Journal of Fluorescence, Vol. 15, No. 5, September 2005 (© 2005)
DOI: 10.1007/s10895-005-2972-x

Double Labeling and Simultaneous Detection of B- and T Cells Using Fluorescent Nano-Crystal (q-dots) in Paraffin-Embedded Tissues

Eran Zahavy,[1] Esther Freeman,[1] Shlomo Lustig,[1] Avi Keysary,[1] and Shmuel Yitzhaki[1,2]

Received June 14, 2005; accepted August 2, 2005

A double immunohistochemical technique for the simultaneous detection of T- and B cells in paraffin-embedded mice tissues have been developed. This procedure is based on using fluorescent nano-crystals (q-dots). The benefit of using q-dots evolves from their unique fluorescence characteristics advantages: such as broad excitation spectrum, narrow emission band and high photo-bleaching threshold compare to organic fluorophores. T cells antigens (CD3) were stained using antibody-coated q-dots with max emission at 655 nm (GαRb-QD655). B cells antigens (CD45R/B220) were stained using streptavidin-coated q-dots with max emission at 585 nm (SA-QD585). The simultaneous detection of T- and B cells was demonstrated in paraffin-embedded lymph node using standard fluorescence microscope.

KEY WORDS: Fluorescence; nano-crystals; quantum-dots; tissues; T cells; B cells.

INTRODUCTION

The ratio between T and B cells and their location are varying within the stage and the type of the disease [1]. For example, changes in lymphocytes subpopulations revealed in tissue sections by confocal microscopy in patients with sequamous carcinoma [2]. Rearrangements of lymphocytes is required for proper generation of immune response, hence it is with great significant to simultaneously detect T- and B lymphocytes in lymphoid tissues. Indeed, such method has been developed using immunogold-silver staining and avidin–biotin–peroxidase complex [3]. This technique had been developed for the staining of frozen sections where proper adjustment of antigens retrieval is less required. Moreover, techniques that are based on enzymatic reaction are prone to non-specific reaction as a result of endogenous enzymes activity and limited by the range of fluorophores exist. Immuno-fluorescent labeling of antigen in tissue sections is a widely used methodology. In principle, these methods include the application of specific antibody following incubation with secondary fluorescent dye conjugate. In most of the cases, the fluorescent dyes are organic molecules such as fluorescein, cyanin dyes, AlexaFluor dyes and more. However, these types of organic dyes are variably limited by relatively rapid and irreversible photobleaching during high intensity illumination. Moreover, conventional fluorescent dyes posses a narrow excitation and broad emission spectra with a relatively small stocks shift, which means that the optimal excitation wavelength is close to the emission peak [4–6]. The last characteristic necessitates a stringent requirement of optical filters for the measurements of fluorescence signals especially when double labeling is required. Unlike organic dyes fluorescent nano-crystal (q-dots) have high photo-bleaching threshold and high quantum yield. Moreover, q-dots have narrow and symmetric emission band that is independent of the excitation wavelength, making it possible to

[1] Israel Institute for Biological Research, Department of Infectious Diseases, Ness-Ziona 74100, Israel.
[2] To whom correspondence should be addressed. E-mail: yitzhaki@iibr.gov.il

excite all colors of q-dots simultaneously with a single excitation light source [7,8]. The q-dots have been modified in several methods [9–12] in order to attach them to biological molecules and in the same time to maintain their spectroscopical characteristics in aqua solution. Surface modification of the q-dots has enabled to utilize the new technology in biological methods [12] such as fluorescence imaging *in vivo* [13–15], immunostaining [10,16,17] and more. Here we report a double labeling and simultaneous T and B cells detection, using fluorescence microscope and a single filter. This was applicable in paraffin-embedded tissues by the finding of antigen retrieval condition that appropriates to both antigens and the use of q-dots.

