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REVIEW

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The development of the DIGE system: 2D fluorescence difference gel analysis technology

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Abstract Two-dimensional (2D) gel electrophoresis is a powerful technique enabling simultaneous visualization of relatively large portions of the proteome. However, the well documented issues of variation and lack of sensitivity and quantitative capabilities of existing labeling reagents, has limited the use of this technique as a quantitative tool. Two-dimensional difference gel electrophoresis (2D DIGE) builds on this technique by adding a highly accurate quantitative dimension. 2D DIGE enables multiple protein extracts to be separated on the same 2D gel. This is made possible by labeling of each extract using spectrally resolvable, size and charge-matched fluorescent dyes known as CyDye DIGE fluors. 2D DIGE involves use of a reference sample, known as an internal standard, which comprises equal amounts of all biological samples in the experiment. Including the internal standard on each gel in the experiment with the individual biological samples means that the abundance of each protein spot on a gel can be measured relative (i.e. as a ratio) to its corresponding spot in the internal standard present on the same gel. Ettan DIGE is the system of technologies that has been optimized to fully benefit from the advantages provided by 2D DIGE.

Keywords 2D DIGE · CyDye DIGE fluors · DeCyder differential analysis software · Fluorescent labeling · Proteomics

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Abbreviations CyDye DIGE fluor Cy2 minimal dye: (3-(4-Carboxymethyl)phenylmethyl)-3′-ethyloxacarbocyanine halide *N*-hydroxysuccinimidyl ester) · CyDye DIGE fluor Cy3 minimal dye: (1-(5-Carboxypentyl)-1′-propylindocarbocyanine halide *N*-hydroxysuccinimidyl ester) · CyDye DIGE fluor Cy5 minimal dye: (1-(5-Carboxypentyl)-1′-methylindodicarbocyanine halide *N*-hydroxysuccinimidyl ester) · CyDye DIGE fluor Cy3 maleimide dye: 1-(6-([2-(2,5-Dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethyl]amino)-6-oxohexyl)-2-[(1*E*,3*E*)-3-(1-ethyl-3,3-dimethyl-5-sulfo-1,3-dihydro-2*H*-indol-2-ylidene)prop-1-enyl]-3,3-dimethyl-3*H*-indolium · CyDye DIGE fluor Cy5 maleimide dye: 1-(6-([2-(2,5-Dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethyl]amino)-6-oxohexyl)-3,3-dimethyl-2-[(1*E*,3*E*,5*E*)-5-(1,3,3-trimethyl-5-sulfo-1,3-dihydro-2*H*-indol-2-ylidene)penta-1,3-dienyl]-3*H*-indolium · DIGE: Difference gel electrophoresis · DIA: Differential in-gel analysis · BVA: Biological variation analysis

Introduction

Two-dimensional (2D) gel electrophoresis is a powerful technology for protein abundance studies and the only method available for simultaneous resolution of thousands of proteins. O'Farrell [1] first described the technique in 1975. The principle of 2D electrophoresis is based on separation of the proteins according to their charge in the first dimension by isoelectric focusing (IEF) and size in the second dimension by SDS-PAGE. Despite being a well-established technique for protein analysis, traditional 2D gel electrophoresis is time-consuming and labor-intensive. Many gels have to be run, analyzed, and compared. In addition, the lack of reproducibility between gels leads to significant system variability making it difficult to distinguish between system variation and induced biological change, which

means that real differences between protein abundance attributed, for example, to a disease state can rarely be predicted with confidence.

The group of Unlu et al. [2] first described a method, 2D difference gel electrophoresis (DIGE), that enabled more than one sample to be separated in a single 2D polyacrylamide gel. The technique involved prelabeling two protein extracts, using two fluorescent cyanine dyes known as Cy3 and Cy5. The labeled samples are then mixed and run on the same 2D gel. The protein samples are then visualized using fluorescence imaging to enable detection of differences between protein abundance in the two samples. In its earliest form the image processing software divided corresponding pixels from two images from the same gel after background subtraction and normalization. The resulting ratio image enabled the visualization of differences between the two samples.

