Double labeling and comparison of fluorescence intensity and photostability between quantum dots and FITC in oral tumors

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Received November 22, 2010; Accepted February 21, 2011

DOI: 10.3892/mmr.2011.457

Abstract. The aim of this study was to evaluate the application of quantum dots (QDs) and the FITC labeling technique in Tca8113 cells, and to compare the fluorescence intensity and photostability of these techniques. First, using one-colour confocal microscopy, QDs and FITC were applied separately to tag HSP70 in Tca8113 strains with indirect immunofluorescence. The expression of HSP70 in Tca8113 cells was observed using confocal laser microscopy, and the fluorescence intensity between QDs and FITC was compared. Using two-colour confocal microscopy, QDs and FITC were applied to tag survivin and HSP70, respectively, in order to compare the fluorescence photostability between QDs and FITC. Using a laser scanning microscope, survivin and HSP70 expression was clearly detected in the Tca8113 cells. The strength of the fluorescence signal from QDs was higher than that from FITC. After 40 min of continuous laser exposure, there was no observable decline in the QD fluorescent markers, whereas the FITC fluorescent signals faded very quickly and became undetectable. QDs have better fluorescence intensity and photostability than FITC, and are more appropriate for studies that require a long dynamic observation of physiological changes in cells.

Introduction

Organic dyes have been widely used to tag biological molecules to study the interactions between various proteins and their impact on cell function. However, the role of organic dyes as a marker in the study of biological molecules and cells during *in vivo* bio-imaging is limited due to certain shortcomings, such as narrow excitation spectra, poor photostability and short fluorescence lifespan (1,2). Functionalized bio-affinity nano fluorescence probes have recently been developed based on semiconductor quantum dots (QDs), and have very broad prospective uses in biomedical applications, particularly in the study of bio-imaging in oncology, due to their unique optical properties. QDs have already been applied to the analysis of membrane proteins in mammary cancer and have been used as a research tool for the molecular imaging of blood cells (3-14). However, there have been no reports on the application of QDs in the study of proteins in human tongue cancer cells. We introduced the QD labelling technique to the study of Tca8113 cells. Survivin and HSP70 in Tca8113 cells were tagged with QDs and FITC, respectively, to compare fluorescence intensity and fluorescence photostability between QDs and FITC, and to study survivin and HSP70 lymphocyte positioning and their roles in the occurrence and development of oral tumors.

Materials and methods

QDs (goat anti-rabbit QD655nm-IgG) were provided by Invitrogen. Mouse anti-human HSP70 antibody was purchased from Abcam, whereas goat anti-mouse FITC-IgG was purchased from Boster Biological Technology, Ltd. RPMI-1640 culture medium and trypsin were from Gibco (USA). The laser confocal microscope used was a Leica TCS SP2 made in Germany.

Cell culture. Tca8113 cells were cultured in RPMI-1640 with 10% foetal bovine serum and were placed in an incubator containing a 5% volume fraction of CO_2 at 37˚C. The culture medium was changed every 2 days and 0.25% trypsin was used to digest and subculture the cells after 2-3 days.

Cell inoculation. The digested cells were inoculated onto a confocal-dedicated utensil at a density of $1x10^5$ cells. After being cultured for 1-2 days, the cells reached 80% confluency. Subsequently, the cells were washed twice with 0.01 mol/l (pH 7.4) TBS and fixed with pre-chilled methanol for 10 min.

One-colour immunofluorescence. The fixed Tca8113 cells were washed with TBS, incubated with Triton X-100 for 10 min and washed. Thereafter, they were incubated at 4˚C overnight with mouse anti-rabbit HSP70 antibody (dilution ratio, 1:100). The cells were washed three times in TBS and incubated with buffer solution in a 37˚C moist box for 10 min, followed by incubation with the equivalent mixed liquid of goat anti-rabbit QD655nm-IgG or goat anti-rabbit FITC-IgG (dilution, 1:100) in a 37˚C moist box for 45 min. Finally, the

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Key words: quantum dots, Tca8113 cells, fluorescence intensity, fluorescence photostability, HSP70, survivin

Table I. Fluorescence intensity comparison between QD655nm and FITC.

Group	QDs	FITC
Fluorescence intensity	0.0928257±0.00193721	0.0891216±0.00165493

P≤0.05 was considered significant. QDs had better fluorescence intensity than FITC.

cells were washed three times with TBST and sent for detection away from light. The blank control group (C and F) was treated with equivalent TBS. Treatment in each group was repeated five times.

