Quantum Dot-labeled Tags Improve Minimal Detection Limit of CA125 in Ovarian Cancer Cells and Tissues

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ABSTRACT

In recent years, a lot of attention has been paid to quantum dot (QD) nanoparticles as fluorescent sensors for sensitive and accurate detection of cancer biomarkers.

Here, using a homemade specific monoclonal antibody against CA125 and QD525- or FITC-labeled probes, expression of this marker in an ovarian cancer cell line and cancer tissues were traced and optical properties of fluorophores were compared qualitatively and quantitatively.

Our results clearly showed that besides lower background and exceptionally higher photobleaching resistance, QD525 exhibited higher fluorescent intensity for both ovarian cancer cell and tissues at different exposure times ($p<0.0001$) and excitation filter sets ($p<0.0001$) exemplified by significantly higher staining index ($p<0.016$). More importantly, the FITC-labeled probe detected antigen-antibody complex at minimum concentration of 0.3 µg/mL of anti-CA125, while reactivity limit decreased to 0.078 µg/mL of anti-CA125 when QD525-labeled probe was applied showing four times higher reactivity level of QD525 probe compared to the same probe labeled with FITC.
Quantum Dots for Improved Detection of CA125

Based on our results, it seems that QDs are inimitable tags for sensitive detection and localization of ovarian cancer micrometastasis and molecular demarcation of cancer tissues in surgical practice, which subsequently figure out accurate therapeutic approaches.

**Keywords:** Carcinoma antigen 125; Minimal reactivity limit; Neoplasm micrometastasis; Ovarian neoplasms; Quantum dots; Sensitivity

**INTRODUCTION**

Ovarian cancer is the most lethal gynecologic malignancy. The most widely used tumor marker in ovarian carcinoma; often considered the gold standard, is CA125. It was first identified in 1981 as a high molecular weight glycoprotein by the murine monoclonal antibody, OC125. CA125 is not expressed on the surface epithelium of normal fetal and adult ovaries thought to be derived from coelomic epithelium. Apparently, CA125 disappears early in the course of formation of the ovarian epithelium and is re-expressed in case of certain neoplastic and reactive lesions due to the metaplasia of the ovarian epithelium.

Despite the advent of new diagnostic modalities, most cases of patients with ovarian cancer are diagnosed at the late-stage (stage III-IV) when cancer has already been spread beyond the ovaries. The five-year survival rate of patients with advanced ovarian cancer is only ~30%; while in those with early stage disease, it can reach 90%. One reason for this high mortality rate is the lack of a highly sensitive and specific diagnostic tool to detect early-stage epithelial ovarian cancer.

The fluorescent labeling of biomolecules using small molecule organic dyes is widely employed in biological imaging and clinical diagnosis. Recently, quantum dot nanoparticles (QDs) have emerged as highly sensitive molecular probes in biomedicine research and imaging techniques based on these particles have showed promising potential applications in cancer research. The majority of QDs are semiconductor nanocrystals with several superior optical properties than traditional organic dyes, such as size-tunable emission wavelength, high fluorescence intensity (FI), strong resistance to photobleaching and chemical degradation, and possibility of simultaneous multiple fluorescence emission under a single excitation source. By having the capacity to be decorated with different molecular species, QDs can be used as versatile nanoscale devices for conjugation with proteins, peptides, and nucleic acids.

As with most cancers, the early detection of ovarian cancer by a sensitive diagnostic tool would have a significant impact on reducing its mortality and in this regard and based on their very high sensitive nature, QDs may aid precise determination of cancer biomarker over-expression at the early stage of the disease. Here we quantitatively assessed sensitivity of CA125 profiling in an ovarian cancer cell line and cancer tissues using QD525-based detection system and compared it with the same system employing emission wavelength-matched fluorophore, FITC. Upon the results presented here, we concluded that QDs based detection systems profoundly decrease minimal detection level of CA125 expression and could be viewed as a reliable alternative to those systems that are being routinely employed for cancer biomarker detection.

**MATERIALS AND METHODS**

**Cell Culture and Preparation**

Human ovarian cancer cell line, OVCAR3, and fibroblast cell line, HFFF-P16, were obtained from National Cell Bank, Iran, Pasture Institute. The cells were cultured in RPMI-1640 containing 10% fetal calf serum (FCS) (Gibco, Waltham, MA, USA) supplemented with additives at 37°C and 5% CO2.

