In vivo molecular and cellular imaging with quantum dots

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Quantum dots (QDs), tiny light-emitting particles on the nanometer scale, are emerging as a new class of fluorescent probe for *in vivo* biomolecular and cellular imaging. In comparison with organic dyes and fluorescent proteins, QDs have unique optical and electronic properties: size-tunable light emission, improved signal brightness, resistance against photobleaching, and simultaneous excitation of multiple fluorescence colors. Recent advances have led to the development of multifunctional nanoparticle probes that are very bright and stable under complex *in vivo* conditions. A new structural design involves encapsulating luminescent QDs with amphiphilic block copolymers and linking the polymer coating to tumor-targeting ligands and drug delivery functionalities. Polymer-encapsulated QDs are essentially nontoxic to cells and animals, but their long-term *in vivo* toxicity and degradation need more careful study. Bioconjugated QDs have raised new possibilities for ultrasensitive and multiplexed imaging of molecular targets in living cells, animal models and possibly in humans.

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Abbreviations

PEG	polyethylene glycol
PSMA	prostate-specific membrane antigen
QD	quantum dot
TOPO	tri-n-octylphosphine oxide

Introduction

Semiconductor quantum dots (QDs) have captivated scientists and engineers over the past two decades owing to their fascinating optical and electronic properties, which are not available from either isolated molecules or bulk solids. Recent research has stimulated considerable interest in developing these quantum-confined nanocrystals as fluorescent probes for biomedical applications. Compared with organic dyes and fluorescent proteins, semiconductor QDs offer several unique advantages, such as size- and composition-tunable emission from visible to infrared wavelengths, large absorption coefficients across a wide spectral range, and very high levels of brightness and photostability [1]. Owing to their broad excitation profiles and narrow/symmetric emission spectra, high-quality QDs are also well suited for combinatorial optical encoding, in which multiple colors and intensities are combined to encode thousands of genes, proteins or small-molecule compounds [2–4].

Despite their relatively large size (2–8 nm diameter), recent research has shown that bioconjugated QD probes behave like fluorescent proteins (4–6 nm) and do not suffer from serious binding kinetic or steric hindrance problems [5•,6••,7••,8,9,10•,11,12]. In this 'mesoscopic' size range, QDs also have a greater surface area and more functionalities that can be used for linking to multiple diagnostic (e.g. radioisotopic or magnetic) and therapeutic (e.g. anticancer) agents. Furthermore, polymer-encapsulated QDs have been found to be essentially nontoxic to cells and animals, an essential requirement for future clinical applications. In this article, we briefly discuss new developments and *in vivo* imaging applications of QD probes that have appeared in the past two to three years. In particular, we discuss the use of multifunctional QDs for simultaneous tumor targeting and imaging in living animals. For further information on QD fundamentals and applications, excellent review articles are available in the literature [1,13,14••].

QD probe development

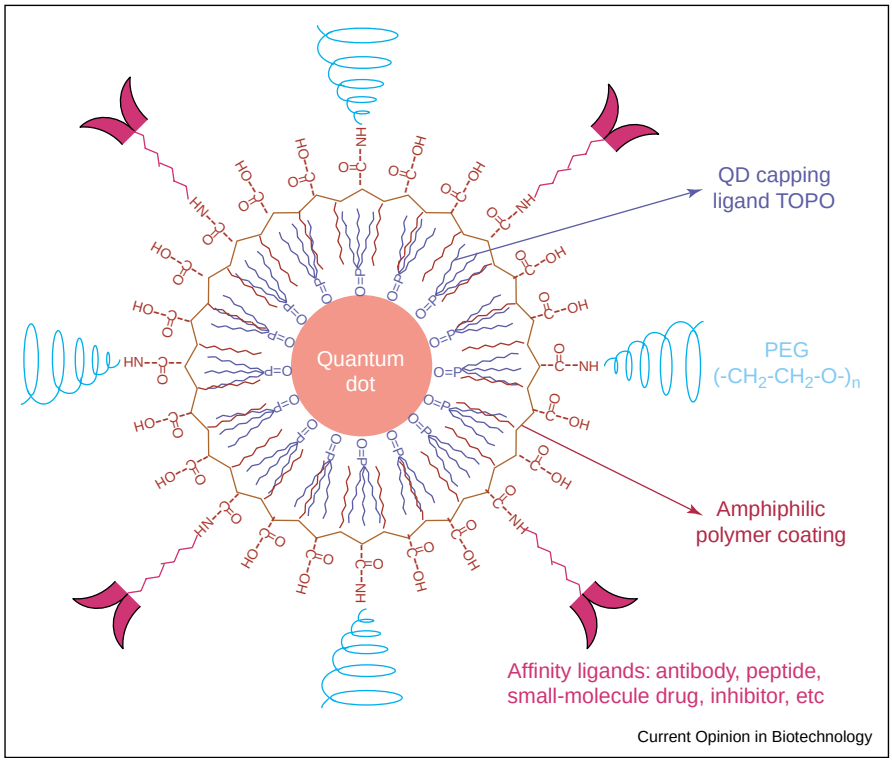
Research in probe development has focused on the synthesis, solubilization and bioconjugation of highly luminescent and stable QDs. The particles are generally made from hundreds to thousands of atoms of group II and VI elements (e.g. CdSe and CdTe) or group III and V elements (e.g. InP and InAs). Recent advances have allowed the precise control of particle size, shape (dots, rods or tetrapods) [15–18] and internal structure (core-shell, gradient alloy or homogeneous alloy) [19–21,22•].

