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In Situ Molecular Profiling of Breast Cancer Biomarkers with Multicolor Quantum Dots**

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Identification of potential diagnostic markers and target molecules among the plethora of tumor oncoproteins requires enabling technology that is capable of analyzing multiple biomarkers in tumor cells and tissues quantitatively.[1] Diagnostic and prognostic classifications of human tumors are currently based on immunohistochemistry (IHC), a technique that has been used in clinical medicine for over 80 years.[2] However, the immunoenzyme (HRP-based) IHC method has a single-color nature and is unable to perform multiplexed molecular profiling. Moreover, IHC remains semi-quantitative and subjective, resulting in considerable inter-observer variation of the results.[3] With new molecular profiling technologies, it is possible to read the molecular signatures of an individual patient's tumor, and to correlate a panel of cancer biomarkers with clinical outcome for personalized therapy. A major difficulty in molecular profiling is that most cancer tumors (especially breast and prostate cancers) are highly heterogeneous, containing a mixture of benign, cancerous, and stroma cells. Current technologies such as RT-PCR, gene chips, protein chips, two-dimensional gel electrophoresis, biomolecular mass spectrometry (e.g., MALDI-MS, ES-MS, and SELDI-MS) are not designed to handle this type of heterogeneous samples.[4,5] Furthermore, a limitation shared by all these technologies is that they require destructive preparation of cells or tissue specimens into a homogeneous solution, leading to a loss of valuable 3D cellular and tissue morphological information associated with the original tumor. In comparison, the development of nanotechnology, especially bioconjugated nanoparticles, can provide an essential link by which biomarkers could be functionally correlated with cancer behavior. Indeed, several groups recently reported the use of quantum dot (QD) probes for immunostaining of fixed cells and tissue specimens enabled by their unique optical properties such as improved brightness, simultaneous excitation of multiple colors, stability against photobleaching, and extremely large Stokes shift.[6–13] However, translational research of the QD-based immunostaining has not received widespread adaptation by clinical studies. A major problem is the lack of technology validation using conventional methods and the validation in large-scale clinical studies. In this Communication, we report the use of multicolor QDs for quantitative and simultaneous profiling of multiple biomarkers using intact breast cancer cells and clinical tissue specimens. We also compare and validate the new QD-based molecular profiling technology with standard western blotting (WB) and fluorescence in situ hybridization (FISH). This new technology could become the first clinical applications of QDs and open a new avenue in molecular pathology.[14]

Multicolor QDs are directly conjugated with antibodies through covalent bonds. Compared with our previously reported carbodiimide-mediated carboxylate and amine condensation,[15] the QDs and antibodies are linked to each other via active ester maleimide-mediated amine and sulfhydryl coupling. Because free sulfhydryl groups are rare in native antibodies and are often unstable in the presence of oxygen, antibodies were first treated with reducing reagents such as dithiothreitol (DTT) to generate free thio groups in the 'hinge' region of antibodies as shown in Figure 1. This procedure results in less nanoparticle aggregation in comparison with carbodiimide-mediated COOH-NH_2 condensation. On the other hand, chemical treatment of antibodies affects their stability and, as a consequence, could impede the antigen-recognition activity.

To demonstrate the feasibility of multiplexed labeling, QDs emitting at 525 nm, 565 nm, 605 nm, 655 nm and 705 nm were directly conjugated to primary Abs against HER2 (QD-HER2), ER (QD-ER), PR (QD-PR), EGFR (QD-EGFR) and mTOR (QD-mTOR). The multicolor bioconjugates were used for simultaneous detection of the five clinically significant tumor markers in breast cancer cells, MCF-7 and BT-474. These two cell lines were selected because they have different expression levels of the five protein