Amersham Biosciences (now part of GE Healthcare) have an exclusive license to this technology from Carnegie Mellon University. The first published evaluation of the technology was performed by Tonge et al. [3], who used the technique to study *N*-acetyl-*p*-aminophenol (APAP) toxicity in mice liver. This work included evaluation of the technology using Cy3 and Cy5 dyes, an imager known as the 2D Master Imager, and ImageMaster 2D-Elite image-analysis software. More recently, Amersham have included a third dye (with similar properties to the Cy3 and Cy5 dyes), known as Cy2, that enables co-electrophoresis of up to three samples on the same 2D gel. In addition, the imager currently used is a variable mode laser imager called Typhoon that gives improved signal and low noise enabling lower intensity spots to be visualized and detected. New image-analysis software, known as DeCyder Differential Analysis software, has also been developed and has been upgraded through several versions with the aim of increasing automation of the analysis and removing user subjectivity.

2D DIGE

Two-dimensional DIGE is based on fluorescence prelabeling of protein mixtures before 2D gel electrophoresis. Protein samples are labeled with up to three spectrally distinct, charge and mass-matched fluorescent dyes known as CyDye DIGE fluors [2, 4, 5]. The labeled proteins are then mixed and separated simultaneously on the same 2D gel. The different protein extracts labeled with different CyDye DIGE fluors can then be visualized separately by exciting the different dyes at their specific excitation wavelengths. This is achieved by use of an imager containing appropriate laser wavelengths for exciting the different dyes and filters for collecting the light emitted. Each dye generates digital images of each individual sample. Amersham Biosciences supplies the Typhoon 9400 variable mode imager, DeCyder differential analysis software, and CyDye DIGE fluors as a system known as Ettan DIGE. An overview of the proteomics 2D workflow is outlined in Fig. 1, and the different elements of Ettan DIGE (sample labeling, image acquisition, and image analysis) are highlighted in orange.

The advantages of improved sensitivity and accuracy provided by the ability to separate more than one sample on a single gel have added a new dimension to 2D electrophoresis. The linearity, sensitivity, and wide dynamic range of these dyes have made 2D DIGE into a quantitative technique. The sensitivity of CyDye DIGE fluor minimal dye Cy2 is 0.075 ng, for Cy3 0.025 ng, and for Cy5 0.025 ng. Compared with silver staining, for which the sensitivity is 1 ng, these dyes are clearly more sensitive. The CyDye DIGE fluor saturation dyes are even more sensitive, with values down to 15 pg. For both Minimal and Saturation dyes the dynamic range is above 3.6 orders of magnitude.

Most critically, this technique has the ability to substantially reduce the effects of gel to gel variation on the quantitation of a protein spot on a gel. Therefore, the

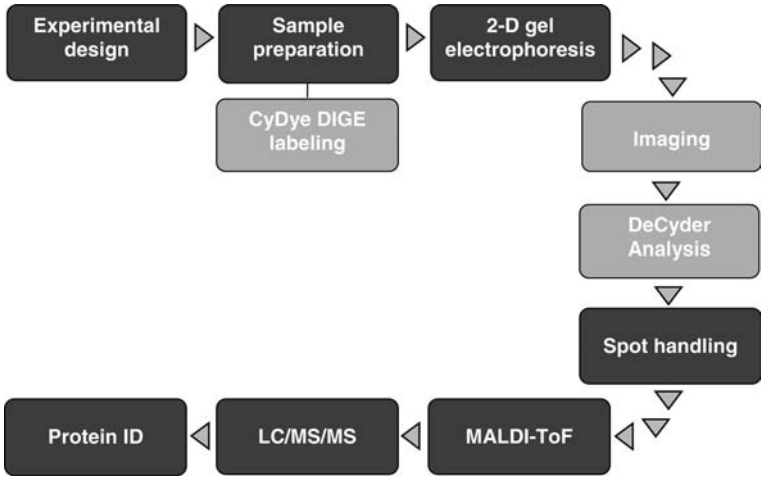

Fig. 1 Overview of the proteomics 2D workflow and 2D DIGE system approach for differential analysis. The elements Ettan DIGE is made up of are highlighted in *orange*

confidence that a difference in fluorescence intensity between two samples is due to biological rather than experimental variation has increased [6]. It has been shown that this technique can generate statistically significant data using fewer 2D gels [7–10].

