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In situ spectral imaging of marker proteins in gastric cancer with near-infrared and visible quantum dots probes

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ABSTRACT

This study presents the investigation of bioconjugating ability of near-infrared (NIR) CdSeTe/ZnS quantum dots (QDs) (710 nm) and visible CdSe QDs (595 nm) in immunofluorescent staining for cancer biomarkers in gastric cancer tissues probed with the homemade Hadamard transform (HT) spectral imaging microscope and a commercial multispectral imaging system. The results show that imunostaining ability of NIR QDs probes is stronger than that of visible QDs when the two kinds of QDs are simultaneously used to probe the cancer biomarkers such as cytokeratin 20 (CK20) and proliferating cell nuclear antigen (PCNA) in gastric cancer tissues. Moreover, when the two QDs probes are used for immunostaining successively for the same target molecules, staining order has great influences on the final results due to their different conjugating ability to the marker proteins. The results imply that NIR QDs hold more promise for real-time imaging of tumor tissues due to its higher sensitivity and contrast. In addition, the results also demonstrate the potential of Hadamard transform spectral imaging as a useful tool in biomedical analysis and quantitative evaluation for tumor tissues.

1. Introduction

Fluorescent semiconductor nanocrystals quantum dots (QDs) are nanoscale crystalline clusters (1–10 nm), which are quite suitable for applying as fluorescent probes for biomedical investigations. QDs have several unique photo-physical properties such as high quantum yield, strong fluorescence, wide excitation spectrum, narrow fluorescence emission peak, multiple-color emission with a single excitation and long fluorescence lifetime, as well as high optical and chemical stability. Studies of Bruchez et al. [1], Chan and Nie [2] have shown that QDs can be used as biological probes applicable to live cell systems. Recently QDs have achieved important results in the biomedical field [3–5]. Moreover, QDs as fluorescence probes bioconjugate with cancer biomarkers have highlighted its advantages [6–8].

While the fluorescence imaging with QDs has been mainly performed in the visible spectral region, biomedical fluorescence imaging, especially noninvasive *in vivo* imaging, requires deep penetration of light into and out of tissues. The penetration depth of light depends on the absorption and scattering properties of the tissue and the absorbance of water. The best penetration is achieved by light with a NIR wavelength ranging from 700 to 1400 nm [9], which is referred as the "diagnostic window" [10,11]. Up till now, most NIR fluorescence imaging has been done with conventional or recently synthesized NIR dye molecules having over 10% of quantum yield [12–14] such as cyanine and alexa fluorophore series [15,16]. However, NIR dyes have the same inherent limitations as visible dyes, namely, low fluorescence quantum yield, broad emission peaks, rapid photodegradation and narrow absorption spectra [17]. Therefore, NIR quantum dots holds great promise for real-time *in vivo* fluorescent imaging [11,18–22].

Gastric cancer is the second leading cause of cancer death worldwide. It is particularly common in Asia and especially in China and Japan [23,24]. Proliferating cell nuclear antigen (PCNA) and cytokeratins (CK) are molecular biomarkers, which can be used for diagnosis and prognosis of gastric cancer. PCNA acts as a process factor for DNA polymerase delta in eukaryotic cells, which is important for both DNA synthesis and DNA repairing [25,26]. PCNA is over-expressed in proliferative cells and objectively reflects the proliferative activity of tumor cells. Therefore, PCNA is widely used in prognosis and diagnosing the proliferation activity of tumor cells [27]. CK are proteins of keratin found in the intracytoplasmic cytoskeleton of epithelial tissues [28]. CK20 is commonly expressed in the gastrointestinal tract. In surgical pathology, the immunoassays of cytokeratins are widely used for various epithelial tumor diagnosis and characterization [29].

The Hadamard transform (HT), which is one of the most common mathematic transforms based on square waves, is well

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suited to multiplexed imaging [30]. In our previous reports, a system of HT microscopic fluorescence imaging which can generate 511×512 pixel image was developed using a movable mask and the system was applied to quantify the DNA content in single cells labeled with fluorescent dyes [31]. More recently, we developed a HT spectral imaging microscope and it was successfully applied to capture four-dimensional (4D) images for samples-location coordinate (X and Y), fluorescence intensity (Z) and wavelength or time to probe important intrinsic or extrinsic biomolecules in cells or tissues [32].

In this study, we used HT spectral imaging technique to investigate the difference bioconjugate efficiency on the cancer biomarkers in gastric cancer tissues between NIR CdSeTe/ZnS QDs and visible QDs probes, and a comparative study was performed with CRi Nuance multispectral imaging technique.

2. Experimental

2.1. Materials and apparatus

Formalin-fixed, paraffin-embedded (FFPE) gastric neoplasms and normal gastric tissue sections were obtained from Hubei Key Laboratory of Tumor Biological Behaviors and Zhongnan Hospital of Wuhan University and were cut into 4 μm sections for QDs-Abs profiling. All the gastric tissue samples were human female patients aged 50 ± 5, and collected in about one-week since their clinical recognition.