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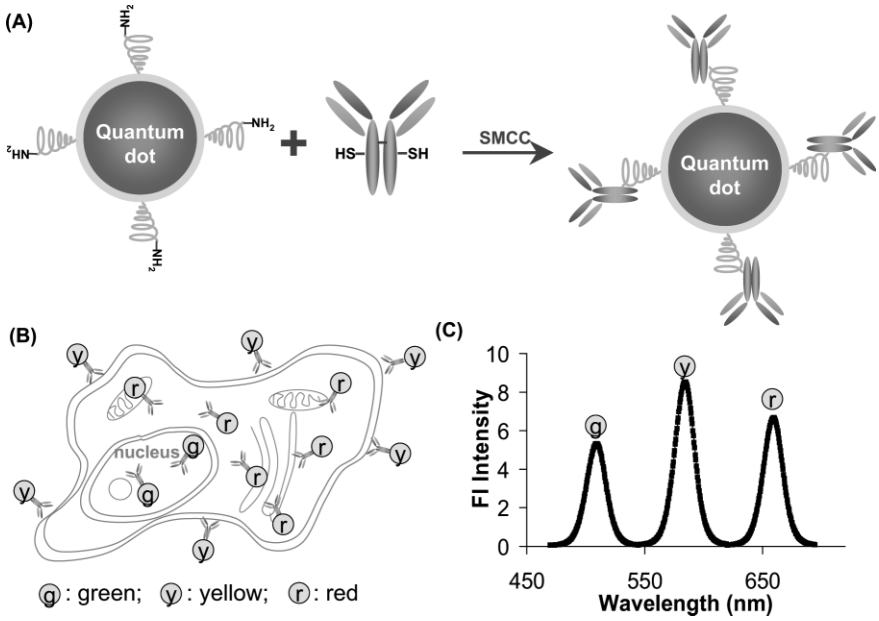

Figure 1. Schematic illustration of bioconjugated QDs for multiplexed in situ molecular profiling. (A) Multicolor QD bioconjugates prepared with SMCC activated QDs and chemically reduced antibodies. (B) Cell staining using multicolor QD-bioconjugates. (C) Quantification of tumor biomarker expression using wavelength-resolved spectroscopy.

markers. It's also worth mentioning that both cell lines were fixed with formalin and embedded in paraffin (formalin-fixed and paraffin embedded or FFPE), which resembles the standard treatment of clinical tissue specimens. Using multispectral confocal microscope,[16] spectrally separated QD fluorescence is clearly visible in both cell lines (Fig. 2). Multi-spectral and confocal imaging requires acquisition of many consecutive spectral cubes and focal-plane images, respectively. The remarkable photostability of QDs allows extended exposure to excitation lights and consequently allows reconstruction of multicolor and high-resolution 3D projection. As revealed in Figure 2A, HER2 and EGFR were located on the cell membrane, mTOR was located in the cell cytoplasm, and both ER and PR were detected predominantly in the cell nuclei. These results demonstrate that QD-Ab bioconjugates can be used to detect proteins regardless of their cellular location. To further explore whether fluorescent signals from labeled proteins can be simultaneously quantified, we performed single-cell spectroscopy using a wavelength resolved single-stage spectrometer. Individual spectra of each labeled protein with peaks in the region of the QD emission maxima (525 nm, 565 nm, 605 nm, 655 nm, and 705 nm) were observed, representing the fluorescent emission of the QD-Abs (Fig. 2B and D). Due to spectral overlap, the fluorescence peak of tumor markers with low expression level could be affected by adjacent colors. This problem, however, could be solved using image-processing techniques, such as spectral deconvolution.[16] The fluorescence intensity patterns should provided a quantitative measurement of the expression level of each analyzed protein, allowing comparison and correlation with conventional proteomic methods.

For a detailed comparison of the QD molecular profiling technology and the standard techniques, we focused on three most important breast cancer biomarkers (ER, PR and HER2), which are routinely detected in surgical pathology laboratories and on which therapeutic decisions are made.[17] MCF-7, BT-474 and MDA-MB-231cells were stained with QD-ER, QD-PR and QD-HER2 and quantified with single-cell spectroscopy (Fig. 3A and B). For statistical significance, the fluorescent spectra of 100 cells in each cell line were measured and the mean levels of HER2, ER and PR expression were evaluated. It should be pointed out that without adjustment to the fluorescence intensities of individual colors, the relative fluorescence intensities of a biomarker cross different cell lines are more meaningful than the comparison of the fluorescence intensities of different biomarkers in one type of cells. This is because the differential optical and structural properties between different color QDs (such as quantum yield, molar extinction coefficient, and surface ligand density). For example, the red QDs could be significantly brighter than the green QDs even they have the same quantum yield because the fluorescence cross section of red QDs are larger than that of green QDs. In addition, different detectors used in different labs could also lead to discordance of experiment results. Therefore, an adjustment of the QD intensity according to their 'brightness' measured by a certain detector is needed when relative intensity is used to compare different markers in a cell line. For the 565 nm, 605 nm and 655 nm QDs used in this experiment, we found that respectively the 605 nm and 655 nm QDs are 4 times and 8 times brighter than the 565 nm QDs of the same concentration. When the individual colors were resolved using a method previously reported[18] and normalized against this 'brightness index' (peak of 565 nm was increased to 8 times its original value; peak of 605 nm was increase to 2 times its original value; and peak of 655 nm was not changed), the results obtained with quantitative spectrometry (Fig. 3B and C) correlate well with biomarker expression patterns obtained with traditional western blotting technique (Fig. 3D), which can probe only one marker at a time.

