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ORIGINAL PAPER

Accurate Sensitivity of Quantum Dots for Detection of HER2 Expression in Breast Cancer Cells and Tissues

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Abstract Here we introduce novel optical properties and accurate sensitivity of Quantum dot (QD)-based detection system for tracking the breast cancer marker, HER2. QD525 was used to detect HER2 using home-made HER2-specific monoclonal antibodies in fixed and living $HER2^{+}$ SKBR-3 cell line and breast cancer tissues. Additionally, we compared fluorescence intensity (FI), photostability and staining index (SI) of QD525 signals at different exposure times and two excitation wavelengths with those of the conventional organic dye, FITC. Labeling signals of QD525 in both fixed and living breast cancer cells and tissue preparations were found to be significantly higher than those of FITC at 460–495 nm excitation wavelengths. Interestingly, when excited at 330–385 nm, the superiority of QD525 was more highlighted with at least 4–5 fold higher FI and SI compared to FITC. Moreover, QDs exhibited exceptional photostability during continuous illumination of cancerous cells and tissues, while FITC signal faded very quickly. QDs can be used as sensitive reporters for in situ detection of tumor markers which in turn could be viewed as a novel approach for early detection of cancers. To take comprehensive advantage of QDs, it is necessary that their optimal excitation wavelength is employed.

Keywords Quantum dots · FITC · Breast cancer · Tumor marker · HER2 · Sensitivity · Photostability · Staining index · Excitation wavelength

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Introduction

The fluorescent labeling of biomolecules using small-molecule organic dyes is widely employed in biological imaging and clinical diagnosis. Organic fluorophores, however, have certain characteristics that limit their advantages in some applications, among them are narrow range of excitation wavelengths and broad emission bands, which make the simultaneous detection of several light-emitting probes difficult due to spectral overlap. Also, many organic dyes exhibit very low photostability [1]. Seeking for light-emitting probes with excellent optical properties, Quantum dot (QD) nanoparticles recently emerged as promising alternatives to conventional fluorophores in fluorescent-imaging applications. QDs are typically formed from a cadmium selenide (CdSe) core and a zinc sulfide (ZnS) shell and generally scale in the range of 2–10 nm [2].

QDs have several superiorities over conventional organic dyes that potentially make them extremely popular alternative for fluorescent-based applications. At first, their narrow and symmetrical emission spectra allow detection of multiple biomarkers with less spectral overlap [3, 4]. Tunable emission spectrum by adjusting the particle size and material composition is an added benefit allowing availability of a panel of rainbow colors [3]. Second, they have broad absorption spectra and exhibit excellent photostability allowing a single light source to be used for multi color excitation. Third, they show higher luminescence and quantum yield than conventional fluorophores under appropriate conditions due to their higher extinction coefficient [5, 6].

These features facilitate the imaging of individual QDs with high signal to noise ratio, even with standard epifluorescent microscopes [7].

Breast cancer is the most-common malignancy of the females worldwide with 1050000 new cases and 372000 deaths annually. Although, incidence rates are higher in more developed countries, accumulative evidence shows steadily increasing rates in less developed countries [8].

HER2 (Human epidermal growth factor receptor-2) also known as c-erbB-2 or HER2/neu is a breast cancer-associated tumor marker which is overexpressed in approximately 20–25 % of breast cancer patients [9, 10]. It is not only widely used as a useful diagnostic tumor marker in the detection but is a specific target of immunotherapy [11, 12]. HER2 expression is also associated with poor prognosis [13, 14] and determines the course of treatment.

As with most cancers, the early detection of breast cancer through biomarkers would have a significant impact on reducing its mortality. Based on their very high sensitive nature, use of QDs could be a promising approach in this regard. It has been reported that QDs aid precise high-throughput determination of protein distribution with both light and electron microscopy [15]. It has also been that quantum dot-conjugated antibodies can be used to study specific sub-populations of breast cancer cells defined by multiple markers in a single tissue section [16]. Here we address the higher accurate sensitivity of QD-525 in comparison to emission wavelength-matched conventional fluorophore, FITC, for detection of breast cancer biomarker, HER2 in breast cancer tissues and cell line and conclude that this capability could be potentially employed for early detection of cancer.

Materials and Methods

Cell Culture

Human breast cancer cell line, SKBR-3, was ordered from American Type Culture Collection (ATCC). We cultured the cells in complete RPMI-1640 supplemented with 10 % (v/v) fetal calf serum (FCS) (Gibco) and additives at 37 °C and 5 % CO2.

Tissue Preparation

We obtained paraffin blocks of breast cancer tissues from pathology department of Shahid Beheshti University of Medical Sciences, prepared three μm sections and tested them for expression of HER2 according to the protocol we published recently [17]. We selected HER2 positive tissues and subjected them to immunofluorescent staining by FITC- and QD525-conjugated probes (see below).