**Tissue Preparation**

Formalin-fixed paraffin embedded (FFPE) samples of normal (n=5) and ovarian carcinoma (n=5) and cryosections (n=3) of fresh ovarian tissues from new cases with suspected ovarian tumor were obtained from cancer institute of Imam Khomeini Hospital and processed for CA125 expression as below. FFPE samples had already been carefully inspected and categorized by an expert pathologist. Diagnosis of ovarian cancer in fresh ovarian tissues was made during surgery in pathology laboratory and confirmed later in
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the same laboratory by inspection of FFPE sections. This study was approved by ethical committee for medical research of Avicenna Research Institute (No: 53) and all participating individuals in the study signed informed consent form.

Production of Anti-CA125 Monoclonal Antibodies (mAbs)

We produced anti-CA125 mAbs against extracellular domain of CA125 as published elsewhere. Briefly, eight week old NMRI mice were immunized by intraperitoneal injection of CA125-positive human ovarian cancer cell line, OVCAR3, followed by a booster intravenous injection of commercial CA125 (Biodesign, Quakertown, PA, USA). After completion of immunization schedule, hybridomas were produced by the standard method (Stahl et al 1980) and screened for reactivity against purified CA125 by ELISA. MABs were purified by affinity chromatography and characterized by multiple protein readout systems as described elsewhere. To be used for immunocytochemical (ICC) and immunohistochemical (IHC) stainings, final clones were also screened for reactivity with OVCAR-3 and CA125-negative fibroblast cell line, HFFF-P16 and one clone with the highest specificity reactivity, namely 10A6, was selected for immunostaining experiments. Reactivity and specificity of the selected clone in IHC staining of FFPE normal and ovarian cancer tissues was confirmed in reference to commercially-available anti-CA125 antibody, OC125 (Invitrogen, Carlsbad, CA, USA) as described elsewhere.

Antibody Labeling with Biotin and Streptavidin

Sheep anti-mouse Ig (Sina Biotech, Tehran, Iran) was labeled with NHS-biotin (Sigma-Aldrich, St. Louis, MO, USA) as described previously. Labeling efficacy was assessed by indirect ELISA. Briefly, ninety-six well plates (Nunc, Roskilde, Denmark) were coated with normal mouse Ig followed by the addition of two fold dilutions (from 1:100) of biotin-conjugated sheep anti-mouse Ig. After incubation for 1h at 37°C, HRP-conjugated streptavidin (Biosource, Camarillo, California, USA) (1:50000 dilution) was added and incubation was continued for further 30 min at 37°C. Color was developed after adding the substrate, 3, 3’, 5, 5’ tetra methyl benzidine (TMB) (US Biological, Salem, MA, USA) and the absorbance was measured at 450nm. The negative controls included omission of first layer (antigen), second layer (biotin- conjugated sheep anti-mouse Ig) or both layers and always was shown to be negative.

QD525-NH2 (Invitrogen) coupling to streptavidin (BD Bioscience, San Jose, CA, USA) was performed according to the protocol we published elsewhere using water soluble heterobifunctional cross linker, BS3, (Biosource), in a two-step reaction procedure. In brief, QD525-NH2 was washed several times with phosphate buffered-saline (PBS), pH 7.4 in a 100 KD ultrafiltration unit (Millipore, Temecula, CA, USA), transferred to siliconized eppendorf tube and mixed with BS3 linker in a final ratio of 8–10 μM QD/1 mM BS3 for 2 h at room temperature. Conjugated QD525 was then purified using a gel filtration Nap5 column (GE Healthcare, Little Chalfont, UK). Colored eluate was mixed with 40-fold excess of streptavidin for 2 h. At the final step, we purified the conjugate from excess streptavidin by ultrafiltration unit (100KD) into 50 mM borate, pH 8.3. Purified QD conjugate was wrapped carefully and stored at 4°C. Labeling of streptavidin with QDS25 was confirmed by SDS-PAGE and assessment of reactivity of the conjugate in immunofluorescent staining of OVCAR3 cells was performed as described below. Streptavidin was also conjugated with FITC (Gibco). Briefly, FITC and streptavidin were dissolved (1 mg/mL) separately in 0.1 M sodium bicarbonate buffer, pH 9. FITC was then added to streptavidin at 1:5 (v/v) ratio and incubated for 2 h at room temperature. Dialysis was performed against PBS and the absorbance was measured at 280nm and 495nm to determine F:P ratio which was calculated to be eight.