In addition, QDs have been synthesized using both two-element systems (binary dots) and three-element systems (ternary alloy dots). Their fluorescence emission wavelength can be continuously tuned from 400 nm to 2000 nm by changing both the particle size and chemical composition, with fluorescence quantum yields as high as 85% at room temperature [23].

High-quality QDs are typically prepared at elevated temperatures in organic solvents, such as tri-n-octylphosphine oxide (TOPO) and hexadecylamine, both of which are high boiling point solvents containing long alkyl chains. These hydrophobic organic molecules not only serve as the reaction medium, but also coordinate with unsaturated metal atoms on the QD surface to prevent the formation of bulk semiconductors. As a result, the nanoparticles are capped with a monolayer of the organic ligands and are soluble only in nonpolar hydrophobic solvents such as chloroform. For biological imaging applications, these hydrophobic dots can be solubilized by using amphiphilic polymers that contain both a hydrophobic segment or sidechain (mostly hydrocarbons) and a hydrophilic segment or group (such as polyethylene glycol [PEG] or multiple carboxylate groups). Several polymers have been reported including octylamine-modified low molecular weight polyacrylic acid, PEG-derivatized phospholipids, block copolymers and polyanhydrides [5•,24••,25••,26]. As illustrated in Figure 1, the hydrophobic domains strongly interact with TOPO on the QD surface, whereas the hydrophilic groups face outwards and render the QDs water-soluble. Note that the coordinating organic ligands (TOP or TOPO) are retained on the inner surface of QDs, a feature that is important for maintaining the optical properties of QDs and for shielding the core from the outside environment. To achieve binding specificity or targeting abilities, polymer-coated QDs are linked to bioaffinity ligands such as monoclonal antibodies, peptides, oligonucleotides or small-molecule inhibitors. Linking to polyethylene glycols or similar ligands can also lead to improved biocompatibility and reduced nonspecific binding.