The greater quantitative accuracy of 2D DIGE is enabled by three main factors:

1. the ability to run multiple samples on the same gel (multiplexing);
2. an internal standard (reference) sample which can be run on all gels; and
3. experimental designs unique to this technique.

Multiplexing

The level of variation in protein spot patterns often seen on a 2D gel is one of the major disadvantages of conventional 2D electrophoresis. Running multiple samples on the same 2D gel means reduction in spot pattern variation in addition to a reduction in the number of gels required in an experiment. Although the generation of statistical data requires the use of multiple gels the ability to compare two or three samples within a single gel is highly advantageous. It means that differences or variation arising from running different samples on different gels could be completely eliminated [6].

Internal standard (reference sample)

The concept and the benefits of using an internal pooled standard for 2D gel electrophoresis were first described by Alban et al. [7]. The internal standard is prepared by pooling equal amounts of protein from each biological sample in the experiment and labeling it with one of the CyDye DIGE fluor dyes, which is usually Cy2 for minimal labeling. This means that every protein from all samples will be represented in the internal standard. The internal standard is then run on every single gel along with each individual sample labeled. Linking every sample in-gel to a common internal standard has several advantages. It means that each sample within a gel can be normalized to the internal standard present on that gel. In addition, the abundance of each protein spot in a biological sample can be measured as a ratio (not a volume) to its corresponding spot present in the internal standard. This enables accurate quantitation and accurate spot statistics between gels and, most importantly, separation of experimental variation from inherent biological variation. This has not been possible with conventional 2D gel electrophoresis, because of the high level of variation associated with running all samples on individual gels. An example of the benefits of using an internal standard is shown in Fig. 2.

Alban et al. [7], demonstrated the power of the 2D DIGE approach and the pooled internal standard experimental design, by spiking a protein sample with different amounts of known proteins. The accuracy and statistical variation of protein quantitation was determined and compared with a 2D DIGE approach without including the internal standard. Their results showed that with the inclusion of an internal standard, they were able to reproduce expected trends in protein abundance, created on 2D gels, using the DeCyder software.

The value of the internal standard was also recently demonstrated in a study by Friedman et al. [8], in which 2D DIGE coupled with mass spectrometry was used to investigate tumor-specific changes in the proteome of human colorectal cancers and adjacent normal mucosa. The Cy3 and Cy5 dyes were used to label the samples and an internal standard sample, formed by pooling an equal amount of all biological samples in the experiment, was labeled with Cy2 and run on each gel. This study concluded that 42 out of 52 statistically significant differences would have been overlooked without the use of the internal standard, because of the extent of variation inherent between the normal and tumor samples.

Knowles et al. [11] also demonstrated the power of the internal standard in an experiment in which cerebral cortex tissue was taken from a model system for the neurokinin 1 receptor. The tissue taken from the neurokinin 1 receptor knockout and wildtype mice was labeled using the Cy3 and Cy5 dyes. In addition, Cy2 was used to label an internal standard sample. Analysis of the results with and without the internal standard using the DeCyder software showed that all but one of the ten spots identified as being significantly different with the internal standard had less significance without the internal standard. In addition, three of the spots identified as being significantly different at the 95% confidence level, were no longer significant without the internal standard and would have been missed. This is mainly because the use of the internal standard reduces the effect of variation observed without the use of the internal standard.

