Multicolor quantum dots for molecular diagnostics of cancer

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In the pursuit of sensitive and quantitative methods to detect and diagnose cancer, nanotechnology has been identified as a field of great promise. Semiconductor quantum dots are nanoparticles with intense, stable fluorescence, and could enable the detection of tens to hundreds of cancer biomarkers in blood assays, on cancer tissue biopsies, or as contrast agents for medical imaging. With the emergence of gene and protein profiling and microarray technology, high-throughput screening of biomarkers has generated databases of genomic and expression data for certain cancer types, and has identified new cancer-specific markers. Quantum dots have the potential to expand this *in vitro* analysis, and extend it to cellular, tissue and whole-body multiplexed cancer biomarker imaging.

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Since 1999, cancer has been the leading cause of death for Americans under the age of 85 years, and the eradication of this disease has been the long sought-after goal of scientists and physicians [1]. Clinical outcome of cancer diagnosis is strongly related to the stage at which the malignancy is detected, and therefore, early screening has become desirable, especially for breast and cervical cancer in women, and colorectal and prostate cancer in men. However, most solid tumors are currently only detectable once they reach approximately 1 cm in diameter, at which point, the mass constitutes millions of cells that may already have metastasized. The most commonly used cancer diagnostic techniques in clinical practice are medical imaging, tissue biopsy and bioanalytic assay of bodily fluids, all of which are currently insufficiently sensitive and/or specific to detect most types of early-stage cancers, let alone precancerous lesions.

Once cancer has been detected, the next challenge is to classify that specific tumor into one of various subtypes, each of which can have drastically different prognoses and preferred methods of treatment. Diagnosis of cancer subtypes is vitally important, yet many types of cancer do not currently have reliable tests to differentiate between highly invasive types and less fatal types, and the final judgment is commonly left to the expert opinion of a pathologist who studies the tumor biopsy. With the advent of high-throughput data analysis of genomic and proteomic classifications of cancer tissues, it is becoming apparent that many subtypes are only distinguished by differences as small as the concentration of a specific protein on a cell's surface. Identification of a cancer by its molecular expression profile, rather than by one specific biomarker, might be necessary to thoroughly classify cancer subtypes and understand their pathophysiology. One cancer subtype may also be heterogeneous over patient populations, making personalized medicine highly desirable in order to treat a patient uniquely for his or her distinct cancer phenotype. However, personalized medicine cannot succeed without developing tools to sensitively detect cancer and reveal clinical biomarkers that can distinguish specific cancer types.

Nanotechnology has been heralded as a new field that has the potential to revolutionize medicine, as well as many other seemingly unrelated subjects, such as electronics, textiles and energy production [2]. The heart of this field lies in the ability to shrink the size of tools and devices to the nanometer range, and to assemble atoms and molecules into larger

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structures with useful properties, while maintaining their dimensions on the nanometer-length scale. The nanometer scale is also the scale of biological function (i.e. the same size range as enzymes, DNA, and other biological macromolecules and cellular components). Many nanotechnologies are predicted to soon become translational tools for medicine, and move quickly from discovery-based devices to clinically useful therapies and medical tests. Among these, quantum dots (QDs) are unique in their far-reaching possibilities in many avenues of medicine. A QD is a fluorescent nanoparticle that has the potential to be used as a sensitive probe for screening cancer markers in fluids, as a specific label for classifying tissue biopsies, and as a high-resolution contrast agent for medical imaging, which is capable of detecting even the smallest tumors. These particles have the unique ability to be sensitively detected on a wide range of length scales, from macroscale visualization, down to atomic resolution using electron microscopy [3]. Most importantly for cancer detection, QDs hold massive multiplexing capabilities for the detection of many cancer markers simultaneously, which holds tremendous promise for unraveling the complex gene expression profiles of cancers and for accurate clinical diagnosis. This review will summarize how QDs have recently been used in encouraging experiments for future clinical diagnostic tools for the early detection and classification of cancer.

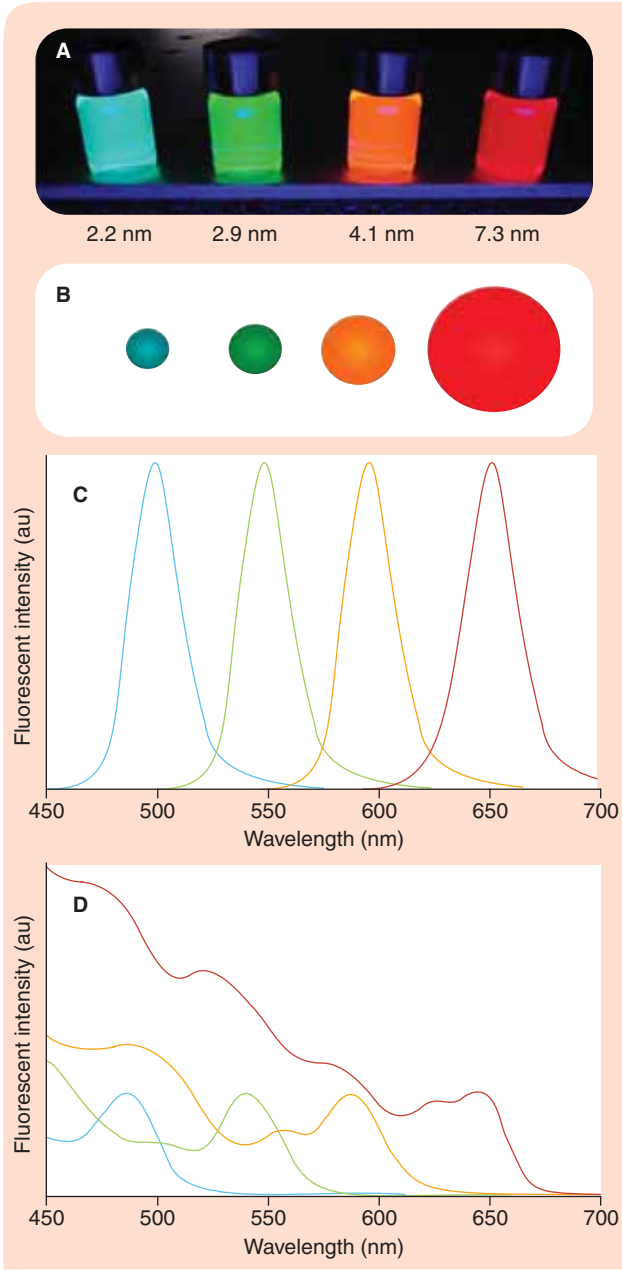

Figure 1. Size-tunable emission of CdSe quantum dots. (A) Fluorescence image of a series quantum dots excited with an ultraviolet lamp. The particle diameters are shown. (B) Schematic illustration of the relative particle sizes. (C&D) The corresponding fluorescence absorption and emission spectra. Replotted from [14].
au: Arbitrary units.

Quantum dot photophysics & chemistry

QDs are nearly spherical, fluorescent nanocrystals composed of semiconductor materials that bridge the gap between individual atoms and bulk semiconductor solids [4,5]. Owing to this intermediate size, which is typically between 2–8 nm in diameter or hundreds to thousands of atoms, QDs possess unique properties unavailable in either individual atoms or bulk materials. In their biologically useful form, QDs are colloids with similar dimensions to large proteins, dispersed in an aqueous solvent and coated with organic molecules to stabilize their dispersion. To understand the origin of their optical characteristics and size-tunable properties, the photophysics of semiconductors and colloidal synthesis techniques will be reviewed.