MATERIALS AND METHODS

Materials

The q-dots conjugates: IgG goat anti-rabbit-coated q-dots with max emission at 655 nm (GαRb-QD655) and streptavidin-coated q-dots with max emission at 585 nm (SA-QD585) were purchased from Quantum Dot Corporation. Rat anti-human CD3 (MCA1477) was purchased from Serotec, rat anti-mouse CD45R/B220 biotin (1665-08) was purchased from SouthernBiotech, rabbit anti-rat IgG (R9255) and goat anti-rabbit-FITC (GαRb-FITC) were purchased from Sigma and Cy2-conjugated goat anti-rat IgG (GαRt-Cy2) from Jackson immunoresearch (#112-225-167),

Sample Preparation

Spleen and axillary's lymph node from adult BALB/c mice were fixed in 10% formaldehyde. Tissues were dehydrated, embedded in paraffin and sectioned to a size of 5 μm. Following deparaffinization and rehydration the paraffin-embedded sections were placed in 1mM EDTA in Phosphate buffer saline (PBS) for 20 min at 95–98°C and allowed to cool in the same buffer for another 20 min. This treatment was necessary to reveal the CD3 antigen on T cells. The sections were washed in distilled water and placed in bath containing PBS.

Staining of Specimens

All incubation steps were performed at 37°C for 1 hr in humid chamber unless indicated otherwise. All dilution steps (antibodies and q-dots) were performed in PBS containing 1% BSA. Sections were washed gently between each incubation step using PBS containing 0.05% Tween 20. Sections were labeled first with rat anti-CD3 for 1 hr following over night incubation at 4°C. The sections incubated with secondary antibody rabbit anti-rat IgG (1:500), washed and incubated for 30 min with GαRb-QD655 (1:500) (Fig. 1). For the staining of B cells (Fig. 1), the specimens were incubated with rat anti-mouse CD45R/B220 biotin (1:100), washed and incubated with SA-QD585 (1:1000) for 15 min. The specimens were washed and mounted with glycerol gelatin (Sigma).

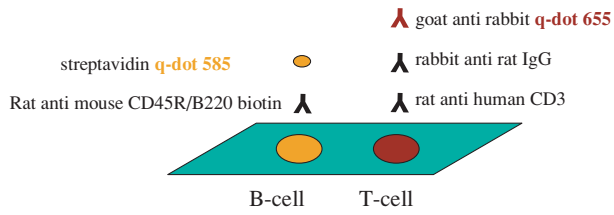

Fig. 1. Schematic representation of B- and T cells staining.

Imaging

Immunofluorescence samples were examined with an epifluorescence Zeiss-Axioskop microscope equipped with filter #09 with excitation band path of 450–490 and emission of long path of 520. Multiple ×40 or ×25 fields were captured from each tissue section. Images were captured using Digital camera Nikon coolpix 995.

RESULTS

Photostability of q-dots Compared With FITC

One of the main advantages of q-dots over organic fluorescence dyes is photostability. In order to demonstrate this, cells from axillaries lymph node fixed to a glass slide and the T cells were stained using anti-CD3 antibody followed by detection of GαRb-QD655 or GαRb-FITC. Continuous illumination resulted in rapid loss of signal in the slide incubated with FITC. After 2 min of illumination it was difficult to detect any FITC fluorescence signal (Fig. 2A). In contrast, there was no loss of intensity in slide treated with GαRb-QD655 even after 10 min of continuous illumination (Fig. 2B).

Simultaneous Detection of T and B Cells in Paraffin-Fixed Tissues

In order to simultaneously detect fluorescnescent-labeled T and B cells on a standard epifluorescence microscope, that is uses a single optical filter for exicitation

