Multiplex Western Blotting Using Quantum Dot Technology

Application Note 106

Abstract

Quantum Dot conjugated antibodies are powerful tools that enable a multiplexed approach when using standard Western blot assay techniques. The ability to utilize optimized filter sets in conjunction with high sensitivity fluorescent imagers significantly enhances the capabilities of fluorescent detection technology. Quantum Dot technology represents a portfolio of powerful imaging reagents that are compatible with both standard Western blot techniques and the FluorChem® line of imagers available from Cell Biosciences. In efforts to determine the merit of using Quantum Dot technology to quantify the detection of several proteins on a single blot, we used routine procedures and methods for detection of the phosphorylation levels of Inhibitor of Kappa Beta Alpha (IκBα) in cell extracts using both standard chemiluminescence and Quantum Dot technology as detection reagents.

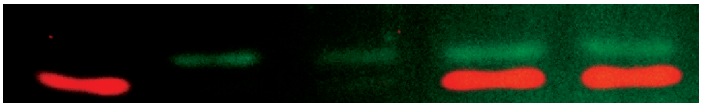

Figure 1. Multicolor Western blot imaged on a low fluorescence (PVDF) membrane (Millipore FL) using standard Quantum Dot emission filters (91-13464-00, 91-13468-00) and the FluorChem HD2 imaging system.

Introduction

Quantum Dots are water-stabilized, multi-layered, nanometer-sized, semi-conductor particles that possess unique physical, optical, and chemical properties for biotechnology applications. Silicon-metal nanocrystal Quantum Dots can replace organic fluorophores in many applications and enable applications in various biological fields including: drug discovery, pre-clinical testing and high throughput screening for drug interactions.

Particle Emission Wavelength	Conjugate Tested	Emission Filte r Catalog Number
585 nm	Goat α-mouse Qdot 585	91-13464-00
655 nm	Goat α-rabbit Qdot 655	91-13468-00

Table 1. Cell BiosciencesEmission Filter compatible with Quantum Dot reagents.

Quantum Dot properties are dependent on the final manufactured size and core composition, and they can be "tuned" for specific emission wavelength and multiplexing application requirements. Quantum Dots fluoresce brightly under ultraviolet irradiation. When Quantum Dots are utilized in blot- or membrane-based applications, this fluorescent excitation can be achieved with simple Ultraviolet (365nm) epi-illumination, as provided in the reflective UV illuminator option available in the FluorChem imaging systems. Detection simply requires an emission filter matched to the emission wavelength of the specific Quantum Dot utilized in the application (Figure 2).

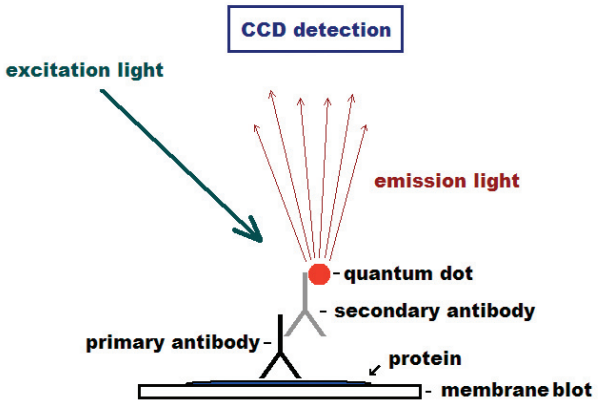

Figure 2. Diagram of Quantum Dot excitation and detection using a CCD based imaging system (FluorChem imager).

Materials and Methods

Cell Culture

Human bladder urothelial cancer (J82) cells were grown to

80% confluence in MEM with 10% FBS. Cells were treated for up to three hours with 25ng/ ml TNFα (R&D Systems, Minneapolis, MN). The media was removed and the cells were washed with ice cold PBS with protease inhibitors (Complete Protease Inhibitor, Roche, Indianapolis, IN).