Immunofluorescent Staining of Ovarian Carcinoma Tissues and OVCAR3 Cell Line

Immunofluorescent staining was performed on FFPE and fresh ovarian cancer tissue samples by labeled streptavidin-biotin (LSAB) method. For staining of FFPE tissues, 3μm sections were prepared, transferred to the poly L-lysine-coated slides and subjected to heat-activated antigen retrieval in citrate buffer (10 mM, pH 6) at 98°C for 30min, followed by three washes with tris-buffered saline, pH 7.4 containing 0.1% bovine serum albumin (TBS-BSA). Endogenous biotin was blocked with biotin blocking system (Dako, Stockholm, Sweden) according to the manufacturer’s instruction. Sections were then washed three times with TBS-BSA and incubated for 15 min with 5% normal rabbit serum. At the next step, the
slides were tilted and incubated for 90 min with anti-CA125 mAb, clone 10A6 (2.5 μg/mL). The slides were washed three times with TBS-BSA and incubated with pre-determined optimal concentration of biotin-conjugated sheep anti-mouse Ig (2.5 μg/mL) for 45 min. Finally, after three washes with TBS-BSA, QD525-conjugated streptavidin (1:50) or FITC-conjugated streptavidin (1:200) were added to slides for 30 min. In negative reagent control slides, primary antibody was substituted by isotype matched irrelevant antibody. After three washes, fluorescent signals were visualized and imaged using BX51 fluorescent microscope (Olympus, Tokyo, Japan). Immunofluorescent staining was also performed on acetone-fixed ovarian cancer tissues and cells. Briefly, cells were detached, washed with PBS and cytospined. Cryosections of fresh ovarian cancer tissues were prepared and transferred to the poly L-lysine-coated slides. After being dried for 30 min at room temperature, slides were fixed for 2 min with ice-cold acetone. Slides were then washed and processed as above.

**Image Acquisition and Analysis**

Digital images were captured with a 12.5 megapixel cooled charge-coupled device (CCD) camera (Olympus). Multiple fields were captured from each tissue or cell line sample. Sections labeled with QD525 were illuminated with excitation filters of either 460-495nm or 330-360nm, while those stained with FITC were excited only with 460-495nm. Fluorescent signals were inspected with an emission filter of 525 nm. Exposure time for each excitation/emission filter set was optimized in reference to negative control slides of the same staining procedure. To compare the fluorescent intensity (FI) of QD525 and FITC, the images for each fluorophore were captured at and analyzed by Image J software, developed by NIH (https://imagej.nih.gov/ij/). For quantification and statistical analysis for every filter set or exposure time, data of minimum 100 cells was analyzed and mean fluorescence intensity (MFI) was then calculated.

**Determination of Minimal Reactivity Limit**

For detection of minimal reactivity limit, immunofluorescent staining of FFPE ovarian cancer tissues was performed using two fold dilution of anti-CA125 mAb, 10A6 from 5 to 0.02 μg/mL. Biotinylated antibody and QD525- or FITC-conjugated streptavidin was then applied as mentioned above. Fluorescent signals were viewed and captured with aforesaid excitation/emission filter sets.

**Comparison of FITC and QD525 Staining Index**

Staining index (SI) is a measure of how well a signal is distinguished from background. To calculate SI, optimal exposure time for each fluorophore was detected as mentioned above. FI of positive and negative controls with equal exposure times were calculated by Image J software. SI for each fluorophore was calculated using the following equation:

\[ SI = \frac{\text{Mean positive FI} - \text{Mean background FI}}{2 \times \text{SD background FI}} \]

**Photostability Comparison of FITC and QD525**

To compare the photostability of FITC & QD525, immunostained slides were continuously illuminated for one hour with excitation filters of 460-495 nm for FITC and 330-360nm for QD525. Images were captured at 1 min intervals for each fluorophore. FI of a minimum 100 cells was calculated by Image J software and analyzed.

**Statistical Analysis**

All statistical analyses were performed using SPSS version 22 (IBM, Armonk, NY, USA). T-test was used to compare the FI of FITC and QD525. \( p<0.05 \) was considered statistically significant.