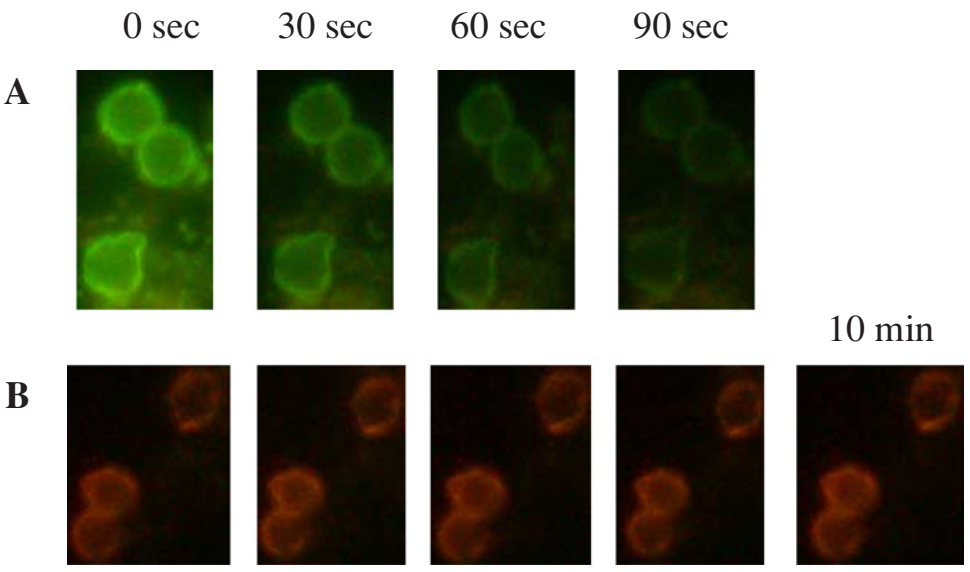

Fig. 2. Photostability of q-dots compared to FITC. Cells from axillaries lymph node were fixed with cold acetone to a glass slide. The slide was incubated with rat anti-CD3, following incubation with rabbit anti-rat. Than the slide was incubated either with GαRb-QD655 (A) or GαRb-FITC (B). Slides were exposed to continuous illumination and images were captured at 0, 30, 60, 90, and 600 s. Objective magnification of 40×.

(450 nm $< \lambda_{EX} <$ 490 nm) and single long-path filter ($\lambda_{EM} >$ 520 nm) for the emission, we have utilized the unique spectroscopical characteristics of q-dots, the broad 1excitation spectrum and the narrow emission band. First T and B cells stained separately in paraffin-embedded lymph node and spleen sections using anti-CD3 or CD45R/B220, respectively. Figures 3A–3C demonstrate the staining of T cells and B cells using GαRb-QD655, SA-QD585 and GαRt-Cy2. When the cells were incubated with SA-QD585 alone or GαRb-QD655, no detectable signal was observed (Fig. 3E), indicating that these conjugates have a very low nonspecific binding in this system. For the staining of B cells no pretreatment was needed. In contrast, in the case of CD3 antigen a pretreatment of antigen retrieval was required otherwise no staining of T cells was observed. In order to detect T and B cells simultaneously in paraffin-embedded tissue a compatible pretreatment procedure for the detection of both antigens was required. We have found that a combination of proteolytic pretreatment using Protease type XXIV and heat treatment is the most useful procedure for the detection of T cells CD3 antigen but it diminished the CD45R/B220 epitope in a way that the antibody can no longer detect it. Heat treatment alone did not harm the immunological reactivity of CD45R/B220 epitope however for efficient staining of CD3 antigen an extended incubation period (over night) was required. We adopted the heat treatment procedure for the simultaneous detection of B and T cells in paraffin-embedded tissues. Figure 3D represents staining of B- and T cells in the lymph node observed under epifluorescence microscope using single filter of excitation band path of 450 nm $> \lambda >$ 490 nm and emission of long path of $\lambda >$ 520 nm. T cells were stained with rat anti-human CD3 antibody, following by rabbit anti-rat and GαRb-QD655 (appear in red). B cells were stained using rat anti-mouse CD45R/B220 biotin conjugate and SA-QD585 (appear in orange).

DISCUSSION

In this paper, we have used the unique spectroscopical properties of q-dots (photostability, broad excitation band, narrow emission) to observe simultaneously T- and B cells in paraffin-embedded tissues on basic epifluorescence microscope. Recently, the application of q-dots to IHC of paraffin-embedded tissues was reported [18,19]. In the first study, Her2 was detected on sections of mouse mammary tumor tissue with q-dots [19], and in the second [18] a combination of tyramide and q-dots had used to provide a sensitive and stable fluorescence signal. These studies used only single antibody for detection hence no adjustment of antigens retrieval was needed. Clearly, the visualization of two q-dots colors simultaneously without switching the filter cube together with the very photostable emission makes the q-dots very convenient and