Photophysical properties

Since QDs are composed of inorganic semiconductors, they contain electrical charge carriers, which are negatively charged electrons and positively charged holes (an electron and hole pair is called an exciton). Bulk semiconductors are characterized by a composition-dependent bandgap energy, which is the minimum energy required to excite an electron to an energy level above its ground state. Excitation can be initiated by the absorption of a photon of energy greater than the bandgap energy, resulting in the generation of charge carriers. The newly created exciton can return to its ground state through recombination of the constituent electron and hole, which may be accompanied by the conversion of the bandgap energy into an emitted photon, which is the mechanism of fluorescence. Due to the small size of QDs, these generated charge carriers are confined to a space that is smaller than their natural size in bulk semiconductors. This quantum confinement of the exciton is the principle that causes the optoelectronic properties of the QD to be dictated by the size of the QD [6–8]. Decreasing the size of a QD results in a higher degree of confinement, which produces an exciton of higher energy, thereby increasing the bandgap energy. The most important consequence of this property is that the bandgap and emission wavelength of a QD may be tuned by adjusting its size, with smaller particles emitting at shorter wavelengths (FIGURE 1). By adjusting

their size and composition, QDs can now be prepared to emit fluorescent light from the ultraviolet (UV), through the visible, and into the infrared spectra (400–4000 nm) [9–13].

Importantly for use as biological probes, QDs can absorb and emit light very efficiently, allowing highly sensitive detection relative to conventionally used organic dyes and fluorescent proteins. QDs have very large molar extinction coefficients, in the order of 0.5–5 × 10^6 $M^{-1}cm^{-1}$ [15], approximately 10–50-times larger than those of organic dyes (5–10 × 10^4 $M^{-1}cm^{-1}$). Combined with the fact that QDs can have quantum efficiencies similar to that of organic dyes (up to 85%) [12], individual QDs have been found to be 10–20-times brighter than organic dyes [16,17], thus enabling highly sensitive detection of analytes in low concentration, which is particularly important for low copy-number cancer markers. In addition, QDs are several thousand times more stable against photobleaching than organic dyes (FIGURE 2A), and are thus well suited for monitoring biological systems for long periods of time, which is important for developing robust sensors for cancer assays and for *in vivo* imaging.

A further advantage of QDs is that multicolor QD probes can be used to image and track multiple molecular targets simultaneously. This is certain to be one of the most powerful properties of QDs for medical applications, since cancer and many other diseases involve a large number of genes and proteins. Multiplexing of QD signals is feasible due to the combination of broad absorption bands with narrow emission bands (FIGURES 1C & D). Broad absorption bands allow multiple QDs to be excited with a single light source of short wavelength, simplifying instrumental design, increasing detection speed and lowering cost. QD emission bands can be as narrow as 20 nm in the visible range, thus enabling distinct signals to be detected simultaneously with very little cross-talk. In comparison, organic dyes and fluorescent proteins have narrow absorption bands and relatively wide emission bands, considerably increasing the difficulty of detecting well-separated signals from distinct fluorophores.

Broad absorption bands are also useful for imaging of tissue sections and whole organisms in order to distinguish the QD signal from autofluorescent background signal (FIGURE 2B). Biological tissue and fluids contain a variety of intrinsic fluorophores, particularly proteins and cofactors, yielding a background signal that decreases probe detection sensitivity. Intrinsic biological fluorescence is most intense in the blue-to-green spectral region, which is responsible for the faint greenish color of many cell and tissue micrographs. However, QDs can be tuned to emit in spectral regions in which autofluorescence is minimized, such as longer wavelengths in the red or infrared spectra. Due to their broad absorption bands, QDs can still be efficiently excited by light hundreds of nanometers shorter than the emission wavelength, compared with organic dyes that require excitation close to the emission peak, burying the signal in autofluorescence. This can allow the sensitive detection of QDs over background autofluorescence in tissue biopsies and live organisms. Sensitivity can also be increased by using time-gated light detection, because the excited state lifetimes of QDs (20–50 ns) are typically 1 order of magnitude longer than that of organic dyes. QD fluorescence detection can be significantly increased by delaying signal acquisition until background autofluorescence is decreased [18].

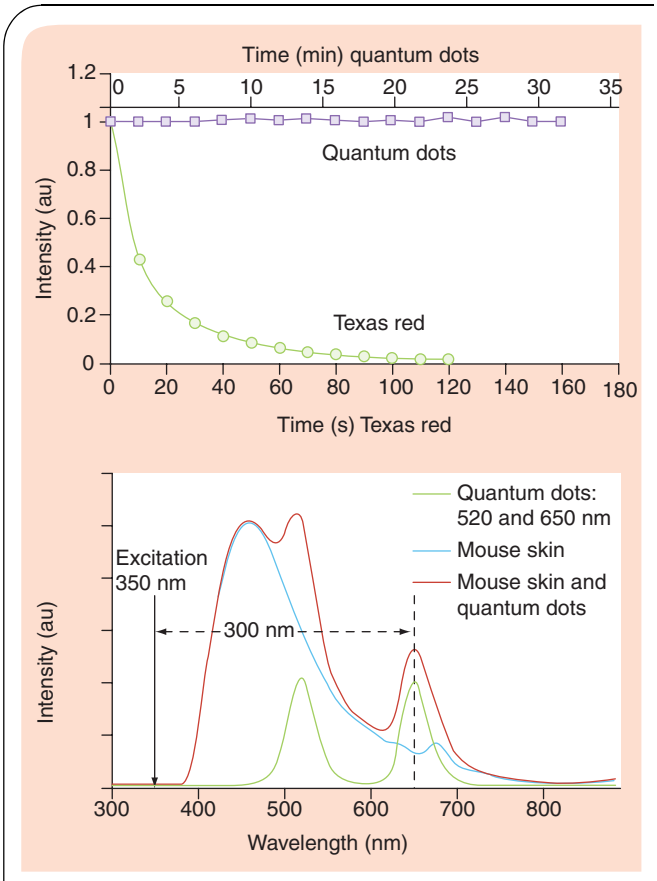

Figure 2. Quantum dots (QDs)'s unique optical properties.
(A) Photostability comparison of QDs versus organic dyes. Photobleaching curves demonstrating that QDs are several thousand times more photostable than organic dyes (e.g. Texas red) under the same excitation conditions. (B) Stokes shift comparison. 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.
au: Arbitrary unit.

Synthesis & bioconjugation

Research in probe development has focused on the synthesis, solubilization and bioconjugation of highly luminescent and stable QDs. Generally made from Group II and VI elements (e.g. CdSe and CdTe) or Group III and V elements (e.g. InP and InAs), recent advances have enabled the precise control of particle size, shape (dots, rods or tetrapods) and internal structure (core-shell, gradient alloy or homogeneous alloy) [5,19,20]. In addition, QDs have been synthesized using both two-element systems (binary dots) and three-element systems (ternary alloy dots).

