Imaging Three–color Western Blots with the Azure c600

Multicolor detection is a powerful application of fluorescent Western blotting. Two or more proteins can be detected on one blot using antibodies labeled with different fluorophores (Figure 1). The ability to simultaneously assay multiple proteins on a Western blot represents a significant advantage of fluorescent detection over chemiluminescent detection, because chemiluminescent detection requires stripping and re-probing a blot or using duplicate blots to detect two proteins, approaches that can introduce errors and variation.

Measuring two or more proteins is important for quantitative Western experiments. With multicolor detection, the amount of a protein of interest can be normalized to that of a housekeeping protein, allowing levels of the protein of interest to be compared between samples. Also, with multicolor fluorescent detection, proteins that are similar in size and not well separated spatially on a gel, such as phosphorylated and non-phosphorylated isoforms of a protein, can be measured at the same time.

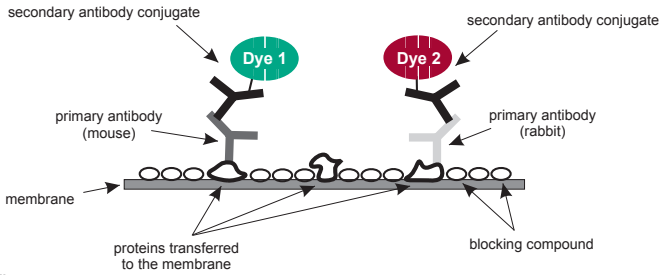

Figure 1

The Azure c600 imaging system is designed for multicolor fluorescent Western blotting, with two near-infrared (NIR) and 3 visible fluorescent channels. This application note demonstrates a procedure to obtain three-color fluorescent blots using WesternDot® reagents. WesternDot® reagents are secondary antibodies conjugated to Qdot® nanocrystals enhanced with VIVID® technology. The WesternDot® reagents WesternDot® 585, WesternDot® 655, and WesternDot® 800 comprise an ideal combination for 3-color Western blotting experiments because these fluorophores have well-resolved, non-overlapping emission spectra (Figure 2). All WesternDot® reagents can be excited with blue or UV light (Figure 2), so imaging each protein independently on a multiplex fluorescent blot requires an instrument such as the Azure c600 that has emission filters specific for the emission of each WesternDot® reagent.

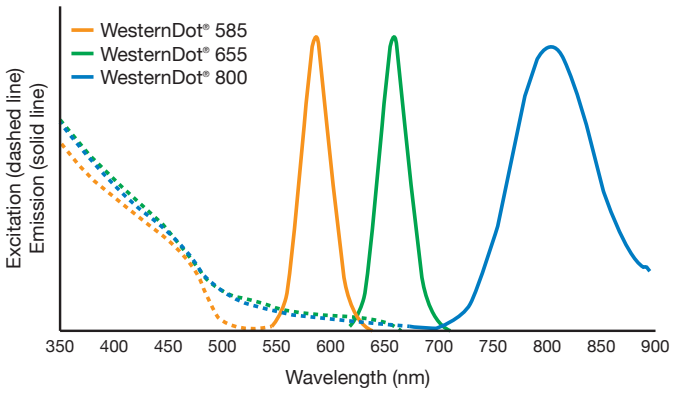

Figure 2

Methods

Approximately 10 µg each of cell lysates from cells induced to express EGFR or phosphorylated-AKT were mixed and loaded on a Novex® Bolt™ 4-12% Bis-Tris Plus 10-well gel. After electrophoretic separation, proteins were transferred to a nitrocellulose membrane using the iBlot® 2 Gel Transfer Device. Western blot processing was carried out with an iBind™ Western Device using the iBind™ FD Solution standard protocol.

The membrane was probed with 2 mls of iBind™ FD solution containing the three primary antibodies: mouse anti-EGFR (diluted 1:200), rabbit anti–phospho-AKT (diluted 1:400), and chicken anti-GAPDH (diluted 1:400). Secondary antibodies used were WesternDot® 585 goat anti-mouse, WesternDot® 800 goat anti-rabbit, and WesternDot® 655 goat anti-chicken (Table 1). All secondary antibodies were diluted 1:100 in iBind™ FD Solution.

The blot was imaged with four imaging systems, as shown in Table 2.

Results

The same three-color blot was imaged with four different imaging systems. The acquired images are shown in Figure 3. The Odyssey instrument (Figure 3a) does not have the capability to detect visible fluorescent dyes, so only one protein, phosphorylated AKT detected in the IR800 channel, is visible. The Typhoon instrument (Figure 3b) has settings for detection of Qdot® 800 and Qdot® 655, and a merged image of both channels is shown. Only GAPDH, detected at 655 nm, is visible; no signal from phosphorylated AKT is seen. The LAS-1000 instrument (Figure 3c) has a blue light source capable of exciting all three WesternDot® reagents, but only one emission filter so all fluorescent signals are detected in a single image. EGFR (detected at 585 nm) and GAPDH are visible, but phosphorylated AKT is not.

