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DRUG DISCOVERY

Automated Processing of 2-D Protein Gels

Assay Tutorial:

2DiD Platform Relieves Imaging & Spot-Picking Bottlenecks in Automated Analysis

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Because most therapeutic agents modulate the functions of specific target proteins, protein-science and biochemistry teams are integral components of pharmaceutical R&D. The primary goals of these teams are to identify targets that play pivotal roles in disease, validate protein function, and build functional assays for screening and lead identification.

But robust assays can only be built upon well-characterized proteins of validated function. Moreover, batch-to-batch variability in amino acid sequence or structure can confound data analysis and, at worst, misdirect lead chemistry.

Many of the tasks involved in protein characterization have evolved into high-throughput technologies, and as a consequence, these aspects of biochemistry are increasingly carried out at core facilities within companies and universities, at "molecular-characterization" or proteomics centers.

Moreover, core-technology centers are under pressure to rapidly deliver proteomics information to expedite the drug discovery process. As a result, robotics and automation tools for proteomic processes have been introduced over the past several years, and platforms continue to evolve with the field.

Thorough study of

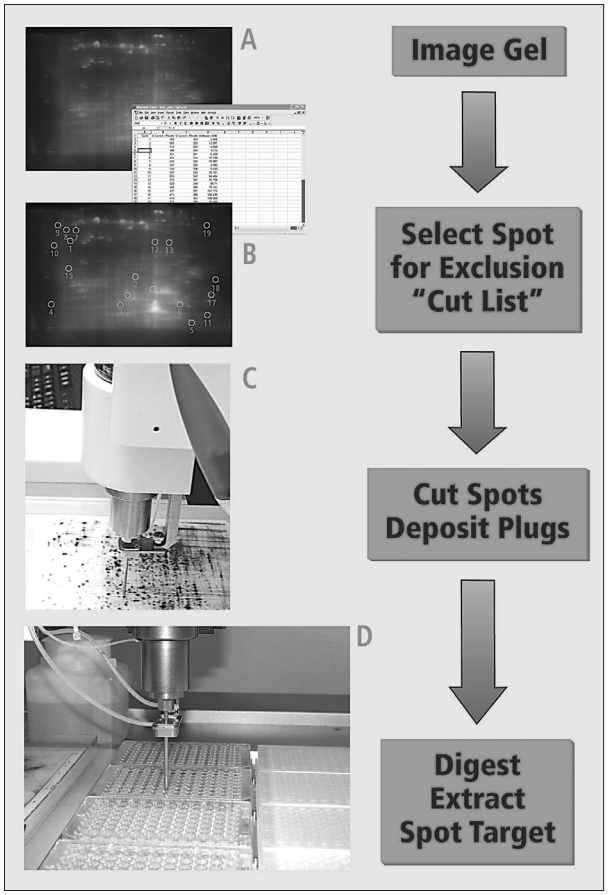

Figure 1. A schematic of the proteomics process from gel imaging to peptide processing. The image is a SYPRO Ruby-stain 2-DE gel (A). The spreadsheet inset shows the list of spots selected in a focused region of the gel (B). A self-washing tool cuts a gel for transfer to a microtiter well (C). The same tool uses a micropipette tip to dispense reagent into the processing wells (D).

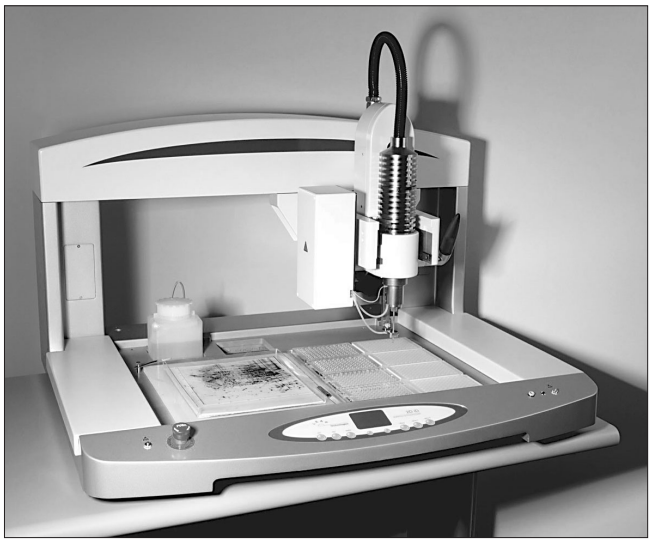

Figure 2. Full view of the 2DiD platform.

proteomes has become a practical reality in biological science, given concomitant advances in several technologies, especially informatics and mass spectrometry (MS). The ability to sequence peptides using mass spectrometry and, subsequently, to identify polypeptides using bioinformatics, has allowed the development of high-throughput tools for proteome analysis.