QDs can be prepared in a variety of media, from atomic deposition on solid-phases to colloidal synthesis in aqueous solution. However, since the size-dependent properties of QDs are most

pronounced when QDs are monodisperse in size, great strides have been made in the synthesis of highly homogeneous, highly crystalline QDs. The highest 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 bases containing long alkyl chains. These hydrophobic organic molecules serve as the reaction medium, and the basic moieties also coordinate with unsaturated metal atoms on the QD surface to prevent the formation of bulk semiconductor. As a result, the nanoparticles are capped with a monolayer of the organic ligands, and are only soluble in hydrophobic solvents, such as chloroform and hexane. The most commonly used and best understood QD system is a core of CdSe, coated with a shell of ZnS to chemically and optically stabilize the core.

For biological applications, these hydrophobic dots must first be made water soluble. Two general strategies have been developed to disperse QDs in aqueous biological buffers, as shown in FIGURE 3. In the first approach, the hydrophobic monolayer of ligands on the QD surface may be exchanged with hydrophilic ligands, but this method tends to cause particle aggregation and decrease the fluorescent efficiency [16]. Furthermore, desorption of labile ligands from the QD surface increases potential toxicity due to exposure of toxic QD elements. Alternatively, the native hydrophobic ligands can be retained on the QD surface, and rendered water soluble through the adsorption of amphiphilic polymers that contain both a hydrophobic segment (mostly hydrocarbons) and a hydrophilic segment (such as polyethylene glycol [PEG] or multiple carboxylate groups). Several polymers have been reported, including octylamine-modified polyacrylic acid [20], PEG-derivatized phospholipids [22], block copolymers [23] and amphiphilic polyanhydrides [24]. The hydrophobic domains strongly interact with alkyl chains of the ligands on the QD surface, whereas the hydrophilic groups face outwards and render the QDs water soluble. Since the coordinating organic ligands (TOPO) are retained on the inner surface of QDs, the optical properties of QDs and the toxic elements of the core are shielded from the outside environment by a hydrocarbon bilayer. Indeed, after linking to PEG molecules, the polymer-coated QDs are protected to such a degree that their optical properties does not change in a broad range of pH (pH 1–14) and salt concentrations (0.01–1 M) [23]. Parak and coworkers have also demonstrated that, for polymer coated QDs, the cytotoxicity is mainly due to the nanoparticle aggregation, rather than the release of Cd ions [24].

To achieve binding specificity or targeting abilities, polymer-coated QDs can be linked to bioaffinity ligands such as monoclonal antibodies, peptides, oligonucleotides or small-molecule inhibitors. In addition, linking to PEG or similar ligands can enable improved biocompatibility and reduced nonspecific binding. Due to the large surface area-to-volume ratio of QDs relative to their small-molecule counterparts, single QDs can be conjugated to multiple molecules for multivalent presentation of affinity tags and multifunctionality. QD bioconjugation can be achieved using several approaches, including electrostatic adsorption [26], covalent-bond formation [16] or streptavidin–biotin linking [27]. Ideally, the molecular stoichiometry and orientation of the attached biomolecules could be manipulated to enable access to the active sites of all conjugated enzymes and antibodies; however, this is very difficult in practice. Goldman and coworkers first explored the use of a fusion protein as an adaptor for immunoglobulin G antibody coupling [28]. The adaptor protein has a protein G domain that binds to the antibody Fc region, and a positively charged leucine-zipper domain for electrostatic interaction with anionic QDs. 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. Surface engineering of nanoparticles is certain to be a greatly studied field in the near future.

Cancer diagnostics with quantum dots

Bioconjugated QD probes have the potential to be useful for cancer diagnosis through many diverse approaches. Their bright and stable fluorescent light emission and multiplexing potential, combined with the intrinsic high spatial resolution and sensitivity of fluorescence imaging, have already demonstrated improvements in existing diagnostic assays. Furthermore, new techniques have been developed based on the unique properties of these nanoparticles.

In vitro *diagnostic assays*

Screening of blood, urine and other bodily fluids for the presence of cancer markers has become a commonly used diagnostic technique for cancer; however, it has been impeded by the lack of specific soluble markers and sensitive means to detect them at low concentrations. The serum assay most commonly used for cancer diagnosis is the prostate-specific antigen screen for the detection of prostate cancer [29]. Although other biomarkers have been identified, including proteins, specific DNA or mRNA sequences and circulating tumor cells, specific cancer diagnosis from serum samples alone may only be possible with a multiplexed approach to assess a large number of biomarkers [30]. QDs could not only serve as sensitive probes for biomarkers, but they could also enable the detection of hundreds to thousands of molecules simultaneously. Experimental groundwork has already begun to demonstrate the feasibility of these expectations, as QDs have been found to be superior to conventional fluorescent probes in many clinical assay types.

Protein biomarker detection

The ability to screen for cancer in its earliest stages necessitates highly sensitive assays to detect biomarkers of carcinogenesis. The current gold standard for detecting low copy-number protein is enzyme-linked immunosorbent assay (ELISA), which has a limit of detection in the pM range. Although these assays are used clinically, they are labor intensive, time consuming, prohibitive of multiplexing and expensive. In this regard, the high sensitivity of QD detection could possibly increase the

