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Quantum dots for cancer molecular imaging

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Current medical practice and biomedical research are quickly moving towards a qualitatively new stage – personalized medicine – which aims at addressing individual diseases in a pathology-specific and patient-specific manner. Such transformation is driven by increasing need in personalized diagnostics and therapy in all areas of medicine, and is especially sought after in treating cancer. While conventional biomedical techniques suffer from significant limitations in characterizing cancer on the molecular level, nanotechnology introduces novel tools for molecular imaging and targeted therapy. Among these, semiconductor nanoparticles (quantum dots, or QDs) represent a class of fluorescent probes that have already shown their utility in conventional biomolecular and cellular imaging applications (*e.g.* cell and tissue staining, Western blot, ELISA, etc.). Moreover, novel applications of *in vivo* fluorescence imaging, live cell single-molecule tracking, and combined drug delivery and imaging are becoming available through utilization of QD bioconjugates. Unique photo-physical properties, such as size-tunable and spectrally narrow light emission, simultaneous excitation of multiple colors, improved brightness, resistance to photobleaching, and extremely large Stokes shift, make QDs well suited for sensitive quantitative molecular profiling of cancer cells and tissues both *in vitro* and *in vivo*. Such functionality holds tremendous promise for unraveling the complex gene expression profiles of cancers improving our understanding of cancer patho-physiology and opening doors towards accurate clinical diagnosis and personalized therapy.

Key words: **Quantum dots - Nanoparticles - Molecular profiling - *In vivo* imaging - Fluorescence - Cancer - Pathology.**

Personalized medicine as a practice of addressing individual diseases in a pathology-specific and patient-specific manner is highly advantageous over traditional generalized methods of diagnosis and therapy, thus remaining a major goal of current biomedical research and clinical practice. Despite many challenges encountered on the way towards personalized medicine, this approach is becoming more feasible due to the development of highly specific and sensitive tools for uncovering physiologically and pathologically relevant molecular information. Scientists working in the field of biomedical research constantly explore new elaborate ways for obtaining comprehensive molecular information in order to better understand and, eventually, control normal and pathologic processes underlying complex physiological phenomena. While conventional biomedical techniques suffer from significant limitations in addressing patho-physiology on molecular level, nanotechnolo-

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gy introduces novel nanoparticle-based tools for molecular imaging, therapy, and targeted drug delivery, thus opening doors to highly sensitive and quantitative diagnostics as well as targeted and personalized treatment.[1-3]

The need in personalized diagnostics and therapy is becoming apparent in all areas of medicine; however, it is especially urgent and sought after in treating cancer. Mechanisms of cancerogenesis and cancer response to therapy remain poorly understood, thus precluding from accurate cancer diagnosis, prognosis, and effective treatment. Elucidation of such mechanisms will undoubtedly provide insights not only for novel approaches to detection and treatment of cancers in early stages, but also for development of effective prophylaxis. Yet, implementation of this task is quite challenging, as each cancer appears to be as unique as a fingerprint.[3] It is evident that different types of cancers have different biomarker expression schemes. Therefore, accurate molecular profiling of individual tumors is one key to effective treatment. Tumor-specific molecular information might identify cellular markers for targeted and effective anti-cancer therapy with minimal adverse side-effects. The ability to thoroughly and quantitatively analyze complex panels of cancer biomarkers of individual tumors is strongly needed for comprehensive understanding of cancer patho-physiology — a prerequisite for accurate cancer diagnostics and effective therapy.

Non-invasive medical imaging techniques (*e.g.* magnetic resonance imaging and positron emission tomography) are routinely used for cancer screening and diagnostics. However, despite considerable achievements in the area of *in vivo* diagnostics, these techniques are not yet sensitive and/or specific enough to assess biomarker expression profiles of cancer cells. Invasive but highly informative and reliable technique of *ex vivo* pathological evaluation of biopsies of primary tumors and their distal metastases remains the basis for addressing cancer diagnostics on the molecular level. In clinics, abnormal expression of cellular markers characteristic to cancer cells is commonly evaluated using standard semi-quantitative immunohistochemistry techniques, such as immunoperoxidase and immunofluorescence methods.[4] Being most well suited for single-color imaging and lacking capabilities for accurate signal quantification these methods have limited use in molecular profiling.[5-7] In order to overcome these limitations, more sensitive and quantitative techniques, such as reverse transcription polymerase chain reaction, gene chips, protein chips, and biomolecular mass spectrometry, can be used to search for multiple proteins as well as RNA and DNA in cancer cells and tumor tissue specimens.[8, 9] However, due to the destructive nature of these procedures, potentially valuable structural cellular and tissue information is lost during sample processing. This is particularly undesirable considering the substantial heterogeneity of tumor composition.[3] Inability to accurately detect and quantify expression of multiple biomarkers in tissue samples with preserved morphology precludes from obtaining a comprehensive "molecular portrait" of cancer cells from analysis of a limited number of sections available from a biopsy (in most cases less than 5-6 sections). Development of new molecular profiling technologies capable of quantitative analysis of molecular signatures of individual patients' tumors would greatly enhance the quality and predictive power of cancer diagnostics.

Quantum dots as a tool for molecular imaging

Nanotechnology offers a handful of novel solutions for molecular characterization of tumors. Based on their unique physical properties, metallic, semiconductor, and magnetic nanoparticles have found use in variety of biomedical applications. Among these, semiconductor nanoparticles (quantum dots, or QDs) have emerged as a promising new tool for detection and quantification of multiple biomarkers in cells and tissue samples *ex vivo*, and even whole organisms *in vivo*.[10] Having size of 2 to 10 nm in diameter, QDs possess unique photo-physical properties drastically different from single atoms or bulk materials due to quantum confinement of charge carriers within a nanoparticle. Moreover, nanometer-scale size of QDs comparable with the size of large proteins allows for integration of nanoparticles and biomolecules yielding biologically functional nanomaterials.[10-12]

Narrow size-tunable light emission and effective light absorption throughout a wide spectrum make QDs more sensitive detection tools than organic fluorophores and provide massive multiplexing capabilities for tumor molecular profiling. The multicolor imaging capability of QDs can be primarily attributed to the presence of very narrow and symmetric light emission bands (20-30 nm in the visible range, and can be as narrow as 14 nm at full-width-at-half-maximum or FWHM),[13-15]which produce no or little cross-talk