We also compared the protein expression results using QD-Abs with WB statistically. ER, PR and HER2 were measured in MCF-7, BT-474 and MDA-MB-231 cell lines and quantified using optical densitometry. To allow the comparison of results obtained by the two different methods (WB and QD), the absolute measurements of the triplet (HER2, ER and PR) were transformed to a relative scale in percent. Figure 3E shows the histogram of ER%, PR%, and HER2%,

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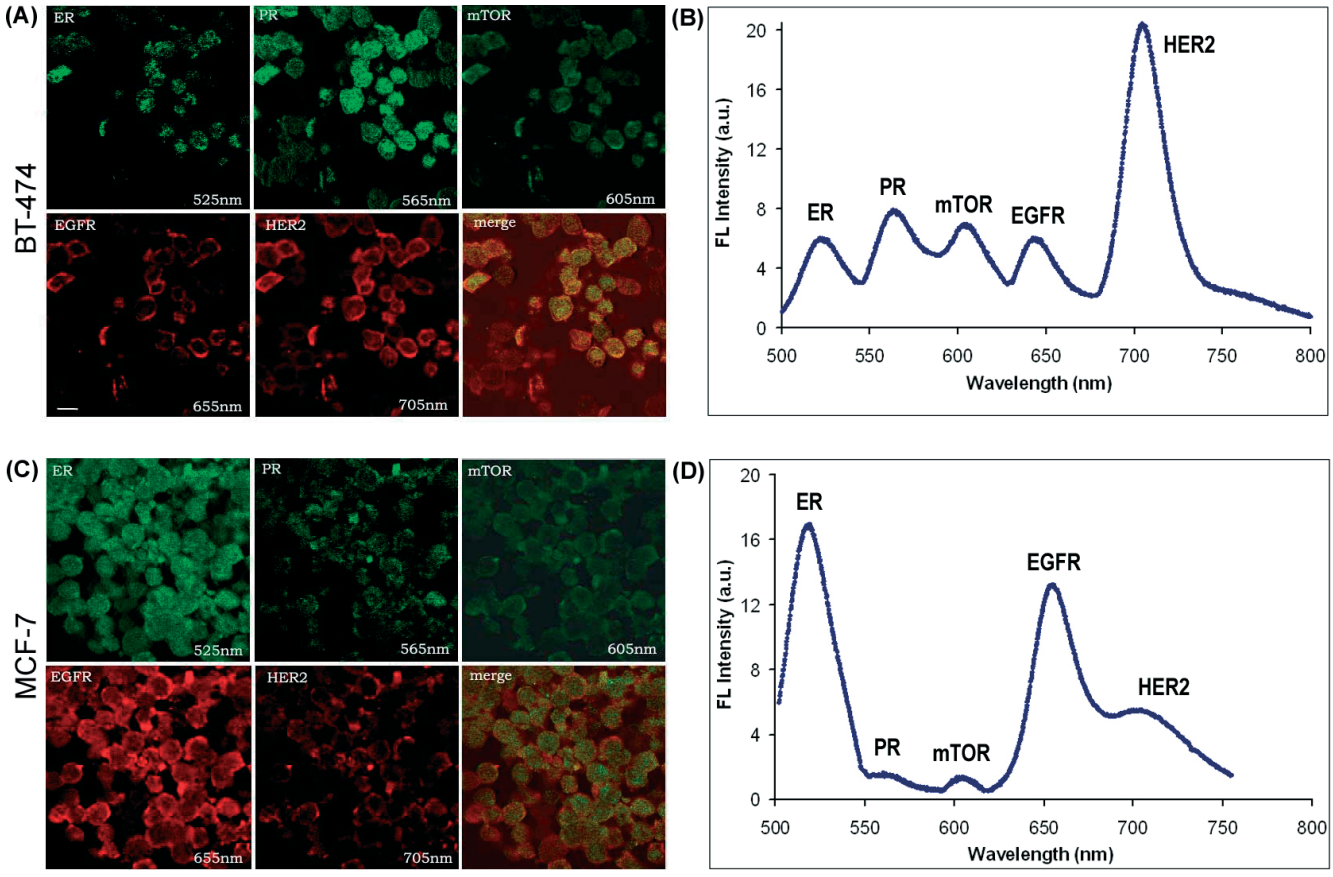