Even so, the expanding field of proteomics faces significant technological hurdles, as a consequence of proteome complexity. Despite decades of protein-chemistry research and technology development, the separation and detection of tens of thousands of proteins remains a challenging hurdle for proteome researchers.

Two-dimensional gel electrophoresis (2-DE) is still the most important tool available for separating mixtures of proteins. At a recent proteomics workshop organized by the American Society of Mass Spectrometry, researchers acknowledged a "love-hate relationship" that researchers have with 2-D gels: although 2-DE can separate thousands of proteins in a single experiment, handling of two-dimensional gels can nonetheless be tedious and laborious.

Thus, although 2-DE is now a standard proteomics technique, it is in need of automation to bring throughput and precision on par with the downstream components of protein identification, like mass spectrometry.

Many of the perceived limitations of 2-DE can actually be solved by good laboratory technique and robust automation tools. This tutorial describes practical issues and methods of processing two-dimensional gels, and introduces a new solution for automated imaging and post-separation tasks.

2-DE Protein Separation

Proteomics research typically involves differential comparison of 2-DE of proteins extracted from tissues or cells in different states, for example, "normal" versus diseased or xenobiotic-challenged tissue. Proteins exhibiting a quantitative perturbation in one state are then identified by mass spectrometry.

Practical proteomics is actually a series of sequential experiments that, minimally, include tissue or cell extraction, protein separation, post-separation processing (usually by MS), data reduction, and protein identification. When using 2-DE, the bottlenecks in this process are protein separation and post-separation processing; the mass-spectrometry and protein-identification steps, on the other hand, have become extremely rapid.

2-DE separates proteins, in the first dimension, by isoelectric point, and in the second dimension, according molecular weight. The many tasks of setting up and performing 2-DE can be somewhat of an art form, and the method is therefore not readily amenable to automation. Even so, for research laboratories initiating proteomic studies, 2-DE should be considered the first choice of separation methods, because it is actually a relatively simple technique, well supported by a number of commercial vendors.

To successfully execute reproducible 2-DE separations, laboratories must be clean and precise. Impure water/buffers, sloppy gel handling (in loading and transport), or a dusty laboratory can lead to poor replication and multiple, false "rediscoveries" of contaminating proteins like keratin and collagen. There are several good informational resources available on the practice and technique of 2-DE separation[1], including vendor Websites, and journals, such as *Electrophoresis* and *Journal of Analytical Chemistry* and *Analytical Biochemistry*.

Post-Separation & Visualization

Following the separation, gel imaging and protein processing must be accomplished with accuracy and precision, to provide reliable samples for MS analysis. Although separation may not be automation-friendly, automation of post-separation procedures is both achievable and recommended, for several reasons. Automation can help prevent manual contamination of the gel, increase precision and throughput, and free researchers to work on data analysis and protein identification.

It is possible to automate all steps, from gel imaging to deposition of

matrix-containing sample onto MALDI surfaces. These steps comprise imaging, spot-volume calculation, spot selection, spot excision, proteolytic digestion, sample cleanup, mixing samples with a MALDI matrix, and spotting of MALDI targets *(Figure 1)*.

Specific Automation Solutions

There are several commercial platforms available that automate at least portions of the overall process, including technologies from **Amersham Biosciences, Applied Biosystems,** joint technology from **Bio-Rad** and **Micromass** (a subsidiary of **Waters Corp.), Bruker Daltonics, Genomics Solutions, PerkinElmer Life Sciences,** and, from a relative newcomer to the field, 2DiD, from **LEAP Technologies'** (Carrboro, NC). Some vendors supply separate imaging systems and liquid-handling stations for processing and preparing digests.

