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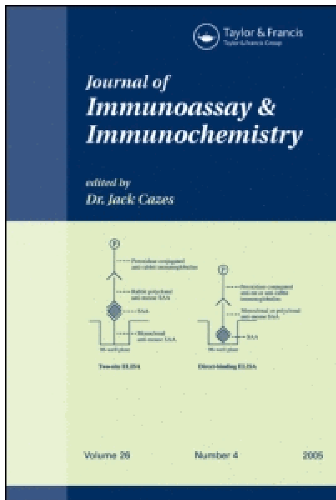

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COMPARATIVE ANALYSIS OF DIRECT FLUORESCENCE, ZENON LABELING, AND QUANTUM DOT NANOCRYSTAL TECHNOLOGY IN IMMUNOFLUORESCENCE STAINING

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□ *A comparative analysis was performed to determine the sensitivity and efficiency of three fluorescent labeling techniques, including direct fluorescent-antibody staining (FA), Zenon labeling, and quantum dot (QD) nanocrystal technology. Two varicella-zoster virus immunoglobin (Ig) G forms, mAb 4F9 and mAb g62, were selected for these studies. The results indicated that: (1) All three methods demonstrated similar brightness and photostability; (2) the time required to conjugate the antibody varied, with Zenon labeling being the quickest; and (3) the stability of each conjugated complex was different, with FITC/rhodamine-conjugated antibody being the most stable.*

Keywords conjugation, immunofluorescence staining, quantum dot, varicella-zoster virus IgG, Zenon labeling

INTRODUCTION

Immunofluorescence staining has become a standard method in many virus laboratories for the rapid and direct detection of viral antigens in clinical samples. The most conventional techniques to detect endogenous proteins using fluorescence are divided into two major types: (1) direct fluorescence antibody staining (FA), using fluorophore-labeled primary antibody as the probe, provides a simple and easy procedure for immunoassay; and (2) indirect fluorescence antibody staining (IFA) in which a secondary antibody labeled with fluorophore is used to recognize a primary antibody. Since various fluorophore-labeled secondary antibodies are commercially available, one can choose the proper antibodies by considering

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the properties of the fluorophores and the animal species of the primary antibodies.[1] However, multiple antibodies derived from the same origin are not applicable for indirect immunofluorescence staining (IFA). For example, in dual staining using primary monoclonal antibodies derived from mouse origin, anti-mouse fluorophore-labeled secondary antibody will simultaneously react with the two primary antibodies and consequently makes it hard to distinguish different antigens. Therefore, in some cases, it is advantageous to use primary antibodies directly labeled with a fluorophore. This direct method (FA) decreases the number of steps in the staining procedure, requiring a single incubation with the labeled reagent and a subsequent wash step.[2] More importantly, it often avoids cross-reactivity and high background problems. The direct method is simple and quick as long as the fluorophore-labeled antibodies are available.[1]

The Zenon labeling method[3–5] is a modification of the FA method and has a wide range of applications, especially for multicolor labeling. It takes advantage of the immunoselectivity of the antibody binding reaction by forming a complex between an intact primary immunoglobulin (Ig) G antibody and a fluorophore-, biotin-, or enzyme-labeled Fab fragment directed against the Fc portion of IgG. Simple mixing of the labeled Fab fragment with the corresponding primary antibody quantitatively produces the Fab–antibody complex in 10 min. Because this labeling is based on immunoselectivity, the Zenon method does not require the removal of exogenous proteins such as serum albumin or amino-containing buffers from the antibody prior to forming the complex. In this method, cross-reactivity is low with antibodies from other species. However, once the conjugates have been formed and excess Fab is taken up by a blocking reagent, the labeled complexes should be used within approximately 30 min, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

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Recent advances in quantum dot (QD) chemistry have made it possible to transfer quantum dots into aqueous buffers and to modify the surface so that biological affinity molecules such as antibodies and nucleic acid probes can be attached and used as direct labels to detect biological markers in various applications.[6–8] Quantum dots are inorganic nanocrystals that fluoresce at sharp and discrete wavelengths depending on their size and have high extinction coefficients and good quantum yields. However, the large size of QDs conjugated to biomolecules ($\sim$10 to 30 nm) prevents efficient traversal of intact membranes, which restricts their use to permeabilized cells or extracellular or endocytosed proteins.[9]

The present study was designed to compare these three "direct" methods, all of which have the advantage of using multiple antibodies derived from the same origin in a simple staining procedure. The objective was to investigate the efficiency and sensitivity of these techniques, which may be helpful in selecting the appropriate method for research and diagnostics.