Figure 2. MCF-7 and BT-474 human breast cancer cells stained with QD-Abs against nuclear hormone receptors (ER and PR), cell membrane surface antigens (HER2 and EGFR) and cytoplasmic mTOR protein. (A) BT-474 cells showed positive labeling of membrane, cytoplasmic and nuclear antigens indicating expression of all five biomarkers; (B) spectra of QD emission of a representative BT-474 cell with emission peaks at 525 nm, 565 nm, 605 nm, 655 nm and 705 nm, confirming the differential expression levels of the tumor biomarkers (not necessarily the same as the average expression levels of a large cell population); (C) MCF-7 cells labeled with the same panel of QD-Ab bioconjugates. Spectral deconvolution reveals positive stains of ER, EGFR, and HER2; (D) spectra of QD emission of a representative MCF-7 cell with intensive fluorescent emission at 525 nm and 655 nm and low signals at 565 nm, 605 nm, and 705 nm (not necessarily the same as the average expression levels of a large cell population). Due to the nature of spectral deconvolution imaging, (A) and (C) are pseudo-color images. Scale bar, 10 μm.

comparing cell line types and methods. Note that biomarker expression levels determined using QD technology and WB for a given cell line tend to aggregate showing excellent correlation between the two methods.

Next, because IHC is also one of the standard methods for detecting cancer-related proteins, we compared the QD-immunostaining technology with traditional IHC using the same set of breast cancer cell lines. Slides from FFPE cell blocks were stained in accordance to standard pathological protocols for ER, PR and HER2 (Fig. 3F). Results of IHC were analyzed by two independent observers and scored with a standard scale from 0 to 3+. Cells with a 3+ score for ER, PR, or HER2 by IHC had 85 to 100 % relative expression of the proteins by QD-based spectroscopy, and cells with 1+ or 2+ score by IHC had 11 to 48% expression as determined by spectroscopic quantification. Interestingly, the use of conjugated QDs and quantitative spectroscopy may be more accurate at quantifying proteins present at low levels compared to IHC. For example, PR expression in MCF-7 cells was classified to 1+ by IHC, which corresponded to 16.8% estimated by the new QD molecular profiling technology; while BT-474 cells with 47.7% of ER expression detected by QDs was also classified to 1+ IHC score. In both cases, however, the expression of the proteins using QDs correlated well with the WB results.

To test whether the QD-based technology correlates with the gene transcription activity of the target proteins, we compared the level of HER2 expression detected by FISH with its protein expression detected by QD-Abs profiling (Fig. 3F). We found a qualitative correlation between the HER2 gene amplification and HER2 protein expression detected using QDs. The amplification ratio of HER2 gene on chromosome 17 centromere in BT-474 cells was 7.0 that corresponded to 100% HER2 expression determined by QD staining. In contrast, 2.5% of HER2 protein expression estimated by QDs in MDA-MB-231 cells corresponds to amplification ratio of 1.31 in FISH studies, which is within the non-amplified range (values > 2.0 were considered amplified). Interestingly, the