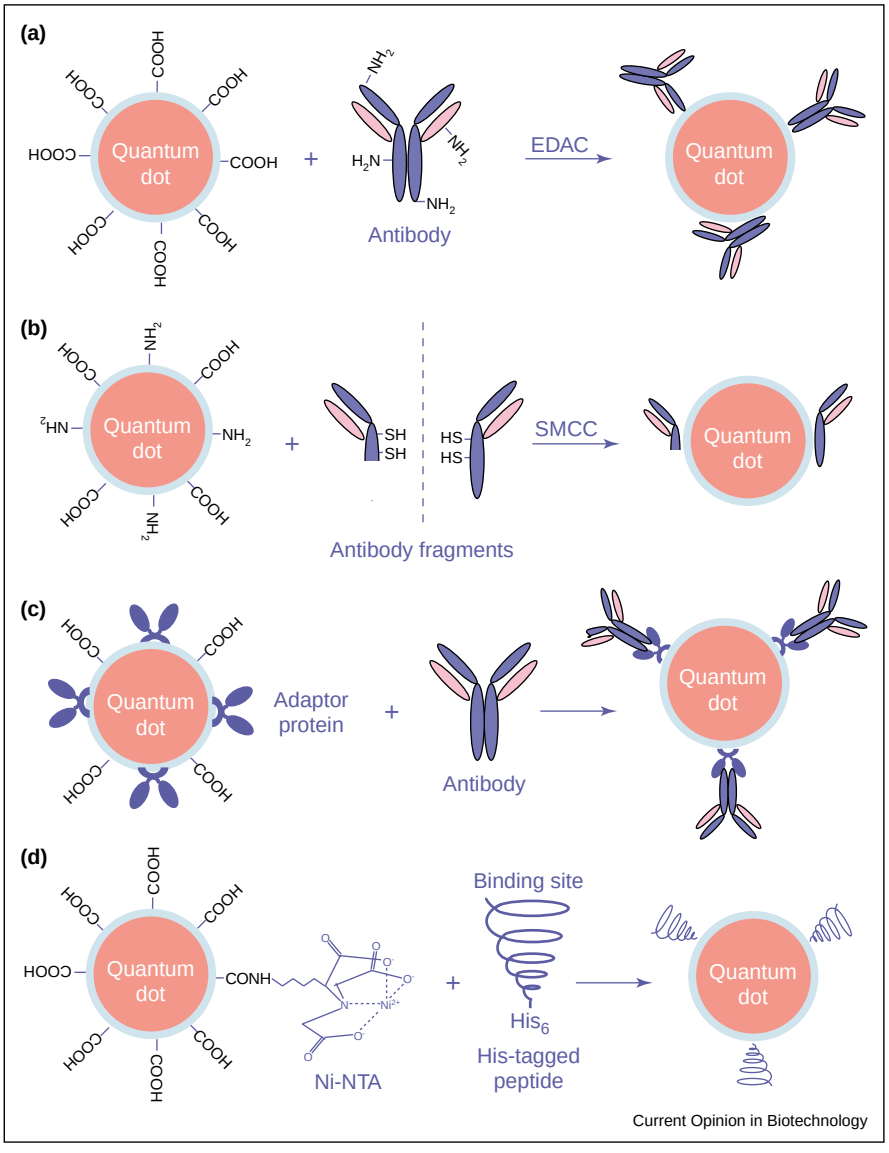
QD bioconjugation can be achieved using several approaches including passive adsorption, multivalent chelation or covalent-bond formation (Figure 2). Two popular cross-linking reactions are carbodiimide-mediated amide formation and active ester maleimide-mediated amine and sulfhydryl coupling. An advantage for the carboxylate-amine condensation method is that most proteins contain primary amine and carboxylic acid groups, and do not need any chemical modification before

Figure 1



The structure of a multifunctional QD probe. Schematic illustration showing the capping ligand TOPO, an encapsulating copolymer layer, tumor-targeting ligands (such as peptides, antibodies or small-molecule inhibitors), and polyethylene glycol (PEG).

Figure 2



Methods for conjugating QDs to biomolecules. **(a)** Traditional covalent cross-linking chemistry using EDAC (ethyl-3-dimethyl amino propyl carbodiimide) as a catalyst. **(b)** Conjugation of antibody fragments to QDs via reduced sulfhydryl-amine coupling. SMCC, succinimidyl-4-*N*-maleimidomethyl-cyclohexane carboxylate. **(c)** Conjugation of antibodies to QDs via an adaptor protein. **(d)** Conjugation of histidine-tagged peptides and proteins to Ni-NTA-modified QDs, with potential control of the attachment site and QD:ligand molar ratios.

QD conjugation. By contrast, free sulfhydryl groups are rare in native biomolecules and are often unstable in the presence of oxygen. Depending on the available chemical groups, other conjugation reactions can also be used. For example, Pellegrino *et al.* [26] reported the use of a pre-activated amphiphilic polymer for nanoparticle solubilization. This polymer contains multiple anhydride units and is highly reactive towards primary amines without the addition of coupling reagents. This procedure deserves further attention, because polyanhydrides represent a class of biodegradable polymers that are under intense development for use in sustained drug delivery and tissue engineering [27,28].

Several strategies can be used to manipulate the molecular orientation of the attached ligands as well as their molar ratios with respect to QDs. But, 'perfect' QD probes with precisely controlled ligand orientations and molar ratios are still not available. Goldman *et al.* [29] first explored the use of a fusion protein as an adaptor for immunoglobulin G antibody coupling. The adaptor protein has a positively charged leucine zipper domain for

electrostatic interaction with QDs and a protein G domain that binds to the antibody Fc region. As a result, the Fc end of the antibody is connected to the QD surface, with the target-specific $F(ab')_2$ domain facing outwards (Figure 2c). In a dramatically different approach, we have linked QDs to a chelating compound (nickel-nitrilotriacetic acid or Ni-NTA) that quantitatively binds to hexahistidine-tagged biomolecules with controlled molar ratio and molecular orientation (Figure 2d). Early studies using genetically engineered peptides showed excellent tumor-targeting abilities (X Gao *et al*., unpublished). This indirect histidine-tag coupling method has several advantages, such as a controlled or known orientation of the binding ligand (as a histidine-tag can be conveniently fused to proteins and peptides at a particular site), compact overall probe size (which should improve binding efficiencies), and low production costs (direct coupling and rapid purification).