EXPERIMENTAL

Conjugation of Fluorescein Isothiocyanate (FITC) or Rhodamine to Antibodies

Monoclonal antibodies (mAbs) to varicalla-zoster virus (VZV) glycoprotein gE (mAb 4F9) and immediate early protein 62 (mAb g62) were selected for labeling. mAb 4F9 reacts with membrane as well as cytoplasmic viral antigen.[10] mAb g62 reacts with both nuclear and cytoplasmic antigens in VZV-infected cells.[11]

Fluorescein isothiocyanate (FITC)-4F9 (5 mg/mL) and rhodamine-g62 conjugated antibodies (5 mg/mL) were prepared as described previously.[12] A ratio of 15 μg FITC (Invitrogen, Carlsbad, CA) per milligram of protein was selected to mix with 4F9 antibody. The mixture was incubated for 1 hr at room temperature with constant agitation. After the incubation period, 1 *M* NH_4Cl (pH 8.0) was added slowly to stop the reaction. The free FITC was then removed from solution by dialysis overnight using phosphate-buffered saline (PBS) (0.01 *M*, pH 7.4). The EZ-label rhodamine protein labeling kit (Pierce, Rockford, IL) was used to conjugate mAb g62; 1 μg of IgG mAb was added to a reaction vial containing NHS-rhodamine red reagent and incubated for 1 hr at room temperature. Free dye was removed using a D-Salt Dextran desalting column (Pierce, Rockford, IL). The labeled antibody was then dialyzed against PBS (0.01 *M*, pH 7.4). Conjugated antibodies were filtered by a 0.2-μm filter and stored at 4°C.

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Conjugation of Zenon Mouse IgG Labeling Reagent to Antibodies

The mouse IgG1 Zenon Alexa Fluo 488 labeling reagent (which creates a green color at 488 nm wavelength) was combined with mAb 4F9, and the mouse IgG1 Zenon Alexa Fluo 555 labeling reagent (which creates a red color at 555 nm wavelength) was combined with mAb g62, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Briefly, 1 μg of each antibody was prepared in PBS solution and incubated with 5 μL of the Zenon mouse IgG labeling reagent for 5 min at room temperature, followed by adding 5 μL of the Zenon blocking reagent into the mixture and incubation for 5 min at room temperature. The labeled complexes were diluted with PBS (to the working concentration of 50 μg/mL) and used immediately.

Conjugation of Quantum Dots to Antibodies

Qdot 565 ITK and Qdot 605 ITK amino quantum dots were used to conjugate mAb 4F9 and mAb g62, according to the manufacturer's

instructions (Invitrogen, Carlsbad, CA). A 2-nmol aliquot of quantum dots in borate buffer was transferred into a 100-kD ultrafiltration unit (Millipore, Billerica, MA) and centrifuged 2 or 3 rounds at 3500 rpm for buffer exchange into PBS (0.01 *M*, pH7.4). After incubation with BS3 (bis-sulfosuccinimidyl suberate) for 30 min at room temperature on a rotator, the mixture was passed over an NAP-5 desalting column pre-equilibrated with PBS. The colored eluent was collected in a glass vial containing excess mouse monoclonal antibody (30 µl of a 5-mg/mL stock for a 2-nmol scale conjugation). After 2 hr of reaction at room temperature, the conjugate was then purified from excess antibody by 5 or 6 rounds of ultrafiltration into 50 m*M* borate (pH 8.3).

Immunofluorescence Staining

Conjugated antibodies were diluted with PBS (0.01 *M*, pH 7.4) at a working concentration of 50 µg/mL and added onto each well of the VZV control slide (BION Enterprise, Des Plaines, IL), which had been fixed and contained one well of uninfected and one VZV-infected cell monolayer, followed by incubation at 37°C for 30 min. Each slide was washed twice with cold PBS, rinsed with water, dried at room temperature, mounted using 50% glycerin/PBS solution, and observed under a fluorescent microscope.

