Imaging and Analysis of DIGE

- Automated scanning of multiplex gels
- Minimization of cross talk between Cy dyes
- Accommodation of samples of varying thickness
- Automated and manual control of detection range
- Automated detection and matching in PDQuest software
- Normalization of data for quantitative analysis
- Color visualization of unique protein spots
- Integrated spot excision
- Automated annotation of identified proteins

Protein Multiplexing With the Molecular Imager® PharosFX™ System and PDQuest™ 2-D Analysis Software

Difference gel electrophoresis (DIGE) is a relatively straightforward application of differential labeling of protein samples using fluorescent dyes. The technique, originally published by Unlu et al. (1997), uses cyanine (Cy) dyes to differentially label proteins separated by 2-D gel electrophoresis. Following labeling and electrophoresis, excellent results can be achieved by scanning the multiplex gels with the PharosFX system and analyzing them with PDQuest 2-D analysis software. The DIGE method is designed to compare two complex protein samples derived from two different growth conditions. One sample is labeled with Cy3 and the other with Cy5. A third dye, Cy2, is used to label a 50:50 mixture of the two samples, which becomes a standard for registering the images obtained with the other two dyes. Equal amounts of the three labeled protein mixtures are combined and run on the same 2-D polyacrylamide gel, eliminating any gel-to-gel variation.

Imaging

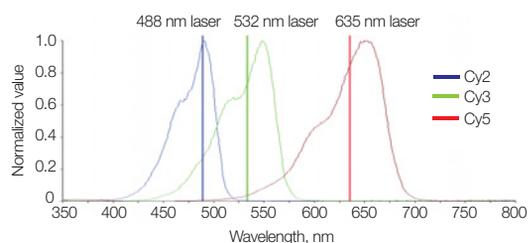**Image Acquisition With the PharosFX System**

The PharosFX multiimager systems are well suited for image acquisition from gels containing Cy-dyed proteins. The laser and filter combinations provided ensure minimal cross talk between the different fluors.

Recommended lasers and emission filters for Molecular Imager PharosFX systems.

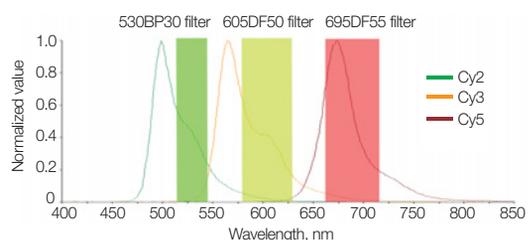| Dye	Laser	Emission Filter	Filter Wheel Location
Cy2	488 nm	530BP30	A2
Cy3	532 nm	605DF50	B3
Cy5	635 nm	695DF55	A4

Lasers

The lasers required for the Cy dyes are the 488 nm external laser for Cy2, the 532 nm internal laser for Cy3, and the 635 nm external laser for Cy5.

Excitation spectra of Cy dyes and excitation wavelengths of the Molecular Imager PharosFX lasers.

Emission Filters

The required emission filters are 530BP30 for Cy2, 605DF50 for Cy3, and 695DF55 for Cy5.

Emission spectra of Cy dyes and bandpass range of the Molecular Imager PharosFX filters.

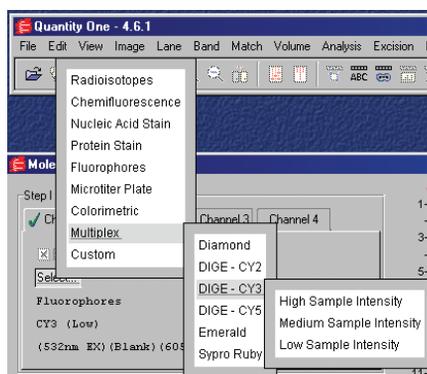**Loading the Gels Into the PharosFX System**

Since proteins are covalently labeled with Cy dyes, post-run staining is not necessary for analysis. Bio-Rad's Ready Gel®, Criterion™, and PROTEAN® II precast gels must be removed from their cassettes and placed on the standard glass sample tray. Gels in glass cassettes may be scanned while in the cassettes as long as the glass has low fluorescence and the plates are not thicker than 1–1.5 mm. Removing at least the top glass plate prior to

BIO-RAD

scanning is recommended. Scans obtained through top glass plates are at least 2-fold less intense than those obtained with the plates removed. Gels removed from their cassettes should be placed on the standard glass sample tray. For gels between thick glass plates, the multi-sample tray II should be used with an appropriate spacer to bring the sample into the proper focal plane for imaging.
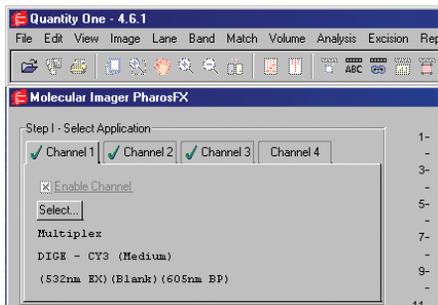
Setting Up the Imaging Software

PharosFX imaging is controlled by Quantity One® software. The acquisition screen contains preset applications to automatically select the correct laser and filter combinations. Select Cy2, Cy3, and Cy5 from the preset applications located in the Quantity One acquisition window.