In collaboration with robotics-engineering firm **BioMachines** (Morrisville, NC), LEAP Technologies has developed an integrated system for imaging and spot-picking *(Figure 2)*. The platform was designed to process one large gel (up to 20 X 25 cm), or several smaller gels simultaneously, as well as multiple microtiter plates for receiving gel plugs. A CCD camera and multifunction tool (for picking and pipetting) are both contained in a single robotic arm.

Out of Sight, Out of the Question

The sensitivity of protein visualization is highly dependent on the specific polypeptide within a spot and on the staining technique. Essentially, the quality of an image is only as good as the quality of the separation and staining of the proteins.

There are a few important points to keep in mind concerning the staining of gels. First, different proteins will react to differing degrees with the various stains. Second, the stain can interfere with mass fingerprinting. Third, automation of gel staining has not yet been practically realized.

Some generalizations can be made concerning limits of detection in relation to stains. The faintest spots generated by staining with Coomassie Blue (R-250), a quantitative technique, typically contain about 200 ng of protein, but the sensitivity is potentially as high as 5 ng. Silver staining (which is, by contrast, a qualitative method) can detect a little as 0.5 ng in a single polypeptide spot.

An increasingly popular fluorogenic reagent is SYPRO™ Ruby (PerkinElmer), which provides sensitivity similar to that of silver staining but which has a quantitative dynamic range of 200- to 1,000-fold. Whatever stain is selected, rigorous technique is required to render reproducible gel images.

Imaging: A Good Picture is Worth Thousands of Proteins

A first step in the analysis of 2-DE is production of a clear image with good contrast. Besides the chemistry aspects outlined above, the image quality will depend on the camera, the illumination mechanism, and the software used for signal averaging and noise reduction.

Imaging can be accomplished using a CCD camera, a laser, or even a simple computer scanner; however, the CCD camera is the most common readout device.

There are two key technical factors to consider when purchasing a CCD imaging system. The digitization (8-, 10-, 12-, or 16-bit) will determine the number of measuring graduations used to separate a very faint signal (spot) from the most intense signal (spot). Thus an 8-bit camera will provide for 256 shades of gray, whereas a 16-bit camera will provide for 65,536 graduations.

Note that, currently, only software tools are capable of distinguishing more than 256 shades. Discrimination of the shade graduation becomes important for accurate quantitative analysis (the more graduations, the better the quantitative accuracy).

Twelve- and 16-bit cameras, however, are very expensive and have slower CCD readout rates, which complicates focusing and sample positioning. Moreover, stained two-dimensional gels typically only present 10-bit–quality images, even from 12- or 16-bit cameras. (This is a function of the read noise and the actual variation of staining intensity.)

In fact, the majority of 2-D gels contain density information that is completely covered by the linear dynamic range of 8-bit images; greater digitization is rarely required.

If 16-bit images are preferred, a solution provided by LEAP's 2DiD is to use a fast 8-bit camera and to rapidly acquire multiple images, averaging the signals to produce a digitized image equivalent to that of a 12- or 16-bit CCD camera. 2DiD's software can then utilize the 16-bit image to optimize accuracy for quantitative analysis.

Another camera specification is resolution; the smallest spot that can be resolved is about five times the size of each pixel (a minimum of five pixels defines a spot). For example, the 2DiD can resolve and identify a spot as small as 60 μm (12 μm X 5) at the highest resolution of 12 μm/pixel. In addition, the technology can image either the entire gel or focused sections of the gel at full resolution. This is important for resolving small and intimately adjacent spots.

Shedding Light on the Problem

Well-controlled illumination of the gel will produce the best-quality images. Considering that each type of stain will require specific lighting, the imaging platform should provide homogeneous illumination, with interchangeable sources of visible and UV wavelengths.

Some platforms have bulb illuminators, positioned either above or below the plane of the gel. In many cases, switching from one light source to another requires manual intervention. A combination white-and-blue LED-based transilluminator integrated into a single system provides uniform illumination on the 2DiD. In addition, switching between wavelengths, adjustments of intensity, and exposure time are all under software control.