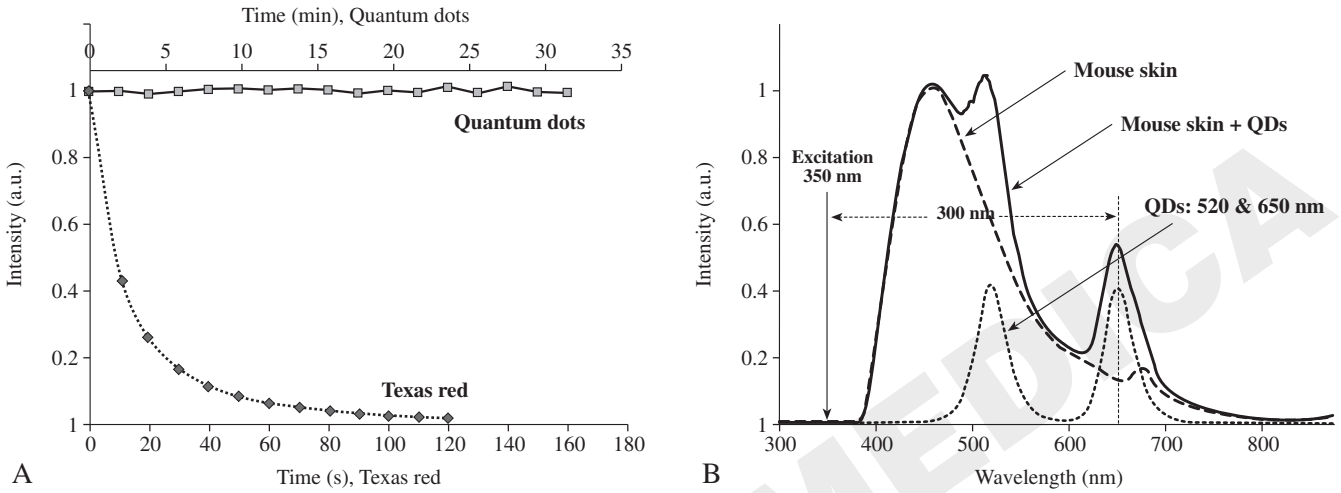

Figure 1.—Unique optical properties of QDs provide enhanced sensitivity, sustained signal intensity, and large Stokes shift for molecular profiling and *in vivo* tumor imaging. A) Photobleaching curves show that QDs are several thousand times more photostable than organic dyes (*e.g.* Texas red) under the same experimental conditions. B) Comparison of mouse skin and QD emission spectra obtained under the same excitation conditions demonstrates that the QD signals can be shifted to a spectral region where autofluorescence is reduced.[10]

between adjacent colors and allow simultaneous detection and quantification of multiple signals. Yezhelyev et al. made use of this property in multiplexed labeling and quantification of five clinically significant breast cancer markers — HER2, ER, PR, EGFR, and mTOR — in breast cancer tissue biopsies.[16] Multiplexing capability of QDs is further complemented by efficient light absorption over a broad spectral range (hundreds of nanometers). As molar extinction coefficient of QDs gradually increases toward shorter wavelength, various QDs can be simultaneously excited by a single high-energy light source (*e.g.* UV lamp),[11] thus eliminating the need for multiple excitation sources, reducing the cost of imaging instrumentation, and simplifying data analysis. In one example, Chattopadhyay et al. utilized a single 408 nm laser source for simultaneous excitation of 8 QD probes in polychromatic flow cytometry analysis of T-cells.[17]

Accurate and consistent quantitative analysis of biomarker expression as well as high-resolution imaging and real-time molecule tracking are readily achievable due to exceptional QD resistance to photobleaching and photodegradation. It has been shown that QDs resist photobleaching for more than 30 minutes, while organic dyes fade by more than 90% in less than one minute under identical experimental conditions (Figure 1A).[18] Unprecedented photostability renders QDs well suited for imaging when long exposure to excitation source is required, while keeping signal intensity constant and allowing for consistent analysis of samples. Tokumasu and Dvorak have reported the use of this property in immunocytochemical studies of human erythrocytes with high-magnification, three-dimensional reconstruction technique, where utilization of QD-bioprobes allowed reliable collection of z-stack image data for 3D reconstruction without loss of image intensity.[19] Cui *et al.* utilized pseudo-TIRF (total internal reflection fluorescence) microscopy for long-term real-time tracking of intracellular transport of QD-labeled nerve growth factor along axons of rat dorsal root ganglion neurons.[20] Lidke *et al.* used QDs conjugated to epidermal growth factor (EGF) to study erbB/HER receptor-mediated cellular response to EGF in living human epidermoid carcinoma A431 cells.[21]Additionally, prolonged light exposure of tissue samples effectively photobleaches autofluorescence resulting from organic molecules and fixation agents while keeping the QD signal constant. It should be noted that bare QDs are not stable under UV illumination for extended periods of time due to photolysis.[22] However, core/shell QDs with stable polymer coating (which are currently used in most biomedical applications) do not exhibit degradation under standard imaging and biologically relevant conditions.[23, 24]

The long excited state lifetime of QDs (20-50 ns), though could be detrimental for light emission dynamics when excitation power is very high, is very useful in separation of QD signal from short-lived autofluorescence background (2 ns) and organic fluorophore signals (1-4 ns) via a technique known as time-domain imaging.[10, 25] This is especially helpful in fluorescence imaging of formalin-fixed-paraffin-embedded (FFPE) tumor biopsies, where autofluorescence from endogenous proteins and fixation agents can be significant. For example, Dahan *et al.* have demonstrated that time-gated imaging significantly and selectively reduces the autofluorescence contribution, achieving enhancement of the signal-to-background ratio by more than an order of magnitude.[26] Time-gated imaging can also be utilized in multiplexed biomarker imaging when both QDs and conventional organic fluorophores are used.[27]

In vivo tumor imaging with QDs benefits from the QD large Stokes shift and long fluorescence lifetime, which allow separation of QD signal from tissue autofluorescence and yield substantial improvement of signal-to-background ratio.[19, 28-34] Red shift produced by QDs is significantly larger than that of organic fluorophores and can be as large as 300-400 nm, depending on the wavelength of the excitation light [35, 36]. Organic dye signals with a small Stokes shift are often buried by strong tissue autofluorescence, whereas QD signals are clearly recognizable above the background (Figure 1B).[10] For example, Gao *et al.* have demonstrated utility of red QDs (emission peak around 640 nm) conjugated to antibodies against prostate-specific membrane antigen (PSMA) for *in vivo* tumor imaging in mice.[24]

Engineering of QD-bioconjugates for cancer imaging

Quantum dots are generally composed of atoms from group II-VI (*e.g.* CdSe and CdTe) and III-V (*e.g.* InP and InAs) elements of the periodic table. Both types of nanocrystals have been synthesized and studied extensively in the past. The first synthesis protocols involved preparation of QDs in aqueous solutions with addition of stabilizing agents, such as thioglycerol and polyphosphate. While being a relatively easy approach for synthesis of water-soluble QDs, such methods provided poor control over the nanoparticle size (with relative standard deviation, RSD, greater than 15%) and yielded QDs with low fluorescence efficiencies, thus greatly compromising the multiplexing capabilities and detection sensitivity of this technology.[11] Realization of QD potential for use in biomedical applications came in 1990s, when high-temperature organometallic procedure for synthesis of highly uniform colloidal CdSe QDs was introduced by Bawendi et al.[37] Further improvement in QD quality was achieved by deposition of a surface-capping layer of a material with larger bandgap, which dramatically increased the quantum yield of QDs, effectively passivated the core surface, protected it from oxidation, and removed most of the defect sites on the core particle surface. As such, the best available QDs with highly refined chemistry are currently made of CdSe cores overcoated with a layer of ZnS.[38] Recently, a leap towards large-scale synthesis of high-quality QDs was made by Peng *et al.*, who have utilized alternative cheap precursor materials (such as CdO) for production of highly crystalline QDs that show excellent quantum yields (85% at room temperature) without an inorganic capping layer.[39-41]

Having developed robust techniques for synthesis of nanoparticles with precisely controlled size, geometry, internal structure, and surface chemistry, scientists have placed increasing efforts towards the design of novel coating and bioconjugation methods for production of small bio-compatible and multi-functional QD-based nanoprobes. Since best available QDs are routinely stabilized by hydrophobic ligands and are, therefore, soluble only in organic solvents, a variety of surface coating and bioconjugation techniques have been implemented (and more are still under development) with the ultimate goal of making QDs soluble, stable, and bio-active in biological buffers, while preserving their original photophysical properties.[25] As new QD-based applications are being explored, more stringent and demanding requirements for QD surface coating arise. For example, the size of QDs should stay small after coating, the surface should be biocompatible and non-immunogenic, reactive groups should be available for conjugation of biomolecules and targeting ligands, and yet QDs should show minimal non-specific interactions with the biological environment. Improvement of existing coating techniques and design of novel application-specific water-solubilization and bioconjugation approaches remains an active area of research.

