Digital imaging for Western blots: why it's essential and what to look for in a digital imaging system

The advent of digital imaging has transformed Western blot detection. Here we outline how Western blotting has changed, and review qualities to look for in a digital imager.

Chemiluminescence and fluorescence

When most people think of imaging their Western blot, they think of going into a darkroom and exposing the blot to film to detect a chemiluminescent signal. A much more powerful alternative is digital imaging, which in the last decade has seen radical advancements of technology, leading to improved speed, sensitivity, and quantitative data when detecting chemiluminescence. This technology has also opened the door to other options such as fluorescent detection for imaging and quantifying Western blots.

Chemiluminescence is a popular detection method for Western blotting because of its inherent sensitivity (Figure 1, Towbin 1979). This technique is a well-established way to detect one protein in a sample. Chemiluminescence is very good at answering the question, "Is my protein there or not?" but it is not very good at addressing other important questions, such as: How much of my protein is present relative to another protein? How much of my protein is in one sample compared to another sample? How do I control for sample loading inconsistencies? While chemiluminescence has become the method of choice for one-signal, one-protein detection (Alegria-Schaffer 2009), the recent introduction of multiplex fluorescence has allowed people to use the spectral properties of fluorescent probes to obtain multiple signals from multiple proteins on one blot (Schutz-Geschwender 2004). In fluorescent detection, the secondary antibody is coupled to a fluorophore with known emission and excitation wavelengths (Figure 2). Multiple secondary antibodies can be detected (multiplex detection) if each antibody is conjugated to a different fluorophore having different spectral characteristics.

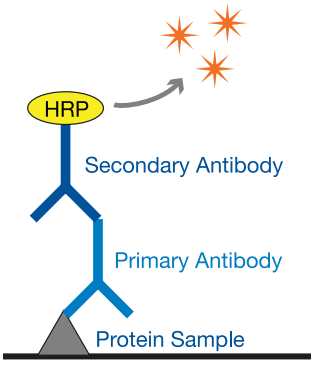

Figure 1. Chemiluminescent Western Blotting. In chemiluminescent detection, the antigen-primary antibody complex is bound by a secondary antibody conjugated to an enzyme, such as horseradish peroxidase (HRP), that generates light in the presence of a luminescent substrate. The light can be detected by exposure to X-ray film, or by CCD imaging systems.

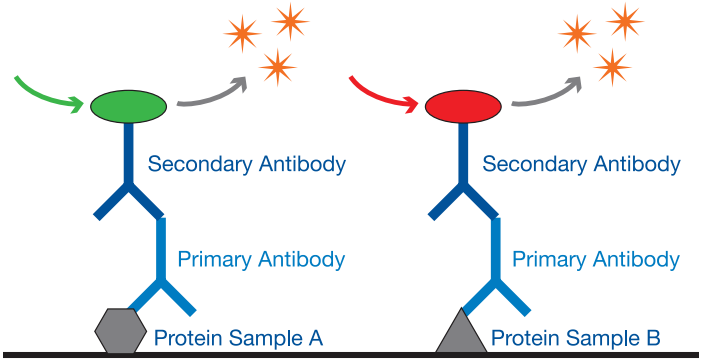

Figure 2. Fluorescent Multiplex Western Blotting. In fluorescent detection, the antigen-primary antibody complex is bound by a secondary antibody conjugated to fluorescent dye. The fluorescence of the dye can be detected by a digital camera in an imaging system equipped with the correct light source to excite the dye, and the correct filters to detect the emitted light. Multiplex detection is possible by using two different fluorescent dyes and an instrument that can excite and detect the light from each.

Why mutiplex?

Why examine more than one protein on a blot? The use of loading controls is a common reason (Figure 3). Loading controls are usually “housekeeping genes”, proteins that exhibit high-level, constitutive expression in the cell type or sample you are examining and are expected to be present at similar levels in all samples. To control for inconsistencies in the amount of protein between lanes on a gel, the signal from the protein of interest is divided by the signal from the loading control for each lane. Using a loading control allows you to compare the amount of protein of interest between samples and have increased confidence that any differences are real.

With chemiluminescence, it is possible to probe for more than one protein when the proteins have different molecular weights and are spatially separated on the blot. However, your protein of interest might not be well separated from your loading control. And, if you probe simultaneously for two different proteins with chemiluminescence, you might mistakenly think a degradation product of the larger protein is the smaller protein, or a multimer of the smaller protein is the larger protein, if the modified products run near the expected molecular weight. For this reason, chemiluminescence is not ideal for multiplexing, even when the two target proteins are different sizes.

Another reason to look at multiple proteins on a single blot is to study post-translational modifications such as phosphorylation, methylation, and glycosylation. Many post-translational modifications do not significantly change the molecular weight of a protein so the unmodified and modified isotypes migrate too closely together to examine using chemiluminescence.