**RESULTS**

**Production of Anti-CA125 Monoclonal Antibodies**

By prime-boost strategy of immunization, several anti-CA125 monoclonal antibodies were produced among them one clone of IgM isotype, namely 10A6 was selected for the experiments conducted in this research. All specifications of this clone including reactivity in ELISA, Western blotting, immunoprecipitation and IHC/ICC and also ability to react with native CA125 in flow cytometry have already been investigated and confirmed. Specifically, 10A6 had comparable reactivity in IHC staining of FFPE ovarian cancer tissues with commercially-available anti-CA125 antibody, OC125. Our antibody failed to react with normal ovarian tissues denoting specificity of our anti-CA125 antibody.\(^{12}\)

**Comparison of Fluorescence Intensity of QD525 and FITC**

Besides being affected by the immunostaining...
protocol, FI is also an intrinsic characteristic of each fluorophore and profoundly affects the sensitivity of fluorophore-base detection systems. The more the FI of a given fluorophore, the more sensitive the detection system is. In order to compare the FI, immunofluorescent staining of FFPE ovarian cancer tissue sections and Acetone-fixed ovarian cancer cell line, OVCAR-3 (Figure 1A) were performed by bioconjugated QD525 and FITC and their FIs were compared at different wavelengths and exposure times.

Figure 1. Comparison of fluorescent intensity of FITC and QD525 in immunofluorescent staining of ovarian cancer tissues and cells.
A) Immunofluorescent staining of ovarian cancer tissues and cells using FITC and QD525 labels: Samples were stained with anti-CA125 mAb and FITC- or QD525-labeled probes. Average fluorescent intensity in each staining method was measured at different exposure times and calculated by Image J software and compared (Right panels). Solid horizontal bars indicate median of each data set. Exposure times are shown in the upper right corner of each figures.
B) Immunofluorescent staining of fresh cryosections of ovarian cancer tissues using FITC and QD525 labels: Samples were stained as above with FITC- or QD525-labeled probes and then illuminated at 460–495 nm (g) or 330–385 nm (f) at optimal exposure times for each filter set, respectively. Irrelevant IgM, as negative control of anti-CA125 mAb, was shown to be negative in both staining methods (h, i).
C) Comparison of staining index of QD525 and FITC: QD525- or FITC-labeled ovarian cancer tissue sections and OVCAR-3 fixed cells were excited at 330–360 nm and 460–495 nm, respectively. Staining index (SI) was calculated based on fluorescent intensity of positive control and background staining. Scale bar: 20 µm for cell line & 50 µm for tissue, *: p<0.016, ****: p<0.0001.
At the first step, MFI was evaluated in optimal exposure time and excitation wavelength of each fluorophore (QD525: 330-360 nm, 111 msec for tissue, 55 msec for cell line and FITC: 460-495 nm, 40 msec for tissue and 166 msec for cell line). Our results showed that in this setting, QD was significantly brighter than FITC (p<0.016 for cell line and p<0.0001 for tissue) (Figure 1A-a). In the next setting, each fluorophore was excited by their optimum excitation wavelength but at the exposure time which was optimum for either FITC or QD525. The results clearly showed that QD525 was at least three to six times brighter than FITC depending on exposure time (p<0.0001 for both cell and tissue) (Figure 1A-b and c). To consolidate the aforesaid results, FITC-labeled slides were excited separately at optimal exposure times for QD525 and FITC. Our results showed that there was no FITC signal at optimal exposure time for QD525 (p<0.0001) (Figure 1A-d).

Fluorescence Intensity (FI) is directly related to maximum extinction coefficient at optimal excitation wavelength. Since maximum extinction wavelength for QD525 is under 400 nm, its FI was measured at excitation wavelengths of 330-360 nm and 460-495 nm with optimum exposure time at each wavelength (330-360 nm: 11 msec for tissue, 55 msec for cell line and 460-495 nm: 100 msec for tissue, 250 msec for cell line). The results showed that MFI of QD525 at 360 nm is significantly higher than that in 488 nm, in spite of being illuminated with a considerably lower exposure time (p<0.0001) (Figure 1A-e). To consolidate the results, brightness of the staining of QD525 and FITC on fresh cryosections of ovarian cancer tissues was compared and as expected QD525 showed considerably higher fluorescent signal compared to FITC (Figure 1B).