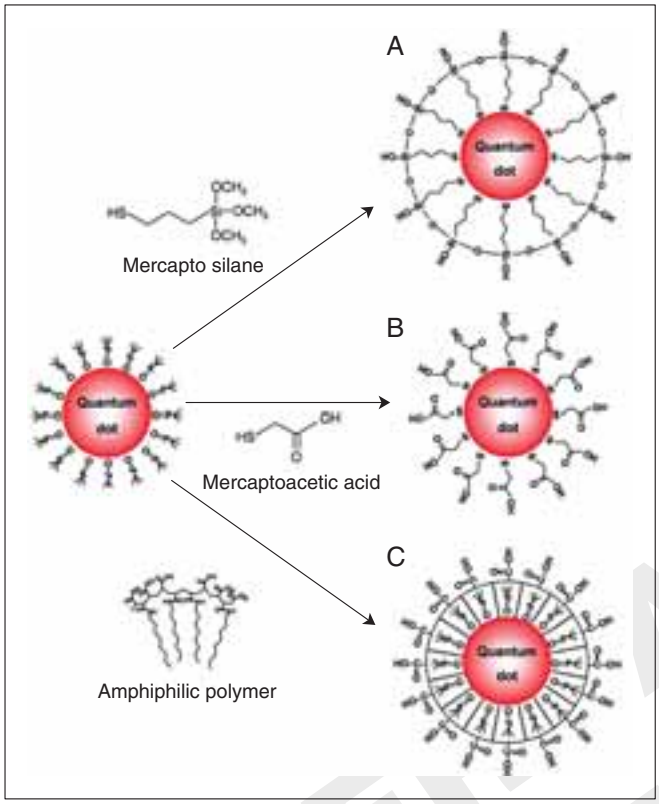

Figure 2.—General strategies for water-solubilization of TOPO-coated QDs are presented. Ligands are drawn disproportionately large for detail; ligand-polymer coatings are usually only 1-2 nm in thickness. A) In a silica shell encapsulation approach TOPO molecules are replaced by mercapto silanes followed by shell cross-linking. B) In a ligand-exchanged approach TOPO coating is replaced by heterobifunctional ligands (such as mercaptoacetic acid) generating hydrophilic QDs exposing carboxylic acids on the surface. C) In the polymer encapsulation procedure, TOPO is retained on the QD surface, and nanocrystals are rendered water soluble through micelle-like interactions with amphiphilic polymers.

QD water-solubilization

One of the first effective water-solubilization strategies via encapsulation of QDs into polymerized silanol shells was developed by Bruchez et al.[13] In this procedure 3-(mercaptopropyl)trimethoxysilane (MPS) is directly absorbed onto the nanocrystals displacing TOPO molecules. Further hydrolysis of silanol groups leads to formation of silica/siloxane shell (Figure 2A). Nanoparticles produced in such a manner maintain a relatively small size and are highly stable against flocculation. However, low reaction yield, poor fluorescence efficiency (~21%), and the tendency to precipitation at neutral pH preclude utilization of this approach for large-scale production and wide-spread use of QD-based probes.[11] In a similar approach, Guo *et al.* have obtained highly stable nanocrystals soluble in polar solvents by encapsulation of QDs in crosslinked dendron boxes.[42] Yet, further surface modification is required to render such QDs water-soluble and capable of bioconjugation.

Another route for water-solubilization of QDs is substitution of the native TOPO coating with small bifunctional ligands presenting both a surface-anchoring group (*e.g.* thiol) and a hydrophilic end group (*e.g.* carboxyl or hydroxyl). Chan and Nie first implemented ligand exchange procedure by coating CdSe/ZnS core/shell QDs with mercaptoacetic acid and demonstrated utility of such nanoparticles for *in vitro* live cell staining and sensitive immunoassays (Figure 2B).[43] In spite of its simplicity, ligand exchange approach results in significant drop in the quantum yield of QDs. The shelf life of such nanoparticles is also very short due to poor stability of mono-thio compounds. Substitution from mono-thio to di-thio ligands can improve the storage stability.[38, 44] For example, Liu *et al.* have utilized di-thiol ligand dihydrolipoic acid conjugated to poly(ethylene glycol) to prepare small (hydrodynamic diameter of 11.4 nm) and stable QDs with some loss of fluorescence efficiency (drop in quantum yield from 65% to 43%).[45] In an alternative approach, Sukhanova *et al.* have water-solubilized QDs with DL-Cysteine and stabilized the particles with poly(allylamine), achieving improvement in QD colloidal stability and increase in quantum yield (from 40% to 65%).[46] Jiang *et al.* have improved the stability of mercaptoundecanoic acid shell by covalently cross-linking neighboring molecules with lysine.[47] However, the dramatic increase in nanoparticle size (from 8.7 to 20.3 nm hydrodynamic diameter) induced by shell cross-linking is undesirable, and further optimization of this procedure is required.

Weiss et al. developed a method for CdSe/ZnS QD surface passivation and water-solubilization using phytochelatin-related peptides.[48] Besides yielding relatively small water-soluble nanoparticles, this approach also provides points of chemical modification and biological functionality on QD surface. Belcher *et al.* have demonstrated that the phage-display libraries can be used to identify, develop, and amplify binding between peptides and inorganic semiconductor substrates.[49] With the use of such libraries and accelerat-

ed evolution techniques it might be possible to select peptide sequences that can specifically bind to any type of nanomaterial, thus providing a universal surface coating approach.[25]

Water-solubilization strategies preserving the native TOPO coating on the QDs (such as overcoating with variants of amphiphilic 'diblock' and 'triblock' copolymers and phospholipids) currently show most promise and utility for biomedical applications.[23, 24, 33] The reaction is mainly driven by absorption of amphiphilic polymer coating onto the TOPO-coated nanoparticles via hydrophobic interactions. In one method QDs are solubilized with an octylamine-modified polyacrylic polymer, which exposes hydrophilic carboxylic acid groups to the solution, thus rendering QDs water soluble (Figure 2C). Another method utilizes coating with polyethylene glycol (PEG)-lipid layer,[23] which has an amphiphilic surfactant structure. Intact TOPO coating maintains the optical properties of nanoparticles and provides better shielding of the core from contact with the outside environment.[18] In fact, polymer-coated QDs linked to PEG molecules are protected to such a degree that their optical properties do not change in a broad range of pH and salt concentrations.[24] The drawback of such approach is increase in final size of nanoparticles (in some cases up to four times) [38] which might be detrimental for quantitative biomarker detection in a crowded biological environment. The thickness of polymer coating might also preclude QDs from fluorescence resonance energy transfer (FRET)-based investigations.[25, 38, 50] Currently, novel encapsulation and bioconjugation approaches are being developed to provide higher nanocrystal protection with thin, biocompatible, and functional coatings.