Novel optical properties

As briefly noted above, QDs are made from inorganic semiconductors and have novel optical properties that can be used to optimize the signal-to-background ratio. QDs have very large molar extinction coefficients in the order of $0.5–5 \times 10^6\ M^{-1}cm^{-1}$ [30], which makes them brighter probes under photon-limited *in vivo* conditions (where light intensities are severely attenuated by scattering and absorption). In theory, the lifetime-limited emission rates for single QDs are 5–10 times lower than those of single organic dyes, because of their longer excited state lifetimes (20–50 ns). In practice, however, fluorescence imaging usually operates under absorption-limited conditions, in which the rate of absorption is the main limiting factor of fluorescence emission. As the molar extinction coefficients of QDs are about 10–50 times larger than those of organic dyes ($5–10 \times 10^4\ M^{-1}cm^{-1}$), the QD absorption rates will be 10–50 times faster at the same excitation photon flux (i.e. the number of incident photons per unit area). Owing to this increased rate of light emission, individual QDs have been found to be 10–20 times brighter than organic dyes (Figure 3a) [31,32]. In addition, QDs are several thousand times more stable against photobleaching than organic dyes (Figure 3b) and are thus well-suited for continuous tracking studies over a long period of time.

The longer excited state lifetimes of QDs provide a means to separate the QD fluorescence from background fluorescence, in a technique known as time-domain imaging [33,34]. Figure 3c shows a comparison of the excited state decay curves of QDs and organic dyes. Assuming that the initial fluorescence intensities of QDs and dyes after a pulse excitation are the same and that the fluorescence lifetime of QDs is one order of magnitude longer, one can estimate that the QD and dye intensity ratio (I_{QD}/I_{dye}) will increase rapidly from 1 at time t = 0 to ~100 in only 10 ns (t = 10 ns). Thus, the image contrast (measured by signal-to-noise or signal-to-background ratio) can be dramatically improved by time-delayed data acquisition.

The large Stokes shifts of QDs (measured by the distance between the excitation and emission peaks) can be used to further improve detection sensitivity. This factor becomes especially important for *in vivo* molecular imaging due to the high autofluorescence background often seen in complex biomedical specimens. As shown in Figure 3d, the Stokes shifts of semiconductor QDs can be as large as 300–400 nm, depending on the wavelength of the excitation light. Organic dye signals with a small Stokes shift are often buried by strong tissue autofluorescence, whereas QD signals with a large Stokes shift are clearly recognizable above the background. This 'color contrast' is only available to QD probes, as the signals and background can be separated by wavelength-resolved or spectral imaging [25••].