Comparison of Staining Indices of QD525 and FITC

One of the major shortcomings of fluorescent microscopy in immunofluorescence analysis is interference by auto fluorescence. This phenomenon may either be intrinsic, due to fluorescent structures in cells on interstitial tissue, or may be induced by fixation media and tissue processing techniques. Such parameters as SI are useful metrics to normalize fluorescent signal over background and to express quantitatively the degree of contrast between FI of positive control and background staining. For this purpose, we excited QD525 or FITC-labeled ovarian cancer tissue sections and OVCAR-3 fixed cell line at 330-360 nm and 460-495 nm, respectively and calculated their SI. It was demonstrated that QD525 had an SI about five times more than that of FITC (p<0.0001) (Figure 1C).

Determining Minimal Reactivity Limit

Detection limit of a given marker is a function of minimal reactivity level of detection system that is a criterion of sensitivity. Anti-CA125 mAb, 10A6, was titrated on ovarian cancer FFPE samples and then the fluorescent signal was detected with probes. FITC-labeled probe detected anti-CA125 antibody at minimum concentration of 0.3 µg/mL, while the reactivity limit decreased to 0.078 µg/mL anti-CA125 when QD525-labeled probe was applied showing four times higher reactivity level of QD525 probe compared to the same probe labeled with FITC (Figure 2).

![Figure 2. Determining minimal reactivity limit of QD525- and FITC-labeled probes in immunofluorescent staining of ovarian cancer tissues.](http://ijaai.tums.ac.ir)
Comparison of Photostability of QD525 and FITC

Quenching fluorescent reporters limits the exposure time over which the fluorescent dye can be followed.

To compare photostability of QD525 and FITC, QD525- and FITC-stained OVCAR-3 cells were continuously illuminated with their optimal excitation wavelength for 1h. Data showed that the FITC signals faded and became undetectable after about 3 min, whereas QD525 signals were fully stable during the entire period of continuous illumination (at least 90 min) (Fig.3A). Fluorescent signals were quantified at each time and plotted versus different time points to show photostability of QD525-labeled probe (Figure 3B).

![Image of fluorescence images and graph showing photostability](image)

Figure 3. Determining photostability of QD525- and FITC-labeled probes in immunofluorescent staining of ovarian cancer tissues and cells.
A) Expression of CA125 on human ovarian cancer cell line, OVCAR-3 and tissues was tracked by immunofluorescent staining using anti-CA125 mAb and FITC- or QD525-labeled streptavidin and imaged continuously till 90 min. Images were captured each minute. B) Mean fluorescent intensity (MFI) of ovarian cancer cells at each time points were calculated and plotted vs. time.
DISCUSSION

As with many cancers, effective treatment of ovarian cancer as the most lethal gynecological malignancy depends upon the early detection of the disease. Over three decades after the first report on the expression of CA125 by ovarian tumors, CA125 is still the only clinically reliable diagnostic marker for ovarian cancer. One major problem associated with detection of cancer biomarkers in tissues is the relatively low sensitivity of detection methods. Since over 90% of ovarian cancers express CA125, we were about to examine to what extent application of QD525-labeled probes could improve the minimal reactivity of the antibodies directed against CA125. In this regard, we first generated anti-CA125 monoclonal antibody and confirmed its reactivity against denatured and native antigen in different protein readout systems.

As a critical key step, early detection of cancer requires a highly sensitive monitoring system that can detect low levels of tumor marker. QDs compared to conventional fluorescent dyes, have unique optical characteristics such as tunable excitation wavelength, higher fluorescence intensity, resistance to photobleaching and chemical degradation, and simultaneous excitation of multiple fluorescent colors under a single excitation source. These properties make QDs as a promising alternative to conventional fluorescent dyes for improving the sensitivity and multiplexing capabilities of molecular histopathology.