Water-soluble CdSeTe/ZnS QDs (emission wavelength ~710 nm and full-width at half-maximum ~68 nm) were purchased from Invitrogen Corporation (U.S.A.) and were used as NIR probes. The surface of the NIR QDs was functionalized with carboxyl group. Visible CdSe QDs emitting at 550 and 595 nm, which were provided by Wuhan Jiayuan Quantum Dots Co., Ltd., were also used as fluorescent probes (FWHM < 30 nm). QDs probes with fluorescence peaks at 550, 595 and 710 nm were prepared after streptavidin was used to conjugate the above three kinds of QDs. Therefore, QDs coupled to streptavidin (QDs-SA) were used as probes immunostaining cancer-marker proteins in this study. Mouse anti-human CK antibody, mouse anti-human PCNA antibody and biotin tagged goat anti-mouse IgG were purchased from Fuzhou MaiXin. Bio. (Fuzhou, China).

The microscopic fluorescence spectra and fluorescence images of the specimens were obtained from the homemade HT fluorescence spectral imaging microscope in our laboratory. The details of the HT imaging microscope have been described in our previous work [32]. The HT fluorescence images and spectra for the tissue sections were captured by a 25×0.65 NA objective throughout the experiment. For acquiring fluorescence spectra and fluorescence imaging the QDs labeled specimens were illuminated with a defocused Argon Laser (35LAP 431, Melles Griot, USA) at 488 nm (4 mW, 4.2×10^3 W/m^2) throughout the experiment. Fluorescence micrographs were captured by DP72 microscope digital camera equipped to a BX51 microscope (Olympus Optical Co., Tokyo, Japan) with blue light from a mercury lamp illuminating the specimens. Digital photos were captured under the same condition with a 10× objective.

Image cubes were captured by CRi's (Cambridge Research & Instrumentation, Inc.) Nuance multispectral imaging system, which consists of an optical head, which was installed on a BX51 (Olympus Optical Co., Tokyo, Japan) microscope's C-mount camera port that includes a liquid crystal tunable filter (LCTF, bandwidth of 20 nm, scanning wavelength range of 500–950 nm) with a spectrally optimized lens system that relays the image to a scientific-grade megapixel CCD. The tunable filter automatically steps in 10 nm increments from 420 to 720 nm while the camera capturing images at each wavelength with 400 ms exposure. The resuled TIFF images (spectral cube, containing the spectra of all pixels) were loaded into the software (Nuance 2.8.0) for further analysis. The software can be used to quantitatively measure the total QD signal (S) and the corresponding area (A) of each unmixing spectral image cube. In this study we use the ratio of S (counts) and A (pixels) to measure the bioconjugation efficiency of QDs for cancer biomarkers.

2.2. Method

NIR QDs are coupled to marker proteins CK and PCNA indirectly through the biotin–streptavidin system. The QDs labeling operation follows the standard protocol of the kit. Mouse anti-human PCNA/CK antibodies are used to connect PCNA/CK in the cell nucleus/membranes, and biotin tagged goat anti-mouse IgG was used to connect the probes of QDs-SA and the mouse anti-human PCNA or CK antibody, respectively. In this experiment, when tissue sections were labeled, visible SA-CdSe QDs were added into the NIR SA-CdSeTe solution and served as indicators for the locations of the lesions.

The specimens were labeled with mixed use of red QDs in visible region and NIR QDs conjugating CK or PCNA. Through the comparison between fluorescence micrographs or the microscopic fluorescence spectra of the visible QDs, NIR QDs and the mixed QDs solution of the two, the labeling ability between the red visible QDs and the NIR QDs were compared. In addition, the same biomarker was labeled repeatedly by using 710 nm QDs and 595 nm QDs to investigate the influence on the multicolor labeling caused by the adding order of different QDs. First, two adjacent sections of a same tissue were stained with excessive 595 nm QDs which are coupled to marker protein PCNA indirectly through the biotin–streptavidin system to make sure that secondary antibodies were connected completely. Secondly, after the two adjacent sections were stained, one of the sections was stained again with NIR QDs-SA. The other one was not stained with any labels. Finally, the two sections were locked with glycerol. After that this step was repeated by reversing the staining order of the two kinds of QDs as control.

3. Results and discussion

3.1. Hadamard transform spectra and fluorescence imaging of NIR QDs labeled gastric tumor tissues

Fig. 1 shows the microscopic fluorescence spectrum and HT image of the gastric cancer tissue (positive sample) and spectrum of normal gastric tissue (negative control) probed by NIR QDs immunofluorescence staining. Curve a shows the fluorescence spectrum of 710 nm QDs probed CK. Curve b shows the spectrum of normal gastric tissue, which clearly exhibits the autofluorescence background from the tissue itself. Curve c, which is the pure fluorescence emission spectrum of CdSeTe QDs probed CK, is produced by subtracting the autofluorescence background (curve b) from curve a. The micrograph in the inset shows the HT fluorescence image of QDs probed CK and it clearly shows that CK locates in the cell membrane, and this is consistent with the expression of CK inside cells. In contrast, the negative control shows no expression of the marker protein (curve b) because no peak at 710 nm appears. These signals can be used as proofs that NIR QDs are fluorescence probes and indeed conjugated to the marker protein CK in tumor tissues.

3.2. Staining titer evaluation between NIR and visible fluorescent QDs probes

In this study, in order to investigate the difference of bioconjugation efficiency between NIR CdSeTe QDs and visible CdSe QDs