Stripping the primary and secondary antibodies off of a blot and re-probing the blot with a second set of antibodies is an option to detect a second protein using chemiluminescence. However, the most common stripping protocols, in which the blot is incubated with a heated solution containing detergent and a reducing agent, can remove sample protein from the membrane and may not completely strip the first set of antibodies (Yeung 2009). Often the second detection is “dirty”, with background noise that reduces sensitivity. And, if the second target protein has a similar molecular weight to the first one, as is the case for many post-translationally modified proteins, there can be a concern that some of the signal observed after re-probing is leftover signal from the first probing. The procedure takes time, could affect your results, and results never are as clean as a fresh blot. A better alternative is needed.

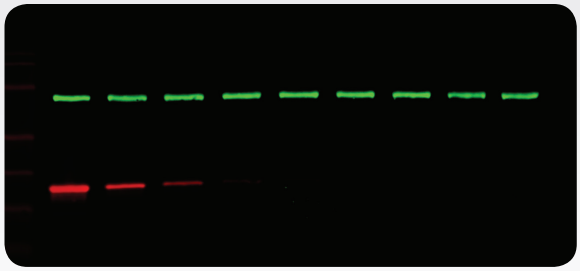

Figure 3. Loading controls enable accuracy when comparing multiple samples. Transferrin was probed for in the 800 channel, while GPDH was probed for in the 700 channel.

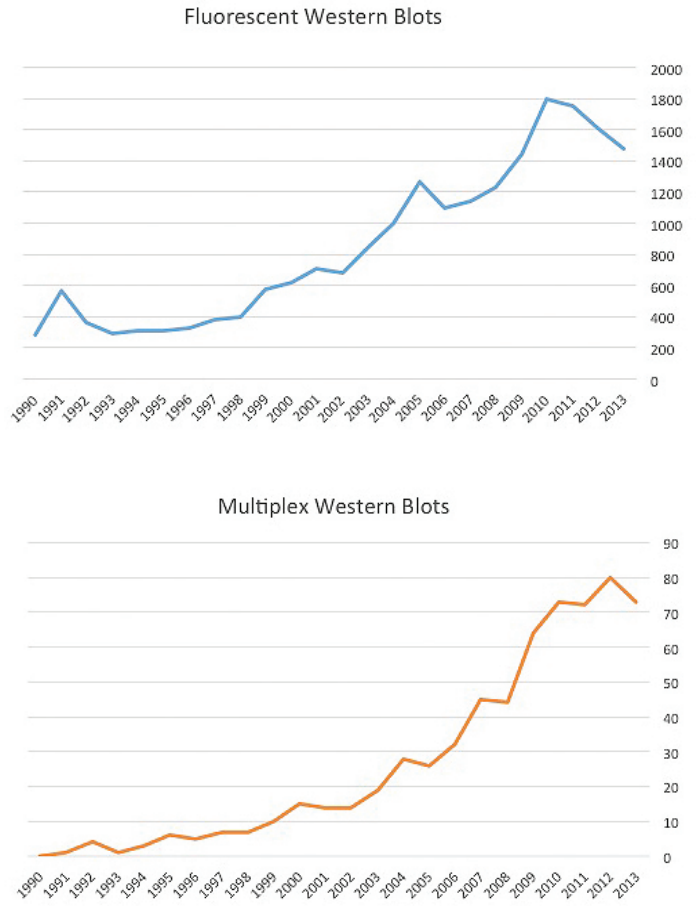

Figure 4. Publication Trends in Western Blotting. Over the past 23 years, the number of publications reporting the use of fluorescent Western blots and of multiplex Western blots has increased tremendously.

Fluorescence detection is a superior method for multiplex detection. The numbers of publications reporting the use of fluorescent Western blots, and of multiplex Western blots, have increased substantially over the past 20 years (Figure 4). Multiplex fluorescent Westerns now allow scientists to probe for multiple proteins at once without stripping and re-probing, and to get clear results regardless of the physical separation of the proteins on the gel (Figure 5).

There are now many commercially available kits on the market to help the customer transition to fluorescent Westerns (Table 1). Of special note are those kits that use infrared fluorescence. Membranes and biomolecules have lower autofluorescence in the longer wavelength near-infrared (NIR) region of the spectrum, so NIR fluorescent Westerns have lower background and higher signal-to-noise ratios than fluorescent blots image in the visible spectrum.

Get out of the darkroom

Most scientists still use a darkroom to process film images of their chemiluminescent Western blots. Film is well known, it's easy, it's sensitive -- why change? Film is expensive. Film developers require toxic chemicals. Film has a small dynamic range and is quickly saturated, so film exposures are not good for quantitative comparisons of chemiluminescent signals. Digital imaging provides a much larger dynamic range so low- and high-intensity bands can be imaged simultaneously. Also, digital imaging produces a file that is immediately ready for publication, while sheets of exposed film must be photographed or scanned to generate a digital image.

