QUANTITATIVE AND MULTIPLEXED IMMUNOCYTOCHEMISTRY USING A MICROFLUIDIC QUANTUM DOT IMMUNO-STAINING SYSTEM

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ABSTRACT

We firstly verify the reason of multiplexing result distortion in sequential multiplexing method of quantum dot (QD) immunohistochemistry (IHC). We found out that competitive binding of secondary antibodies (Abs) induces unfavorable binding of next sequence QDs on the primary Ab in previous sequence. In this study, a microfluidic QD multiplexing method was introduced which can multiplex Abs simultaneously without any unfavorable binding of QDs. We showed multiplexing capability of 16 Abs on the SK-BR-3 cell block. Consequently, this method enables relative quantification of proteins such as estrogen receptor (ER), ErbB2, progesterone receptor (PR) and Ki-67.

KEYWORDS: Sequential multiplexing, Quantum dot, Immunocytochemistry, Microfluidics.

INTRODUCTION

Quantum dot (QD) nanoparticle, which has superior optical properties, has been received increasing attention from biologists and pathologists as a promising labeling molecule which is able to realize multiplexed protein identification and quantitative analysis in immunohistochemistry (IHC) and immunocytochemistry (ICC). Sequential QD multiplexing method is widely used in many laboratories due to its flexibility and high fluorescence signal using QD-secondary Ab conjugates (Figure 1A).

However, distorted protein multiplexing results in the sequential multiplexing based on QD-IHC have been reported in the recent papers. Fluorescence resonance energy transfer (FRET) effect between two different QDs was suspicious for explaining this problem [1]. Some reports assumed that this is due to the cross reactivity of QD-secondary Ab conjugates [2]. Nevertheless, there is no reliable evidence to support these hypothesis. This problem limits the usage of QDs in quantitative multiple protein analysis.

The cocktail method, which exploits different species of primary Abs and secondary Abs as shown in Figure 1B, has been introduced in QD-IHC to avoid unfavorable binding problem in sequential multiplexing method. Although this method could reduce the total process time, only two or three biomarkers can be multiplexed due to the limited number of antibody species.

Herein, we first demonstrate the reason of sequential QD-IHC multiplexing result distortion with QD-ICC, and introduce a new method which can realize quantitative and multiplexed QD-IHC and ICC method using a microfluidic immunostaining system with reduced reaction time and reagent consumption.

Figure 1. Schematic process of conventional (A) quantum dot (QD) sequential multiplexing method and (B) the QD cocktail multiplexing method.
**EXPERIMENTAL**

The cell block preparation was specifically described in our previous work [3]. Briefly, SK-BR-3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU ml\(^{-1}\) penicillin, and 100 mg ml\(^{-1}\) streptomycin. To make a cell block, the harvested cells were centrifuged. After the fixation process in formalin, it was suspended in agar. Paraffin embedding process was followed to produce a cell block. The cell blocks were then sectioned with 4 μm thickness. The sections were mounted and baked onto positively charged slides, and they were performed to dry for 1 h at room temperature, followed by 1 h in a convection incubator at 60°C.

Both sequential multiplexing and cocktail multiplexing methods were performed to stain ErbB2 protein with QD565 and Ki-67 protein with QD655. In the sequential multiplexing method, anti-ErbB2 rabbit primary Abs, anti-Ki-67 rabbit primary Abs, and anti-rabbit goat secondary Ab-QD conjugates were used to stain two proteins in the order of ErbB2 and Ki-67. A mixture of anti-ErbB2 mouse Ab and anti-Ki-67 rabbit Ab were used in the cocktail multiplexing method to stain two proteins with their corresponding QD conjugates. For the microfluidic multiplexed QD-ICC method, a microfluidic device was mounted on the SK-BR-3 cell block slide. A more detailed process of device preparation and reversible device bonding was described in our previous work [3]. ER, ErbB2, PR, and Ki-67 rabbit Abs were injected to each inlet with β-actin mouse Ab to perform relative quantification by normalizing each protein expression with β-actin, which is one of the housekeeping gene expressed protein. This process is described in Figure 2.

**RESULTS AND DISCUSSION**

We hypothesized that distorted result in the sequential QD multiplexing is caused by the unfavorable binding of next sequence QD-secondary Ab conjugates to the previous sequence primary Ab. The localization of ErbB2 and Ki-67 is cell membrane and nucleus, respectively, so unfavorable binding can be verified by protein localization. Figure 3C–E used rabbit Abs and Figure 3F–H used both of rabbit Ab and mouse Ab. Because QD655 was incubated in the second sequence, red color was expressed not only in the nucleus but also in the membrane region (Figure 3E). This indicates that distorted QD multiplexing result is caused by competitive binding of QD conjugates of the next sequence. However, there was no unfavorable binding of QDs in Figure 3H, which used a cocktail method.

![Figure 2. Schematic of multiplexed immunocytochemistry (ICC) method using a microfluidic QD immunostaining system.](image)

![Figure 3. Fluorescent images of SK-BR-3 cell blocks stained by primary Abs and QD conjugates in a microchannel. (A) ErbB2 with QD565. (B) Ki-67 with QD655. (C–E) Conventional QD sequential and (F–H) cocktail multiplexing results in the sequence of ErbB2 with QD565 and Ki-67 with QD655 (D,G: QD565 image, E,H: QD655 image). White arrows in panel E indicate unfavorable binding of QDs.](image)
Multiple microchannels enable the realization of multiple Ab incubation on the same cell block. Eight channels were applied on the SK-BR-3 cell block with four rabbit primary Abs (ER, PR, ErbB2, and Ki-67), one mouse primary antibody (β-actin), anti-rabbit QD525, and anti-mouse QD655 (Figure 4). This result represents that 16 Abs can be multiplexed with this method, without unfavorable binding problem. Also, eight Abs can be multiplexed with reference protein which enables relative quantification of proteins. In Figure 5, four protein expressions were compared quantitatively with this system by using spectrum analysis.

CONCLUSION

In summary, we experimentally showed the conventional multiplexing problem in QD-IHC or QD-ICC techniques. To solve the unfavorable binding problem and limitation of multiplexing capability, a microfluidic QD multiplexing method was proposed. Eight primary Abs can be multiplexed quantitatively with this method, without any unfavorable binding of QDs.

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