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Comparative Analysis of Three Direct Labeling Methods

To study the stability and reactivity of each conjugated antibody, all conjugated antibodies were filtered by a 0.2-µm filter and stored at 4°C. The reactivity of each labeled antibody was tested by immunofluorescence staining weekly during a 2-month incubation period. The fluorescent intensity of each slide preparation was graded on a 4+ scale as previously described.[13,14] Photostability was monitored by timing the exposure. Higher photostability allowed more time for observation and image capture. Time required for antibody conjugation was calculated according to the procedure of each method as described earlier. Estimated cost for each method included reagent cost and hourly labor cost.

RESULTS AND DISCUSSION

Immunofluorescence Staining of VZV-Infected Cells with Conjugated Antibodies

Reactivity of conjugated antibodies was tested using VZV slides by immunofluorescence staining as described earlier. Dual staining with

FITC-4F9 and rhodamine-g62 demonstrated the localization of green cytoplasmic and membrane staining (VZV gE) and red nuclear staining (VZV IE62) in VZV infected cells, as shown in Figure 1A.

The fluorescent cytoplasm and membrane antigens (VZVgE) as well as nuclear antigens (VZV IE62) stained by conjugated Zenon complexes are shown in Figure 1B.

Due to quantum confinement, different sized quantum dots emit different colors when irradiated with ultraviolet (UV) light.[15] VZV-infected cells stained with a mixture of Qdots conjugated to mAb 4F9 and mAb 62 fluoresced green for cytoplasmic and membrane staining of VZV gE and

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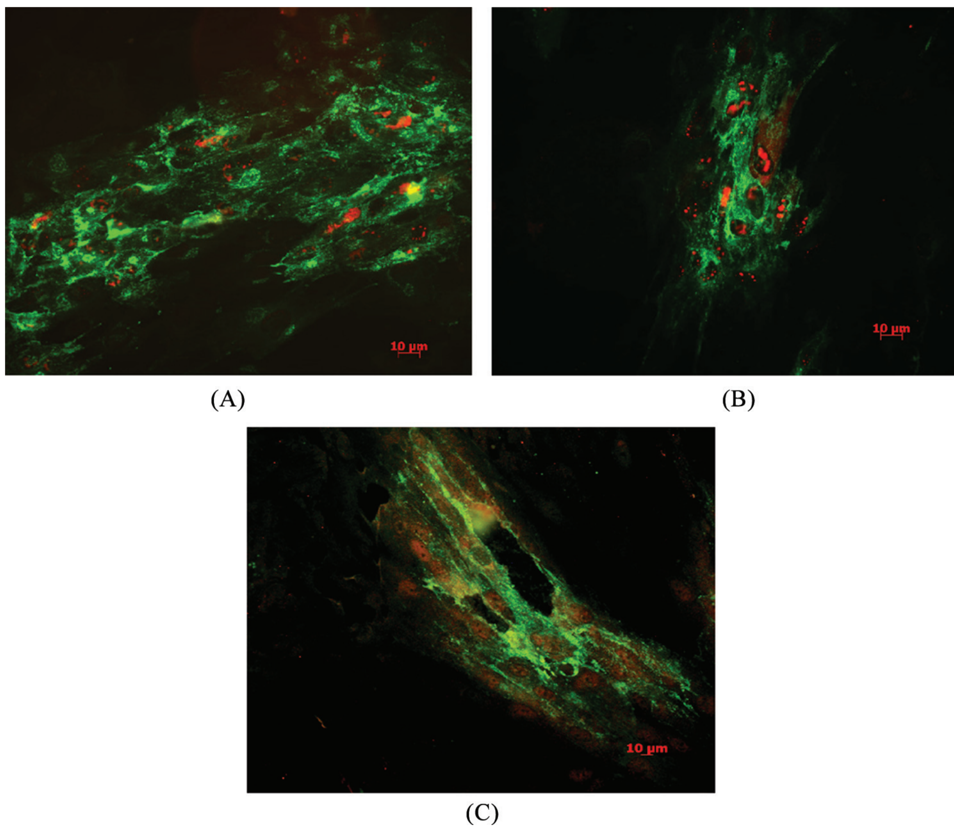