Production of Monoclonal Antibody Against HER2

We selected two peptides from extracellular domain of HER2 and produced monoclonal antibodies (mAbs) against them according to the protocol we published elsewhere [18]. Briefly, we conjugated the peptides with KLH, emulsified in Freund's adjuvant (Sigma) and injected the conjugates intraperitoneally to female Balb/c mice. After completion of immunization schedule, we selected mice with higher titers of specific antibodies for fusion. At the next step, we produced hybridomas by fusion of spleen cells with SP2/0 cell line and screened culture supernatants of hybridoma clones by ELISA against peptides and subcloned the positive clones by limiting dilution. We purified antibodies produced by final clones over affinity columns and confirmed their reactivity by different assays including ELISA, western blot (WB) and immunoprecipitation (IP).

Antibody Biotinylation

We dialyzed rabbit mouse Ig-specific antibody (Avicenna Research Institute) against several changes of 0.1 M sodium carbonate buffer, pH 9.5 at 4 °C. After dialysis, we determined antibody concentration by UV spectrophotometry and adjusted it to 2 mg/ml. Meanwhile, we prepared solution of NHS-

biotin (Sigma) in DMSO at a final concentration of 2.2 mg/ml and added it with gentle stirring to the antibody solution at 1:10 ratio (v/v). Reaction was continued at room temperature for 4 h. Then, we dialyzed antibody-biotin conjugate against several changes of PBS buffer, pH 7.4 at 4 °C. Biotinylation of antibody was confirmed by both western blotting using streptavidin-HRP as detector (Biosource) and ELISA.

Labeling of Streptavidin with FITC

For FITC conjugation, was dialyzed 1 mg of streptavidin solution (Invitrogen) against 0.1 M sodium carbonate buffer, pH 9 overnight at 4 °C and adjusted the final concentration to 1 mg/ml. Immediately before use, we dissolved FITC (Sigma) in DMSO at a concentration of 1 mg/ml and added 125 μl of the FITC solution to the streptavidin slowly with stirring at room temperature. Incubation was continued at 4 °C for 2 h in the dark. We removed excess fluorophore by gel filtration in Sephadex G-25 equilibrated with PBS. At the next step, we collected the colored fractions and pooled. The ratio of streptavidin to FITC was 1:5 as determined by measuring the absorbance.

Conjugation of Streptavidin with QD525

For coupling of QD525 to streptavidin, we transferred two hundred and fifty μl of 2 μM QD525® ITK Amino (PEG) (Invitrogen) into the 100 KD ultrafiltration unit (Millipore) and washed it several times with phosphate buffered-saline (PBS), pH 7.4. We then transferred QD525 to siliconized eppendorf tube and admixed them with BS3 linker (Thermo Scientific) in a final ratio of 8–10 μM QD/1 mM BS3. Reaction was continued over mixer for 2 h at room temperature. After equilibration of a gel filtration Nap5 column (GE Healthcare) with five runs of PBS exchange, we purified the QDs from excess cross-linker over it and collected and colored the eluate into a siliconized eppendorf tube containing a 40-fold excess of streptavidin (10–12 mg/mL stock). The tube content was mixed gently and the reaction continued at room temperature for 2 h. Then, we quenched the reaction with 1 M glycine by adding glycine to a final concentration of approximately 50 mM for 15 min. At the final step, we purified the conjugate from excess streptavidin by ultrafiltration unit (100kD) into 50 mM borate, pH 8.3. This typically took 5 or 6 rounds of buffer exchange. Purified QD conjugate was wrapped carefully and stored at 4 °C. To confirm correct labeling of streptavidin with QD525, we used two approaches. First, electrophoresis pattern of conjugate was investigated by SDS-PAGE. The results showed that streptavidin-QD525 conjugate always stood on the interface between stacking and separating gels confirming proper conjugation. Second, as shown below, we evaluated the reactivity of this conjugate in immunofluorescent staining.

Immunolocalization of HER2 on Fixed Breast Cancer Cell Line (SKBR-3)

We harvested SKBR-3 cells and attached them to poly L lysine-coated slides after cytospin centrifugation. After drying for 10 min, we fixed the cells with 4 % (v/v) formaldehyde for 10 min at room temperature and washed the slides immediately with Tris-buffered saline (TBS) containing 1 % (w/v) bovine serum albumin (BSA) (TBS-BSA). At the next step, we permeablized the cells with 0.05 % (v/v) Triton X-100 in TBS for 20 min, washed the slides as above and blocked non-specific binding sites for 20 min with TBS containing 5 % (v/v) rabbit serum and 2 % (w/v) BSA. We then incubated the cells with 5 μg/ml HER2-specific mAb, clone 3E3 for 2 h at room temperature. After washing, incubation was continued with 5 μg/ml biotin-conjugated rabbit antibody against mouse Ig for 1 h. Then, we washed the cells and incubated them with optimal dilutions of streptavidin-QD525 or streptavidin- FITC for 30 min. After washing three times with TBS-BSA, we mounted the slides with PBS-glycerol and imaged cells under a fluorescent microscope (Olympus BX51).

Live Imaging of $HER2^+$ Breast Cancer Cell Line (SKBR-3)

To label HER2 on living cells, we harvested cultured live SKBR-3 cells in logarithmic phase, washed with cold PBS and incubated them sequentially with 5 μg/ml HER2-specific monoclonal antibody; clone 3E3 for 45 min, 5 μg/ml biotin-conjugated rabbit antibody to mouse Ig for 30 min, and optimally diluted QD-streptavidin or FITC-streptavidin for 30 min. Between all steps, we washed the cells 2 times with cold HANK'S balanced salt solution (HBSS). All antibodies and conjugates were diluted in HBSS and all steps were carried out at 4 °C. At the next step, we suspended the labeled cells in PBS and placed a drop of cell suspension on a glass slide, covered the cells with cover slip and imaged slides immediately under an upright fluorescence microscope. Negative controls were processed as mentioned above.