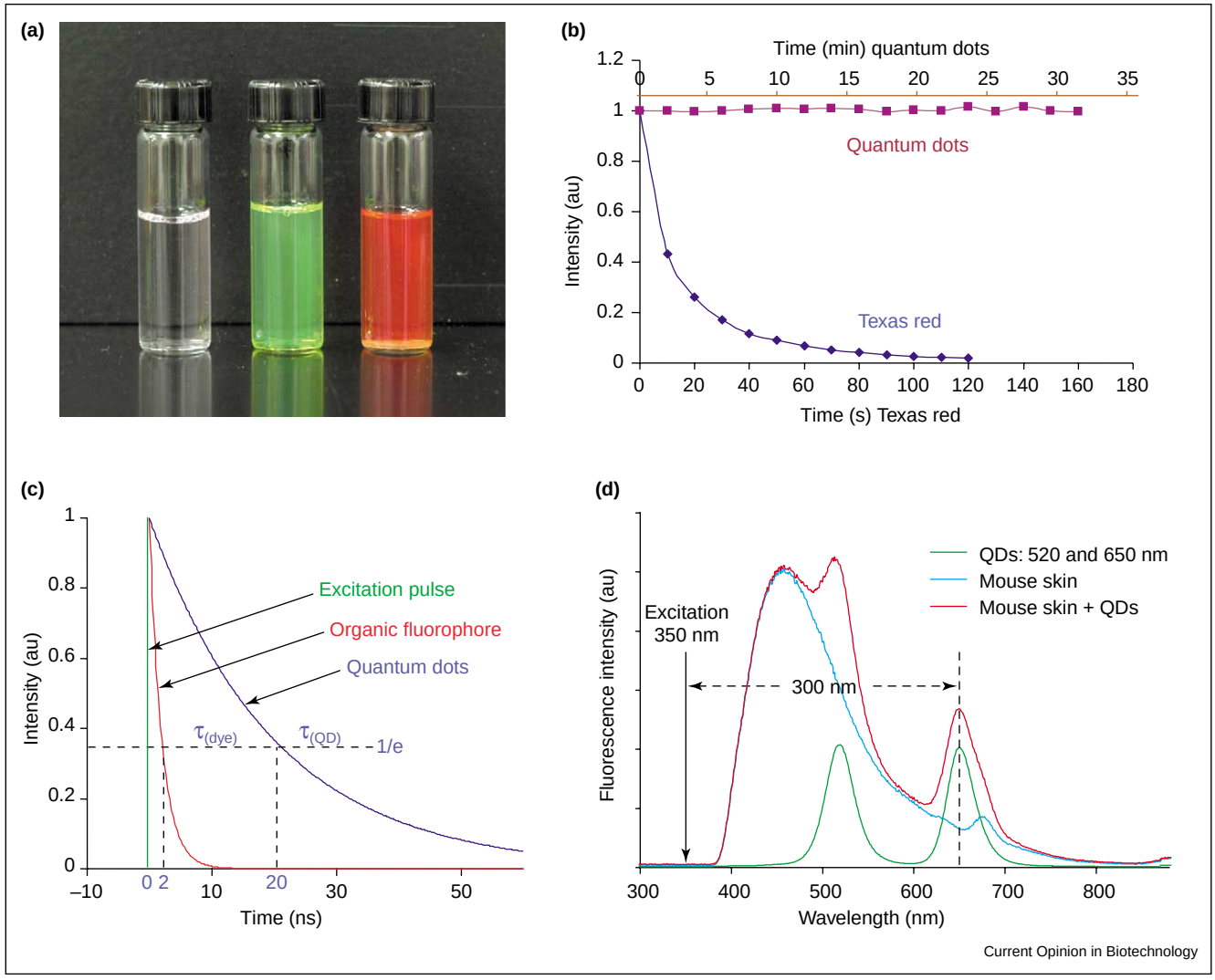
A further advantage of QDs is that multicolor QD probes can be used to image and track multiple molecular targets simultaneously. This is a very important feature, because most complex human diseases such as cancer and atherosclerosis involve a large number of genes and proteins. Tracking a panel of molecular markers at the same time will allow scientists to understand, classify and to differentiate complex human diseases [35]. Multiple parameter imaging, however, represents a significant challenge for magnetic resonance imaging, positron emission tomography, computed X-ray tomography, and related imaging modalities. By contrast, fluorescence optical imaging provides both signal intensity and wavelength information, and multiple wavelengths or colors can be resolved and imaged simultaneously (color imaging). Therefore, different molecular or cellular targets can be tagged with different colors. In this regard, QD probes are particularly attractive, because their broad absorption profiles allow simultaneous excitation of multiple colors and their emission wavelengths can be continuously tuned by varying particle size and chemical composition. For organ and vascular imaging in which micrometer-sized particles could be used, optically encoded beads (polymer beads embedded with multicolor QDs at controlled ratios) could allow multiplexed molecular profiling *in vivo* at high sensitivities [35–40].

In vivo molecular and cellular imaging

Cellular imaging and tracking

The use of QDs for sensitive and multicolor cellular imaging has seen major recent advances, owing to significant improvements in QD synthesis, surface chemistry and conjugation (Figure 4). Wu *et al*. [5•] linked polymer-protected QDs to streptavidin and showed detailed cell skeleton structures using confocal microscopy. The improved photostability of QDs allowed acquisition of many consecutive focal-plane images and