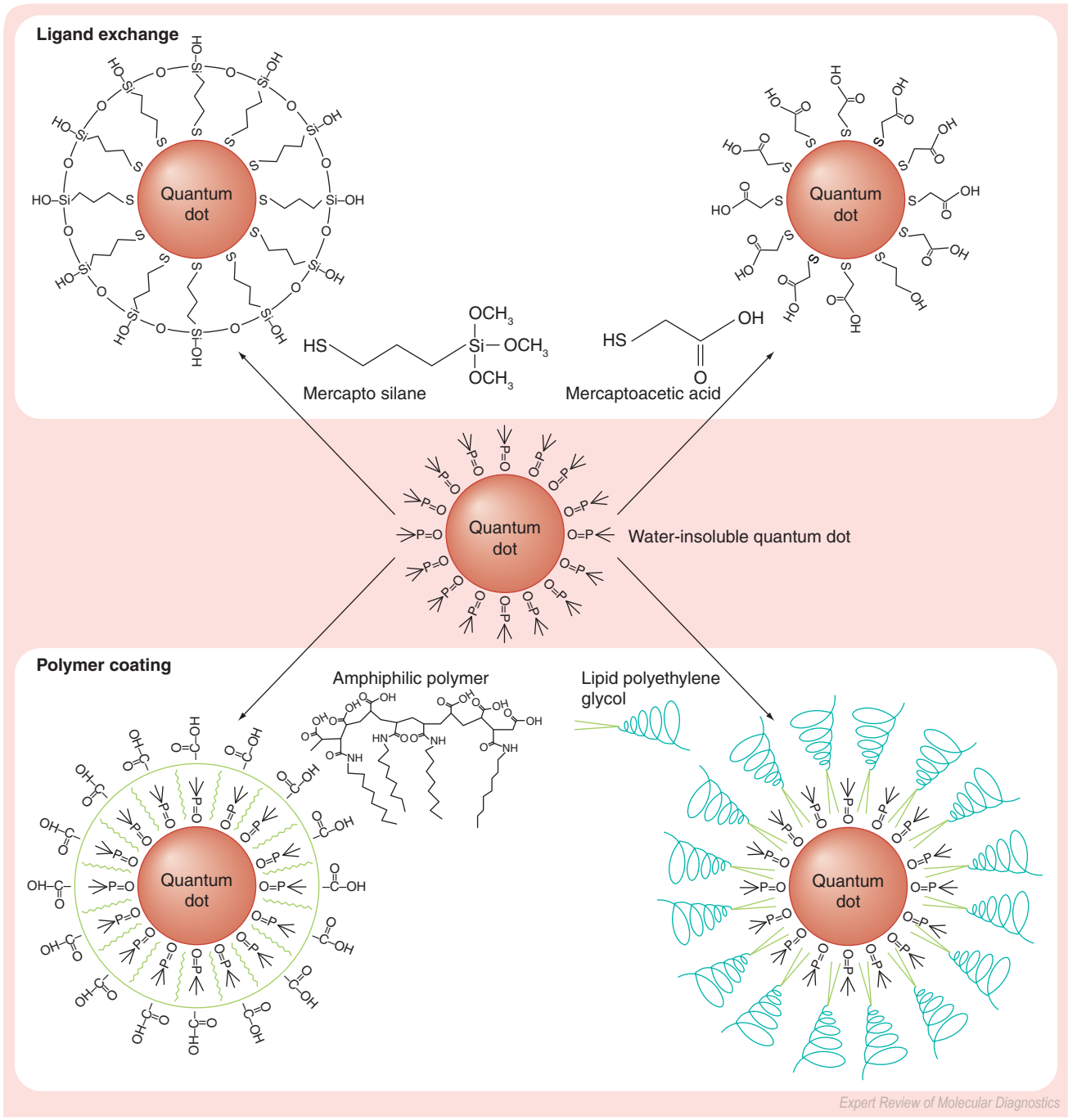

Figure 3. Diagram of two general strategies for phase transfer of tri-n-octylphosphine oxide (TOPO)-coated quantum dot (QD) into aqueous solution. Ligands are drawn disproportionately large for detail, but the ligand–polymer coatings are usually only 1–2 nm in thickness. The top panel illustrates the ligand-exchange approach, where TOPO ligands are replaced by heterobifunctional ligands, such as mercapto silanes or mercaptoacetic acid. This scheme can be used to generate hydrophilic QD with carboxylic acids or a shell of silica on the QD surfaces. The bottom panel illustrates the polymer-coating procedure, where the hydrophobic ligands are retained on the QD surface and rendered water soluble through micelle-like interactions with an amphiphilic polymer or lipids.

clinical relevance and routine use of diagnosis based on low copy-number proteins. QDs have been successfully used as substitutes for organic fluorophores and colorimetric reagents in a variety of immunoassays for the detection of specific proteins; however, they have not demonstrated an increase in sensitivity (100 pM) [28,30]. Increasing the sensitivity of these probes may only be a matter of optimizing bioconjugation parameters and assay conditions, although the multiplexing capabilities of these probes have already been demonstrated. Goldman and coworkers simultaneously detected four toxins using four different QDs, which emitted between 510 and 610 nm, in a sandwich immunoassay configuration with a single excitation source [32].

Although there was spectral overlap of the emission peaks, deconvolution of the spectra revealed fluorescence contributions from all four toxins. However, this assay was far from quantitative, and it is apparent that fine tuning of antibody cross-reactivity will be required to make multiplexed immunoassays useful. Similarly, Makrides and coworkers demonstrated the ease of simultaneously detecting two proteins with two spectrally different QDs in a western blot assay [33].

Biosensors are a new class of probes developed for biomarker detection on a real-time or continuous basis in a complex mixture. Assays resulting from these new probes could be invaluable for protein detection for cancer diagnosis due to their high speed, ease of use and low cost, enabling them to be used for quick point-of-care screening of cancer markers. QDs are ideal for biosensor applications due to their resistance to photobleaching, thereby enabling continuous monitoring of a signal. Fluorescence resonance energy transfer (FRET) has been the most prominent mechanism to render QDs switchable from a quenched off state to a fluorescent on state. FRET is the nonradiative energy transfer from an excited donor fluorophore to an acceptor. The acceptor can be any molecule (e.g., a dye or another nanoparticle) that absorbs radiation at the wavelength of the emission of the donor (the QD). Medintz and coworkers used QDs conjugated to maltose-binding proteins as an *in situ* biosensor for carbohydrate detection (FIGURE 4A) [34]. Adding a maltose derivative covalently bound to a FRET acceptor dye caused QD quenching (~60% efficiency), and fluorescence was restored upon addition of native maltose, which displaced the sugar–dye compound. QD biosensors have also been assembled that do not require binding and dissociation to modulate quenching and emission. The same group conjugated a donor QD to a photoresponsive dye that becomes an acceptor after exposure to UV light, and becomes FRET-inactive following white-light exposure, thus allowing light exposure to act as an on–off switch [35]. Before this work can be translated to a clinical tool, these probes must be optimized for higher detection sensitivity, which will require higher quenching efficiencies.

Nucleic acid biomarker detection

Early detection and diagnosis of cancer could be greatly improved with genomic screening of individuals for hereditary predispositions to certain types of cancers, and by detecting mutated genes and other nucleic acid biomarkers for cancer in bodily fluids. The current gold standard for sensitive detection of nucleic acids is PCR combined with a variety of molecular fluorophore assays, commonly resulting in a detection limit in the fM range. However, like ELISAs, the clinical utility of nucleic acid analysis for cancer diagnosis is precluded by its time and labor consumption, and poor multiplexing capabilities. Many types of new technologies have been developed recently for the rapid and sensitive detection of nucleic acids, most notably reverse transcriptase PCR and nanoparticle-based biobarcodes [36], each of which have a limit of detection in the tens of molecules. However, QDs could have an advantage in this already technologically crowded field, due to their multiplexing potential. Gerion and coworkers reported the detection of specific single nucleotide polymorphisms of the human p53 tumor suppressor gene using QDs in a microarray assay format [37], although the level of sensitivity (2 nM) was far from matching current standards. Importantly, this work demonstrated the capacity to simultaneously detect two different DNA sequences using two different QDs.

Recently, Zhang and coworkers developed a QD biosensor for DNA, analogously to the aforementioned protein biosensor (FIGURE 4B) [38]. However, in this case, fluorescence emission was monitored from the quenched QD donor, as well as from an acceptor reporter dye bound to the target DNA. Since QDs have broadband absorption compared with organic dyes, excitation of the QD at a short wavelength did not excite the dye, thereby allowing extremely low background signals. This enabled the highly sensitive and quantitative detection of as few as 50 DNA copies, and was sufficiently specific to differentiate single nucleotide differences. However, this strategy is not ideal for high-throughput analysis of multiple biomarkers because sensitive detection required the analysis of single QDs, followed by statistical data analysis.

High-throughput multiplexing

Rather than using single QDs for identifying single biomarkers, it has been proposed that different colors of QDs can be combined into a larger structure, such as a microbead, to yield an optical barcode. With the combination of six QD emission colors and ten QD intensity levels for each color, 1 million different codes are theoretically possible. A vast assortment of biomarkers may be optically encoded by conjugation to these beads, thereby opening the door to the multiplexed identification of many biomolecules for high-throughput screening of biological samples. Pioneering work was reported by Han and coworkers in 2001, in which 1.2-µm polystyrene beads were encoded with three colors of QDs (red, green and blue) and different intensity levels (FIGURE 4C) [39]. The beads were then conjugated to DNA, resulting in different nucleic acids being distinguished by their spectrally distinct optical codes. These encoded probes were incubated with their complementary DNA sequences, which were also labeled with a fluorescent dye as a target signal. The hybridized DNA was detected through co-localization of the target signal and the probe optical code, via single-bead spectroscopy, using only one excitation source. The bead code identified the sequence, while the intensity of the target signal corresponded to the presence and abundance of the target DNA sequence. This uniformity and brightness of the QD-encoded beads were substantially improved by Gao and Nie recently using mesoporous materials [39,40].