Cell Extract Collection

Nuclear and cytosolic extracts were prepared using the Pierce NE-PER Kit and protein concentrations were determined with a Micro BCA Kit (Pierce, Rockford, IL), as per manufacturer's instructions.

Immunoblotting

Immunoblots were prepared using 30 µg cytosolic extract loaded onto a 10% tris-glycine SDS gel and electrophoresed at 125V for 1.5 hours in tris-glycine SDS running buffer (BioRad, Hercules, CA). Proteins used with chemiluminescent detection were transferred to a 0.45 µm nitrocellulose membrane using a standard electro-blotting apparatus and ½X tris-glycine SDS running buffer with 20% methanol. Proteins used with Quantum Dot detection were transferred to a 0.45 µm Immobilon-FL membrane (Millipore, Bedford, MA) using a standard electro-blotting apparatus and after pre-soaking the membrane for 30 minutes in methanol.

Primary Antibody Techniques

Primary antibody concentrations were identical for both Quantum Dot and chemiluminescent blots. The following primary antibodies were used: 1:1000 mouse anti-pIκBα Ser 32/ 36 (Cell Signaling Technology, Danvers, MA, #9246), 1:1000 rabbit anti-IκBα (Cell Signaling Technology, #4812), and 1:10,000 anti-β-actin (Abcam #AC-415). All primary antibody incubations were performed overnight.

Quantum Dot Western Blot Techniques

Blots used for Quantum Dot detection were blocked with Seablock (Pierce, Rockford, IL) overnight at 4° C. Membranes were incubated with mouse anti-pIκBα Ser 32/ 36 and rabbit anti-IκBα (Cell Signaling Technology, #4812) overnight at 4° C. After extensive washing (5 X 20 min) the membrane was incubated overnight at 4° C with a mixture of Quantum Dot conjugated secondary antibodies [anti-rabbit Qdot 655 (Invitrogen #1142-2) and anti-mouse Qdot 585 (Invitrogen #1101-1)]. Images were acquired using the FluorChem HD2 imaging system (Cell Biosciences) with the reflective UV lights for excitation and with appropriate emission filters for Quantum Dot detection (91-13464-00, 91-13468-00).

Chemiluminescence Western Blot Techniques

Membranes used for chemiluminescent detection were blocked with 2% BSA in PBS with 0.05% Tween for 2 hours at room temperature. Membranes were incubated with primary antibody (mouse anti-pIκBα Ser 32/ 36) overnight at 4° C. Membranes were incubated with 1:10,000 secondary antibody [anti-mouse HRP (Promega #7622101)] for 2 hours at room temperature. After washing, Amersham ECL substrate was added and the membrane was imaged with the FluorChem HD2. The membrane was then stripped in 100 mM β-mercapto-ethanol, 2% w/v SDS, 62.5 mM Tris HCl pH 6.7 for 30 min at 50° C. After blocking, the detection process was repeated using 1:1000 rabbit anti-IκBα (Cell Signaling Technology, #4812) as primary antibody and 1:10,000 anti-rabbit HRP (Santa Cruz Biotechnology #sc-2301) as secondary antibody. Chemiluminescent images were again acquired with the FluorChem HD2 on the [normal/ high] setting with an exposure time of 5-10 minutes. Images were quantified using the AlphaEase FC software provided with the FluorChem HD2 system.

Results

The FluorChem line of imagers supports detection of multiple Quantum Dot reporters on a single Western or dot blot (Figures 1 and 3). Quantum Dot-based assays exhibit high sensitivity, which conserves sample availability by enabling detection of low sample protein concentrations and quantities, as well as eliminating the requirement for membrane-stripping in order to visualize multiple bands/ targets on a single blot (1). In order to demonstrate the performance of Quantum Dot technology using the FluorChem system, a series of comparisons were made using a well characterized IκBα cell signaling pathway (2).