Laser confocal fluorescence microscopy detection and Image-Pro Plu analysis. The wavelength of the excitation spectra was 488 nm and the constant temperature system was adjusted to 30˚C. Thirteen photofluorograms were taken from each sample and the 65 photofluorograms from each group were analysed by Image-Pro Plu.

Two-colour immunofluorescence. The fixed Tca8113 cells were incubated at 4˚C overnight with the equivalent mixed liquid of rabbit anti-human survivin antibody and mouse anti-human HSP70 antibody (dilution, 1:100). The cells were washed three times in TBS and incubated with buffer solution in a 37˚C moist box for 10 min, followed by incubation with the equivalent mixed liquid of goat anti-rabbit QD655nm-IgG and goat anti-mouse FITC-IgG (dilution, 1:100) in a 37˚C moist box for 45 min. Then, the cells were washed three times with TBST. After the last wash, the TBS-T was not completely absorbed, and the cells were sent for detection away from light. The negative control group (A) was treated with the same amount of mouse anti-human HSP70 antibody (dilution, 1:100) as the experimental group, whereas the negative control group (B) was treated with the same amount of rabbit anti-human survivin antibody (dilution, 1:100); the blank control group was treated with equivalent TBS.

Laser confocal fluorescence microscopy detection. The samples were observed in the same region under the same focal length using a dynamic observation system. Photograph images were automatically captured every 16,031 msec for 40 min, and then the 152 photofluorogram and their corresponding statistical data of fluorescence intensities were analysed by the software accompanying the laser confocal fluorescence microscopy.

Statistical analysis. Results are expressed as the means ± SD. Comparisons between two groups were conducted using the t-test. P-values of ≤0.05 were considered significant.

Results

HSP70 labelled by QD655nm or FITC in one-colour immunofluorescence. Under a laser confocal microscope, it was observed that the QD655nm-marked HSP70 was clearly expressed in the cytoplasm and nucleus of the Tca8113 cells, and mainly distributed in the cytoplasm (Fig. 1A). Compared to FITC-tagged HSP70 (Fig. 1D), QD655nm-tagged HSP70 had the same lymphocyte positioning; however, the photofluorogram of the QD655nm-tagged HSP70 (Fig. 1A) had better specificity and stronger fluorescence intensity.

QD655nm has better fluorescence intensity than FITC. Sixty-five photofluorograms from each group were analysed by Image-Pro Plus. The results are expressed as the means ± SD. Comparisons between the two groups were conducted using the t-test. P≤0.05 was considered significant (Table I).

Survivin and HSP70 were concurrently labelled by QD655nm and FITC in two-colour immunofluorescence. Fig. 2A-D shows the experimental group under four channels of the laser confocal microscope. Fig. 2A was captured under the multi-colour channel. There were three different colours of fluorescence, red, green and yellow, in the image. Yellow fluorescence resulted from the overlap of red and green fluorescence, indicating that both the proteins were distributed in the region. Green fluorescence, representing FITC-tagged HSP70, was distributed in the nucleus, indicating that HSP70 was present in the absence of survivin in these regions. Red fluorescence, which indicated QD655nm-labelled survivin, was observable in the cytoplasm, revealing that survivin was present in the absence of HSP70 in these regions.

Fig. 2B and C were observed through the red and green monochrome channels, respectively. FITC-labelled HSP70 was clearly expressed in the cytoplasm and nucleus (green fluorescence). QD655nm-tagged survivin was mainly distributed in the cytoplasm (red fluorescence). Fig. 2D shows the cellular morphology through an ordinary light microscope.

Images of the control group under the laser confocal microscope are shown in Fig. 2E-G. Fig. 2E shows the control group (A) under the monochrome channel. FITC-marked HSP70 was clearly expressed in the cytoplasm and nucleus (green fluorescence); Fig. 2F shows the control group (B) in the monochrome channel. QD655nm-labeled survivin was mainly distributed in the cytoplasm (red fluorescence). Fig. 2G shows the blank control group, in which no fluorescence was observed.