Additionally, film can't image multiplex fluorescence. Only digital imaging systems give you this capability.

Important characteristics to look for when purchasing a digital imager

High resolution. High-resolution CCD cameras enable you to see fine detail in your image when you zoom in. The larger the number of pixels, the greater the resolution.

Wide dynamic range. Dynamic range is the measure of signal to noise of a system. Without a wide dynamic range, your strong signals will saturate before you can detect your weak bands, making quantitation impossible (Figure 6).

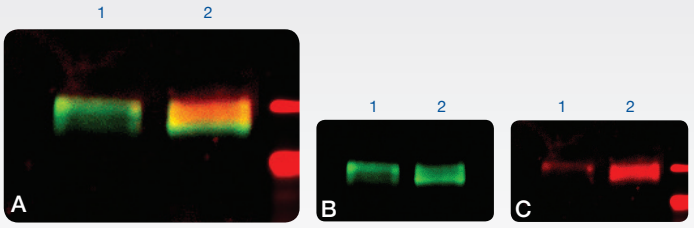

Figure 5. Simultaneous detection of EGFR and phospho-EGFR. Control cells (lane 1) and cells treated with EGF (lane 2) were imaged. EGFR was detected in the green channel (panel B), and phospho-EGFR was detected in the red channel (panel C). Panel A shows the green and red channels superimposed.

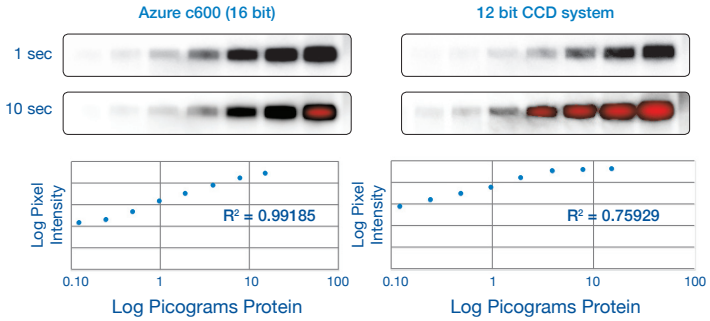

Figure 6. 16 bit Imaging for a Wide Dynamic Range. A Western blot was imaged on both the Azure c600 (a 16 bit system) and a competitive 12 bit system. While the 10 second exposure appears similar on the different systems, the 12 bit system produces an image that is saturated, and not suitable for analysis.

Manufacturer	Kit
Azure	AzureSpectra Fluorescent Western Blotting Kits
LI-COR	LI-COR® Odyssel Western Blotting Kits
Life Technologies	WesternDot® Fluorescent Immunodetection Kits
ProteinSimple	SpectraPlex Fluorescent Western Blot Kit
Abcam	Optiblot Fluorescent Western Blot Kit
Advansta	WesternBright™ MCF and MCF-IR
Rockland Immunochemicals	MaxTag™ for IRDYE® Immunoblotting
Antibodies online/ Genscript	ONE-HOUR Western Fluorescent Kit

Table 1: Commercially available kits.

A small F stop/wide aperture. Especially for chemiluminescence, the F stop is an important value to consider. The smaller the F stop, the wider the aperture, and the more light that can be let in. Small F stops drastically reduce exposure times.

Dye flexibility. The availability of compatible dyes is also important to those who want to do multiplex fluorescent Westerns. You will want to look at a system that has multiple excitation sources and multiple detection wavelengths to be compatible with a wide range of dyes. Additionally, you will want to make sure that the best light sources have been selected. For example, IR dyes are typically excited with lasers, in part because lasers only emit light close to the excitation peak of the dye. Check to make sure the instrument is able to excite and detect the dyes you work with.

Imaging flexibility. Many Western blot imaging systems also offer DNA and protein gel documentation, through such features as dual-wavelength UV transilluminators, white lights, and blue lights. This allows labs to image their routine DNA gels and protein gels stained with common fluorescent and colorimetric stains.

Sensitivity. Another important feature is the sensitivity of the instrument. If you request a demo of the machine, what is the limit of detection? Does it meet your needs?

Ease of use. Finally, only at a demo can you really address the ease of use. While "ease of use" might not sound important, think about the future. Do you want to have to train every new person in the lab on the instrument, or do you want a system that they can figure out by themselves?

In summary, digital imaging provides benefits for both chemiluminescent and fluorescent Western imaging. When choosing a digital imaging system, specs are important, but also test the system yourself to make sure it is well designed and easy to use.

References

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