IκBα is a key transcription factor that plays a role in inflammation, immunity and cancer. IκBα controls the transcriptional capabilities of the nuclear factor NFκβ and the phosphorylation state of IκBα can influence NFκβ nuclear translocation. Upon stimulation with TNFα, IκBα is

phosphorylated and dissociates from NFκβ, which marks IκBα for proteosomal degradation. Once freed, NFκβ is translocated to the nucleus where it transcribes a variety of genes, including IκBα (2). In order to understand IκBα protein levels both phosphorylated and native IκBα protein abundance must be determined. Changes in IκBα protein abundance following TNFα exposure were quantified using both chemiluminescent and Quantum Dot technology.

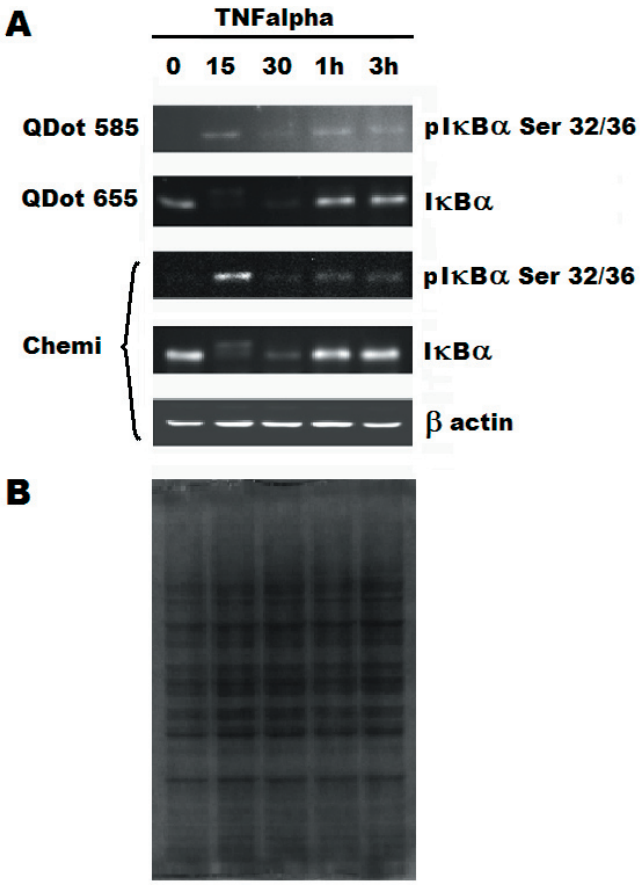

Figure 3A. Comparison between Quantum Dot and chemiluminescent detection technology. Quantum Dot technology gives comparable detection of target proteins when compared to chemiluminescence. 3B. Coomassie stain of a representative gel demonstrating consistent loading. Images obtained with the FluorChem HD2 imaging system.

Primary antibodies to total IκBα and phosphorylated IκBα were utilized in conjunction with appropriate secondary antibodies to evaluate levels of phosphorylated and non-phosphorylated IκBα protein. Figure 4 shows the changes in phosphorylated IκBα (pIκBα) abundance following TNFα treatment. As expected, J82 cells stimulated with TNFα show a rapid, transient increase in pIκBα abundance followed by a decrease in pIκBα protein levels (Figures 3A and 4). Total IκBα protein abundance decreases after initial exposure of J82 cells to TNFα. (Figures 3A and 5). This pattern follows previous reports of IκBα protein abundance after TNFα exposure and overall quantization is similar when using either detection method (chemiluminescence vs Quantum Dot technology) (3, 4).