QD655nm has better photostability than FITC with 40 min of continuous laser exposure. The red fluorescence of QD655nm experienced no significant decline after continuous excitation by a high-intensity laser for 40 min (Fig. 3M-R), whereas the FITC green fluorescence was almost quenched (Fig. 3G-L). With the quenching of the FITC, the fluorescence of the three colours (red, green and yellow) observed under the multi-colour channel gradually faded into monochromatic red fluorescence (Fig. 3A-F).

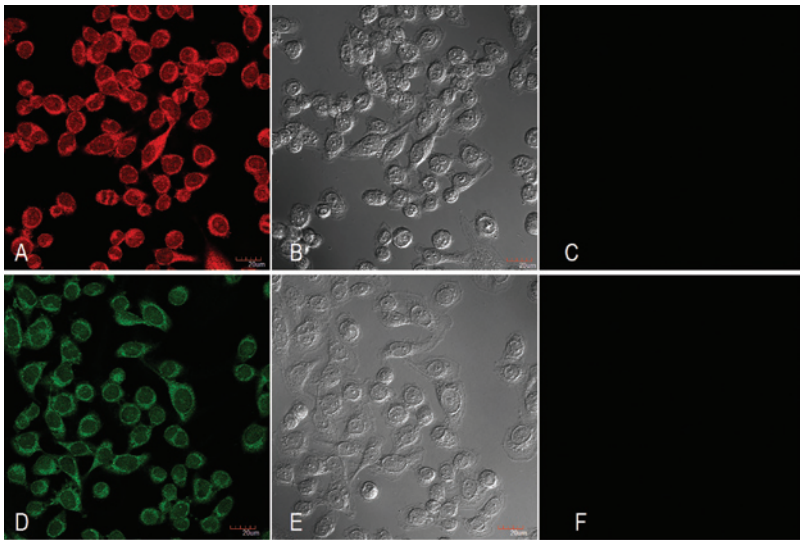

Figure 1. Fluorescence imaging of HSP70 tagged by QD655nm or FITC in one-colour immunofluorescence. (A) QD655nm-tagged HSP70 and (D) FITC-tagged HSP70 had the same lymphocyte positioning, but the photofluorogram of the QD655nm-tagged HSP70 had better specificity and stronger fluorescence intensity.

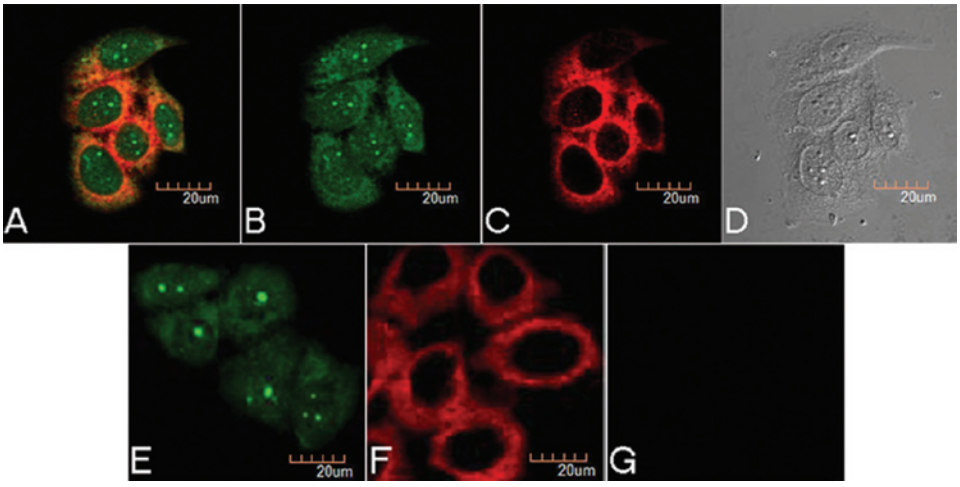

Figure 2. Fluorescence imaging of survivin and HSP70 tagged by QD655nm and FITC in two-colour immunofluorescence. (A-D) Fluorescence imaging of four different channels in the experimental group. Cells were incubated with a mixture of HSP70 primary antibody and survivin primary antibody overnight. (E) Only green fluorescence was observed in the negative control group (A). Cells were incubated with a mixture of equivalent HSP70 primary antibody and TBS overnight. (F) Only red fluorescence was observed in the negative control group (B). Cells were incubated with a mixture of equivalent survivin primary antibody and TBS overnight. (G) No fluorescence was observed in the blank control group. Cells were incubated with equivalent TBS overnight.