Figure 3

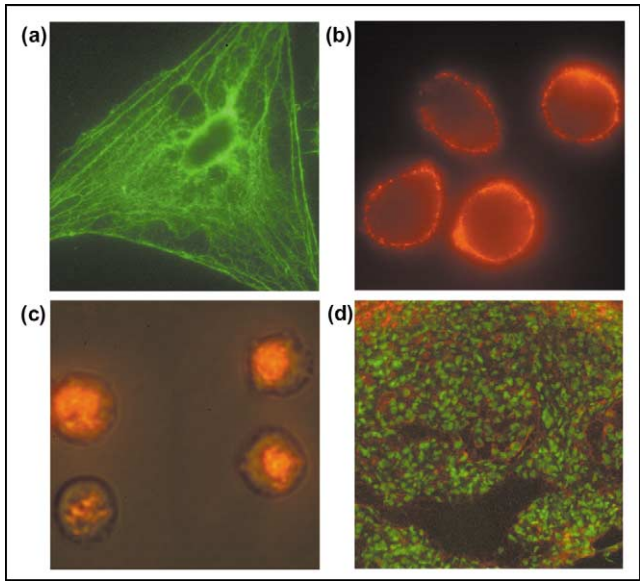


Novel optical properties of QDs for improving the sensitivity of *in vivo* bioimaging. **(a)** Comparison of fluorescence light emission from the organic dye tetramethylrhodamine isothiocyanate (TRITC; left vial), green QDs (middle vial) and red QDs (right vial) under normal room light illumination and at the same molar concentration (1.0 μM). Bright fluorescence emission is observed from the QDs but not from the dye, owing to the large absorption cross-sections of QDs. **(b)** Photobleaching curves showing that QDs are several thousand times more photostable than organic dyes (e.g. Texas red) under the same excitation conditions. **(c)** A comparison of the excited state decay curves (monoexponential model) between QDs and common organic dyes. The longer excited state lifetimes of QD probes allow the use of time-domain imaging to discriminate against the background fluorescence (short lifetimes). $\tau_{(dye)}$ and $\tau_{(QD)}$ are the delay times for the fluorescence signals to decrease to 1/e of their original values, where e is the natural log constant and is equal to 2.718. **(d)** Comparison of mouse skin and QD emission spectra obtained under the same excitation conditions, demonstrating that the QD signals can be shifted to a spectral region where autofluorescence is reduced.

their reconstruction into a high-resolution three-dimensional projection. The high electron density of QDs also allowed correlated optical and electron microscopy studies of cellular structures [41]. Going one step further, Dahan, Jovin and their coworkers achieved real-time visualization of single-molecule movement in single living cells [6••,7••], a task that would be extremely difficult or impossible with organic dyes. The single-molecule sensitivity achieved should open new avenues for studying receptor diffusion dynamics, ligand–receptor interactions, biomolecular transport, enzyme activity and molecular motors.

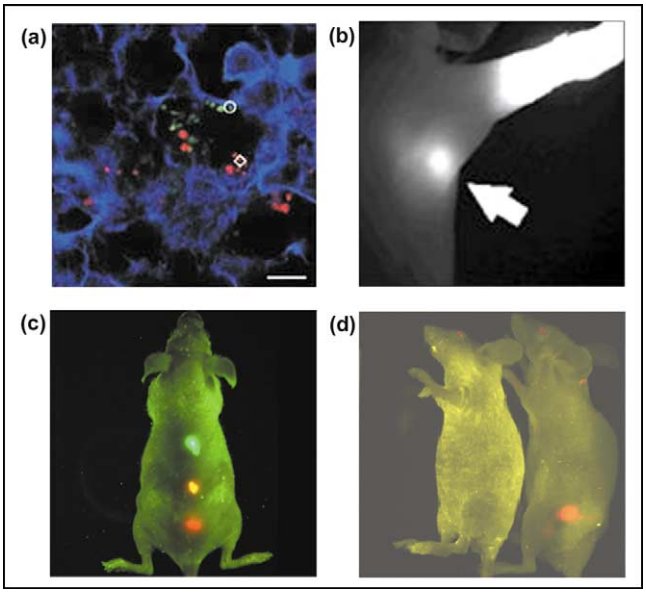
For long-term cell imaging and tracking, Dubertret *et al.* [24••] encapsulated QDs with PEG-derivatized phospholipid micelles and injected them into frog embryos. The resulting PEG-coated dots were highly stable and biocompatible with normal embryo development for up to 4 days [24••]. Other recent studies also took advantage of the extraordinary photostability of QD probes, and

Figure 4



Fluorescence micrographs of QD-stained cells and tissues. **(a)** Actin staining (green QDs) on fixed 3T3 fibroblast cells. **(b)** Live MDA-MB-231 breast tumor cells labeled with a red QD–antibody conjugate targeting the urokinase plasminogen receptor. **(c)** Intracellular labeling of live mammalian cells using QD–Tat peptide conjugates [25••]. **(d)** Frozen tissue specimens stained with QDs (targeting the CXCR4 receptor, red) and a nuclear dye (green).