Immunofluorescent Staining of Breast Cancer Tissues

Sections of formalin-fixed paraffin-embedded (FFPE) breast cancer tissues were prepared, deparaffinized, hydrated and subjected to antigen retrieval in citrate buffer (10 mM, pH 6) at 98 °C for 30 min. Following three washes with TBS, we blocked endogenous biotin with biotin blocking system (Dako) according to manufacturer's instruction. Then, we washed the sections three times with TBS-BSA and blocked non-specific binding sites for 15 min with 5 % (v/v) normal rabbit serum. At the next step, we incubated the slides for 90 min with HER2-specific mAb clone 3E3 at a concentration of 2.5 μg/ml. After washing with TBS-BSA, we added biotin-

labeled rabbit antibody to mouse Ig at a concentration of 2.5 μg/ml to the slides for 45 min, washed them three times with TBS-BSA and incubated the sections for 30 min with optimum dilutions of FITC- or QD525-conjugated streptavidin. The slides were then mounted and imaged.

Image Acquisition and Fluorescent Intensity Analysis

We captured all images by DP70 CCD camera (Olympus). Two sets of excitation and emission filters (U-MWU2; ex: 330–385 nm, em: 420 nm, Dicromatic filter: 400 nm and U-MWIB3; ex: 460–495 nm, em: 510 nm, Dicromatic filter: 505 nm) were used for QD525, while the signal of FITC was tracked with its nominal excitation filter and emission filter sets, U-MWIB3; ex: 460–495 nm, em: 510 nm, Dicromatic filter: 505 nm. In negative reagent controls, we substituted primaries with equivalent concentrations of mouse immunoglobulin.

To compare the quantum yield of QD525 and FITC, we illuminated stained cells or tissues with aforesaid excitation filter sets in different exposure times. For each filter set and exposure time, we captured multiple images and measured the fluorescent intensity of green light by OLYSIA (Olympus) software. For each probe in each excitation filter set and exposure time, we measured and compared the intensity of at least 100 individual cells. Because QD525 and DAPI have the same optimal wavelength range of excitation, we excited double stainings with QD525 and DAPI with 330–385 nm filter and imaged them simultaneously except when QD525 was excited with 460–495 nm filter in which we separated DAPI channel from above images with channel tools of Imagej software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2011) and merged the DAPI images with QD525 images using color function and color merge plugin of this software. In the case of double staining with FITC and DAPI, images were merged as above.

Comparison of Staining Indices (SI) Between QD525 and FITC

We measured fluorescence intensity of FITC- and QD525-labled SKBR-3 cells (positive FI) in conjunction with their negative controls (background FI) as above and calculated SI of each probe with the following equation:

$$\textit{Staining Index} = (\text{Mean positive FI} - \text{Mean background FI}) \div (2 \times \text{SD of background FI})$$

Photostability Comparison Between QD525 and FITC

To compare the photostability of QD525 and FITC, we continuously illuminated immunostained SKBR-3 cells and breast cancer tissues for 15 min. We then captured images at 1 min intervals and analyzed mean fluorescent intensity of each cell with OLYSIA software.

Statistical Analysis

Based on non parametric distribution of data, we used Mann Whitney test with α set at .05 for comparison of fluorescent intensity of FITC- and QD525-labeled cells. Differences at P values <0.05 were considered significant.

Results

Production of HER2-specific Monoclonal Antibodies

For detection of HER2 overexpression, we produced monoclonal antibodies against two peptides from extracellular domain of HER2 and evaluated their reactivity by different immunological assays [18]. We produced a total of 5 hybridoma clones from two separate fusions, and antibody isotyping revealed that all clones were of IgM isotype. These mAbs showed excellent reactivity in WB and IP by detecting the 185 KD band of HER2. Here we describe the QD-based application of one of these clones, namely 3E3, in immunohistochemistry and immunofluorescence for detection of HER2 in situ.

Detection of HER2 with QD525- and FITC-labeled Probes on Fixed Breast Cancer Cell Line, SKBR-3

To compare the staining power of QD525 and FITC which have the same emission wavelengths, we labeled HER2 positive breast cancer cell line, SKBR-3 with HER2-specific monoclonal antibody, 3E3, and tracked the signal by labeled-streptavidin biotin (LSAB) method using streptavidin-conjugates of both fluorophores. The results clearly showed that both probes were able to specifically detect expression of HER2 on SKBR-3 cells, but the staining pattern differed in some respects. When illuminated with 488 nm excitation filter, which is maximum excitation wavelength for FITC, QD525 was superior to FITC in terms of brightness (Fig. 1). Indeed, QD525 showed less background compared to FITC, as in QD525 staining fluorescent signal was, to large extend, confined to the cell membrane, whereas FITC images had non-specific signals in cytoplasm. We repeated the experiment with indirect immunostaining method using QD525- and FITC-conjugated mouse Ig-specific antibodies (produced in this project) and the same results were achieved (Data not shown). When we excited QD525 with wavelengths under 400 nm the optimal illumination wavelength of quantum dots, the fluorescent intensity, as described below, increased dramatically (Fig. 1).