QD bioconjugation

Quantum dots uniquely combine small protein-scale size and large surface area, representing versatile nanoscaffolds for attachment of multiple proteins, peptides, and nucleic acids, thus enabling design of multifunctional nanoparticle-biological hybrids.[51-53] Variety of bioconjugation approaches have been developed and utilized in QD-based tumor imaging applications. Covalent bond formation between reactive functional groups (*e.g.* primary amines, carboxylic acids, alcohols, and thiols) is one of the most popular bioconjugation methods. Many proteins contain primary amine groups that can be linked to carboxyl-coated QDs via carbodiimide-mediated amide formation (*i.e.* EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, condensation reaction; Figure 3A). This reaction does not require additional chemical modification of proteins, preserving their natural structure. However, inability to control molecular orientation and number of the attached proteins yields QD population with varying biological activity, thus reducing stoichiometry of QD labeling. Nonetheless, Bagalkot *et al.* utilized EDAC coupling to decorate the surface of polymer-encapsulated QDs with double-stranded RNA aptamers targeting PSMA and demonstrated efficient drug delivery and live cell imaging on prostate cancer cells.[54] Another common covalent bonding procedure involves active ester maleimide-mediated amine and sulfhydryl coupling. Site-specific incorporation of sulfhydryl groups in biomolecules provides better control over biomolecules orientation on the surface of nanocrystals (Figure 3B). However, often required reduction of biomolecules with DTT (dithiothreitol) might substantially decrease their biological activity.[55] Despite this limitation Yezhelyev et al. have utilized SMCC (4-(maleimidomethyl)-1-cyclohexancarboxylic acid N-hydroxysuccinimide ester) procedure for QD-antibody conjugation and have successfully developed a QD-based assay for quantitative detection of three breast cancer markers – estrogen receptor, progesterone receptor, and ERBB2 — in paraffin-embedded human breast-cancer cells.[56]

As an alternative to covalent bonding to functionalized QD coating, biomolecules can be linked directly to the nanocrystal surface via a thiol-exchange reaction. Here, mercapto-coated QDs are mixed with thiolated biomolecules or biomolecules containing polyhistidine (HIS) residues, and a small fraction of the surface ligands is replaced by the molecule of interest.[38] As with SMCC reaction, rational design of poly-HIS tags and targeted incorporation of thiolated anchors allow more control over the final bioconjugate assembly. In one example, Lao *et al.* have designed a tripartite fusion protein consisting of an N-terminal HIS-tag, a stimulus-responsive elastin-like peptide (ELP), and a C-terminal IgG-binding protein L.[57]In this construct, HIS-tag effectively binds to QD surface, orienting the remainder of the ligand away from the nanoparticle surface; ELP causes reversible aggregation in high salt buffers, aiding in QD-bioconjugate purification; and protein L binds to IgG light chains with high affinity, allowing preparation of aggregate-free QD-antibody conjugates.

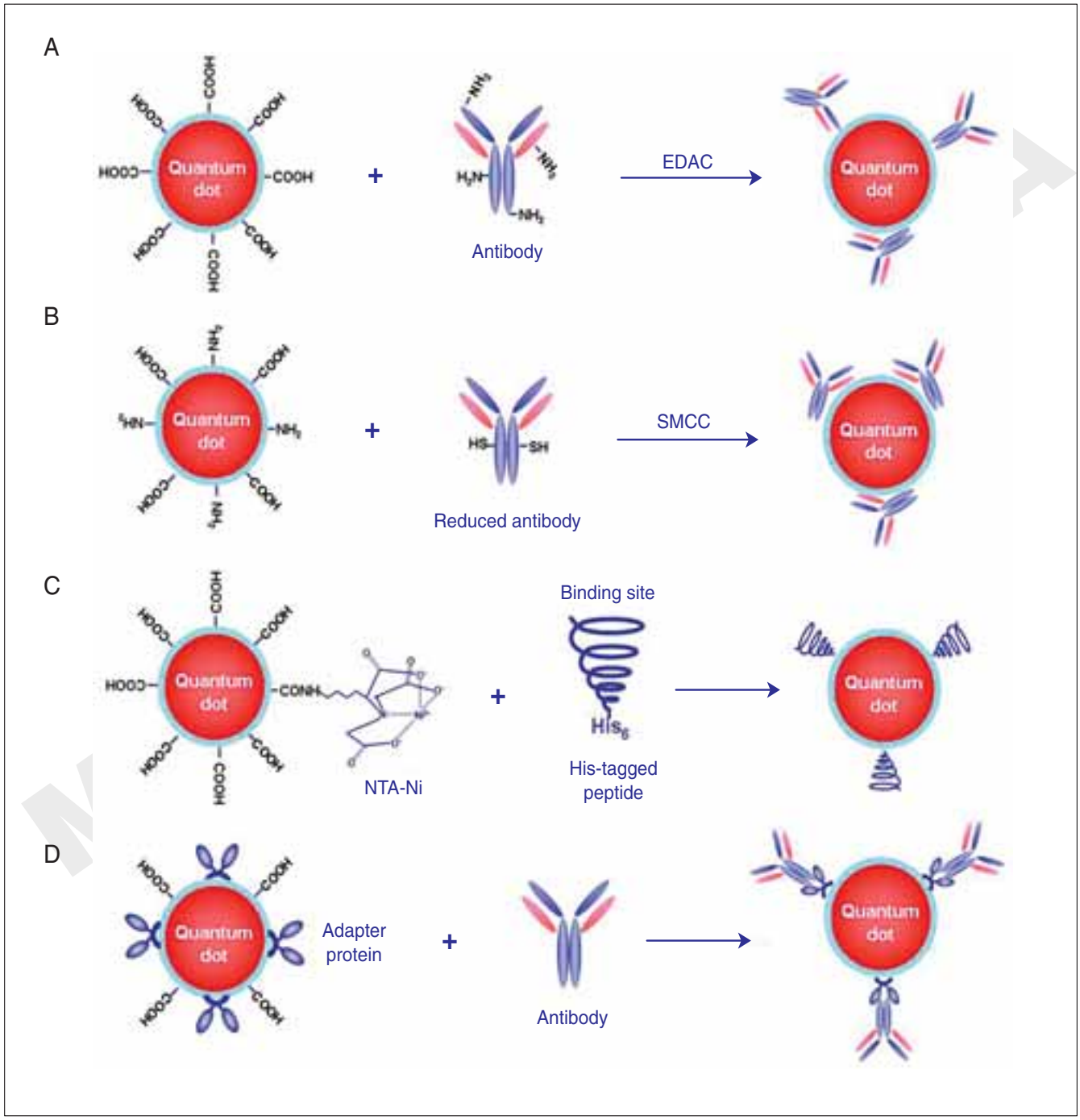

Figure 3.—Methods for QD-biomolecule conjugation. A) Traditional covalent cross-linking chemistry using EDAC as a catalyst produces bioconjugates with random orientation of ligands. B) Conjugation of antibody fragments to QDs via reduced sulfhydryl-amine coupling mediated by hetero-bifunctional crosslinker SMCC controls ligand orientation, but disrupts its natural structure. C) Conjugation of histidine-tagged peptides and proteins to Ni-NTA-modified QDs provides potential control over the attachment site and QD-to-ligand molar ratios. D) Conjugation of antibodies to QDs via an adaptor protein preserves the natural structure of the ligand and controls its orientation, yet producing large multi-layer bioprobes.[10]