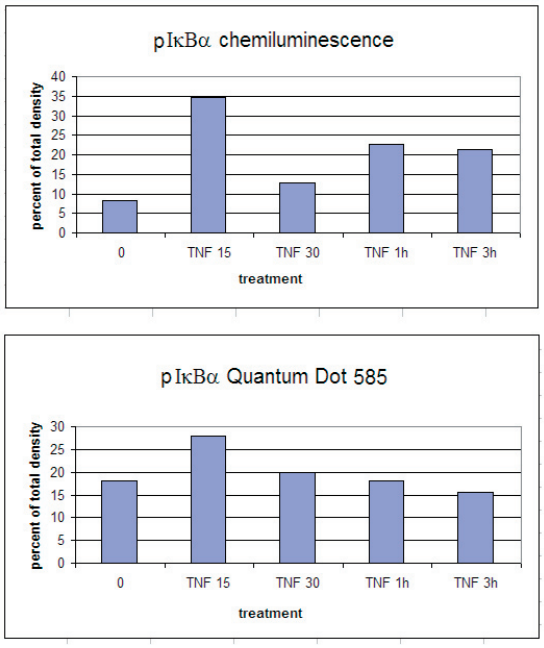

Figure 4. J82 cells stimulated with TNFα exhibit changes in (phosphorylated) pIκBα protein abundance. pIκBα detected using either chemiluminescence or Quantum Dot 585 technology. Imaged using the FluorChem HD2 imaging system (Cell Biosciences).

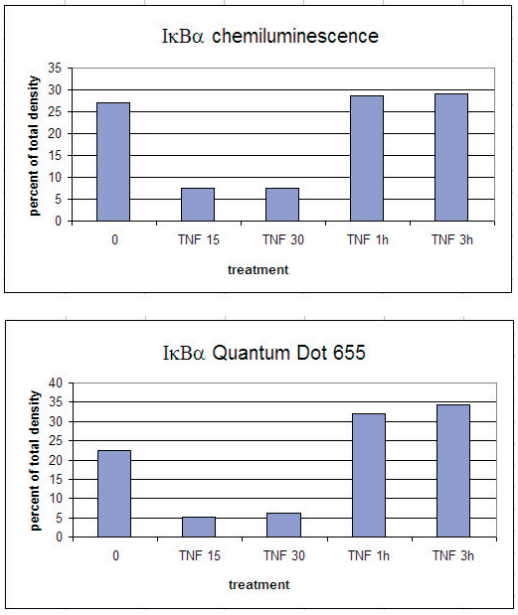

Figure 5. J82 cells stimulated with TNFα exhibit changes in total IκBα protein abundance. IκBα detected using either chemiluminescence or Quantum Dot 655 technology. Imaged using the FluorChem HD2 imaging system (Cell Biosciences).

Discussion

The main advantage of Quantum Dot technology is the ability to probe a membrane and identify multiple protein variants, irrespective of the molecular weight of the target protein. Figures 4 and 5 demonstrate equivalent detection of pIκBα and IκBα protein abundance when utilizing the Cell Biosciences FluorChem HD2 imaging system irrespective of the detection reagents utilized. The FluorChem imaging systems are configured to accommodate both chemilumiluminescent and fluorescent assays.

Quantum Dot technology is effective for the identification of abundant proteins with a narrow spatial separation on standard and low autofluorescence membranes. For example, the ability to distinguish proteins with a very similar molecular weight such as phosphorylated and non-phosphorylated isoforms of a single protein without aggressive techniques such as membrane stripping is made possible by Quantum Dot technology. In addition, simultaneous multiplexed probing of a membrane for multiple proteins greatly increases the efficiency, speed, and ease of identifying and quantifying the abundance of multiple target proteins on a single membrane.

References

1. Makrides S.C., Gasbarro C., Bello J.M., Bioconjugation of quantum dot luminescent probes for Western blot analysis.2005. Biotechniques. 39(4):501-6.
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4. Chen Z.J,, Parent L., Maniatis T., Site-specific phosphorylation of IkappaBalpha by a novel ubiquitination-dependent protein kinase activity. 1996. Cell. 84(6): 853-62.

The FluorChem family of bioimaging systems are designed for applications in Chemiluminescent, Fluorescent, and Colorimetric imaging. The FluorChem line combines sensitivity, resolution and dynamic range providing customers the best in class imaging capabilities for Gel, Membrane and Microplate based assays.

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