Preset applications for Cy2, Cy3, and Cy5.
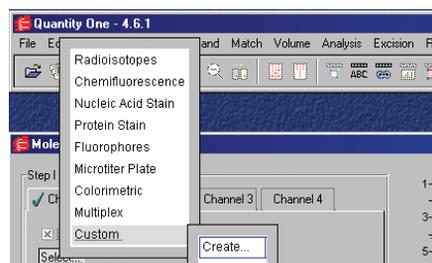
Automated Scanning

Once the applications are created, images can be acquired sequentially or automatically. Make sure that the acquisition frame is tightly wrapped around the gel image, and do not change the frame between successive scans. Since all three images are obtained from the same gel, it is essential that the gel not be moved between scans and that the acquisition frame be the same for all scans. For sequential scanning, simply select the Cy3 setting in channel 1 and scan. Repeat for Cy5 and Cy2. For automated scanning, set three of the channel tabs to conform to the different Cy dyes (e.g., set channel 1 for Cy3, channel 2 for Cy5, and channel 3 for Cy2). The order does not matter. The Enable Channel box must be selected in each channel to automate the acquisition. A green check mark appears for each enabled channel.
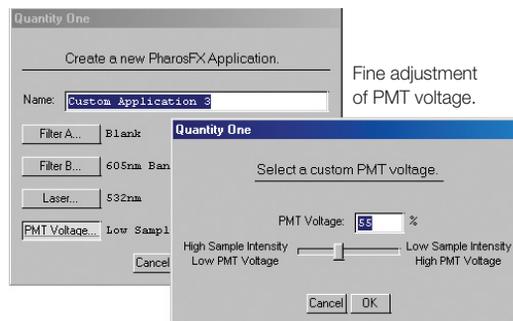

Automated sequential scanning.

Optimizing Imaging

To save time by avoiding scanning areas outside the gel, a quick initial scan at 800 µm resolution should be done to find the location of the gel and the approximate intensity of the spots. Select the Highlight Saturated Pixels box to aid in interpreting sample intensity. No more than a few of the brightest spots should contain saturated pixels. If an image contains too many saturated pixels, reduce the photomultiplier tube (PMT) voltage. Change the PMT voltage through the Select Application pop-up window by selecting either a different preset sample intensity setting (e.g., high, medium, or low) or a custom voltage for more refined control. Keep in mind that high sample intensity (saturated pixels) requires a lower PMT voltage, and a low sample intensity requires a higher PMT voltage.

Custom application creation.

Fine adjustment of PMT voltage.

Preparative Gels and Protein Identification

For optimal mass spectrometry results, it is recommended that you use SYPRO Ruby-stained preparative gels to obtain enough protein for identification.

Load the SYPRO Ruby-stained gel into the PharosFX imager as described earlier. In the PharosFX acquisition window, select the SYPRO Ruby preset application and scan as before for the Cy dye-stained gels.

Analysis

Image Analysis With PDQuest 2-D Analysis Software

PDQuest software analyzes multichannel experiments with standard gel matching procedures. Since all of the images come from the same gel, matching is straightforward and well suited to the automatic functions of PDQuest. Experiments created to compare the Cy3 and Cy5 images use the Cy2 image as the master image for the matching of spots across the images. Since the multichannel method is meant to be quantitative, intensity normalization between images is important to compensate for variations in spot intensity caused by systematic variations such as different characteristics of the lasers, filters, and dyes.

Transform the Images

Open all of the images pertinent to the experiment and transform as desired for visualization (Edit > Image > Transform).

Crop the Images

Trim away extraneous areas of the gel images using the Advanced Crop function (Edit > Image > Advanced Crop). Cropping will be efficient as long as the gel was not moved during image acquisition. It is recommended that you apply the Advanced Crop tool without the use of the placement crosshair. Accurate cropping is most important before using the Multi-Channel Viewer to assess the similarities and differences among the three images.
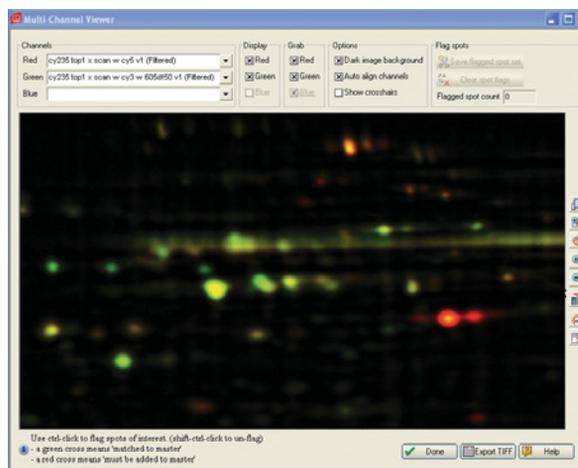
Spot Detection and Matching

Using the Experiment Wizard, select Multichannel Experiment, assign the Cy2 image as the internal standard, and proceed with the Spot Detection Wizard to detect spots on all the gel images.