Yet, the major downside of a thiol-exchange approach is the necessity for unstable surface coatings (such as mercapto compounds), which significantly reduce the brightness and stability of such bioconjugates in aqueous solutions. To overcome this limitation Gao *et al.* have developed an indirect HIS-tag coupling method by linking stable polymer-coated QDs to a chelating compound Ni-NTA (nickel-nitrilotriacetic acid), which binds to polyHIS-tagged biomolecules in a quantitative and controlled manner (Figure 3C).[10]

Electrostatic interactions between nanocrystals and biomolecules have been widely utilized for non-covalent self-assembly of engineered proteins on the surface of QDs. For example, in a simple and straightforward procedure Goldman *et al.* deposited avidin – a highly positively charged glycoprotein – on the surface of negatively charged QDs for further conjugation to biotinylated antibodies,[58] while Medintz *et al.* have used this procedure to decorate biotinylated cowpea mosaic virus with QDs.[59] Similarly, Mattoussi et al. have described the utility of a chimeric fusion protein for indirect coupling of native unmodified immunoglobulin G antibodies to QDs (Figure 3D).[60] Preservation of native structure and excellent control over ligand orientation make this approach attractive for preparation of high-quality QD bioconjugates. However, the size of such bioconjugates becomes large due to a number of thick layers deposited on the QD surface, imposing a number of limitations on use of QD probes in tumor molecular imaging applications (*e.g.* increased non-specific binding, slower diffusion to a target, decreased ability to penetrate biological membranes, and steric hindrance between QD probes).

Recent achievements in merging nanoparticle encapsulation and bioconjugation and design of pre-functionalized surface coatings promise to provide more compact, stable, and biocompatible nanoparticles with controlled density and orientation of ligands attached. Amphiphilic polymers with maleic anhydride backbone are being actively explored for this purpose. In organic anhydrous solvents, such polymers encapsulate TOPO-coated QDs and introduce reactive anhydride groups on the surface. In basic aqueous buffers anhydride rings are quickly hydrolyzed, yielding negatively charged carboxylic acid groups and rendering QDs water soluble.[61] More importantly, anhydride groups are highly reactive towards amine-containing molecules, thus allowing to covalently conjugate a variety of biomolecules to polymer chains without the need for post-encapsulation modification.[62, 63]

Up to this day, a great number of different surface coating and bioconjugation techniques have been developed. The complexity of these procedures as well as the quality of resulting QD bioconjugates and degree of reaction control range widely. Some methods, such as direct covalent conjugation of ligands via SMCC or EDAC reaction and indirect coupling via streptavidin-biotin bond, have found wide use in many biomedical applications and especially in the area of *in vitro* and *ex vivo* multicolor cell and tissue imaging.[34] Yet, presently there is no universal method best suitable for all biomedical applications.

QD applications in tumor molecular imaging and profiling

Despite the relatively recent introduction of nanoparticles into biomedical research, QDs have already proven to be well suited for sensitive quantitative molecular profiling of cancer cells and tissues, holding tremendous promise for unraveling the complex gene expression profiles of cancers, accurate clinical diagnosis and personalized treatment of patients.[3, 64] Currently, QDs have found use in most of the conventional biomedical tools where fluorescence or colorimetric imaging of target biomarker is utilized (*e.g.* cancer cell and tissue staining, Western blot, ELISA, etc.) and have launched novel applications (*e.g. in vivo* tumor imaging, single-molecule tracking, combined drug delivery and imaging, etc.) utilizing their unique photo-physical properties. The number of biomedical applications of QDs continues growing, ranging from ultrasensitive detection *in vitro* to targeted drug delivery and imaging *in vivo*.

Molecular imaging of fixed cancer cells and tissues

Various labeling techniques for detection of DNA, mRNA, proteins, and other biomolecules in fixed cancer cells and tissue specimens are currently used in clinical practice and research. Most of these methods are based on specific interaction between the target and its ligand (*e.g.* antigen and antibody, or DNA and complementary oligonucleotide) with visualization of the target position via fluorescence, brightfield, electron, or other types of microscopy. Conventional immunohistochemistry methods have been successfully used for reliable and consistent staining of cli-

nical tissue specimens; yet these methods suffer from significant limitations in characterizing cancer on molecular level. At the same time, QD-based probes with unique optical properties, stable and biocompatible coatings, and functionalized surfaces have already shown their outstanding performance in multiplexed fluorescent detection and quantitative analysis of proteins and nucleic acids in cancer cells and tissue sections.

Applications of QDs in high-resolution correlated fluorescence/electron microscopy

Electron microscopy is an imaging technique that utilizes electrons rather than photons to probe a sample, thus providing high magnification and allowing analyzing the localization of proteins on a sub-cellular level. Being electron dense nanocrystals with highly controlled and defined sizes and shapes, QDs represent suitable probes for multiplexed target detection with electron microscopy. Further, dependence of emission color on QD size allows for correlation between low-resolution fluorescence images and high-resolution TEM images, thus providing sub-cellular, cellular, and tissue-level information from a single specimen.

Giepmans *et al*. have evaluated performance of antibody-conjugated QDs in correlated fluorescence and electron microscopy.[29] Tissue specimens were processed and stained in accordance with standard two-step immunohistochemistry procedure. Initial optimization of staining parameters was achieved by using fluorescence microscopy. Further examination with transmission electron microscopy (TEM) revealed intracellular localization of QD probes with respect to sub-cellular structures. Preservation of the QD fluorescence for correlated fluorescence/electron microscopy proved to be one of the major limitations of this technology. As preparation for the TEM requires postfixation in osmium tetroxide, which quenches QD fluorescence, and skipping osmication step might decrease TEM resolution, correlated fluorescence/TEM imaging will find limited use until new QD-compatible protocols for TEM sample preparation become available. Nonetheless, Giepmans et al. have reported successful detection of at least three QD-labeled biomarkers distinguishable at both fluorescence and TEM levels with good resolution.[29] Additional multiplexing functionality of this technique can be obtained from discrimination of QDs based on their elemental composition. Nisman *et al.* have proposed the use of electron spectroscopic imaging (ESI, a technique for generating elemental maps of materials with high resolution and detection sensitivity) for mapping the distribution of quantum dots in cells and tissues based on QD internal chemistry.[32]

Fluorescence/TEM correlated imaging is not meant to be used for high-throughput quantitative screening of clinical samples, but rather as a tool for ultrasensitive detection of target markers and for the detailed study of distribution and relationships between different biomarkers on a sub-cellular level. While this technique has not been applied in clinical oncology yet, it will certainly play a major role in uncovering complex molecular pathways underlying development of cancer and other pathological processes in future.

Molecular profiling of tumor tissue sections and microarrays with QD bioprobes

Tissue microarrays have been extensively used for high-throughput histological analysis of tumor biopsies. However, utilization of standard immunohistochemistry techniques for microarray evaluation limited this technique to qualitative single-biomarker analysis. QDs have a potential to significantly enhance the performance of tissue microarray analysis by providing access to quantitative and multi-color labeling. Wu *et al.* have investigated the utility of QDs conjugated to streptavidin and IgGs for simultaneous labeling of membrane-associated Her2 receptor and of a nuclear antigen in breast cancer cells [33]. In comparison to Alexa dyes, QDs have proven to be much more photostable and have produced higher signal intensity. However, while staining of cell surface antigens was reliable and effective, staining of cytoplasmic and nuclear markers was more variable. This issue is directly associated with the large size of QD probes and can be potentially resolved by stronger sample permeabilization and by optimization of staining conditions.