Experimental design

The high level of interest in the field of proteomics has seen an explosion in the amount of data being generated by different groups. The number of publications and information in relevant databases, for example PubMed and HighWire, has vastly increased from a couple of publications to over 70. If these data are to be shared between different groups, the integrity of this ever increasing mountain requires strict control. This has meant that many groups are now employing the skills of statisticians to guide the designs of their experiments.

Two-dimensional DIGE enables the use of experimental designs that would not be possible using conventional 2D electrophoresis. Swatton et al. [9] successfully utilized some of the benefits of the unique experimental designs possible with 2D DIGE in an experiment in which human post mortem samples were analyzed. To compare brain samples of 20 normal individuals with those from 20 schizophrenic

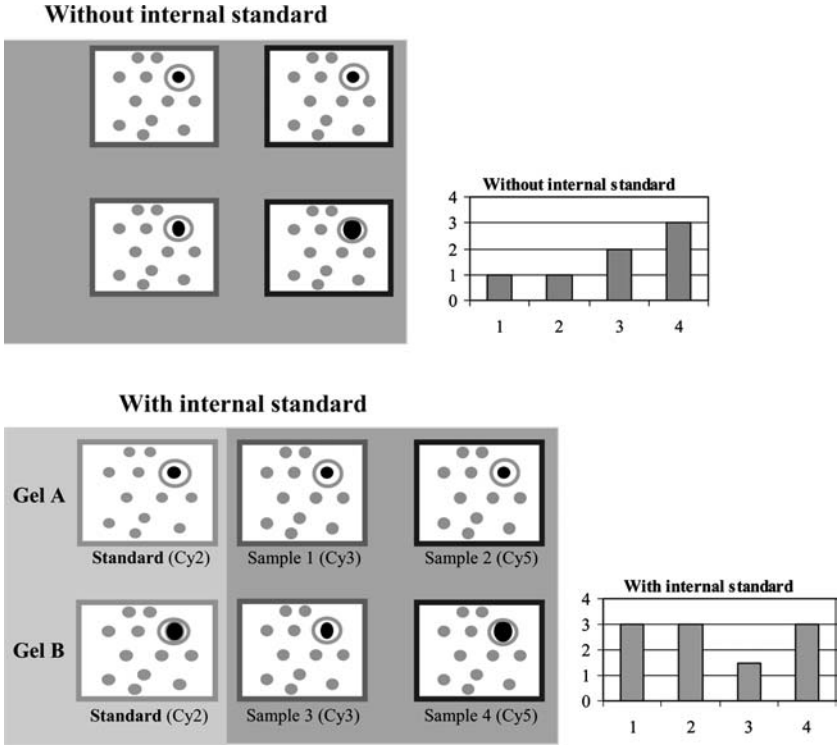

Fig. 2 Comparison of gel electrophoresis with and without internal standard. Four different samples and one internal standard on two different gels. The diagrams show quantitation from the *circled spot* in samples 1–4 with and without normalization to the internal standard. The internal standard creates a link between all gels, removing gel-to-gel variation, and reveals biological change with statistical accuracy. Not using the internal standard can lead to wrong biological conclusions

individuals, an internal standard was used in the experiment. The internal standard was labeled with Cy2 and run on each gel. In addition, reciprocal labeling in which half of each group was labeled with Cy3 and the other half with Cy5 was also utilized. This study found 215 proteins that changed in abundance between normal and schizophrenia individuals with a confidence level of 95% significance and above. Mass spectrometry (MS) analysis identified 79% of these proteins and found them to be involved in metabolism/cellular respiration, axonal/neuronal growth, protein trafficking/turnover, and cellular signaling.

Tonge et al. [3] also used this experimental design in which reciprocal labeling is used. Control and paracetamol (APAP) treated mouse liver homogenates were labeled with Cy3 and Cy5 (half of each group was labeled with Cy3 and the other half labeled with Cy5). This study identified 66 proteins of interest. Eleven of the 20 proteins identified by MS had not been previously linked to APAP toxicity (the other nine had previously been identified by 2D gel analysis with colloidal Coomassie blue staining). In addition, the experimental design used enabled this group to determine that none of these differences was due to preferential labeling.