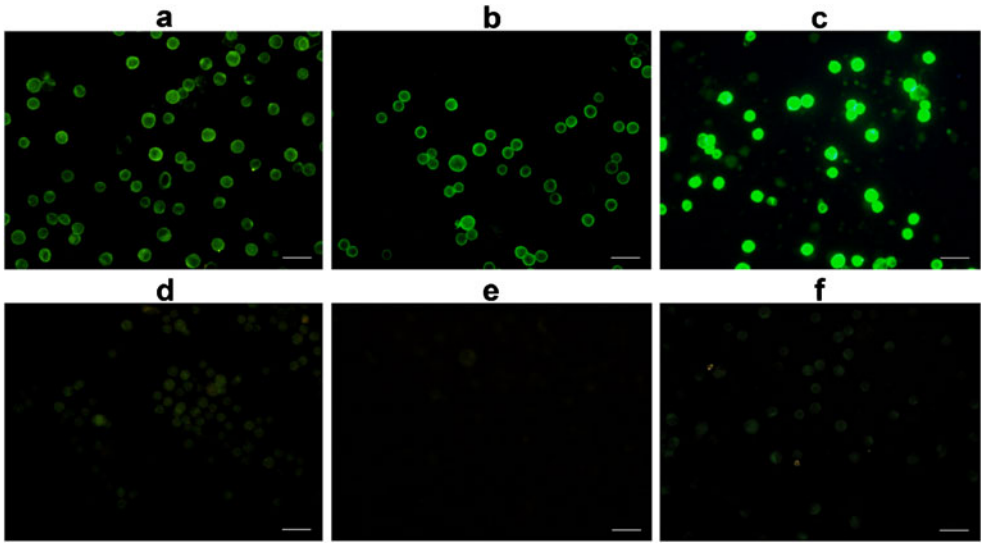

Fig. 1 Tracking of HER2 expression on SKBR-3 cell line using FITC- and QD525-based probes. Expression of HER2 on SKBR-3 cells was traced with LSAB method using HER2-specific monoclonal antibody and FITC- or QD525-labled streptavidin. FITC-stained cells were illuminated with 460–495 nm filter (**a**), while QD525 was excited with both 460–495 nm (**b**) and 330–385 nm filters (**c**). **d**–**f**: Negative controls of cells tracked with FITC, QD525 (ex: 460–495 nm) and QD525 (ex: 330–385 nm), respectively. Scale bar: 20 μm

Live Imaging of HER2 with QD525 and FITC

Live imaging is a measure of capacity of a system for in situ monitoring of living cells. In antibody-based systems, not only is it affected by sensitivity, but it reflects the ability of an antibody to bind cell surface markers. In order to test the binding capacity of our anti-HER2 mAb, 3E3, to cell surface HER2 and to compare the signal strengths of FITC- and QD-sterptavidin conjugates, we performed live imaging of SKBR-3 cell line. Results of such experiment clearly showed that 3E3 was able to bind extracellular domain of HER2 on living SKBR-3 cells. In line with immunofluorescent staining of fixed cells, fluorescent signal emitted by QD525 was considerably higher compared to FITC (Fig. 2).

Immunofluorescent Staining of Breast Cancer Tissues

We immunostained FFPE sections of breast cancer tissues with HER2-specific mAb, 3E3, and tracked the fluorescent signal using FITC- and QD525-labled probes. Staining pattern of positive cells in breast cancer tissues in terms of intensity and background for FITC and QD525 was similar to that of FITC- or QD525-labeled breast cancer cell line. Cancerous cells exhibited stronger fluorescent signal when illuminated under 488 nm and traced with QD525 compared to FITC. Using the optimum excitation wavelength for each fluorophore, the superiority of QD525 to FITC in terms of FI was more highlighted (Fig. 3) (see below).

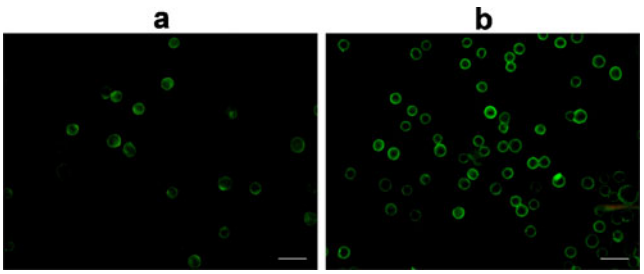

Fig. 2 Live immunofluorescent staining of HER2 on SKBR-3 cell line using FITC- and QD525-labeled probes. SKBR-3 cells were labeled with HER2-specific monoclonal antibody. Fluorescent signal was evaluated after staining the cells with LSAB method using FITC- (**a**) or QD525-labled streptavidin (**b**). Scale bar: 20 μm