The high-throughput potential of this technology was realized by combining it with flow cytometry. For example, DNA sequences from specific alleles of the human cytochrome P450 gene family were correctly identified by hybridization to encoded probes [42]. It is worth mentioning that the long excited state of QDs and the blinking effect (isolated QDs show intermittent fluorescence emission, thus appearing to blink) do not interfere with bead decoding [41]. If three or more colors

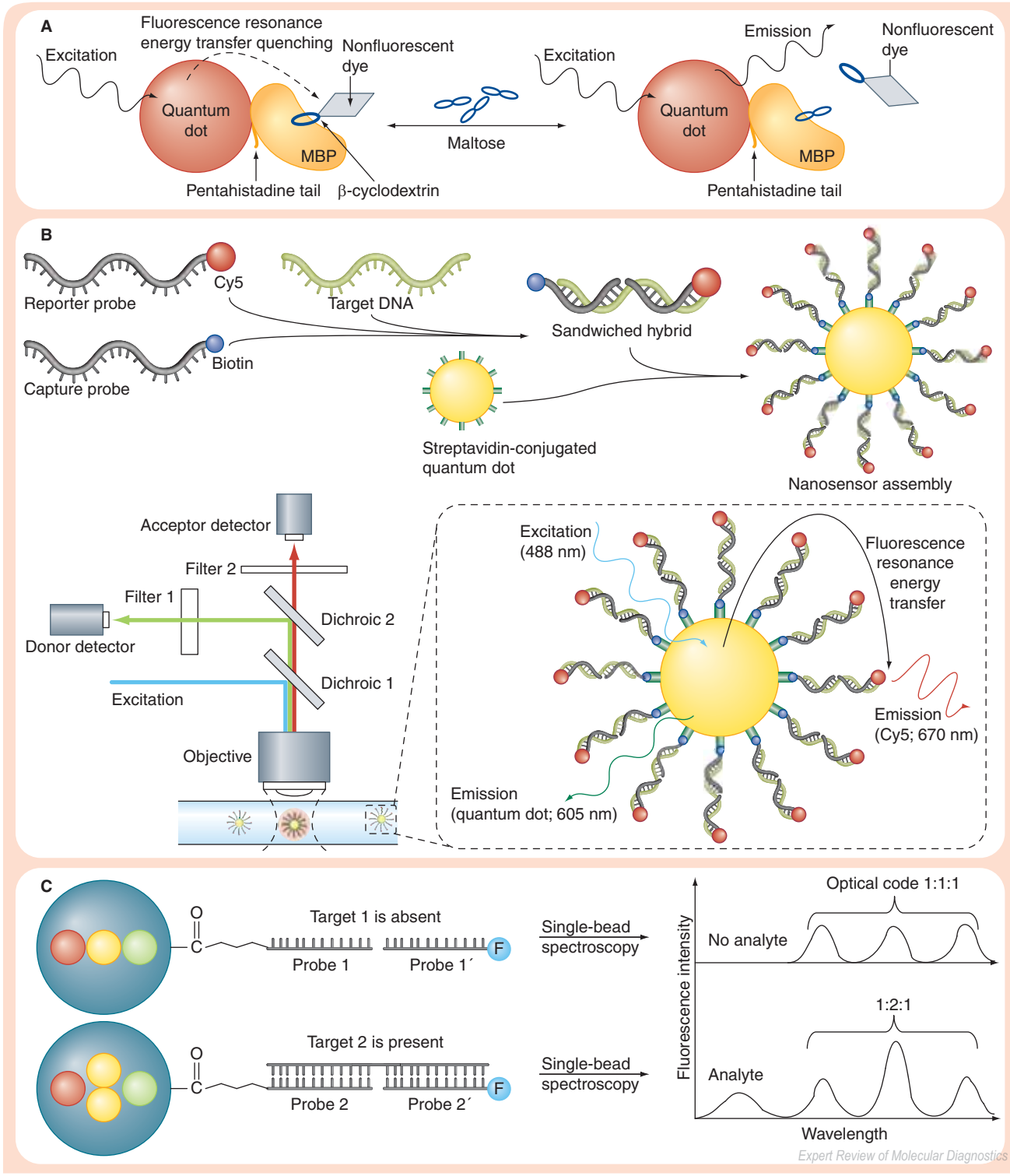

Figure 4. Quantum dot (QD)-based biosensors and optical barcodes. (A) Competitive FRET assay for maltose detection. QDs are initially quenched by nonfluorescent dyes bound to cyclodextrin. When maltose is present, it replaces the cyclodextrin–dye complexes, and the QD fluorescence is recovered [34]. **(B)** Single QD DNA sensors. (Top) Conceptual scheme showing the formation of a nanosensor assembly in the presence of targets. (Bottom left) Experimental setup. (Bottom right) Fluorescence emission from Cy5 on illumination of QD caused by FRET between Cy5 acceptors and a QD donor [38]. **(C)** DNA hybridization assays using QD barcode beads. When the target molecule is absent, only the QD barcode signals are detected by single bead spectroscopy or flow cytometry because hybridization does not occur. When the target molecule is present, it brings the barcode probe (Probe 2) and reporter probe (Probe 2') together, which results in detection of both the barcode fluorescence and the reporter signal. The reporter signal not only indicates the presence or absence of the analyte, but also its abundance. The reporter probes (Probes 1' & 2') can be labeled with either an organic fluorophore or a single QD (shown as a blue sphere).

are used for microbead encoding, this identification would be considerably more difficult with organic dyes because their emission peaks overlap, thus obscuring the distinct codes, and multiple excitation sources are required. Once encoded libraries have been developed for identification of nucleic acid sequences and proteins, solution-based multiplexing of QD-encoded beads could quickly produce a vast amount of gene and protein expression data. These data could not only be used to discover new biomarkers for disease, but also open the door to simple and fast genotyping of patients and cancer classification for personalized medical treatment. Another approach to multiplexed gene analysis has been the use of planar chips, but bead-based multiplexing has the advantages of greater statistical analysis, faster assaying time and the flexibility to add new probes at lower costs [43].

Cellular labeling

Pathological evaluation of biopsies of primary tumors and their distal metastases is the most important cancer diagnostic technique in practice. After microscopic examination of the tissue, the pathologist predicts a grade and stage of tumor progression, and thus, the cancer can be classified to determine a prognosis and appropriate treatment regimen. However, evaluation is based primarily on qualitative morphological assessment of the tissue sections, sometimes with fluorescent staining of the tissue for specific cancer biomarkers. This field is highly subjective, and diagnoses of identical tissue sections may vary between pathologists. A more objective and quantitative approach based on biomarker detection would increase diagnostic accuracy. Previous success has been made with colloidal gold and dye-doped silica nanoparticles; however, immunogold staining is essentially a single-color assay, whereas dye-doped silica nanoparticles are limited by the unfavorable properties of organic fluorophores. In comparison, QDs would be better candidates for quantitative staining of tissues for biomarkers due to their ability to detect multiple analytes simultaneously and because they have already been proven to be outstanding probes for fluorescent detection of proteins and nucleic acids in cells.