Figure 5



In vivo targeting and imaging with QDs. **(a)** *Ex vivo* tissue examination of QD-labeled cancer cells trapped in a mouse lung [44•]. **(b)** Near-infrared fluorescence of water-soluble type II QDs taken up by sentinel lymph nodes [49••]. **(c)** *In vivo* simultaneous imaging of multicolor QD-encoded microbeads injected into a live mouse [25••]. **(d)** Molecular targeting and *in vivo* imaging of a prostate tumor in mouse using a QD–antibody conjugate (red) [25••].

achieved real-time tracking of molecules and cells over extended periods of time [6••,7••,9]; for example, QDs were rapidly taken up by lymph nodes and were observable for more than 4 months in mice [42•].

Semiconductor QDs have also been employed as cell 'taggants' for *in vivo* imaging of pre-labeled cells (Figure 4c) [24••,25••,43,44•,45,46]. The results indicate that large amounts of QDs can be delivered into live mammalian cells via three different mechanisms: non-specific pinocytosis, microinjection, and peptide-induced transport (e.g. using the protein transduction domain of HIV-1 Tat peptide, Tat-PTD) [25••]. A surprising finding was that two billion QDs could be delivered into the nucleus of a single cell, without compromising its viability, proliferation or migration [24••,44•,47]. The ability to image single-cell migration and differentiation in real time is expected to be important to several research areas such as embryogenesis, cancer metastasis, stem-cell therapeutics and lymphocyte immunology.

Lymph node and vascular mapping

In vivo imaging with QDs has been reported for lymph node mapping, blood pool imaging, and cell subtype isolation (Figure 5a–c). Ballou and coworkers injected PEG-coated QDs into the mouse blood stream and investigated how the surface coating would affect their circulation lifetime [42•]. In contrast to small organic dyes, which are eliminated from the circulation within minutes after injection, PEG-coated QDs were found to stay in the blood circulation for an extended period of time (half-life more than 3 h). This long-circulating feature can be explained by the unique structural properties of QD nanoparticles. PEG-coated QDs fall within an intermediate size range: they are small enough and sufficiently hydrophilic to slow down opsonization and reticuloendothelial uptake, but are large enough to avoid renal filtration. Webb and coworkers took advantage of this property, and reported the use of QDs and two-photon excitation to image small blood vessels [48•]. They found that the two-photon absorption cross-sections of QDs are two to three orders of magnitude larger than those of traditional organic fluorophores.

For improved tissue penetration, Kim *et al.* prepared a novel core-shell nanostructure called type II QDs [49••] with fairly broad emission at 850 nm and a moderate quantum yield of ~13%. In contrast to conventional QDs (type I), the shell materials in type II QDs have valence and conduction band energies both lower than those of the core materials. As a result, the electrons and holes are physically separated and the nanoparticles emit light at reduced energies (longer wavelengths). Their results showed rapid uptake of bare QDs into lymph nodes, and clear imaging and delineation of involved sentinel nodes (which could then be removed).

This work points to the possibility that QD probes could be used for real-time intra-operative optical imaging, providing an *in situ* visual guide so that a surgeon could locate and remove small lesions (e.g. metastatic tumors) quickly and accurately. At present, however, high-quality QDs with near-infrared-emitting properties are not yet available. Most materials (e.g. PdS, PdSe, CdHgTe and CdSeTe) are either not bright enough or not stable enough for biomedical imaging applications. As such, there is an urgent need to develop bright and stable near-infrared-emitting QDs that are broadly tunable in the far-red and infrared spectral regions. Theoretical modeling studies by Lim *et al.* [50] indicate that two spectral windows are excellent for *in vivo* QD imaging, one at 700–900 nm and another at 1200–1600 nm.

Tumor targeting and imaging

Akerman *et al.* [51••] first reported the use of QD–peptide conjugates to target tumor vasculatures, but the QD probes were not detected in living animals. Nonetheless, *in vitro* histological results revealed that QDs homed to tumor vessels guided by the peptides and were able to escape clearance by the reticuloendothelial system. Most recently, Gao *et al.* [25••] reported a new class of multifunctional QD probes for simultaneous targeting and imaging of tumors in live animals. This class of QD conjugates contains an amphiphilic triblock copolymer for *in vivo* protection, targeting ligands for tumor antigen recognition, and multiple PEG molecules for improved biocompatibility and circulation. The use of an amphiphilic triblock copolymer has solved the problems of particle aggregation and fluorescence loss previously encountered for QDs stored in physiological buffer or injected into live animals [51••,52,53]. Detailed studies were reported on the *in vivo* behavior of QD probes, including their biodistribution, nonspecific uptake, cellular toxicity and pharmacokinetics.