Comparison of Fluorescent Intensities Between QD525 and FITC

The minimal detection limit of any immunofluorescent-based technique is reflected by fluorescent intensity of detection system. Thus we compared the fluorescent intensity of QD525 and FITC in immunofluorescent staining of breast cancer marker, HER2 in both breast cancer cell line and tissue. This factor was quantified and compared in different exposure times for both fluorophores. The results showed that when both fluorophores were excited at 488 nm, the average FI of QD525 at 1:4 sec exposure time is significantly higher compared to that of FITC ($P<0.0001$) (Fig. 4a). This comparison at other exposure times (1:2.8 and 1:8 sec) yielded the same results (Fig. 4b for 1:2.8 sec). Because the maximum excitation wavelength for QD525 is under 400 nm, its FI was measured at 360 nm and compared with that at 488 nm. The results showed that the average FI of QD525 is at least four time higher when excited at 360 nm compared to 488 nm ($P<0.0001$) (Fig. 4c)

When we excited QD525 and FITC at their optimal excitation wavelengths (360 nm and 488 nm, respectively), QD525 exhibited very high FI that was more than four times stronger than that of FITC. ($P<0.0001$) (Fig. 4d). At

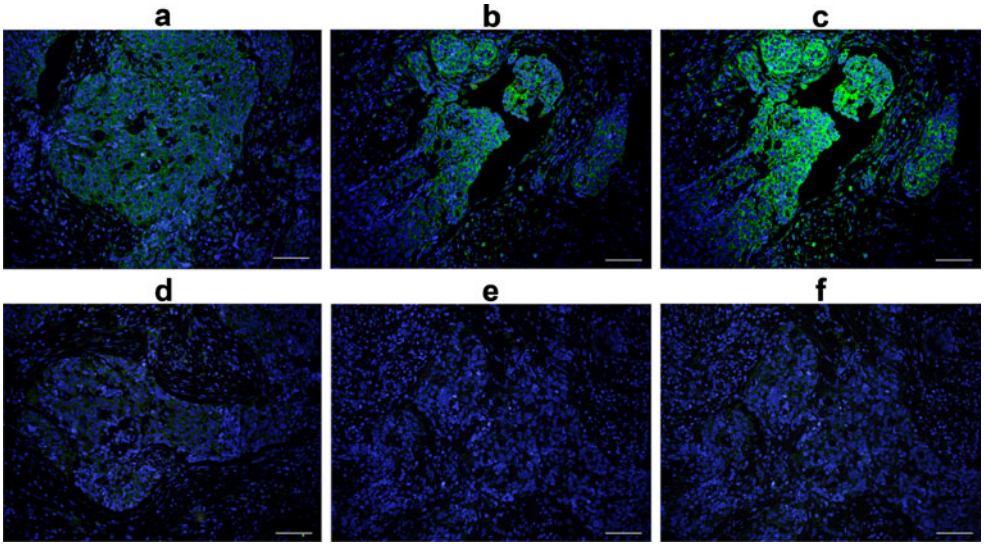

Fig. 3 Immunofluorescent staining of breast cancer tissue using FITC- and QD525-based probes. Paraffin-embedded formalin fixed breast cancer tissues were cut in to 5 μm sections and stained with HER2-specific monoclonal antibody and FITC- or QD525-based probes. FITC-stained cells were illuminated with 460-495 nm filter (**a**), while QD525 was excited with both 460–495 nm (**b**) and 330–385 nm filters (**c**). **d**–**f**: Negative controls of tissues tracked with FITC, QD525 (ex: 460–495 nm) and QD525 (ex: 330–385 nm), respectively. Scale bar: 50 μm

1:60 sec exposure, we observed no signal from FITC, while QD525 fluoresced brilliantly. (Fig. 4e)

Comparison of Staining Indices of QD525 and FITC

Staining index (SI) is a measure of how well a signal is distinguished from background. SI takes into account the spread of the negative population and describes positive and negative population separation. We excited QD525- or FITC-labeled SKBR-3 cells at their optimal excitation wavelengths, 365 nm and 488 nm, respectively, and calculated their SI. SI comparison demonstrated the superior signal separation capacity of the QD525, where QD525 had an SI of about 5 times greater than that of FITC.

Photostability of Quantum Dots

Photostability is a very useful characteristic of a fluorescent probe allowing more time a microscopic field of a sample is illuminated constantly without significant loss of fluorescent intensity. Time course of images for QD525- and FITC-labeled SKBR-3 cell line and breast cancer tissue under continuous illumination at 488 nm for 15 minutes (Fig. 5a) showed that the FITC signals faded quickly and reached to the level of negative control signal after about 5 min, whereas QD525 signals showed no tangible change during the entire 15 min illumination period. The results of fluorescent intensity calculation clearly showed that the signal intensity of FITC is negatively influenced by the illumination time, while that of QD525 remained constant irrespective of the time they were illuminated (Fig. 5b).

Discussion

Breast cancer is the most common malignancy diagnosed among women in most parts of the world. As with most progressive malignancies, the most important determining factor of survival rate in patients with breast cancer is the stage in which the disease is diagnosed. Early diagnosis of breast cancer translates in clinic to longer survival and less extensive treatments.