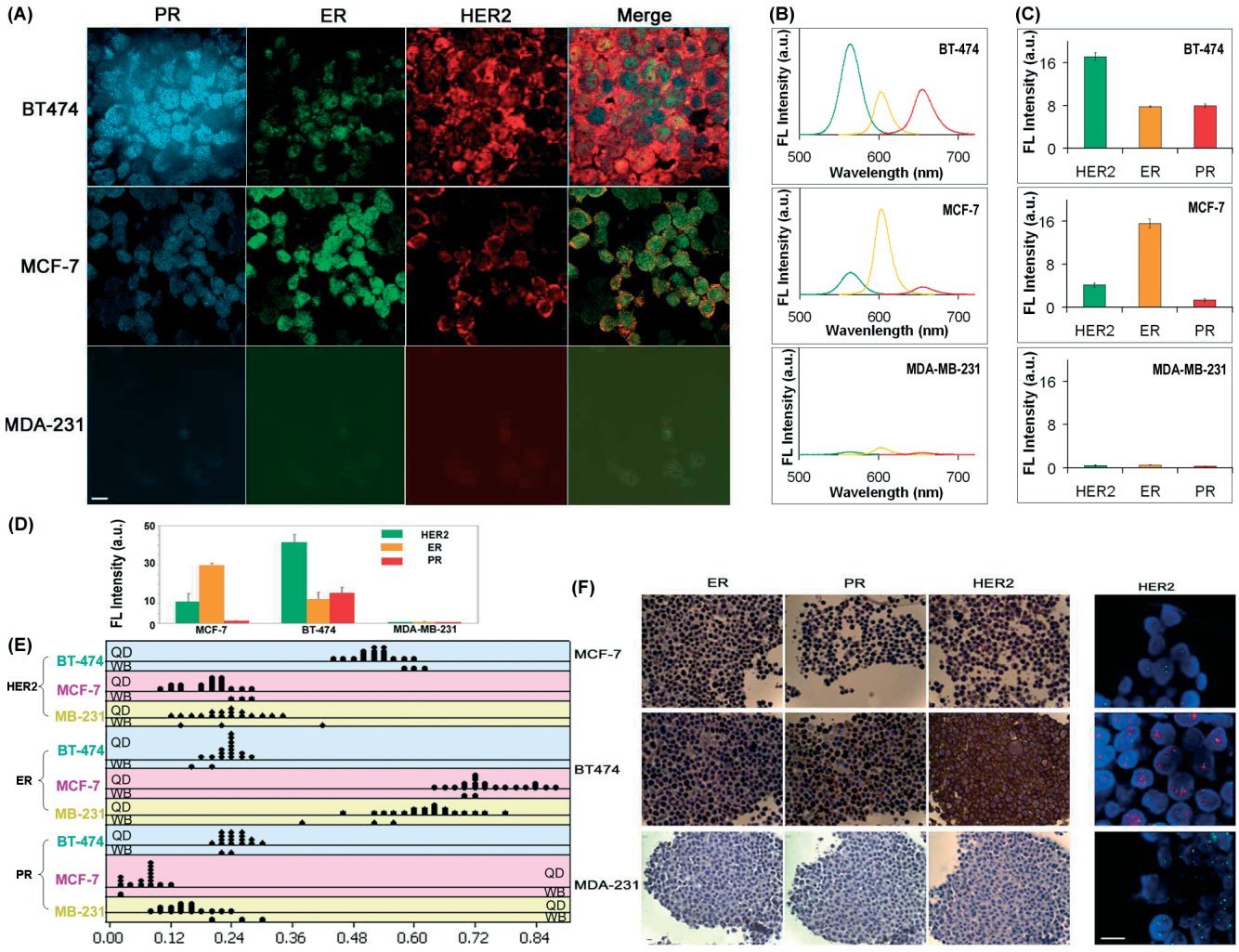

Figure 3. Comparison of quantitative detection of HER2, ER and PR by QD-Abs with conventional techniques. (A) Expression of ER, PR and HER2 signals in MCF-7, BT-474 and MDA-MB-231 breast cancer cells detected by fluorescence microscopy (pseudo-color); (B) Representative spectra of single cell spectroscopy measurement of the protein biomarkers. Individual colors were resolved using a previously described method [18]. The 565-nm and 605-nm peaks were enhanced to 8 times and 2 times of their original values to compensate the differential brightness of multicolor QDs; (C) average expression levels of HER2, ER and PR obtained from the results of spectral analysis of 100 single cells; (D) ER, PR, and HER2 expression measured with WB in MCF-7, BT-474 and MDA-MB-231 cells; (E) Comparative statistical analysis of QD-Abs profiling data with western blotting results. This multivariate analysis showed a good level of agreement between the two techniques; (F) Expression of ER, PR and HER2 estimated by IHC in the breast cancer cell lines (left panels) and amplification of the HER2 gene in breast cancer cell lines assessed by FISH. Scale bar 10 μm.