Ghazani and coworkers have demonstrated application of QD labeling for quantitative analysis of tumor biopsies in tissue microarrays.[28] Three-color staining of lung carcinoma xenografts for epidermal growth factor receptor (EGFR), E-cadherin, and cytokeratin has been achieved by utilization of 655, 605, and 565 nm QD-based assays, and specificity of staining has been confirmed by standard immunofluorescence imaging with Alexa 488 dye. Superior QD signal intensity and photostability allowed for effective removal of autofluorescence background and reliable quanti-

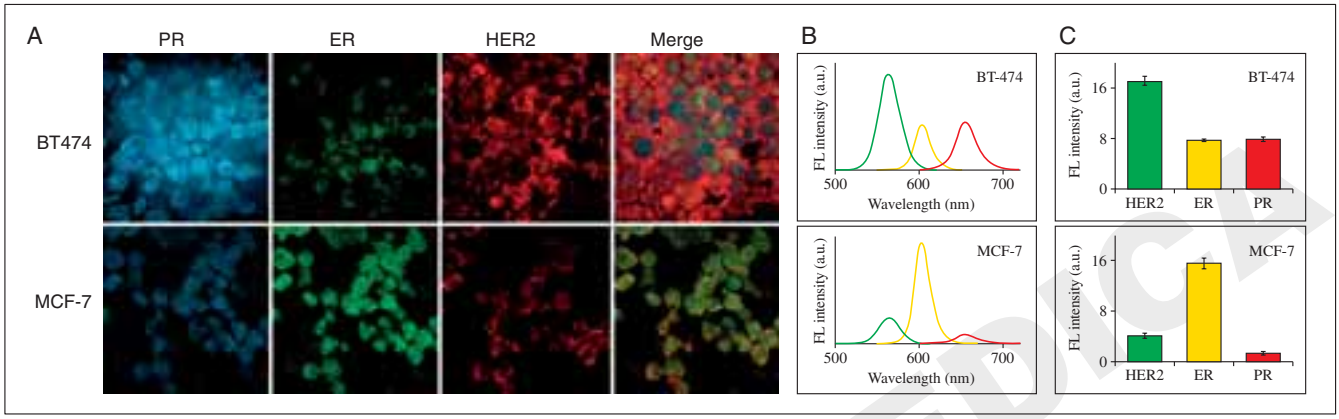

Figure 4.—Quantification of expression levels of three representative breast cancer markers – PR, ER, and HER2 – in FFPE breast cancer cells. A) Fluorescence images are deconvolved into three individual components. B) Representative spectra obtained with single-cell spectroscopy are used in quantitative analysis of biomarker expression levels. 565nm and 605nm peaks are enhanced by a factor of 8 and 2 respectively to compensate the differential brightness of multicolor QDs. C) Statistical analysis of biomarker expression obtained by averaging spectra of 100 single cells.[16]

zation of signal intensity. One important limitation of QD technology pointed out in this study was that quantitative comparison of different biomarkers in multiplexed staining could be severely compromised by the strong signal enhancement of red QDs and reduction of 565 nm signal.[28] This discordance in fluorescence properties of individual probes directly relates to light absorption properties of QDs and can be accounted for in signal analysis algorithm. Yet, in order to obtain a uniform staining of multiple targets within one sample it is advisable that brighter red QDs be used for labeling of low-copy number biomarkers, while dimmer blue QD conjugates be used for targets present in large quantities. Optimization of antibody concentrations and other staining conditions can also be used to prevent saturation of a detector by one highly bright signal.

Recently, Yezhelyev *et al.* have demonstrated the use of QD bioconjugates for multiplexed labeling and quantification of five clinically significant breast cancer markers – HER2, ER, PR, EGFR, and mTOR.[16] The performance of QD-antibody conjugates was evaluated on FFPE breast cancer cells and validated by conventional techniques for three representative biomarkers – ER, PR, and HER2 (Figure 4). In order to account for signal enhancement of red QDs and compare expression levels of biomarkers within one sample, acquired data was adjusted according to the relative intensities of QDs (QD655:QD605:QD565 = 8:2:1 as measured in a separate experiment for equal QD concentrations). Utility of this technology for clinical evaluation of tumor tissue specimens has been demonstrated by detection and quantification of the panel of all five biomarkers on FFPE breast cancer tissue biopsies.

QD-OLIGONUCLEOTIDE BIOCONJUGATES FOR *IN SITU* HYBRIDIZATION

Fluorescence in situ hybridization (FISH) is a common method of determining gene expression level or messenger RNA (mRNA) distribution using fluorescent-labeled DNA or RNA probes. Driven by binding of oligonucleotide probes to complimentary mRNA molecules in 1:1 probe-to-target ratio, this technique offers high level of specificity, yields direct quantitative correlation between gene amplification (i.e. number of mRNA molecules present) and signal intensity, and provides accurate information about mRNA localization within the cell. However, low copy number of target mRNA and quick photobleaching of organic fluorophores heavily compromise the quantitative potential and sensitivity of FISH. QD-oligonucleotide bioconjugates could become brighter FISH probes that are easy to detect and quantify.[12]

Xiao and Barker have used highly stable PEGylated QD-Streptavidin bioconjugates for visualization of biotinylated oligonucleotide probes in FISH analysis of amplification of clinically important ERBB2 gene.[65]

Substantially higher signal-to-noise ratio has been observed for QD bioconjugates compared to organic dyes (Texas Red and fluorescein), while specificity of hybridization has been demonstrated for all three probes. Unfortunately, due to utilization of a universal biotin-streptavidin linkage, the 2-step experimental procedure used implies that only one target can be detected per sample, rendering multiplexed FISH impossible. In a similar approach, Tholouli *et al.* have modified the hybridization protocol to allow for simultaneous detection of several mRNA molecules while still using biotin-streptavidin linkage for QD-oligonucleotide conjugation.[66] Biotinylated DNA probes were pre-incubated with QD-Streptavidin conjugates to allow for detection of 3 mRNA targets in a 1-step FISH procedure. Naturally, pre-conjugation of multiple oligonucleotides to QDs significantly increases the overall size of the probe, thus requiring stronger specimen permeabilization with enzymes (*e.g.* proteinase K), which necessarily degrades cell and tissue architecture and destroys most of the protein-based biomarkers useful for immunohistochemical studies.