With the hope of early detection of breast cancer, the main interest in the field is focusing on the discovery of potential biomarkers and in this regard such techniques as proteomics and gene expression profiling have been employed [19]. Human epidermal growth factor receptor-2 (HER2/erbB-2) is the best characterized breast cancer marker which is widely used both in diagnosis and treatment. HER2 overexpression is associated with tumor aggressiveness and poor prognosis [13, 14]. Demonstration of HER2 expression in breast cancer has tremendous implication for treatment and outcome in patients whose tumors overexpress this marker. Patients with high expression of HER2 benefit most from therapeutic effects of humanized HER2-specific antibody (Trastuzumab, Herceptin) in terms of survival rate and recurrence of the disease [11, 20]. Therefore, HER2 testing has utmost importance for the management of patients with breast cancer. Indeed, regarding the fact that only 25–30 % of breast tumors overexpress HER2 [9, 13], accurate assessment of HER2 expression will determine which patients will benefit from HER2 targeted immunotherapy.

Our HER2-specific monoclonal antibody was proved to be very sensitive and specific able to detect HER2 in different immunoassays. Its ability to label HER2 in living cells demonstrates that it could be potentially used for targeted therapy.

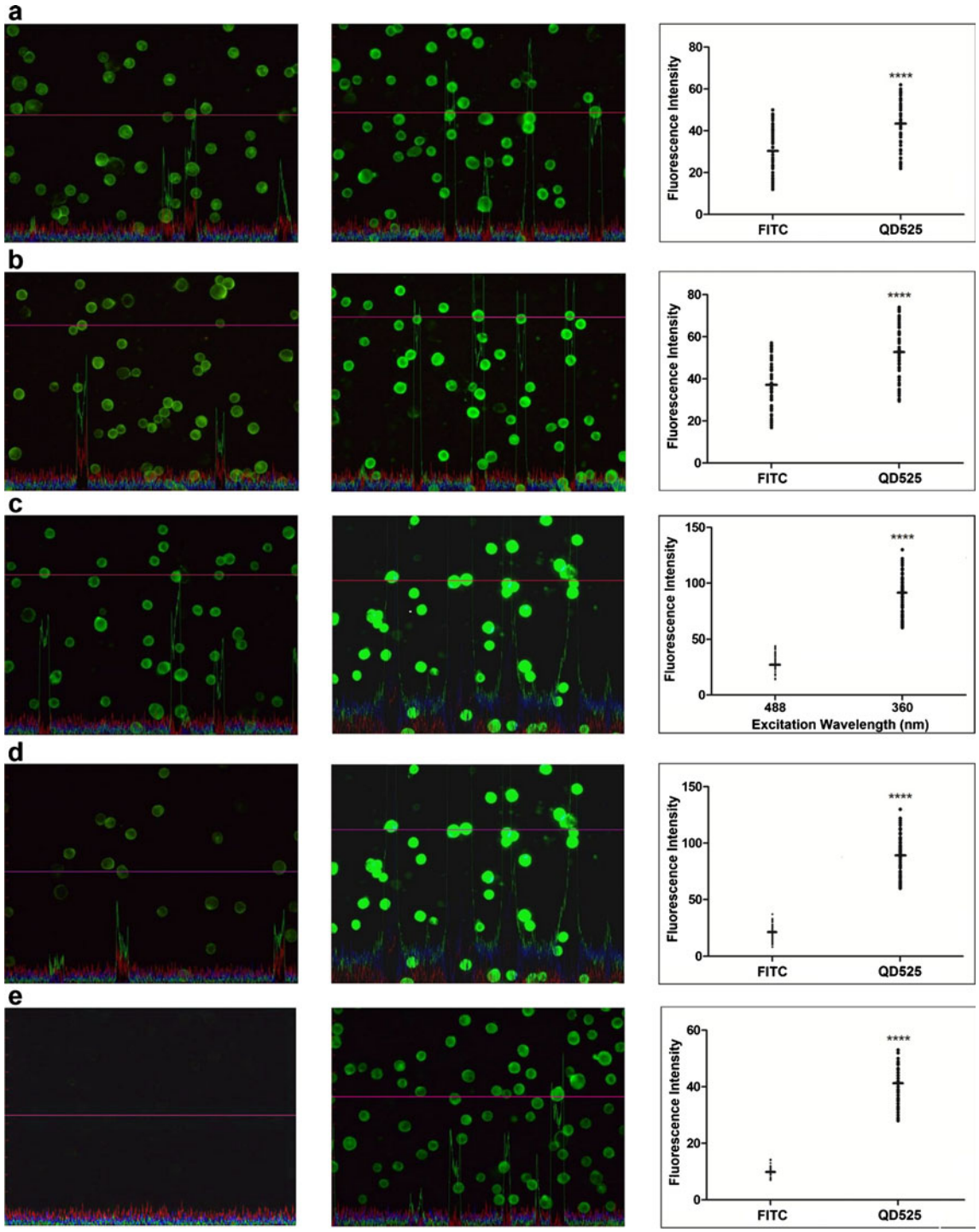