Labeling of fixed cells & tissues

The feasibility of using QDs for biomarker detection in fixed cellular monolayers was first demonstrated by Bruchez and coworkers in 1998 [17]. By labeling nuclear antigens with green silica-coated QD and F-actin filaments with red QD in fixed mouse fibroblasts, these two spatially distinct intracellular antigens were simultaneously detected. This article and others have demonstrated that QDs are brighter and dramatically more photostable than organic fluorophores when used for cellular labeling [16,21]. Many different cellular antigens in fixed cells and tissues have been labeled using QDs (FIGURE 5A), including specific genomic sequences [44,45], mRNA [46], plasma membrane proteins [21,47,48], cytoplasmic proteins [17,21] and nuclear proteins [16,20], and it is apparent that they can function as both primary and secondary antibody stains. In addition, high-resolution actin filament imaging has been demonstrated using QDs (FIGURE 5B) [21], and the fluorescence can be correlated directly to electron micrograph contrast due to the high electron density of QD [49,50]. It has now become clear that QDs are superior to organic dyes for fixed cell labeling. However, the translation from fixed cell labeling to staining of formaldehyde-fixed, paraffin-embedded tissue sections of tumor biopsies is not simple due to the high autofluorescence and the loss of antigen presentation associated with the embedding and fixation processes. Nonetheless, tissue-section labeling with QDs has been successful for biomarker-specific staining of rat neural tissue [51], human skin basal cell carcinomas [47], and human tonsil tissue [52]. The recent advances in

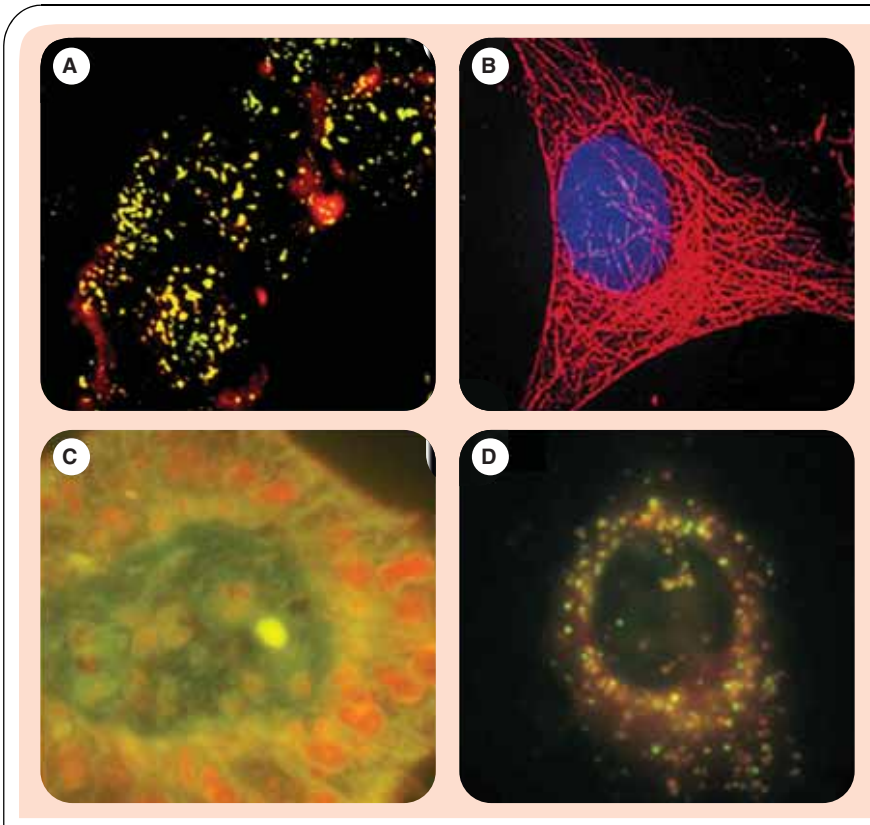

Figure 5. Molecular imaging of cells and tissues. (A) 3D imaging of intracellular localization of growth hormone and prolactin and their mRNA using quantum dots (QDs) and confocal laser scanning microscopy [46]. (B) Microtubules in NIH-3T3 cells labeled with red color QDs [21]. (C) QD immunostaining of formalin-fixed, paraffin-embedded human prostate tumor specimens. Mutated p53 phosphoprotein overexpressed in the nuclei of androgen-independent prostate cancer cells is labeled with red color QDs. The Stokes shifted fluorescence signal is clearly distinguishable from the tissue autofluorescence. (D) Intracellular delivery of QDs with mitochondria localization peptide [55].

immunohistochemistry for protein detection and fluorescence *in situ* hybridization for nucleic acid detection using QD probes could revolutionize clinical diagnosis of biopsies due to the large number of biomarkers that could be simultaneously monitored (FIGURE 5C).

Live cell imaging

In 1998, Chan and coworkers demonstrated that QDs conjugated to a membrane-translocating protein, transferrin, could cause endocytosis of QDs by living cancer cells in culture [16]. The QDs retained their bright fluorescence *in vivo* and were not noticeably toxic, thus revealing that QDs could be used as intracellular labels for living cell studies (FIGURE 5D). Most subsequent live cell studies with QDs have focused on labeling of plasma membrane proteins [53,54] and evaluating techniques for traversing the plasma membrane barrier [55], and it is becoming evident that QDs will become powerful tools for unveiling cellular biology, and for optically tagging cells to determine lineage and distribution in multicellular organisms [22]. In this fast moving and exciting field, QDs have already been used to calculate plasma membrane protein diffusion coefficients [54] and observe a single ErbB/Her receptor (a cancer biomarker) and its internalization after binding to epidermal growth factor [53]. Furthermore, QD probes of living cells have prompted the discovery of a new filopodial transport mechanism [53,56]. While most of these studies have centered on biological discovery, a new clinically relevant assay for cancer diagnosis has already been developed from these living cell studies. Alivisatos and coworkers created a cell motility assay, in which the migration of cells over a substrate covered with silica-coated QD was measured in real time [57]. As the cells moved across their substrate, they endocytosed the QDs, causing an increase in fluorescence inside of the cells and a nonfluorescent dark path in their trails [58]. These phagokinetic tracks were used to accurately assess invasive potential of different cancer cell types, as motility of cells is strongly associated with their malignancy *in vivo*. This new assay could aid in the clinical classification of cancers with ambiguous subtypes, and further separate subtypes into more discretely defined categories for better diagnosis.

In vivo *imaging*

Despite the large number of identified cancer biomarkers, targeted molecular imaging of cancer has yet to reach clinical practice, although it has been successful in animal models. The four major medical imaging modalities rely on signals that can transmit through thick tissue, using ultrasonic waves (ultrasound imaging), x-rays (computed x-ray tomography), gamma rays (positron emission tomography), or radio waves (magnetic resonance imaging [MRI]). Image contrast from these techniques is generated from the differences in signal attenuation through different tissue types, which is predominantly a function of tissue structure and anatomy. Many tumor types can be identified purely based on their image contrast, and exogenous contrast agents are commonly intravenously infused in patients with tumors of poor contrast. However, none of these acquired images can convey molecular information of the cancer that is possible with quantitative *in vitro* assays and tissue biopsy evaluation. In addition, detection of multiple markers is extremely difficult with these imaging techniques, and none of these modalities has innately high spatial resolution capable of detecting most very small, early-stage tumors. Generating spatially accurate images of quantitative biomarker concentration would be a giant leap toward detection and diagnosis of cancers, especially for finding sites of metastasis.

