Three-Color Imaging with the Molecular Imager® FX Pro Plus™ System and Quantity One® Software

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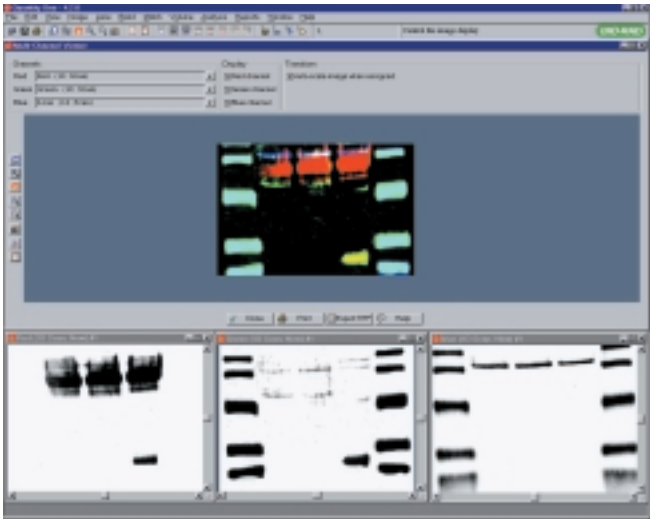
Introduction

A fluorescent imaging system such as the Molecular Imager FX Pro Plus multi-imaging system is ideal for imaging and quantitating fluorescent samples. As an illustration of its capabilities, we imaged a western blot that had been hybridized with 3 different primary antibodies and 3 different secondary antibodies. These secondary antibodies were each conjugated to fluorescent dyes that are optimally excited with 3 different laser lines to minimize fluorescent crosstalk. Our results using the FX Pro Plus and the latest version of Quantity One software demonstrate that fluorescent detection methods can provide a 10-fold greater linear dynamic range with only 2- to 4-fold less sensitivity than chemiluminescent detection.

Molecular Imager FX Pro Plus System

The FX Pro Plus is designed for imaging isotopic samples through storage phosphor technology as well as fluorescent samples through direct fluorescent methods using an internal and optional external lasers. Fluorescent samples are scanned by a laser that is positioned over the sample and is moved across it in a raster pattern. The excitation of the fluorescent sample by the laser is diverted through a fiber optic bundle and delivered through an emission filter and then to a detector called a photomultiplier tube. For this set of experiments, the FX Pro Plus was equipped with an internal 25 mW 532 nm laser, and external 15 mW 488 nm and 10 mW 635 nm lasers. For optimal excitation and detection of FITC, Cy™3, and Cy5 fluorescent samples, scans were taken sequentially to minimize crosstalk that would have been generated between the samples. The peak excitation and emission maxima, respectively, are 494 and 518 for FITC, 550 and 565 for Cy3, and 650 and 670 for Cy5.



Quantity One software for analysis of a 3-color western blot. Top, merged 3-color image; bottom (left to right), red, green, and blue channels.

Quantity One Software

Quantity One software is a package that consists of an acquisition and analysis program that is the main graphical user interface used in Bio-Rad's imaging instrumentation. This software runs on Macintosh® and Windows® (95, 98, 2000, NT) operating systems. The core package of this software program allows the user to quantitate images generated by the various systems, such as the Molecular Imager FX Pro Plus. Once an image is scanned with the FX Pro Plus and a digital file is created, quantitation can be performed using volumetric or profile analysis on that image. The latest release of Quantity One (version 4.2) is capable of merging multiple images and analyzing that color-merged image.

Excitation and Emission Settings

For details of our methodology, refer to Gingrich et al. (in press). Once the blots were hybridized with the appropriate primary and secondary antibodies, the blots were imaged using the Molecular Imager FX Pro Plus. FITC-labeled samples were imaged using the 488 nm external laser with a 530DF30 emission filter. The Cy3-labeled samples were imaged using the internal 532 nm laser with a 605DF50 emission filter, and the Cy5-labeled samples were imaged using an external 635 nm laser with a 695DF50 emission filter.

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Quantity One software for analysis of a 3-color western blot. Top, merged 3-color image; bottom (left to right), red, green, and blue channels.

Bleedthrough of Fluorescent Dyes

The amount of bleedthrough or crosstalk was compared with the 3 different fluorescent samples. In addition to the western blots, dot blots were generated using FITC-, Cy3- and Cy5-conjugated secondary antibodies excited with lasers at optimal excitation wavelengths. When the dot blots were imaged with the appropriate laser lines, minimal fluorescent bleedthrough was observed. The greatest bleedthrough was observed when the Cy3-labeled sample was excited with the 488 nm laser (Gingrich et al. in press). Even for this case, the Cy3 signal was ~2% of the FITC signal when equal amounts of protein were loaded. In the best case, <0.1% bleedthrough of either FITC or Cy3 was present in the Cy5 channel. This demonstrates that fluorescent quantitation from any of these 3 labels is not significantly affected by fluorescent signal from either of the other fluorophores.

Fluorescent and Chemiluminescent Western Limit of Detection

How sensitive is detection using fluorescence versus chemiluminescence? Blots were incubated with ECL™ chemiluminescent substrate (Amersham Pharmacia Biotech) and imaged either on the Fluor-S® MAX CCD imaging system or on X-ray film. From our experience, the sensitivity of fluorescent detection is approximately 2- to 4-fold less compared to using chemiluminescent substrates such as ECL.

Fluorescent and Chemiluminescent Western Linearity

How linear is the signal from a fluorescent compared to a chemiluminescent western blot? We have found that Cy5, Cy3, and FTIC are linear over 4 orders of magnitude, while ECL is linear over 3 orders. In addition, fluorescent detection is linear up to ~20 ng of antigen, while chemiluminescent detection is linear up to only ~2 ng of antigen.

Summary

The main advantage fluorescent detection has over chemiluminescent detection for western blotting is the ability to multiplex. Multiplexing allows the comparison of levels of several target proteins on the same blot. Although fluorescent westerns are not as sensitive as chemiluminescent westerns, fluorescent westerns can provide better linearity and thus better protein quantitation.

Reference

Gingrich JC et al., Multiplex detection and quantitation of proteins on western blots using fluorescent probes, Biotechniques, in press.

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