Here, we developed and validated the QDs-based immunostaining protocol for quantifying CA125 expression in an accurate and sensitive approach on ovarian cancer cells and tissues. In the first step, we compared fluorescence intensity of QD525 and its emission wavelength-matched fluorescent dye, FITC for detection of CA125 at different exposure times. There are tradeoffs between exposure time, image brightness and fluorescent intensity values up to a point. But, it should be kept at optimal level to avoid photobleaching, photo toxicity especially for living cell imaging and also to minimize autofluorescence and background. Accordingly, we set optimal exposure times for each fluorophore at their optimal excitation wavelengths by corresponding negative control slides and then studied in a crisscross manner the FI of each fluorophore. We observed that at the exposure time optimal for QD525, QD525 exhibited FI of about six times more than that of FITC, while at optimal exposure time for FITC, the FI of QD525 was only near three times more than that of FITC. This inconsistency could be attributed to the fact that CCD camera detectors have a limited capacity to hold electrons; if this capacity is reached, the corresponding pixel will be “saturated” and any photons reaching the detector after saturation will not be counted. So, fluorescence is the result of real staining choose an exposure time that avoids over saturation.

The results we presented here on superiority of QD525 over FITC in terms of FI are in line what we reported previously for detection of HER2 on breast cancer tissues and cells. When we studied FITC FI at the optimal exposure time for QD525, no signal was observed, further consolidating the superiority of QD525 over FITC.

Although semiconductor QDs have broad excitation wavelengths, they fluoresce more efficiently when excited at wavelengths below 400 nm. In this regard, we compared FI of QD525-labeled CA125 in ovarian cancer tissue and cells with that of FITC. As expected, FI was profoundly increased when QD525 was excited at 330-360 nm implying that sensitivity of CA125 detection in ovarian cancer tissues is further increased when QDs are excited at their optimal excitation wavelengths. Metastasis is a hallmark of most invasive cancers and detection of micrometastasis has considerable impact on the course of treatment. Such a high sensitivity of QDs is of utmost importance and may influence the subsequent therapeutic approach as far as reliable tracing of ovarian cancer micrometastasis is concerned. Indeed, molecular demarcation of cancer tissues in surgical practice could potentially be feasible using QD-labeled tumor-specific reports.

In immunodetection studies, immunostaining technique and properties of tracer profoundly affect minimal detectable level of antigen-antibody complex. The combination of conventional IHC procedures with QD-based fluorescent dyes could considerably improve the resolution and sensitivity of the method and provides possibility of simultaneous imaging of several markers. Accordingly, we showed, using the same detection system, that QD525 compared to FITC could minimize four folds the minimal reactivity of anti-CA125 in ovarian cancer tissues.

Fluorophore detection in a given experiment can be obscured by high background fluorescence, which is
most commonly caused by insufficient removal of unbound fluorescent probe or sample autofluorescence. Staining index is a useful metric for normalized fluorescent signal over background. In this study, quantification of staining index for fluorophores clearly showed that QD525 exhibited very low background and hence considerably higher staining index compared to FITC.

Chemical structure of fluorophores greatly affects their photostability and photobleaching profiles. The comparison of these physico-chemical characteristics is a valuable approach to identify the best fluorophores in such experiments as ICC, IHC and flow cytometry. For this purpose, we illuminated two fluorophores continuously for at least 90 minutes and captured images to quantify their photostability. The results showed that FITC entirely bleached after 2 minutes while, fluorescent signal of QD525 showed no obvious alteration over time. Such a high photobleaching resistance can significantly improve the precision of diagnosis where the observer needs to meticulously observe the pathological details of a tissue in an extended period of time.

Although, application of the QD-labeled fluorophores for specific detection of CA125 was first introduced by Wang et al the results of our study consolidated the aforesaid report by quantification of QD optical properties and providing compelling evidence of utility of QD-based detection system in clinical practice. Although detection of cancer biomarkers in serum is a non-invasive screening approach for cancer detection and monitoring, none of the anti-CA125 antibody pairs produced in this research project was able to detect CA125 in sandwich ELISA.

In Conclusion, the application of QDs in cancer investigations has dramatically increased since the first introduction of these nanoparticles in 1998 due to their unique size-dependent optical properties. In continuation of our previous report on potential usefulness of QDs for accurate detection of Her2 expression, here we showed that QD-based detection systems possess undeniable superiority over wavelength-matched conventional dyes and quantitatively and qualitatively improve detection of CA125 expression in both ovarian cancer cells and tissues. Based on our results, it seems that QDs are inimicable tags for sensitive detection and localization of ovarian cancer micrometastasis, which subsequently suggests accurate therapeutic approaches. To take full spectrum advantages of QDs, it is not only necessary to focus on optical properties but also on the interference by environmental parameters and digital image acquisition that could potentially affect the precision and accuracy of quantitative fluorescent microscopy measurements.

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