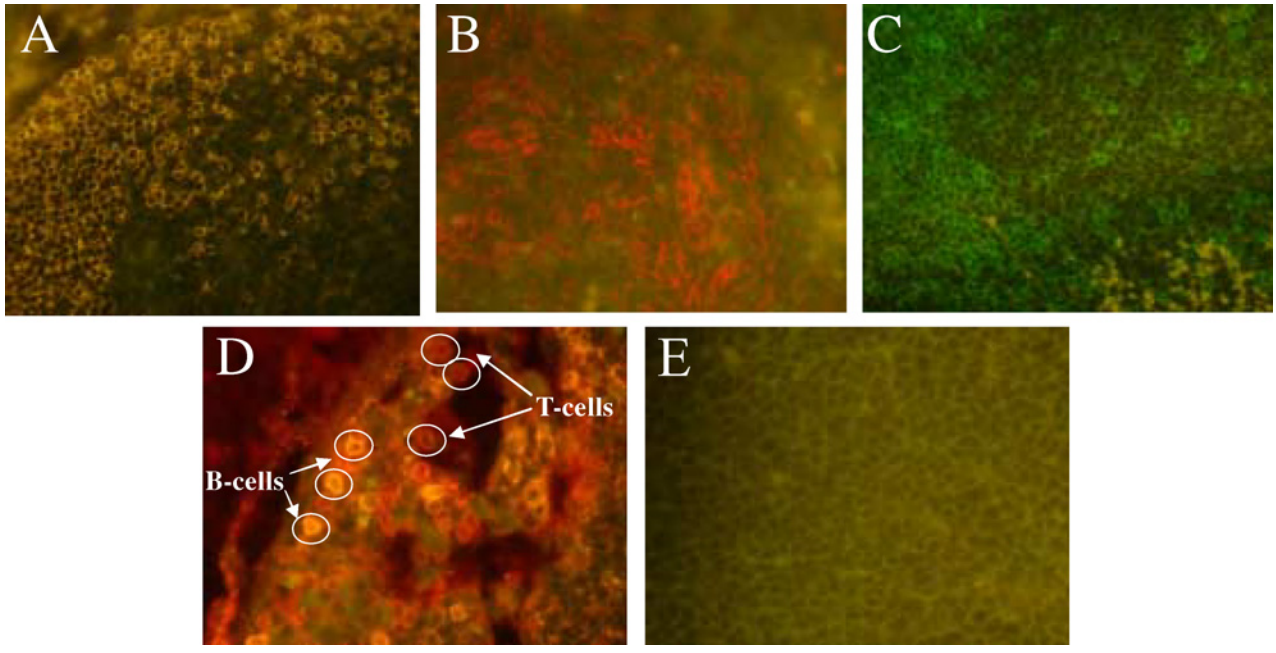

Fig. 3. Simultaneous detection of T- and B cells in paraffin-fixed tissues. Paraffin-embedded sections of lymph node (A, B, D, and E) and spleen (C) underwent antigen retrieval before immunostaining procedures. (A) B cells stained using rat anti-mouse CD45R/B220 biotin and SA-QD585. (B) T cells stained using rat anti-human CD3, rabbit anti-rat IgG and GαRb-QD655. (C) T cells stained using rat anti-human CD3 and GαRt-Cy2. (D) Simultaneous staining of B and T cells was stained as described in A and B. Negative control (E) was stained with SA-QD585 and GαRb-QD655 without the use of primary antibodies.

simple to use. The large range of q-dots dyes and their characteristics allowed analyzing numerous antigens simultaneously. This is especially important in complicated samples when large parameters need to be analyzed.