Normalization

Normalization of image data is important for meaningful quantitative comparisons of the different images. Select Total Quantity in Valid Spots in the new Experiment Wizard when prompted. If any of the images contain a significant amount of saturated pixels, select Total Density in Gel Image instead. Compare the Cy3 and Cy5 images with the Scatter Plot view (Analyze > Scatter Plot). Activate and inactivate normalization to view the effect of normalization.

Multi-Channel Viewer

The Multi-Channel Viewer (View > Multi-Channel Viewer) gives an initial visualization of matched and unique spots in the three Cy dye-labeled images, but it is not necessary for the analysis. Overlay the desired images and select the Auto-Scale Image When Assigned checkbox to adjust the images to similar brightness and contrast. The following color scheme is suggested: blue for the Cy2 image, green for the Cy3 image, and red for the Cy5 image.
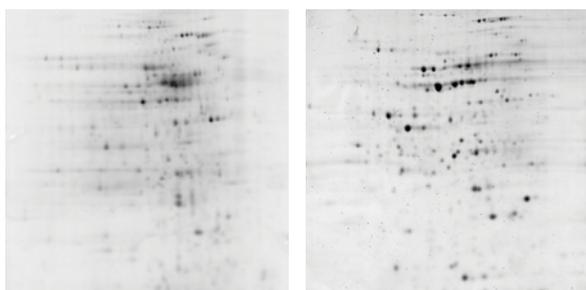

Multi-Channel Viewer allows color-coded comparison of differential expression.

Analysis

Use Analysis Set Manager to create analysis sets that contain spots exhibiting significant protein regulation between the Cy3 and Cy5 samples (Analyze > Analysis Set Manager > Create). The Spot Review tool (Analyze > Spot Review) can be used to review these analysis sets and to verify that all the spots in each analysis set are defined as desired. A subset of these spots can be selected by clicking on their graphs and then selecting Create Analysis Set to establish the desired multichannel analysis set.

Preparative Gels

Carry out a separate spot detection on the SYPRO Ruby-stained gel. It is not necessary for preparative gels to match perfectly to the corresponding multichannel gels — in most cases the preparative and analytical gel images will appear very different. However, it should be possible to locate the proteins of interest that were targeted in the analytical gels in the preparative gels. The proteins of interest will be those in the multichannel analysis sets. Since Cy dye-labeled and SYPRO Ruby-stained gels are visibly different, they must be matched manually.
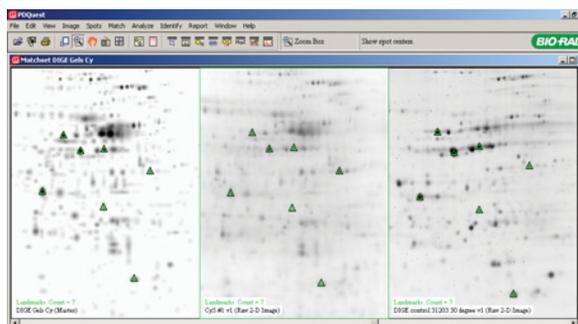
Cy3-labeled analytical gel.

SYPRO Ruby-stained preparative gel.

Applying landmarks to match Cy5 analytical gel with SYPRO Ruby preparative gel.

Matching Cy Dye-Labeled Images With the SYPRO Ruby-Stained Gel

Add the SYPRO Ruby image to the Multichannel Experiment using Configure Experiment (Edit > Configure Experiment > Members). Try matching the SYPRO image to the master image using the Automated Matching method first (Edit > Match > Automated Matching). If automated matching does not produce useful results, add some landmarks and rerun automated matching. Use Classic Match Gel as the extended match method (Edit > Match > Classic Match Gel). Begin by manually landmarking the SYPRO Ruby image to the multichannel master image (Edit > Match > Landmark). Select as landmarks spots that are clearly in equivalent positions in the Cy2- and SYPRO Ruby-stained gels whether or not they have the same intensities. Match the SYPRO Ruby image to the multichannel master image (Edit > Match > Classic Match Gel).

Locate Spots of Interest in the Preparative Gel

In order to map the spots of interest from the analytical gels to the preparative gel, verify that the spots are matched to the SYPRO Ruby-stained gel using the Spot Review tool. Open the Spot Review tool (Analyze > Spot Review). Select the analysis set containing the interesting multichannel spots. Verify that these spots are matched to the preparative SYPRO Ruby-stained gel. If the spots are not matched to the preparative gel, use manual matching (Edit > Match > Manual Match) to match these spots. Once the multichannel spots are matched to the SYPRO Ruby-stained gel, proceed with isolation of the targeted analysis set spots using the EXQuest™ spot cutter. PDQuest software will direct automatic excision of the targeted proteins, track the excised spots through microplate digestion and mass spectrometric protein identification, and allow automatic annotation of the gel image with the resulting protein identifications.

Reference

Unlu M et al., Difference gel electrophoresis: a single gel method for detecting changes in protein extracts, Electrophoresis 18, 2071–2077 (1997)

Cy is a trademark of Amersham Biosciences. SYPRO is a trademark of Molecular Probes, Inc.

Bio-Rad
Laboratories, Inc.