amplification ratio of the HER2 gene in MCF-7 cells also showed a negative result (1.3 amplification ratio), but the HER2 protein expression assessed by QDs was found to be at the level of 24.4%. As noted above, IHC and WB confirmed the expression of HER2 in MCF-7 cells. This suggests that the use of conjugated QDs can detect low levels of HER2 protein expression, although the clinical relevance of this finding deserves further investigation.

These systematic comparisons using cell lines indicate that the new QD-based molecular profiling technology is capable of imaging and quantifying multiple tumor biomarkers in intact tumor specimens. We, therefore, seek to apply this technology to clinical FFPE breast tumor specimens. Because the long-term in vivo toxicity effect of CdSe/ZnS QDs is largely unknown presently, molecular profiling of tumor specimens could become the first clinical applications of QD bioconjugates. This is particularly important to cancer prognosis and treatment given the fact that many cancers are being diagnosed at smaller sizes. The ability to detect multiple target proteins on small pieces of cancer tissues could offer more effective therapeutic decisions. Besides the multiplexing capability, QDs also possess other unique optical properties that render them the better fluorophores for molecular profiling on tissues.[19] First, QDs are brighter fluorescence probes than organic fluorophores, which could enhance the detection of low-abundance targets. Second, QDs are several thousand times more stable against photobleaching than dye molecules, and are thus well suited for quantitative imaging and spectros-

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copy. An interesting application of this optical property for the reduction of high autofluorescence background in FFPE tissue samples is to illuminate the QD-stained specimens over extended period of time before recording images and spectra (typically ranging from seconds to minutes depending on the intensity of light source). By doing this, the autofluorescence could be reduced (photobleached in a similar way to organic fluorophores), but the QD fluorescence should remain constant. Note that this technique is not available to organic fluorophores, because they are not nearly as photostable as QDs. Along the same line, the autofluorescence could be further reduced by using QDs with large Stokes shifts (measured by the distance between the excitation and emission peaks). The Stokes shifts of semiconductor QD fluorescence can be as large as 300–400 nm, depending on the wavelength of the excitation light; whereas that of organic dye fluorescence and autofluorescence is generally around 20–50 nm.

Because of these unique optical properties, we proceeded to use QD-Abs for detection of ER, PR, mTOR, EGFR and HER2 in FFPE human breast cancer specimens. Using laser scanning microscopy we were able to detect the fluorescent signals, corresponding to the five expressed proteins (Fig. 4). Only ER and PR were visualized by confocal microscopy in tumor biopsy 1, which corresponded to the two peaks detected on the spectrogram (top panels). Spectroscopic measurement of biopsy 2 demonstrated expression of mTOR and EGFR, which was confirmed by confocal microscopy (middle panels). Finally, biopsy 3 expressed variable amounts of ER, PR, mTOR, and HER2, which were detected by both spectroscopy and confocal microscopy (bottom panels).

Similar to the technology validation experiments performed on cells, we also selected ER, PR and HER2 for the comparison of QD technology with IHC in two additional breast tumors with differential expression of the target proteins. The IHC values for the three tumor markers are known (HER2 3+; ER 3+; PR 3+ and HER2 –; ER 2+; PR 2+). For QD fluorescence, we were not able to measure the fluorescence from individual cells because they are connected to each other (unlike the spatially separated cultured cells). Therefore, the QD emission from the tissue samples were obtained by measuring 40 randomly selected spots in the tissue sections, for better representation of the protein expression levels of the tumor specimens. Quantification of QD-Ab labeling demonstrated definitive differences in HER2 and ER expression between the 2 tumors. The first tumor expressed a large amount of HER2 (245.7±57.1) and ER (164.6±36.9), there was very little expression of HER2 (14.3±5.1) and a significantly lower expression of ER in the second tumor (99.9±22.7). When compared with the IHC results using relative scales (the highest expression level for each biomarker was set as unity), the average values of the biomarker expression measured with QDs showed an excellent correlation.