Of the four imaging systems used, only the Azure c600 (Figure 3d) detected all three WesternDot® conjugates. The c600 has default settings for common fluorescent dyes and can also be manually configured. To image the WesternDot® conjugates custom settings were used; the blot was excited using the settings for imaging Cy2 (460 nm), and detected using three different emission filters (Table 2). All three channels are merged in the image shown (Figure 3d), with WesternDot® 655 shown in blue, WesternDot® 585 in red, and WesternDot® 800 in green.

The ability to detect three proteins on a single blot dramatically increases the amount of information that can be obtained from a Western blot experiment. For example, two proteins and a loading control can be detected simultaneously, allowing quantitative comparisons to be made between proteins and between samples. WesternDot® reagents are available with a variety of species specificities, for flexibility in designing three-color experiments. With the Azure c600 and WesternDot® reagents, three-color Western blots are at your fingertips.

Primary antibody	Secondary antibody	Excitation/Emission
Mouse anti-EGFR	WesternDot® 585 goat anti-mouse	405-485 nm/585 nm
Rabbit anti-phospho-AKT	WesternDot® 800 goat anti-rabbit	405-485 nm/800 nm
Chicken anti-GAPDH	WesternDot® 655 goat anti-chicken	405-485 nm/655 nm

Table 1: Protein targets, primary antibodies, and secondary antibodies

Imaging system (manufacturer)	EGFR (WesternDot® 585)	GAPDH (WesternDot® 655)	Phospho-AKT (WesternDot® 800)
Odyssey (LI-COR)	NA	NA	800 channel
Typhoon™ FLA 9000 (GE Healthcare)	NA	Qdot® 655, PMT 1000	Qdot® 800, PMT 1000
LAS-1000 (Fujifilm Life Science)	Blue-light excitation 3-second exposure	Blue-light excitation 3-second exposure	Blue-light excitation 3-second exposure
c600 (Azure)	Excite Cy2/ detect orange filter	Excite Cy2/ detect red filter	Excite Cy2/ detect 800 filter

Table 2: Imaging systems and settings used for each target protein

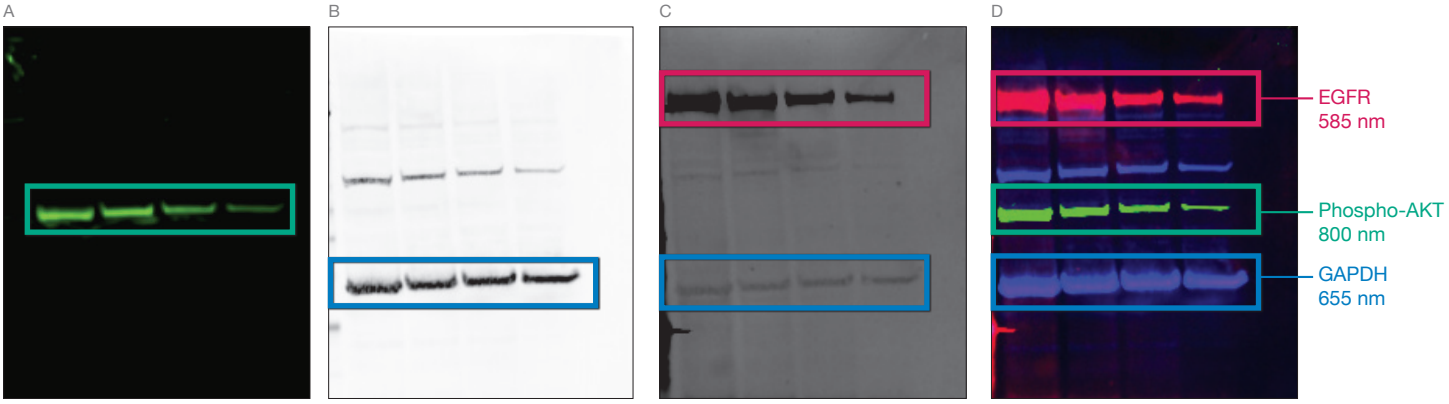

Figure 3. Images of the same three-color Western blot, detected with WesternDot® reagents WesternDot® 585, WesternDot® 655, and WesternDot® 800, collected using four different imaging systems. a. LI-COR Odyssey; b. GE Healthcare Typhoon 9000; c. FUJIFILM LAS-1000; d. Azure c600.

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