Under *in vivo* conditions, QD probes can be delivered to tumors either by a passive targeting mechanism or through an active targeting mechanism. In the passive mode, macromolecules and nanometer-sized particles are accumulated preferentially at tumor sites through an enhanced permeability and retention effect [54,55]. This effect is believed to arise from two factors. First, angiogenic tumors produce vascular endothelial growth factor, which hyperpermeabilizes the tumor-associated neovasculature and causes the leakage of circulating macromolecules and small particles. Second, tumors lack an effective lymphatic drainage system, which leads to subsequent macromolecule or nanoparticle accumulation. For active tumor targeting, Gao *et al.* [25••] used antibody-conjugated QDs to target a prostate-specific membrane antigen (PSMA; Figure 5d). Previous research identified PSMA as a cell-surface marker for both prostate epithelial cells and neovascular endothelial cells [56]. PSMA has been selected as an attractive target for both imaging and therapeutic intervention of prostate cancer [57]. Accumulation and retention of PSMA antibody at the site of tumor growth is the basis of radioimmunoscintigraphic scanning and targeted therapy for human prostate cancer metastasis [58].

Toxicity and potential clinical use

The potential toxic effects of semiconductor QDs have recently become a topic of considerable importance and discussion. Indeed, *in vivo* toxicity is likely to be a key factor in determining whether QD imaging probes would be approved by regulatory agencies for human clinical use. Recent work by Derfus *et al.* [59•] indicates that CdSe QDs are highly toxic to cultured cells under UV illumination for extended periods of time. This is not surprising because the energy of UV irradiation is close to that of a covalent chemical bond and dissolves the semiconductor particles in a process known as photolysis, releasing toxic cadmium ions into the culture medium. In the absence of UV irradiation, QDs with a stable polymer coating have been found to be essentially nontoxic to cells and animals (no effect on cell division or ATP production; D Stuart *et al.*, unpublished). *In vivo* studies by Ballou and coworkers also confirmed the nontoxic nature of stably protected QDs [42•]. Still, there is an urgent need to study the cellular toxicity and *in vivo* degradation mechanisms of QD probes. For polymer-encapsulated QDs, chemical or enzymatic degradation of the semiconductor cores is unlikely to occur. But, the polymer-protected QDs might be cleared from the body by slow filtration and excretion out of the body. This and other possible mechanisms must be carefully examined before any human applications in tumor or vascular imaging.

Conclusions

QDs have already fulfilled some of their promises as a new class of molecular imaging agents. Through their versatile polymer coatings, QDs have also provided a 'building block' to assemble multifunctional nanostructures and nanodevices. Multimodality imaging probes could be created by integrating QDs with paramagnetic or superparamagnetic agents. Indeed, researchers have recently attached QDs to Fe_2O_3 and FePt nanoparticles [60,61] and even to paramagnetic gadolinium chelates (X Gao, S Nie, unpublished). By correlating the deep imaging capabilities of magnetic resonance imaging with ultrasensitive optical imaging, a surgeon could visually identify tiny tumors or other small lesions during an operation and remove the diseased cells and tissue completely. Medical imaging modalities such as magnetic resonance imaging and positron emission tomography can identify diseases non-invasively, but they do not provide a visual guide during surgery. The development of magnetic or radioactive QD probes could solve this problem.

Another desired multifunctional device would be the combination of a QD imaging agent with a therapeutic agent. Not only would this allow tracking of pharmacokinetics, but diseased tissue could be treated and monitored simultaneously and in real time. Surprisingly, QDs may be innately multimodal in this fashion, as they have been shown to have potential activity as photodynamic therapy agents [62]. These combinations are only a few possible achievements for the future. Practical applications of these multifunctional nanodevices will not come without careful research, but the multidisciplinary nature of nanotechnology may expedite these goals by combining the great minds of many different fields. The success seen so far with QDs points towards the success of QDs in biological systems, and also predicts the success of other nanotechnologies for biomedical applications.

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- •• of outstanding interest

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