CyDye DIGE fluors

Amersham currently supply two matched sets of CyDye DIGE fluors, known as CyDye DIGE fluor minimal and CyDye DIGE fluor saturation (or scarce sample) dyes. Three minimal dyes—Cy2, Cy3, and Cy5—enable co-electrophoresis of up to three samples on the same gel and are suitable for detecting differences between protein abundance on analytical gels when 50 μg protein is used.

Two saturation dyes—Cy3 and Cy5—enable co-electrophoresis of up to two samples on the same gel and are suitable for detecting differences in protein abundance on analytical gels when only 5 μg protein is used.

Minimal labeling

Lysine labeling is referred to as minimal labeling because the ratio of dye to protein is kept very low so that the only protein molecules visualized on the gel are those that are labeled with a single dye molecule. In-house experiments were performed with synthetic peptides containing one or multiple lysine residues in a consecutive sequence. Mass spectrometric analysis of the optimized labeling reactions showed that only one dye molecule is present per protein molecule. Even if multiple lysines are labeled, the percentage of double-labeled species is too small to be visualized. As a result, only 3–5% of the total protein present in the sample is labeled. In addition, the recommended experimental design compensates for any preferential labeling, although previous work does not support this effect. The relatively high lysine content of most proteins makes this amino acid suitable for this labeling strategy, in which very small amounts of dye are used. The use of a saturation labeling strategy for labeling all lysines in the protein extract would require a large amount of dye, which would lead to problems related to protein insolubility

[2, 10]. Each CyDye DIGE fluor minimal dye has an NHS-ester reactive group, which covalently attaches to the epsilon amino group of lysine residues in proteins via an amide linkage.

The single positive charge on the CyDye DIGE fluor minimal dyes replaces the single positive charge present in lysine at neutral or acidic pH, thus ensuring that the pI of the protein is not significantly altered. In addition, the CyDye DIGE fluor minimal dyes are size-matched, adding approximately 500 Da to the labeled protein. Consequently, because the CyDye DIGE fluor minimal dyes are matched for charge and molecular weight, the same protein labeled with any of these dyes will migrate to the same position on a 2D gel [9]. This labeling generates a spot pattern on the gel that is the same as that seen with post-staining techniques.

An example of an experimental design in the labeling of samples with CyDye minimal dyes is presented in Table 1.

Saturation labeling

The saturation dyes label all available cysteine groups on each protein under the conditions used. For this reason the dyes used for this method are known as saturation dyes. To achieve optimum labeling of cysteine residues, a high dye-to-protein labeling ratio is required. The relatively low prevalence of cysteine residues in proteins, in addition to the fact that its chemistry is amenable to chemical modification, makes this amino acid suitable for this labeling strategy, where very high amounts of dye are used [10].

This set of dyes has a maleimide reactive group which is designed to form a covalent bond with the thiol group of cysteine residues on a protein via a thioether linkage. The saturation dyes have a neutral charge and, as with the minimal dyes, are matched in molecular weight (adding approximately 677 Da to the labeled protein) [10]. Consequently, as the CyDye DIGE fluor saturation (also referred as scarce sample) dyes are matched for charge and molecular weight, the same protein labeled with any of these dyes will migrate to the same position on a 2D gel. These dyes can be used to visualize small amounts of proteins and as little as 5 μg protein can be used for each labeling reaction. These dyes are recommended for applications in which precious or a very small amount of sample is available. For example, Kondo et al. [12] used the CyDye DIGE fluor saturation dyes to successfully compare normal and diseased protein samples obtained by laser microdissection.

An example of an experimental design in the labeling of samples with CyDye saturation dyes is presented in Table 2.