In summary, we have demonstrated the use of QDs for multiplexed detection of five tumor biomarkers in both cultured human breast cancer cells and on single paraffin embedded clinical tissue sections. Simultaneous quantification of ER, PR, and Her2 receptors correlated closely with the results from traditional methods including IHC, western blotting and FISH, suggesting that the QD-based technology are well suited for molecular profiling of tumor biomarkers in vitro, which could become the first translational application of QDs. Despite these encouraging results we also note that further improvements are needed before this novel technology will receive widespread adaptation in cancer diagnosis and prognosis.[20] For example, the technology can be further improved from its present state by (i) more compact QD probes for

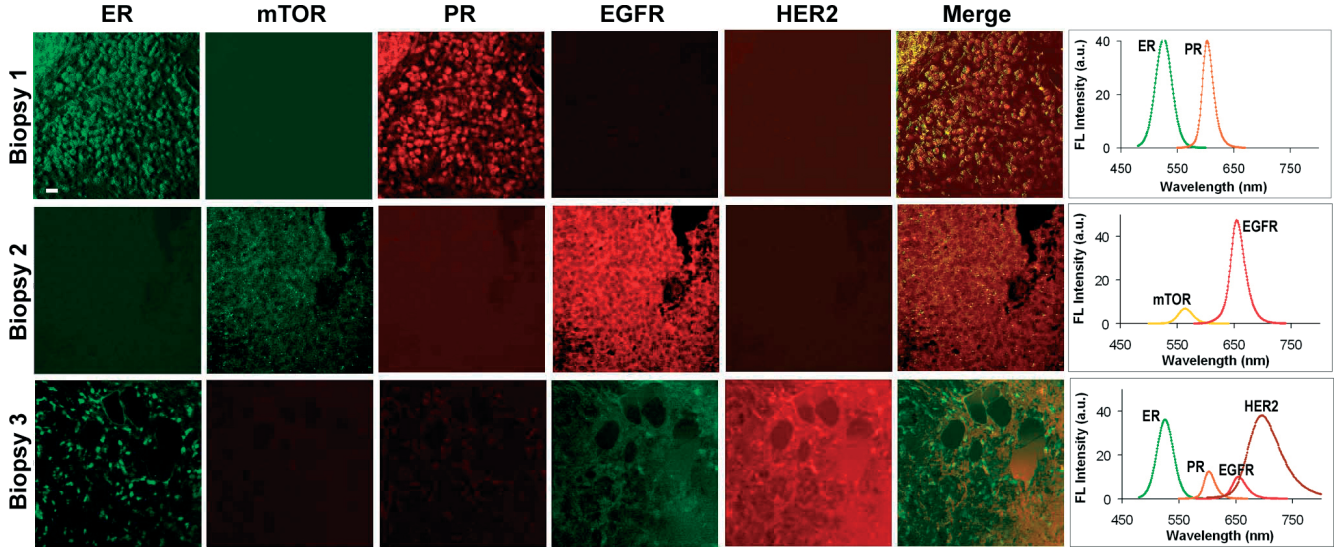

Figure 4. Multiplexed molecular profiling of FFPE tissue sections. Different patterns of nuclear, cytoplasmic and cell membrane fluorescent signals were detected by microscopy (left panels, pseudo-color) and expression of these biomarkers was quantified by wavelength-resolved spectroscopy (right panels). Again, the emission spectra were resolved into individual channels and compensated for the differential brightness between different colors. Scale bar, 20 μm.

Table 1. Quantitative profiling of human breast tumors by QD-Abs.