Another important fact to consider when selecting an imaging system is that multiple or prolonged exposure of SYPRO Ruby-stained spots to UV light will bleach the chromophore. Therefore, the image will be either gradually or immediately lost, depending on the time and intensity of exposure.

The 2DiD has the capability to take multiple images of a single SYPRO-stained gel without causing significant bleaching of the fluorescent dye.

Software

Different analysis packages provide a range of capabilities and are priced according to the level and versatility of the processing solutions. Capabilities can include automatic spot-picking, integration of spot volumes, cataloging of spot information, gel matching, statistical tools, and advanced information management.

A captured image of a 2-D gel was processed using **Nonlinear Dynamics'** (Newcastle upon Tyne, U.K.) Progenesis software to generate a volume plot *(Figure 3)*.

Major suppliers of automated protein-analysis software include Bio Rad, **Compugen, GeneBio,** Nonlinear Dynamics, and PerkinElmer, among others. Performance of various software packages has been compared in the literature.[2]

Before deciding upon any platform, users should ensure that image files generated by the imaging system are portable to available analysis software.

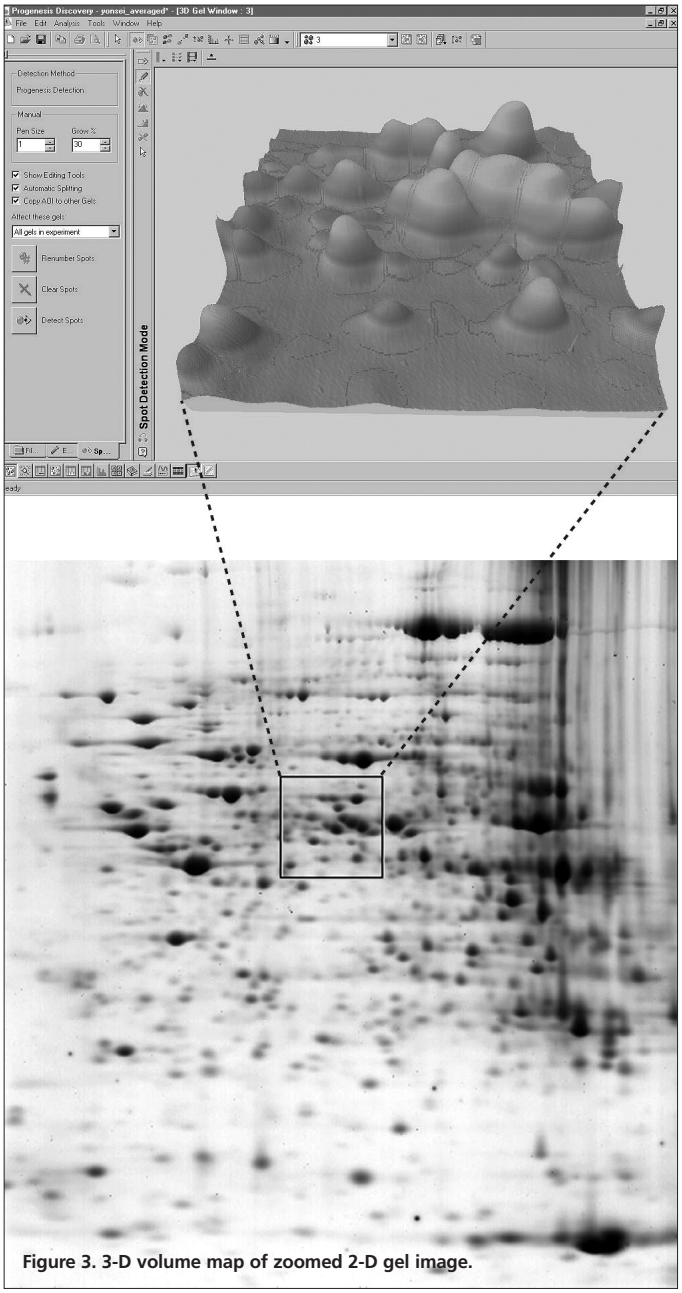

Figure 3. 3-D volume map of zoomed 2-D gel image.