Table 1 An example of an experimental design implemented in DeCyder differential analysis software to derive statistical data on differences between control and treated samples labeled with CyDye DIGE fluor minimal dyes

Gel number	Cy2	Cy3	Cy5
1	Pooled standard	Control 1	Treated 2
2	Pooled standard	Control 2	Treated 3
3	Pooled standard	Control 3	Treated 1
4	Pooled standard	Treated 5	Control 4
5	Pooled standard	Treated 6	Control 5
6	Pooled standard	Treated 4	Control 6

Example of an experimental design for CyDye DIGE fluor minimal dyes

Imaging

Fluorescence-labeled proteins in the 2D gels are scanned using a Typhoon variable mode imager.

The Cy2, Cy3, and Cy5 dye images are scanned sequentially with 488, 532, and 633 nm lasers, respectively, and emission filters of 520 (band pass 40), 580 (band pass 30), and 670 nm (band pass 30), respectively. The CyDye DIGE dye filter and laser combinations are selected to give the optimum results with minimal cross-talk between fluorescent channels.

An advantage of using the Typhoon is that the 2D gels can be scanned after electrophoresis still assembled within the low-fluorescence glass plates. This ensures that the gels are managed easily and not damaged during imaging and also minimizes the possibility of contamination.

Yan et al. [13] found that the gel image generated using the Typhoon scanner was superior in quality to the image generated on the 2D-Master Imager. Typhoon was found to enable linear detection over a wide dynamic range and improved sensitivity for Cy3 and Cy5. In addition, the fact that the Typhoon scanner is a point scanner means that the effects of photo bleaching associated with imagers in which constant gel illumination is required are not observed.

Table 2 An example of an experimental design implemented in DeCyder differential analysis software to derive statistical data on differences between control and treated samples labeled with CyDye DIGE fluor saturation dyes

Gel number	Cy3	Cy5
1	Pooled standard	Treated 1
2	Pooled standard	Treated 2
3	Pooled standard	Treated 3
4	Pooled standard	Treated 4
5	Pooled standard	Treated 5
6	Pooled standard	Treated 6
7	Pooled standard	Control 1
8	Pooled standard	Control 2
9	Pooled standard	Control 3
10	Pooled standard	Control 4
11	Pooled standard	Control 5
12	Pooled standard	Control 6

Example of an experimental design using CyDye DIGE fluor saturation dyes from the CyDye DIGE fluor labeling kit for scarce samples

DeCyder differential analysis

Image analysis is a lengthy and user-variable process and is often a major bottleneck in proteomics. DeCyder differential analysis software has been developed as part of the DIGE system and therefore, all the unique advantages of the 2D DIGE technique are used in the software. DeCyder differential analysis software is fully automated image analysis software for spot detection and accurate measurement of protein differences with statistical confidence.

The reproducibility of the dye technology, with the accuracy of the DeCyder differential analysis software, enables measurement of very small protein abundance differences with high confidence. DeCyder can routinely detect, within minutes, less than 10% differences between protein expression in samples with more than 95% confidence. By using the internal standard approach to derive statistical data within and between gels, the gel to gel variation is effectively eliminated. This software gives highly accurate and reproducible results from 2D DIGE. This has been demonstrated in several applications such as those by Freidman et al. [8], Gharbi et al. [5], Alban et al. [7], Gade et al. [14], and Yan et al. [13].

The DeCyder platform consists of four modules that are schematically represented in Fig. 3: differential in-gel analysis (DIA), biological variation analysis (BVA), batch processor and XML toolbox.

DIA (differential in-gel analysis)

The DeCyder DIA module processes the images from a single gel, performing background subtraction, detection, and quantitation, in-gel normalization, and gel artifact removal, all automated for high-throughput and reduced user-specific variation. Detection is performed using the novel co-detection algorithm in the software, which generates identical spot segmentation (detection) on up to three images from the same gel (Fig. 4). The DIA module quantifies the spot volumes for each image and expresses these values as a ratio, comparing spot volumes on the sample image with corresponding spot volumes from the internal standard image. This ratio can then be used for inter-gel protein abundance comparisons.