Optical imaging, particularly fluorescence imaging, has high intrinsic spatial resolution (theoretically 200–400 nm), and has recently been used successfully in living animal models; however, it is limited by the poor transmission of visible light through biological tissue. There is a near-infrared optical window in most biological tissue that is the key to deep-tissue optical imaging [59]. This is because Rayleigh scattering decreases with increasing wavelength, and because the major chromophores in mammals (hemoglobin and water) have local minima in absorption in this window. Few organic dyes are currently available that emit brightly in this spectral region, and they suffer from the same photobleaching problems as their visible counterparts; although this has not prevented their successful use as contrast agents for living organisms [60]. One of the greatest advantages of QDs for imaging in living tissue is that their emission wavelengths can be tuned throughout the near-infrared spectrum by adjusting their composition and size, resulting in photostable fluorophores that can be highly stable in biological buffers [61]. Visible QDs are more synthetically advanced than their near-infrared counterparts, which is why most of the living animal studies implementing QDs have used visible light emission. However, even these have demonstrated great promise, due to their ability to remain photostable and brightly emissive in living organisms.

Vascular imaging

QDs have been used to passively image the vascular systems of various animal models. In a report by Larson and coworkers, intravenously injected QDs remained fluorescent and detectable when they circulated to capillaries in the adipose tissue and skin of a living mouse, as visualized fluorescently [62]. This report made use of two-photon excitation, in which near-infrared light is used to excite visible QDs, allowing for deeper penetration of excitation light, despite strong absorption and scattering of the emitted visible light. Lim and coworkers intravenously injected near-infrared QDs to image the coronary vasculature of a rat heart [63]. The circulation lifetime of an injected molecule is dependent on the size of the molecule and its chemical properties. Small molecules, such as organic dyes, are quickly eliminated from circulation minutes after injection due to renal filtration. QDs and other nanoparticles are too large to be cleared through the kidneys, and are primarily eliminated by nonspecific opsonization (a process of coating pathogenic organisms or particles so they are more easily ingested by the macrophage system) by phagocytotic cells of the reticuloendothelial system (RES), which is mainly located in the spleen, liver and lymph nodes.

Ballou and coworkers demonstrated that the lifetime of QDs in the bloodstream of mice is significantly increased if the QDs are coated with PEG polymer chains [64], an effect that has also been documented for other types of nanoparticles and small molecules. This effect is caused by a decreased rate of RES uptake, which is partly due to decreased nonspecific adsorption of the nanoparticle surface and decreased antigenicity [65]. Recently, PEG-coated QDs have been used to image the vasculature of subcutaneous tumors in mice. Stroh and coworkers used two-photon microscopy to image the blood vessels within the microenvironment of a tumor [66]. Simultaneously, autofluorescence from collagen allowed high-resolution imaging of the extracellular matrix, and transgenic genetic modification of green-fluorescent protein revealed perivascular cells (FIGURES 6A & B). Stark contrast between cells, matrix and the erratic, leaky vasculature was evident, which suggests the use of fluorescence contrast imaging for the high-resolution, noninvasive imaging and diagnosis of human tumors.

Lymph node tracking

The lymphatic system is another circulatory system that is of great interest for cancer diagnosis. Cancer staging, and therefore prognosis, is largely evaluated based on the number of lymph nodes involved in metastasis close to the primary tumor location, as determined from sentinel node biopsy and histological examination. It has been demonstrated that QDs have an innate capacity to image sentinel lymph nodes, as first described by Kim and coworkers in 2003 [58]. Near-infrared QDs were intradermally injected into the paw of a mouse and the thigh of a pig. Dendritic cells nonspecifically phagocytosed the injected QD, and then migrated to sentinel lymph nodes that could then be fluorescently detected even 1 cm under the skin surface (FIGURE 6C). Their results demonstrated rapid uptake of QDs into lymph nodes, and clear imaging and delineation of involved sentinel nodes (which could then be excised). This work demonstrates that QD probes could be used for real-time intraoperative optical imaging, providing an *in situ* visual guide enable a surgeon to locate and remove small lesions (e.g. metastatic tumors) quickly and accurately. The authors later demonstrated the ability to map esophageal and lung lymph nodes in pigs [67,68], and also revealed preferential lymph nodes for drainage from the pleural space in rats [69]. Another interesting aspect of this research is that the QDs remained fluorescent after the biopsies were sectioned, embedded, stained and frozen, thus enabling microscopic detection of the QDs postoperatively, and providing pathologists with another visual aid in judging tissue morphology and cellular identity.

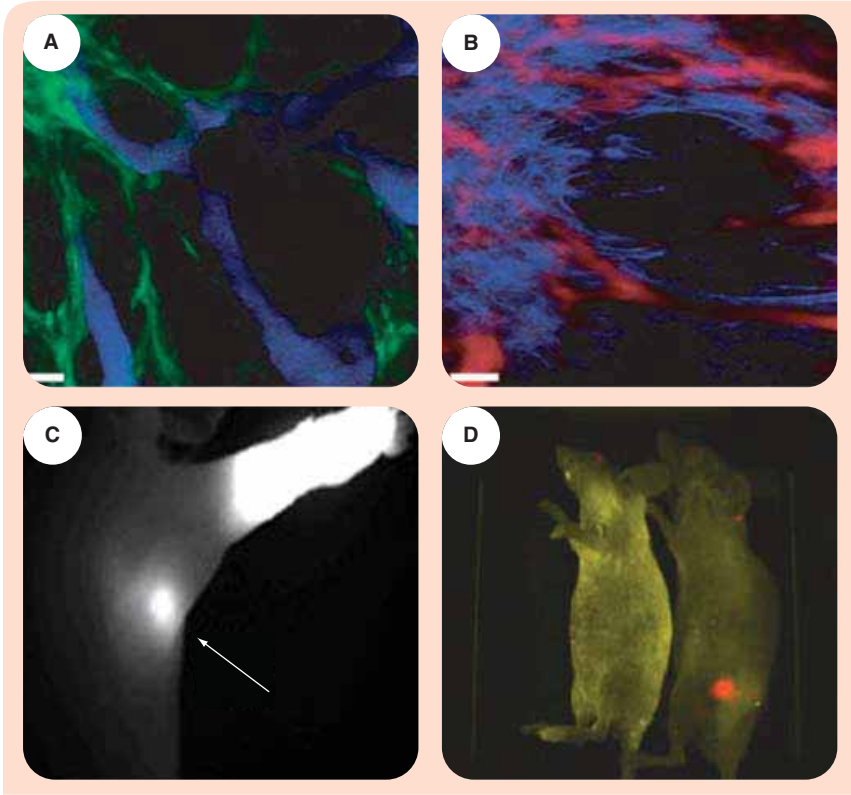

Figure 6. *In vivo* targeting and imaging with quantum dots (QDs). (A) Simultaneous visualization of blue QD vessel marker and green-fluorescent protein-expressing perivascular cells [66]. (B) Blood vessels highlighted with red QDs and second harmonic generation signal of collagen in blue [66]. (C) Near-infrared fluorescence of water-soluble Type II QDs taken up by sentinel lymph nodes [61]. (D) Molecular targeting and *in vivo* imaging of a prostate tumor in mouse using a QD–antibody conjugate (red) [23].