	HER2			ER			PR		
	QD		IHC	QD		IHC	QD		IHC
	a.u.	%†	%	a.u.	%	%	a.u.	%	%
Sample 1	245.7±57.1	96.0	90–100	164.6±36.9	64.3	70–90	255.1±63.1	100	90–100
Sample 2	14.3±5.1	5.3	0	99.9±22.7	37.9	30–50	261.2±82.6	100	100

deeper tissue penetration; (ii) better conjugation chemistry for well-controlled bio-ligand orientation and the number of bio-ligands per nanoparticle (directly affect the stoichiometry between targets and probes); (iii) inclusion of housekeeping markers for standardization; and (iv) large-scale clinical testing. With these improvements, we envision that the QD-based molecular profiling technology could offer a new avenue in determining the molecular fingerprints of individual cancers, on which effective treatment decisions can be made.

Experimental

QD-Abs Conjugation: Antibodies targeting Her2, ER, PR, mTOR and EGFR (all from US Biological, Swampscott, MA) were conjugated to the QDs using 525 nm, 565 nm, 605 nm, 655 nm, and 705 nm Qdot Antibody Conjugation Kits (Invitrogen, Carlsbad, CA) respectively, using the procedure provided by the manufacturer. Briefly, BSA free antibodies (300 μL) at a concentration of ~1 mg mL^{-1} were reduced with 20 mM of DTT to expose free sulfhydryls and purified by size exclusion chromatography. At the same time, QDs were activated using the hetero-bifunctional crosslinker, 4-(maleimidomethyl)-1-cyclohexancarboxylic acid N-hydroxysuccinimide ester (SMCC). The activated QDs were mixed with the reduced Abs for 1 h. The reaction was quenched by adding 100 μM of mercaptoethanol. The bioconjugates were concentrated using ultrafiltration and purified using size exclusion chromatography.

Labeling of Breast Cancer Cells: Breast cancer cell lines (MCF-7, BT-474 and MDA-MB-231) were obtained from American Type Culture Collection (ATCC, Manassas, VA). For multiplexed staining using QDs, FFPE cell blocks were deparaffinized by incubation with xylene and series of alcohol dilutions followed by 30-min incubation in humidified chamber at 37 °C with 1% of Triton-X. For antigen retrieval, cells were washed with phosphate buffered saline (PBS) and incubated with 1 mM EDTA at pH 8.0 for 40 min at 95 °C, and were cooled down to room temperature. After blocking with 2% goat and 5% bovine serum diluted in PBS for 1hour , cells were stained with a mixture of conjugates targeted for ER, PR, HER2, mTOR, EGFR (40nM each) for 1 hour and washed 5 times in PBS. Stained cells were mounted on slides in Aqueous Mounting Medium (Biomeda, Foster City, CA) with a coverslip.

Labeling of Clinical Tumor Samples: Human breast tumor specimens were kindly provided by Drs. T. Styblo and B. Senquer (Department of Surgery). For multiple QD-Abs profiling, FFPE breast tumor sections were cut into 5 μm thin sections and stained using the same protocol as the cell blocks described above.

Laser-Scanning Confocal Microscopy. Images of QD-conjugated Abs were acquired using a Zeiss LSM 510 META confocal microscope (Thornwood, NY) mounted on an Axioplan 2 microscope. To simultaneously visualize multicolor QDs, the QDs were excited with a laser line at 405 nm. Their fluorescence emission was separated using the META detector, which allows for spectral discrimination of overlapping emission signals. To do this, samples containing single QD-Abs for every color were used to generate a lambda stack. This spectral library was stored and then used as references to digitally separate the spectral components for each pixel of the fluorescence micrograph.

Quantitative Spectrometry. Spectroscopic measurements of cells and tissues were performed with modified procedures reported previously.[13] Briefly, broad-band excitation in the UV or blue spectral region was provided by a 100 W mercury lamp. A longpass dichroic filter (Chroma Technologies, Brattleboro, VT) was used to reject the scattered light and to pass the Stokes-shifted fluorescence signals. Single-cell spectroscopy was accomplished by using the fluorescence microscope equipped with a pinhole, a single-stage spectrograph (SpectroPro 150, Roper Scientific, Duluth, GA) and a thermoelectrically cooled charge-coupled device (CCD) detector. For measurement of biomarker expression in cultured cells, spatially-separated individual cells were manually positioned in the 'hot-spot' defined by the position and size of the pinhole. For determination of the biomarker expression in tissue specimens, QDs spectra from 40 randomly selected areas were measured. Data from the spectroscopy measurements was converted to ASCII format for further quantification and statistical analysis.

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