Fig. 4 Fluorescent intensity comparison between FITC and QD525. Acetone-fixed SKBR-3 cells were stained with HER2-specific monoclonal antibody and FITC- or QD525-labeled probes using LSAB method. FITC-stained cells were illuminated with 460–495 nm filter, while those labeled with QD525 were excited either with 460–495 nm or 330–385 nm filters. Average fluorescent intensity of at least 100 cells in each staining method at different exposure times were measured by OLYSIA software and compared (Right panel). Solid horizontal bars indicate median of each data set. **a**) FITC- (left) and QD525-labeled cells (middle): excitation at 460–495 nm for 1:4 sec. **b**) FITC- (left) and QD525-labeled cells (middle): excitation at 460–495 nm for 1:2.8 sec. **c**) QD-525-labeled cells: excitation at 460–495 nm (left) and 330–385 nm (middle). **d**) FITC- (left) and QD525-labeled cells (middle): FITC excitation at 460– 495 and QD525 excitation at 330–385 nm, both for 1:8 sec. **e**) FITC- (left) and QD525-labeled cells (middle): FITC excitation at 460– 495 and QD525 excitation at 330–385 nm, both for 1:60 sec. Scale bar 20 μm, ****$p<.0001$

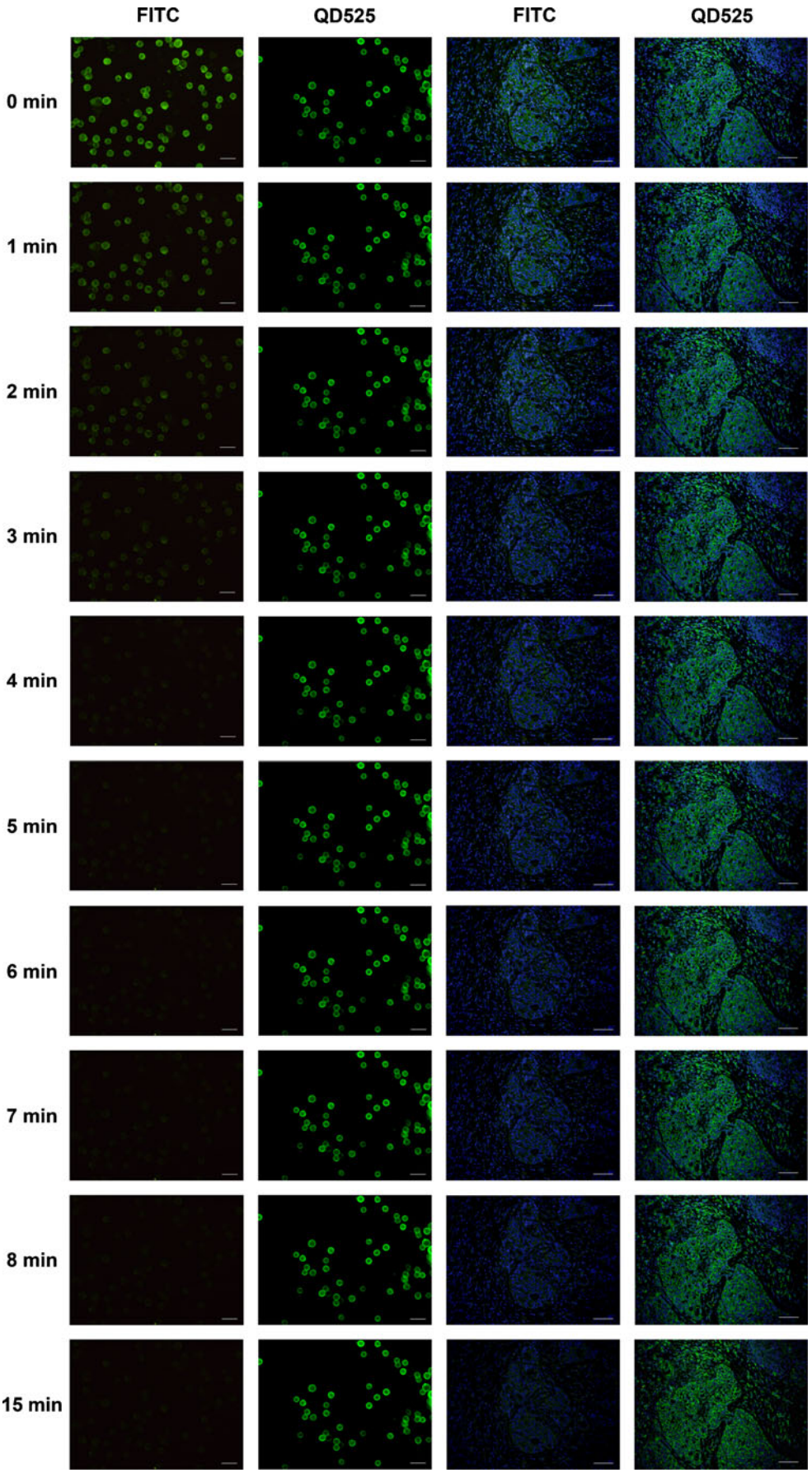

Fig. 5 Photostability of FITC- and QD525-stained breast cancer tissue and cell line. Expression of HER2 on human breast cancer cell line, SKBR-3, and tissues were tracked with LSAB method using HER2-specific monoclonal antibody and FITC- or QD525-labled streptavidin and imaged continuously under 460–495 nm illumination for 15 min. Images were captured each minute. Right panel shows average fluorescent intensity of FITC- (rectangle symbol) and QD525-labled (triangle symbol) SKBR-3 cells at different illumination periods