Chan *et al.* have developed a more controlled procedure for pre-conjugation of exactly one oligonucleotide probe per QD via biotin-streptavidin linkage.[67] Starting with commercial QD-streptavidin conjugates, excess streptavidin sites are blocked with biocytin (water soluble biotin derivative), and only a few biotinylated oligonucleotides are allowed to bind. Further purification of QD-oligo conjugates in agarose gel electrophoresis yields relatively small mono-oligonucleotide FISH probes suitable for multiplexed mRNA detection under mild specimen permeabilization. High-resolution multiplexed FISH has been demonstrated in simultaneous detection of four mRNA targets using two different QD probes and two different organic fluorophore probes within a single mouse midbrain neuron (Figure 5). Ability to use protein-compatible specimen permeabilization techniques has allowed Chan et al. to successfully combine QD-based FISH and QD-based immunohistochemistry to compare cellular distribution patterns of the vesicular monoamine transporter (Vmat2) mRNA and immunoreactivity of tyrosine hydroxylase in dopaminergic neurons [67]. These results offer the possibility of correlating gene expression at the mRNA level with the number of corresponding protein copies in tumor cells and tissue specimens as well.[12]

Recently, combined QD-based FISH/immunohistochemistry technique has been improved by Matsuno *et al.* who demonstrated the use of confocal laser scanning microscopy for three-dimensional imaging of the intracellular localization of growth hormone (GH), prolactin (PRL), and of their mRNAs.[30] The exceptional photostability and signal intensity of QDs have been utilized for reconstruction of high-resolution three-dimensional images of tissue samples. However, the use of 2-step FISH procedure and universal biotin-streptavidin linker for QD-oligonucleotide conjugation limits this technique to qualitative detection of only one mRNA plus a few proteins (detected by QD-antibody conjugates) per section. With incorporation of new probes suitable for multiplexed FISH and immunohistochemistry this technology will allow three-dimensional mapping of the relative position of biomarkers and corresponding mRNAs inside cells and tissues with high resolution and sensitivity, thus providing access to studies of intricate signaling pathways and mechanisms of oncogenesis.

In vivo tumor imaging

Non-invasive *in vivo* tumor imaging represents the major goal of current biomedical research as it provides access to high-throughput patient screening, accurate cancer diagnosis, and real-time assessment of therapy efficiency. Conventional medical imaging techniques, such as ultrasound imaging, magnetic resonance imaging, and positron emission tomography, in most cases lack sensitivity for early cancer detection and specificity for conveying cancer molecular information. QDs possess high brightness and multiplexing capabilities along with large Stokes shifts, thus representing a promising tool for *in vivo* tumor molecular imaging and profiling. Yet, *in vivo* imaging with QDs presents a number of unique challenges not encountered in QD-based molecular imaging of fixed cells and tissue specimens. Among these, short-term and long-term toxicity and immunogenicity of nanoparticles remain a major concern.[68]

Early studies of QD toxicity by Derfus *et al.* indicated significant cytotoxicity of unprotected CdSe-core QDs due to nanoparticle photo-oxidation upon exposure to UV light and release of toxic Cd^{2+} ions.[22] Capping of CdSe core with ZnS layer and deposition of a stable coating dramatically reduced QD toxicity in cell cultures. Yet, live organisms are more complex than single cells, providing numerous mechanisms for QD accumulation, degradation, and excretion. Several reports have indicated an important role

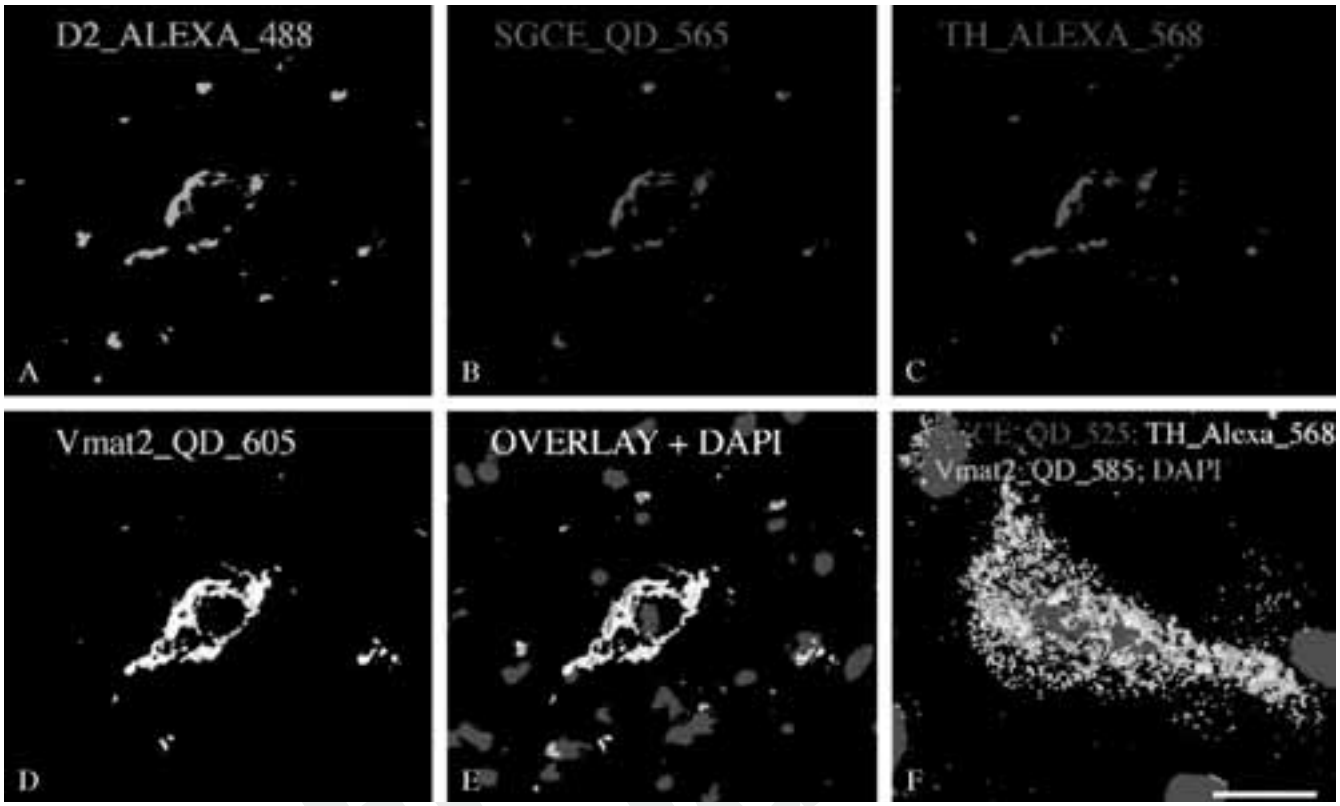

Figure 5.—Multiplex FISH using QDs and organic fluorophores is demonstrated. A-D) Four different mRNA targets are detected and separated by spectral imaging. Dopamine D2 receptor mRNA is labeled with Alexa488, ε-sarcoglycan (SGCE) mRNA – with QD565, tyrosine hydroxylase (TH) mRNA – with Alexa568, and vesicular monoamine transporter (Vmat2) – with QD605. (E) Overlay of (A–D). (F) A higher magnification image of a single neuron labeled for three different mRNAs (SGCE, TH, and Vmat2) using two QDot probes and one organic fluorophore. Nuclei are counterstaining in blue.[67]

of QD surface coating and particle size on the biodistribution and toxicity. Pharmacokinetics studies performed on rat models by Fischer *et al.* have shown that QDs coated with bovine serum albumin (BSA) are efficiently eliminated from the bloodstream by liver uptake, while QDs lacking BSA on their surface are cleared at slower rate.[69] Ballou *et al.* have emphasized the importance of coating with high molecular weight PEG to reduce accumulation of QDs in liver and bone marrow and indicated that intact fluorescent QDs remained in bone marrow and lymph nodes for several months after injection.[70] As routs of *in vivo* QD degradation and long-term effect of nanoparticle accumulation in organs have not been studied in details, Choi *et al.* have suggested that only QDs capable of clearing through renal excretion are used for *in vivo* imaging applications.[71] Systematic investigation of the renal clearance of QDs on rat and mice models has defined the renal clearance threshold of 5.5 nm. Further, only zwitterionic and neutral surface coatings prevented adsorption of serum proteins and increase in QD size, thus outlining the general strategy for design of *in vivo* QD probes.[71]