Tumor targeting & imaging

Akerman and coworkers first reported the use of QD–peptide conjugates to target tumor vasculatures, but the QD probes were not detected in living animals [70]. Nonetheless, *in vitro* histological results revealed that QDs homed to tumor vessels guided by the peptides, and were able to escape clearance by the RES. Most recently, Gao and coworkers reported a new class of multifunctional QD probe for simultaneous targeting and imaging of tumors in live animals [23]. This class of QD conjugate contains an amphiphilic triblock copolymer for *in vivo* protection, targeting ligands for tumor antigen recognition, and multiple PEG molecules for improved biocompatibility and circulation. Tissue section microscopy and whole-animal spectral imaging enabled monitoring of *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 (FIGURE 6D). In the passive mode, macromolecules and nanometer-sized particles are accumulated preferentially at tumor sites through an enhanced permeability and retention

effect, which is a result of the permeable vasculature of the tumor and lack of effective lymphatic drainage. Active tumor targeting was achieved using QDs conjugated to an antibody specific to the prostate-specific membrane antigen, which was previously identified as a cell-surface marker for both prostate epithelial cells and neovascular endothelial cells.

Toxicity & clinical potential

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 and coworkers indicates that CdSe QDs are highly toxic to cultured cells under UV illumination for extended periods of time [71]. This is not surprising because the energy of UV irradiation is close to that of a chemical bond, which can induce photolytic dissolution of semiconductor particles in a process termed photolysis, thereby 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 [22,23,56,62,64,66,67,72,73]. 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. It is possible that the polymer-protected QDs might be cleared from the body by slow filtration and excretion. Although this should not impede the progress of cellular and solution-based assays using QDs, toxicity must be carefully examined before any human applications in medical imaging are considered.

Expert commentary

Nanotechnology has recently unveiled a host of new tools in the pursuit of improved cancer diagnosis. QDs have the unique distinction of being applicable in nearly all facets of clinical diagnosis, from blood screening to medical imaging. QDs will also undoubtedly play an important role as tools of pure biology, as they have already been used as probes for many different types of molecules *in vitro* and *in vivo*. However, much development and standardization will be necessary to convert these sensitive probes into clinical tools that reliably screen for the early detection of carcinogenesis. This can be expected to occur rapidly, as QD probes have advanced significantly since their seminal use in biological systems in 1998, and can now be highly monodisperse, stable, brightly emissive and protected by an assortment of polymeric coatings. One of the first realms of clinical cancer diagnosis that could be impacted by QDs is their use as *in vitro* probes for multiple biomarkers, which is an area that is not affected by potential toxicity. Their use in fast, sensitive clinical assays will be expedited if QD biosensors can be assembled with high quenching efficiencies and high target specificity, and if stable and bright near-infrared QDs can be synthesized for analysis of biomarkers in whole blood. Use of QD-encoded beads for gene and protein profiling is on the clinical horizon, and is only hindered by the technical challenge of developing libraries for screening a large number of targets.

For *in vitro* analysis, QDs are competing with a large number of other highly promising and already established probes, such as small fluorophores, biobarcodes and microarrays of DNA, protein and tissue. However, the superiority of QD probes for cellular labeling is already abundantly obvious. Clinical cancer diagnosis might have the most to gain from QDs as cellular labels for tissue biopsy analysis. Although only small steps have been taken to translate success with fixed cells in culture to fixed tissue sections, QDs could enable the sensitive and quantitative *in situ* detection of mutated tumor suppressor genes, oncogenes and low copy-number transcription products and proteins. Furthermore, the use of QDs as clinical contrast agents for medical imaging could revolutionize cancer diagnosis; however, this is far from being realized due to the toxic nature of most semiconductor compounds. If high-quality QDs can be prepared from relatively nontoxic compounds (e.g., silicon), or if the toxic components can be inertly protected from exposure, then their clinical relevance could be foreseeable. However, for the time being, QDs will provide a highly sensitive model for the distribution, metabolism and long-term fate of many types of nanomaterials in living animals. New types of potentially toxic nanomaterials with intriguing properties are continually being reported, and hopefully, technology to encapsulation these materials and render them to be nontoxic will advance just as quickly.

Five-year view

Advances in nanoparticle synthesis and surface chemistry over the past 5 years have produced a variety of QD reagents, which recently became commercially available to the general scientific community. The next wave of research activities is likely to be the novel applications of QDs to solve important biological and medical problems. The areas of greatest impact include intracellular imaging of live cells, in which there are currently no sensitive and robust probes available. In fact, one of the only true discoveries reported so far using QDs has been in this domain, with the report of a new type of retrograde transport along cellular filopodia [55]. QD probes should also open new doors to understanding the pathophysiology of cancer, as they have already been used to study the migration of cancer cells *in vitro* [57], monitor the metastasis of QD-labeled cells *in vivo* [73], and microscopically examine the microenvironment of cancer tissue *in vivo* [66]. The near future is also likely to see advances in the use of QDs to image and screen for cancer. As surface engineering of QDs advances, their utility for specific, high-affinity detection of cancer biomarkers will also progress, because the active, functional component of a nanoparticle is its surface.

The long-term goal of medical nanotechnology is to develop multifunctional nanostructures, that are capable of finding diseased tissue, treating the disease and reporting progress in real time. These nanomachines will not be established until

the distant future, but the technology needed to assemble these machines is already being designed. Nanoparticles such as QDs can already be assembled into larger and more complex objects with multiple functions, such as composites of QDs and superparamagnetic iron oxide nanoparticles, which are capable of magnetically separating cells, and providing contrast for fluorescence imaging and MRI [74]. In theory, these conjugates could serve as MRI contrast agents for the detection of deep-tissue tumors, thereby enabling a surgeon to excise the entire tumor, as verified in real time through fluorescence imaging. QDs have also been conjugated to therapeutic agents, which could soon enable the real-time monitoring of pharmacokinetics and disease treatment. Interestingly, QDs may be inherently therapeutic, as they have been shown to be photosensitizers for the generation of reactive oxygen species, which could induce apoptosis in cancer cells [75] . Although these are only basic forms of the intelligent, multifunctional nanomachines that are hypothesized for the future of medicine, they may already be close to having clinical relevance, and may soon become part of a physician's nanotechnology toolbox.

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Key issues

- There are currently very few sensitive tests for early-stage cancers, and detecting cancer before it has progressed to a highly invasive stage is essential for a high survival rate.
- Quantum dot (QD) probes have potential for use as sensitive probes for detecting cancer biomarkers in bodily fluids, fixed tumor tissue, and living animals and humans.
- Cancer is a disease that is associated with a change in a large number of genes and an alteration in the expression of many different proteins.
- QDs have the ability to detect a large number of biomarkers simultaneously due to their unique optical properties.
- Potential toxicity of QD probes must be examined thoroughly before clinical use.
- Surface engineering and bioconjugation strategies are new fields in nanotechnology, and advances are certain to aid in the progress of QDs as clinical labels.

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