BVA (biological variation analysis)

When spot detection and quantitation of a single gel has been performed, data are transferred to the BVA module for inter-gel analysis. DeCyder BVA processes multiple gel images, performing matching of multiple images from different gels for comparison to provide statistical data on different protein abundance levels between multiple groups. This module utilizes the benefits provided by the pooled internal standard experimental design by performing gel-to-gel matching on pooled internal standard images only (Figs. 5, 6). This process enables comparison of protein abundance between samples on different gels. Moreover, DeCyder BVA enables analysis of experimental designs with different degrees of complexity from a simple control/treated experiment through to a multi-condition experiment addressing factors such as dose and time, all performed in a single analysis.

The presence of the same pooled internal standard on every gel enables accurate normalization of the individual experimental samples, reducing gel-to-gel and software analysis variation. This approach results in unparalleled accuracy enabling experimental conclusions to be drawn with high confidence.

Batch processor

The batch processor implements both DeCyder DIA and BVA processes, and performs fully automated spot detection, quantitation, matching and comparison of multiple 2D DIGE gel images without user intervention. The Batch Processor links both the DeCyder DIA and BVA modules to perform spot detection and inter-gel matching of images, present in as many as 500 gels.

XML toolbox

When using DeCyder differential analysis software, large amounts of data are generated and it is useful to be able to save these data in a format that can be efficiently stored and easily accessible. Data generated in the different software modules, DIA and BVA, can be saved using a common file format called DeCyder extended

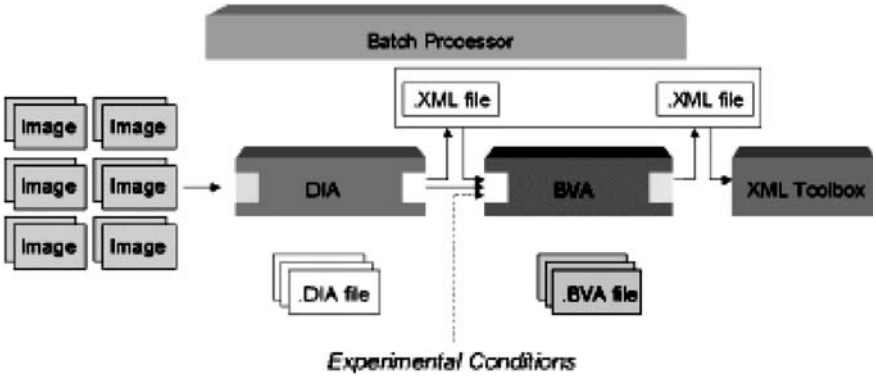

Fig. 3 Scheme showing the image analysis workflow in DeCyder differential analysis software

