding and colonization, angiogenesis, and interaction between the tumor and its local environment [46•]. Since the initial discovery of green fluorescent protein from the jellyfish *Aequorea victoria*, numerous mutant FPs with a wide range of emission wavelengths have been created through extensive genetic engineering. Tsien and coworkers [47] combined iterative somatic hypermutation and fluorescence-activated cell cytometry to produce FP variants with red and NIR emission (648 nm) that will be better suited to *in vivo* fluorescence imaging. The availability of these new FP mutants greatly overcomes the previously limited choice of emission color and will facilitate newer applications for *in vivo* imaging [48•].

β-Galactosidase

β-Galactosidase (β-gal) is another commonly used reporter with many fluorogenic and chromogenic substrates already developed for its detection. For example, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) is routinely used for β-gal expression staining, and fluorescein di-β-D-galactoside (FDG) is used for live cell imaging. Recently, an improved substrate (2-Me-4-OMe-Tokyo-Green O-β-galactoside or TG-βGal) has been reported for live cell imaging with single-phase kinetics and has been found superior to FDG [49]. To extend the utility of β-gal from *in vitro* and live cell imaging to *in vivo* imaging, Tung *et al.* [50] demonstrated that a previously available substrate 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) β-D-galactopyranoside (DDAOG) can be used to image β-gal expression in a living mouse. The cleavage of DDAOG by β-gal releases the fluorophore DDAO that emits maximally at 659 nm with a 50 nm red shift from the substrate DDAOG. However, the narrow separation of the DDAO emission spectrum from its excitation spectrum significantly limits the sensitivity of the reporter. Another important development that has greatly improved the sensitivity of the β-gal reporter is the invention of a sequential reporter-enzyme technology for *in vivo* imaging of β-gal. Blau *et al.* [51] took advantage of the high *in vivo* sensitivity of a bioluminescent reporter firefly luciferase (FLuc) and combined it with a commercially available substrate, a caged D-luciferin-D-galactoside conjugate (Lugal), to image β-gal *in vivo*. β-Gal first cleaves Lugal to release D-luciferin that can then be catalytically oxidized by FLuc to generate light. These recent additions help unify β-gal as a single reporter for *in vitro*, cell culture, and *in vivo* imaging.

β-Lactamase

TEM-1 β-lactamase (Bla), a small (29 kDa), monomeric bacterial enzyme, is another popular reporter and has been extensively used for detecting or imaging biological processes and interactions in living mammalian tissue culture cells; for example, Bla has been used to study the activity of promoter/regulatory elements, for imaging RNA splicing, monitoring viral infection, detecting protein–protein interactions, and visualizing cell-surface proteolytic activity of proteases. Several fluorogenic substrates have been reported [52–55]. Recently, we have designed a cell-permeable NIR fluorogenic substrate for Bla imaging in live cells [55]. Our design utilized a fluorescence resonance energy transfer (FRET) pair, Cy5-QSY21; the substrate cephalosporin was connected to the NIR fluorophore Cy5 at the 7-amino group and to the quencher QSY21 at the 3′ site. A D-glucosamine analog was also conjugated to the probe to facilitate the cellular uptake. C6 cells expressing Bla can be imaged with this probe in culture. The probe was also modified for the successful imaging of Bla expression in a C6 glioma tumor in living mice (B Xing, H Yao, J Rao, unpublished).

Receptor imaging mediated by the biotin–streptavidin interaction

In contrast to the three optical reporter systems mentioned above, a new reporter technique based on the tight interaction between biotin and streptavidin has recently been described by Tannous *et al.* [56] for imaging reporter-labeled cells in culture and *in vivo*. The reporter is a recombinant membrane protein (BAP-TM) that contains a biotin acceptor peptide (BAP) which can be biotinylated by endogenous biotin ligase. Subsequently, labeled streptavidin probes were injected to selectively bind biotinylated reporter proteins. This versatile technique can be applied to other *in vivo* imaging modalities such as MRI.

Emerging new directions

High-resolution *in vivo* fluorescence imaging

Unlike fluorescence microscopy, *in vivo* fluorescence imaging detects bulk signals from thousands of cells with much lower resolution. By contrast, fluorescence microendoscopy uses small size optical probes (typically 0.25–1 mm in diameter) that are minimally invasively inserted into solid tissues to achieve high-resolution imaging deep within tissues [57]. Combined with fluorescent probes, this technology could allow the interrogation of the biology at the single-cell and single-molecule level in living tissues.

Multimodality imaging

The coregistration of *in vivo* fluorescence imaging with anatomical imaging modalities such as MRI and X-ray computed tomography (CT) helps traverse the shortcomings of fluorescence imaging, such as limited tissue penetration of photons and low three-dimensional spatial resolution, and provides complementary information. The development of multifunctional probes is attracting increasing attention and several studies have already appeared — from iron-oxide- and dendrimer-based dual MRI–fluorescence imaging contrast agents to a fused X-ray CT-fluorescence imaging system from Kodak [58,59].

FLIM imaging

The majority of *in vivo* fluorescence imaging studies currently measure the change in fluorescence intensity. Unlike intensity measurements, fluorescence lifetime imaging (FLIM) is less dependent on the local concentration of the probe and is inherently robust in the presence of absorption and scattering, although it can be highly sensitive to changes in the environment [60]. Several recent studies have begun to explore the utility of whole-body FLIM in living small animals through the development of three-dimensional fluorescence lifetime tomography and by imaging tumor xenografts with an NIR peptide probe [61,62].

Conclusions

In vivo fluorescence imaging visualizes the fluorescent emissions from fluorophores in whole-body living small animals. With the increasing availability of fluorescent probes and reporters, it is gaining momentum as an important translational tool between basic research and clinical application. Although traditional small-molecule NIR dyes continue to be used, the development of fluorescent organic, biological, and inorganic nanoparticles for *in vivo* fluorescence imaging offers powerful tools for many exciting applications. These nanoparticles serve as a platform to build multifunctional probes for multimodality imaging. The invention of novel imaging strategies and reporter techniques will greatly improve the specificity and sensitivity of the probes. Advances in the development of high-resolution and lifetime-based *in vivo* fluorescence imaging could further empower this imaging technology. The continued evolution of these new technologies will allow more molecular targets and diseases to be interrogated *in vivo*.

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