Presence of tissue barriers between tumor sites and imaging equipment complicates the utilization of fluorescence microscopy for *in vivo* imaging as biological tissues efficiently absorb and scatter visible light along with producing intense autofluorescence over a broad spectrum. For example, in early studies Akerman *et al.* used QD-peptide bioconjugates for targeted imaging of tumor vasculature.[72] However, utilization of green and red QDs precluded from deep-tissue imaging in live animals, and post-mortem histological examination of tissue specimens was used to

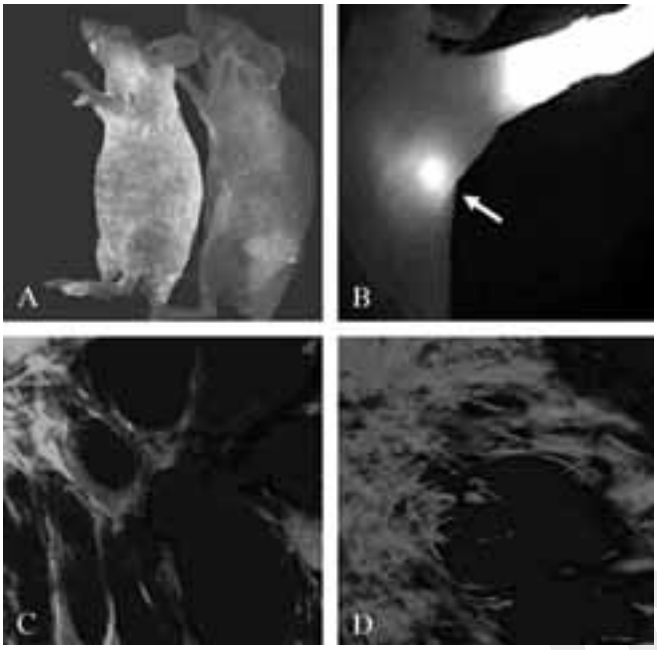

Figure 6.—*In vivo* tumor imaging with QDs. A) Antibody-conjugated red QDs are used to image prostate cancer in mouse model. Spectral demixing algorithm allows to separate QD (red) signal from tissue autofluorescence (green). QD signal is clearly distinguishable in tumor-bearing mouse (on the right), while only tissue autofluorescence is observed in control mouse (on the left).[24] B) Sentinel lymph node mapping with NIR QDs provides nearly background-free image and allows for image-guided surgery.[74] C, D) Multi-photon microscopy study of tumor morphology using QDs for labeling of tumor vasculature (blue QDs in (C) and red QDs in (D)), GFP for labeling perivascular cells (green in (C)), and second harmonic generation signal from collagen to visual0ize extracellular matrix (light-blue in (D)) provides access to multiplexed *in vivo* imaging.[78]

evaluate QD biodistribution. Gao *et al.* have achieved targeted *in vivo* tumor imaging with red QDs stabilized by PEG molecules and conjugated to antibodies against PSMA, but utilization of spectral demixing algorithm was required for separation of QD signal from tissue autofluorescence (Figure 6A).[24] Further, significant signal attenuation by biological tissues reduces sensitivity of this technique. In attempts to minimize interference of tissues Lim *et al.* in modeling studies have identified two spectral windows in far-red (700-900 nm) and infrared (1200-1600 nm) regions suitable for nearly background-free deep-tissue imaging.[73] Kim *et al.* took advantage of this in mapping sentinel lymph nodes (SLN) with near-infrared (NIR) QDs providing accurate identification and image-guided resection of SLN — an indispensable tool in surgical treatment of metastatic cancer (Figure 6B).[74] Targeted *in vivo* imaging of human glioblastoma vasculature in mouse model was demonstrated by Cai et al., who used NIR CdTe/ZnS QDs conjugated to targeting peptide against integrin $\alpha_v\beta_3$.[75] Recently, Diagaradjane *et al.* reported on *in vivo* imaging and quantitative analysis of EGFR with NIR QDs (emission peak at 800 nm), showing QD capability to distinguish EGFR overexpression in tumor site compared to normal expression levels in surrounding tissues.[76] As relatively large size of NIR QDs reduces their extravasation capabilities, targeting of lymph nodes and tumor vasculature remains one of the potentially clinically useful applications of such probes.

Two-photon microscopy despite some technical limitations represents a powerful tool for *in vivo* tumor imaging. This technique uses low-energy photons (in red and infrared regions) for excitation of QDs emitting in visible range, given that both photons are absorbed almost simultaneously in a single quantum event. Therefore, attenuation of excitation light by tissues can be reduced dramatically, while allowing utilization of QDs emitting over full visible spectrum. Moreover, high two-photon cross-section of QDs provides access to deeper-tissue imaging and reduces associated autofluorescence of organic molecules. First study of QD-based multiphoton fluorescence *in vivo* imaging was reported by Larson *et al.*, when green CdSe/ZnS QDs were used for imaging of capillaries under the dermis layer of skin.[77] In a recent *in vivo* study of tumor morphology Stroh *et al.* utilized two-photon microscopy for simultaneous imaging of tumor vessels (stained with blue QDs) and perivascular cells (expressing GFP, Figure 6C).[78] Further incorporation of second harmonic generation signal emanating from collagen provided information about distribution and morphology of extracellular matrix (Figure 6D).

Conclusions

Quantum dots have emerged as a new class of molecular imaging agents and have already fulfilled some of their promises in cancer research, molecular diagnostics, and non-invasive imaging. Design of compact, stable, and biocompatible coatings functionalized with targeting agents have converted QDs into multifunctional nanodevices suitable for *in vitro* as well as *in vivo* applications. However, further improve-

ments are needed for this technology to receive widespread adaptation. For example, the relatively large size of QD bioconjugates hampers their deep tissue penetration, reduces extravasation from the bloodstream, and precludes from imaging of intracellular targets. *In vivo* application of QDs is further complicated by the adsorption of biomolecules on the surface of nanoparticles, thus further increasing their size, reducing renal clearance rate, and promoting QD uptake by the reticulo-endothelial system. Development of compact QD bioconjugates with "non-sticky" (presumably zwitterionic or neutral) surface coatings would resolve this problem. The QD-ligand conjugation chemistry still requires further improvement as the control over the number and orientation of bioligands is essential for staining stoichiometry. The lack of standardization procedures makes large-scale studies of cancer pathophysiology problematic due to possible variations in data interpretation by different labs. Finally, in order to become applicable for *in vivo* imaging in human subjects, detailed systematic study of QD toxicity and immunogenicity must be performed and safety criteria for QD bioprobes design should be developed. With these improvements, QD-based molecular imaging will allow researchers to thoroughly investigate cancer pathophysiology using *in vitro* and *in vivo* models and will provide an opportunity for development of cancer-specific and patient-specific personalized treatment schemes.

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