REFERENCES

1. S. Poppema, A. K. Bhan, E. L. Reinherz, R. T. McCluskey, and S. F. Schlossman (1981). Distribution of T cell subsets in human lymph nodes. *J. Exp. Med.* **153**, 30–41.
2. B. Vidal-Rubio, M. Sanchez-Carril, J. Oliver-Morales, A. Gonzales-Femandez, and F. Gambon-Deza (2001). Changes in human lymphocyte subpopulations in tonsils and regional lymph nodes of human head and neck squamous carcinoma compared to control lymph nodes. *BMC Immunol.* **2**, 2
3. H. Sako, Y. Nakane, K. Okino, K. Nishihara, M. Kodamam, M. Kawata, and H. Yamada (1986). Simultaneous detection of B-cells and T-cells by a double immunohistochemical technique using immunogold-silver staining and the avidin–biotin–peroxidase complex method. *Histochemistry* **86**, 1–4.
4. J. B. Birk (1970). *Photophysics of Aromatic Molecules*, Wiley/Interscience, London.
5. R. P. Haugland (2002). *Handbook of Fluorescent Probes and Research Products*, 9th ed., Molecular Probes, USA.
6. B. Valeur (2002). *Molecular Fluorescence: Principles and Applications*, Wiley-VCH, Weinheim.
7. M. A. Hines and P. Guyot-Sionnest (1996). Synthesis and characterisation of strongly luminescing ZnS-capped CdSe nanocrystals. *J. Phys. Chem.* **100**, 468–471.
8. A. P. Alivisatos (1996). Perspectives on the physical chemistry of semiconductor nanocrystals. *J. Phys. Chem.* **100**, 13226–13239.
9. M. Han, X. Gao, J. S. Su, and S. Nie (2001).Quantum-dot-tagged microbead for multiplexed optical coding of biomolecules. *Nat. Biotech.* **19**, 631–635.
10. E. R. Goldman, G. P. Anderson, P. T. Tran, H. Mattoussi, P. T. Charles, and J. M. Mauro (2002). Conjugation of luminescent quantum dots with antibodies using an engineered adaptor protein to provide new reagents for fluoroimmunoassays. *Anal. Chem.* **74**, 841–847.
11. D. Gerion, F. Pinaud, S. C. Williams, W. J. Parak, D. Zanchet, S. Weiss, and A. P. Alivisatos (2001). Synthesis and properties of biocompatible water-soluble silica-coated CdSe/ZnS semiconductor quantum dots. *J. Phys. Chem. B* **2001**, 8861–8871.
12. M. Bruchez Jr., M. Moronne, P. Gin, S. Weiss, and A. P. Alivisatos (1998). Semiconductor nanocrystals as fluorescent biological labels. *Science* **281**, 2013–2016.
13. X. Michalet, F. Pinaud, L. A. Bentolila, J. M. Tsay, S. Doose, J. J. L. Sundaresan, A. M. Wu, S. S. Gambhir, and S. Weiss (2005). Quantum dots for live cells, *in vivo* imaging and diagnostics. *Science* **307**, 538–544.
14. M. E. Akerman, W. C. W. Chan, P. Laakkonen, S. N. Bhatia, and E. Ruoslahti (2002). Nanocrystal targeting in vivo *Proc. Natl. Acad. Sci. U.S.A.* **99**, 12617–12621.
15. B. Dubertret, P. Skourides, D. J. Norris, V. Noireaux, A. H. Brivanlou, and A. Libchaber (2002). *In vivo* imaging of quantum-dots encapsulated in phospholipid micelles. *Science* **298**, 1759–1762.
16. E. R. Goldman, E. D. Balighian, H. Mattoussi, M. K. Kuno, J. M. Mauro, P. T. Tran, and G. P. Anderson (2002). Avidin: A natural bridge for quantum dot—Antibody conjugates. *J. Am. Chem. Soc.* **124**, 6378–6382.

17. B. Sun, W. Xie, G. Yi, D. Chen, Y. Zhou, and J. Cheng (2001). Microminiaturized immunoassays using quantum dots as fluorescent label by laser confocal scanning fluorescence detection. *J. Immunol. Meth.* **249**, 85–89.
18. J. M. Ness, E. S. Akhtar, C. B. Latham, and K. A. Roth (2003). Combined tyramide signal amplification and quantum dots for sensitive and photostable immunofluorescence detection. *J. Histochem. Cytochem.* **51**, 981–987.
19. X. Wu, H. Liu, J. Liu, K. N. Haley, J. A. Treadway, J. P. Larson, N. Ge, F. Peale, and M. P. Bruchez (2003). Immunofluorescence labeling of cancer marker Her2 and other cellular targets with semiconductor quantum dots. *Nat. Biotech.* **21**, 41–46.