FIGURE 1 Immunofluorescence staining of VZV-infected cells with FITC-4F9 (anti-VZV gE) and rhodamine-g62 (anti-VZV IE62) antibodies (A); Zenon Alex Fluo 488 mouse IgG1 conjugated to mAb 4F9 and Zenon Alex Fluo 555 mouse IgG1 conjugated to mAbg62 (B); and Qdot 565 ITK amino quantum dots conjugated to mAb 4F9 and Qdot 605 ITK amino quantum dots conjugated to mAb g62 (C). VZV control slides were stained with these conjugated antibodies at 37°C for 30 min, respectively. The slides were washed twice with PBS, rinsed with water, dried at room temperature, mounted using a 50% glycerin/PBS solution, and observed under a UV fluorescent microscope (Zeiss). Bars, 10 μm.

red for nuclear staining of VZV IE62 when UV irradiated, as shown in Figure 1C.

Comparative Analysis of Three Direct Labeling Methods

A comparative analysis of these three methods is shown in Table 1. The results presented in this study indicated similar brightness among these three methods. All organic fluorophores undergo irreversible photobleaching during prolonged illumination.[16] It is reported that Qdots, unlike organic dyes, have broad absorption spectrums allowing all colors to be excited with a single wavelength of light, and do not photobleach significantly.[17–19] Our studies substantiated this finding and showed that a higher photostability was obtained with Qdots labeling, which allowed more time for observation and image capture.

As shown in Table 1, the time required to prepare the antibody conjugations was different. Zenon technology only required 10 min to form the fluorophore-labeled Fab–antibody complex. Conjugation of Qdots to antibody required several hours. On the other hand, FITC and rhodamine conjugation required 1 hr of constant agitation at room temperature followed by dialysis overnight. Although FITC and rhodamine conjugation required the longest time, the conjugation complex was the most stable. The FITC-conjugated antibody was stable for at least 2 years at −20°C. The quickly conjugated Zenon complexes were to be used within 30 min in order to obtain best results. Our results indicated that the reactivity of Zenon complexes was completely diminished in 3 weeks (Table 1).

In addition, the results presented in Table 1 indicated that the estimated cost for FA is lowest for reagents and hourly labor costs. Although Zenon complexes and Qdots labeling also work well for

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TABLE 1 Comparative Analysis of Three Direct Labeling Methods

Method	Brightness[a]	Photostability[b] (score 0–5)	Conjugation time[c]	Reactivity stability[d]	Estimated cost[e]
FA	4+	3	18 hr	2 years	Low
Zenon labeling	4+	3	10 min	3 weeks	Medium
Qdots labeling	3+	4	5 hr	2 months	High

[a]Grading intensity: 4+, glaring fluorescence, and 3+, bright fluorescence.

[b]Higher photostability allows more time for observation and image capture.

[c]Time required for antibody conjugation. Zenon labeling required 10 min to form the complex; conjugation of Qds to antibody, 5 hr; FITC (Rhodamine) conjugation with antibody, 18 hr.

[d]Stability of conjugated antibodies was determined by testing the reactivity of each antibody at different time intervals after conjugation.

[e]Estimated cost included reagent and labor cost. Estimated cost for FA, Zenon, and Qdot was $160, $300, and $500, respectively.

immunofluorescence staining, they are more expensive and have a shorter shelf life compared to FA. Therefore, depending on the requirements and budget for a particular project, the most cost-effective method is conventional fluorophore labeling with fluorescein isothiocyanate or the equivalent, but Zenon complexes are rapidly generated for fast results.

CONCLUSIONS

In conclusion, this study indicated that: (1) All three methods demonstrated similar brightness and photostability; (2) the time required to conjugate the antibody varied, with Zenon labeling being the quickest; and (3) the stability of each conjugated complex was different, with FITC/rhodamine-conjugated antibody being the most stable.

DISCLAIMER

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the funding agency.

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