The software accompanying the laser confocal microscope recorded the fluorescence intensities, which were collected every 16 sec during excitation by a strong laser for a duration of 40 min (Fig. 4). During the first 5 min of excitation, the fluorescence intensities of QD655nm dropped from 299.478 to 278.517 mW. At 9.5 and 12.8 min, the fluorescence intensities were 267.634 and 265.761 mW, respectively, which showed that the intensity of the QD signal tended to stabilize. During the course of the 40 min of continuous exposure, changes in fluorescence intensity were not observed. However, during the first 5 min of irradiation with FITC, the fluorescence intensity declined from 463.298 to 299.478 mW, while at 9.5 and 12.8 min, the fluorescence intensities were 177.740 and 99.740 mW, respectively, indicating a sharp decline in intensity with continued excitation. From 27 to 40 min, the fluorescence intensity dropped from 30.931 to 20.977 mW, becoming very weak. This indicated that FITC has low stability, weak resistance to photobleaching and a short fluorescence lifespan.

Discussion

QDs, which are semiconductor nanocrystals with a diameter less than or close to that of a Bohr radius, are a new type of fluorescence marker developed in recent years. They are composed of II-VI (such as CdSe and CdTe) or III-V (such as InP and InAs) group elements. QDs have more outstanding optical properties than conventional organic fluorescence materials because of their unique quantum size and surface effects. Their excitation spectra are broad and the fluorescence spectra peak position is regulated by changing

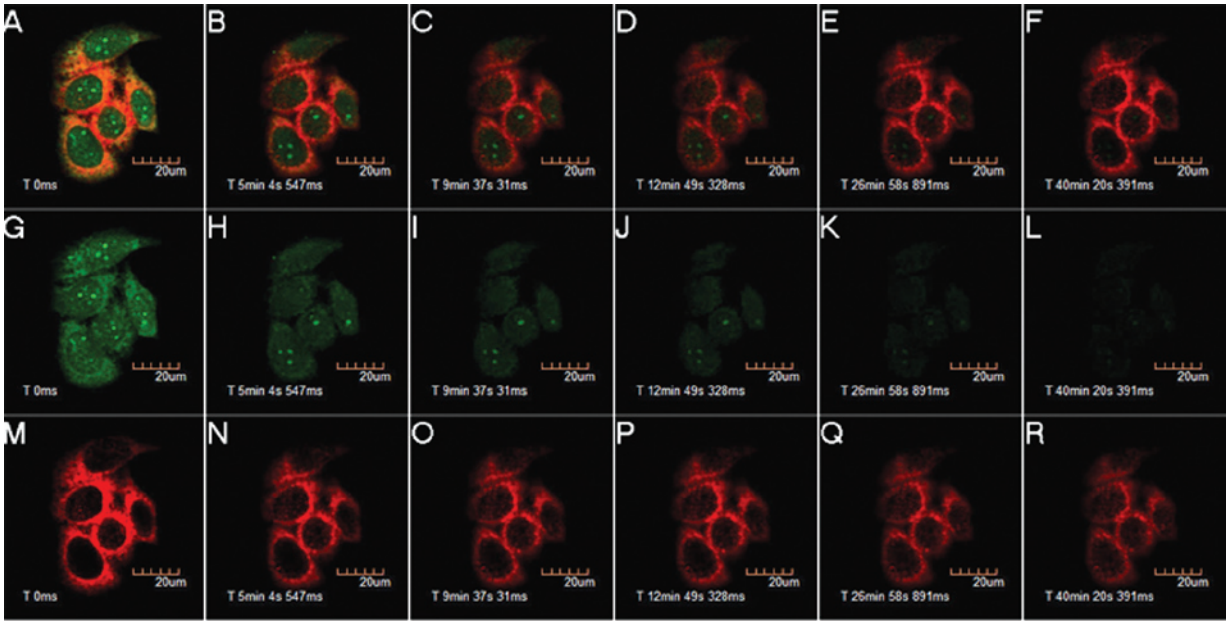

Figure 3. Fluorescence imaging of survivin and HSP70 with 40 min of continuous high-intensity laser excitation. The red fluorescence of QD655nm experienced no significant decline after being continuously excited by a high-intensity laser for 40 min (M-R), whereas the FITC green fluorescence was almost quenched (G-L).