Detection of a cancer in its early stage requires a very sensitive detection system allowing lower levels of tumor marker to be detected accurately. Quantum Dots are tunable, highly sensitive fluorescent probes which could be potentially used in this regard. In this study, we compared the optical properties of QD525 and FITC which is the most widely used fluorophore used in immunofluorescent applications. To this end, we coupled streptavidin with QD525 using a linker with a long arm spacer, BS3, which allows free spatial movement of the conjugate and used this probe in LSAB immunofluorescent staining of both human breast cancer cell line and tissues. In this way, we showed that QD525 are marginally superior to FITC in terms of FI (median FI=30 vs. 43 at 1:4 sec exposure time) when illuminated at 488 nm. This was expectable as fluorophores do not exhibit their maximum extinction coefficients when excited with their non-optimal wavelengths. When we excited each probe in its maximum excitation wavelength, however, QD525 showed excellent signal which was about four fold stronger than FITC. This result was repeated when different exposure times were used. More interestingly, when we set the exposure time to optimum signal to noise for QD525 at 360 nm, the signal obtained by FITC at 488 nm was at the same level as that for negative control. These observations theoretically mean that QD525, as a sensitive reporter system, is able to report HER2 expression with the same signal intensity as does FITC when the antigen density is only one forth of that detected by FITC. This was the case when both breast cancer cell line, SKBR-3, and tissues were examined implying that QD-based probes could potentially enable us in paraclinical settings to improve sensitivity of diagnostic approaches, thereby they assist the clinicians in early diagnosis of breast cancer. This is also a very important issue when decision on Herceptin therapy has to be made.

Our results clearly showed that the QD525 conjugate could efficiently label the living cells as well with higher signal and lower background compared to its wavelength-matched organic fluorophore, FITC. It was recently reported that single QD tracking could be readily used on living cells to decipher complex cellular events such as cell membrane dynamism, signal transduction or intracellular transport [21].

As an alternative to tissue-based diagnostic methods, detection of serum soluble HER2 extracellular domain and establishment of its potential clinical usefulness has been the focus of many studies [22]. Based on the recent introduction of QDs for immunofluorometric quantification of biomolecules [23], it is conceivable that QD-based probes could be robust reporters for sensitive detection of serum soluble HER2.

The exact superiority of QD525 over FITC in terms of fluorescent intensity is more highlighted when fluorophore/streptavidin ratio is considered. In fact, this ratio for FITC is 16 times greater than that of QDs and so it could be estimated that unconjugated QDs are 64 time (4×16) brighter than FITC. As the emission wavelength of QDs increases, there is a steeper increase in their extinction coefficient compared to conventional fluorophores. This means that by using QDs with higher emission wavelengths, the sensitivity of QD-based diagnostic methods increases exponentially.

As a matter of fact, the more sensitive a diagnostic technique is, the more background is likely to happen. Signal to noise ratio could not give the precise picture of the sensitivity of a fluorescence-based diagnostic method in most settings. The rational behind this is that the spread of the negative, or "noise," population will also diminish the ability of a diagnostic system to distinguish the signal and noise populations. Staining index [24] measures the ability of a fluorescence-based method to distinguish negative and positive populations by incorporating both the spread of the negative peak and the difference (rather than ratio) of the means of the positive and negative populations. To see whether higher fluorescent signal of QD525 is concomitant with higher background, we measured staining index. Our results clearly showed that despite the higher fluorescent intensity, QD525 exhibits very low background and hence considerably higher staining index compared to FITC. Higher quantum yield combined with lower background are the two fundamental advantages of any fluorescent probe which make it an attractive candidate for molecular diagnosis.

Another feature that is critical for most fluorescent applications is photostability. We showed that FITC tended to bleach steadily by continuous illumination and became undetectable after about 5 min. In the case of QDs, however, signals showed no obvious change during the entire 15 min illumination period even under excitation at 360 nm. This feature makes QDs suitable candidates for applications requiring continuous tracking of the probe. This is the case for conditions where precise phenotype of the positive cells in a microscopic slide is to be investigated by pathologist.

In an elegant work by Wu et al. [25], QDs conjugated to immunoglobulin G (IgG) and streptavidin were used to label the breast cancer marker, HER2 on the surface of cancer cells. Here we added further information to the field on novel conjugation method of QDs to biomolecules, application of home-made HER2-specific mAbs in molecular tracking of HER2 on human breast cancer tissues by these conjugates, a thorough scrutiny of QD525 staining index and optical behavior in different wavelengths in comparison to wavelength-matched organic dye, FITC. In a very recent report by Chen et al. [26], QD-based techniques were found to be more sensitive and accurate compared to conventional immunohistochemical methods for the detection of HER2 in clinical breast cancer diagnosis.

Conclusion

Given the exceptional optical properties of semi-conductor QDs and their definite superiority over conventional fluorophores in terms of resistance to photobleaching, higher fluorescent intensity, higher staining index and lower minimum detection limit, this type of fluorescent probes can be used as sensitive reporters for in situ detection of tumor markers which in turn could be viewed as a novel approach for early detection of cancers. To take full spectrum advantages of QDs, it is necessary that their optimal excitation wavelength is employed. It seems likely that QDs will become a dominant fluorescent reporter in cancer detection over the next several years.

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