Protein Recovery from 2-DE

In laboratories that use 2-DE for analysis of differential expression and protein identification, one experiment may comprise up to several dozen 2-D-gels (for instance, two treatments plus a control, with two sample dilutions and triplicate gels, results in 18 2-D-gels in total). Typically, the intent is to identify up- or downregulated proteins.

Such experiments regularly involve thorough imaging of each gel prior to the picking and cutting of polypeptide spots. Each gel is positioned at least once for imaging, and then positioned on a spot-picking device. Thus, the spots captured in the initial digital image must then be accurately relocated for processing.

With the 2DiD, imaging and picking are accomplished on the same platform, and spot relocation is accomplished by triangulation; the coordinates of several "landmark" spots on each gel image are

Laboratory preparation of samples for mass spectrometry.

stored with the image file for future positioning and spot location.

Once a "cut list" is generated, gel plugs must be cut and moved to a tube or well in a multiwell plate. Accurate picking and transfer of the plug is highly dependent on the location-accuracy of the picking device and on the method for seizing and expelling the plug.

When selecting a platform for cutting and transferring gel plugs, spatial resolution and reproducibility of the picking tool must be evaluated. Positive verification that the plug is actually transferred from the gel to a vessel is also an issue, especially when processing hundreds of polypeptide spots.

To address this, the 2DiD picks a plug using three mechanisms: the picking tool cores the gel with a Z motion, cuts a plug with a twisting (theta) motion, and grabs the plug with vacuum. A sensor confirms both plug excision and deposition by the probe tool, thus eliminating "misses" from a plug being left behind or lost at the wash station. Because the device is a combined imager and picker, it can be programmed to reimage the gel after processing, to compare the cut list with the actual locations picked.

Recovery of low-abundance proteins and avoiding contamination can be a confounding task. Contamination can result from extraneous particles (dust), or carryover from previously cut spots. Carryover is prevented by rigorous washing or by exchange of the cutting tip on robotic tool.

The typical washing method employs a separate wash vessel containing a cleaning buffer; the tip is submerged and aspirated with several volumes of the buffer. Alternatively, the cutting tool can be washed internally ("on the fly"), thus avoiding a separate washing step. (An example of the 2DiD self-washing tool is shown in *Figure 1*).

Post-Picking Chemistry

The steps following spot-picking must result in clean peptide fragments for MS analysis. A number of vendors supply liquid-handling stations to carry out polypeptide extraction, proteolysis, peptide cleanup, and delivery to the sample to the MS.

If peptide-mass fingerprinting is the technique that will be used to identify proteins, the peptide samples are deposited on MALDI plates. The chemistry steps may include the following: washing of the gel plug with various buffers, reduction of disulfide bonds at 60°C, alkylation of cystine residues with iodoacetamide, removal of excess reagents, equilibration with digest buffer, addition of digestion enzyme, incubation at 37°C for digestion, and elution of peptides from gel plug.

Because there is a large variety of wet methods for preparing peptides from gel plugs, it is important to evaluate the versatility of the processing stations with regard to pipette capability and vessel compatibility (tubes and microtiter plates), and to map out procedures and put the process to the test. Pipette-tip exchange and washing must be included in analyses of the process.

The 2DiD was developed for carrying out multiple dispensing and transfer steps, which can be programmed by the user.

Conclusion

Each laboratory using 2-DE will have its own particular needs for automating proteomics processes. To best determine those needs, researchers should answer, at a minimum, the following questions:

1) How many protein samples will be separated by 2-DE, and how many polypeptide spots will be processed in any given period (per day, week, month)?

2) What are the bottlenecks in the process?

3) What is the requisite dynamic range for protein detection and analysis?

4) Is the objective differential analysis or protein identification?

5) Which protein stain is most suitable?

6) What type of mass spectrometry, if any, will be used for polypeptide analysis?

7) Will 2-D gels be reimaged after storage?

8) What are the practical realities, in terms of space and expenditure?

9) Is there a need for integrated imaging-picking platform or for separate systems?

There are numerous solutions to imaging and processing gels; the best solution will be identified after rigorous analysis of specific processes and available platforms.

Recent introductory and laboratory texts may prove helpful in determining specific procedures and needs.[3,4] GEN

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