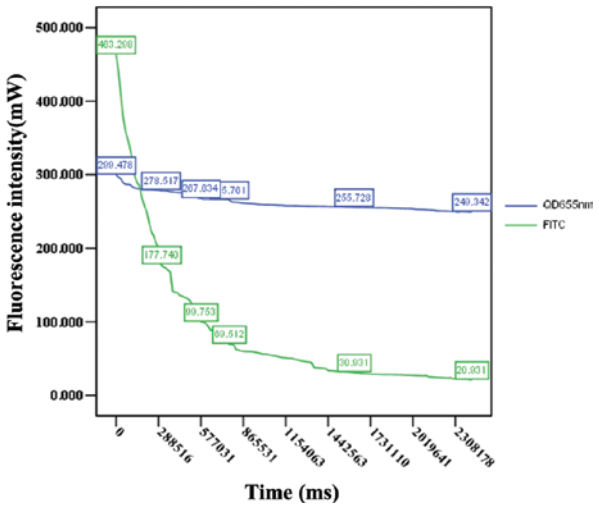

Figure 4. Comparison of photostability between QD655nm and FITC. QD has better fluorescence photostability than FITC. The red fluorescence of QD655nm experienced no significant decline after being continuously excited by a high-intensity laser for 40 min, whereas the FITC green fluorescence was almost quenched. The images marked on the chart are the values of the fluorescence intensity at the six above-mentioned times (T_1=0 msec; T_2=5 min, 4 sec, 547 msec; T_3=9 min, 37 sec, 31 msec; T_4=12 min, 49 sec, 328 msec; T_5=26 min, 58 sec, 891 msec; T_6=40 min, 20 sec, 391 msec).

the particle size and composition of the QDs. The Stokes displacement (the D-value between the excitation peak and emission peak wavelengths) is so large (300-400 nm) that the overlap of the emission and excitation spectra can be avoided, thus allowing for spectroscopy detection even in cases of a low signal. QDs have a strong stable fluorescence intensity and are resistant to photobleaching (15-18). The QD, which is currently the focus of several studies, is a shell-core structure composed of a CdSe core and a ZnS shell with a diameter of 2-6 nm. In this experiment, we adopted a QD compound (QD-IgG) that binds to biomolecules by antigen-antibody reactions. The nature of the compound composed of a QD and a biomolecule is stable, thus it is conducive to laboratory observation.

Heat shock proteins (HSP), also known as 'chaperonins', play a vital role in cell growth, development, differentiation, gene transcription and other functions. Studies have shown that HSP70 has anti-tumor activity and participates in anti-tumor immunity (19). Survivin is a new member of the inhibitor of apoptosis protein family and is the most powerful inhibitor of apoptosis known. Currently, survivin is the subject of intensive research (20).

In this experiment, we performed an analysis of the phosphostability of QDs and FITC by simultaneously labelling survivin and HSP70 proteins in Tca8113 cells with QD655nm and FITC, respectively, using two-colour immunofluorescence. QD655nm-tagged survivin and FITC-tagged HSP70 were excited at an excitation wavelength of 488 nm. Using a laser confocal microscope, three different colours of fluorescence, red, green and yellow, were observed. QD655nm-tagged survivin presented as red fluorescence and was mainly distributed in the cytoplasm, while FITC-labelled HSP70 presented as green fluorescence with bright fluorescence in both the cytoplasm and nucleus. The overlap of these two proteins was shown as yellow fluorescence, indicating that the two kinds of proteins were both expressed in this region.

We then performed a photostability comparison between QD655nm and FITC. After 40 min of continuous high-intensity confocal laser excitation, the red fluorescence of QD655nm experienced no significant weakening, whereas the FITC green fluorescence was almost quenched. The excitation wavelength used for QD655nm was 488 nm rather than its maximum, whereas the excitation wavelength used for FITC was its maximum. Hence, it could be assumed that if a shorter excitation wavelength was used, there would be a greater difference between the intensities of QD655nm and FITC (21,22).

The photostability of quantum dots is well known (21,22), but the data and the photofluorograms presented in this study are clear. They demonstrate that QD655nm has a high fluorescence signal and superordinary photostability in fluorescence imaging, which can be used to detect the position of intracellular proteins.

In conclusion, QDs have better fluorescence intensity and photostability than FITC, and are more appropriate for studies that require a long dynamic observation of physiological changes in cells.

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