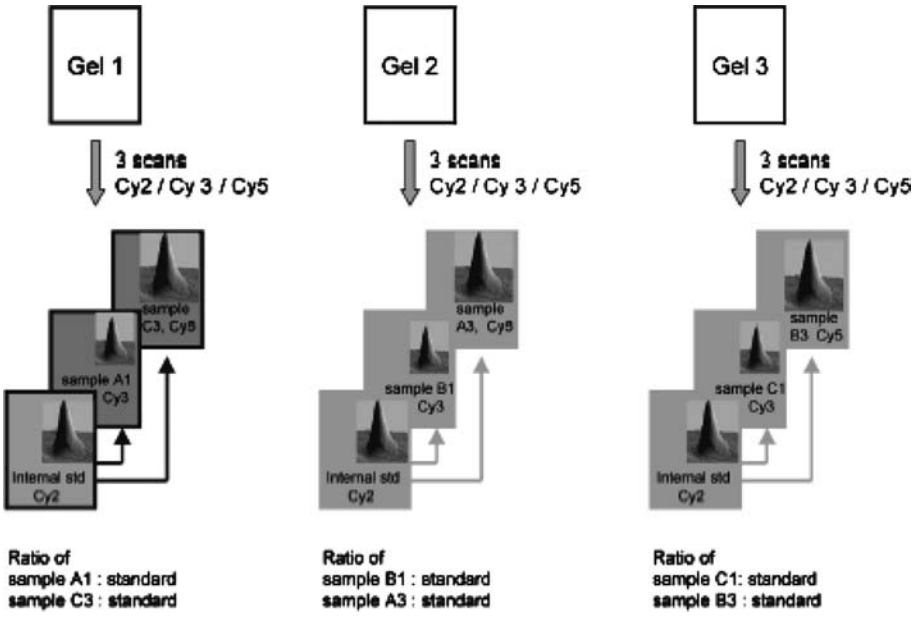

Fig. 4 Detection and quantitation of protein abundance using co-detection algorithms in DeCyder analysis in each gel. From each gel, three scan images are generated, CyDye DIGE fluor Cy2 minimal dye for the internal standard, CyDye DIGE fluor Cy3 and Cy5 for minimal dyes for experimental samples. The protein abundance for each spot in each sample is expressed as a ratio relative to the internal standard

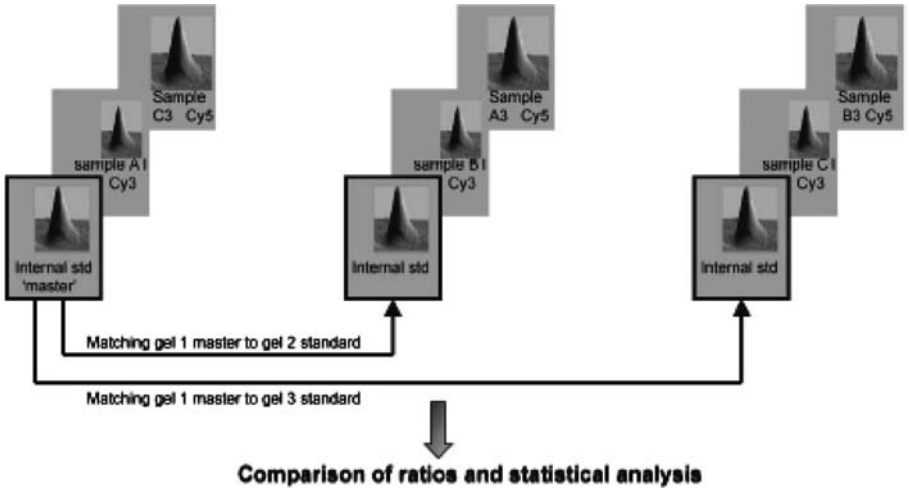

Fig. 5 Matching of multiple gels by the internal standard spot patterns. Internal standard spot patterns are matched across all the gels so that the position of each protein spot is mapped to the identical spot on the master gel

markup language (DeCyder XML). These files contain all information generated during the processing of the images in a structured file format, which makes it easy to access data from DeCyder differential analysis software workspaces. DeCyder XML toolbox has a range of tools for extraction of user-defined data from the different XML files produced within DeCyder differential analysis software. The extracted data can then be converted into text files, HTML files, or other data formats to facilitate automatic report generation.

Application of 2D DIGE in proteome studies

The term proteome was coined in the mid 1990s as the ensemble of proteins related to a genome. Proteomics is the study of the proteome and can be divided into profiling, functional and structural proteomics [15]. The increase in the use of 2D DIGE in proteomics can be measured by the fact that the number of publications using 2D DIGE between January 2003 and June 2004 has more than tripled from 20 to approximately 70.

The 2D DIGE technology has been employed in several biological applications to examine the protein profiles of various tissues, cell lines and cell types including those from bacteria [7, 13, 14], yeast [16], plants [17–19], fruit fly [20, 21], insect [22], mouse and rat liver [3, 23–26], rat kidney [27], rat heart [28], rat lung [29], cat brain [6, 30], mouse and rat brain [11, 31–33], guinea pig brain [34], human brain [9] and human cancer cells [4, 5, 8, 35–40].

The following section of this review will describe in detail one of the applications of 2D DIGE to proteome profiling.

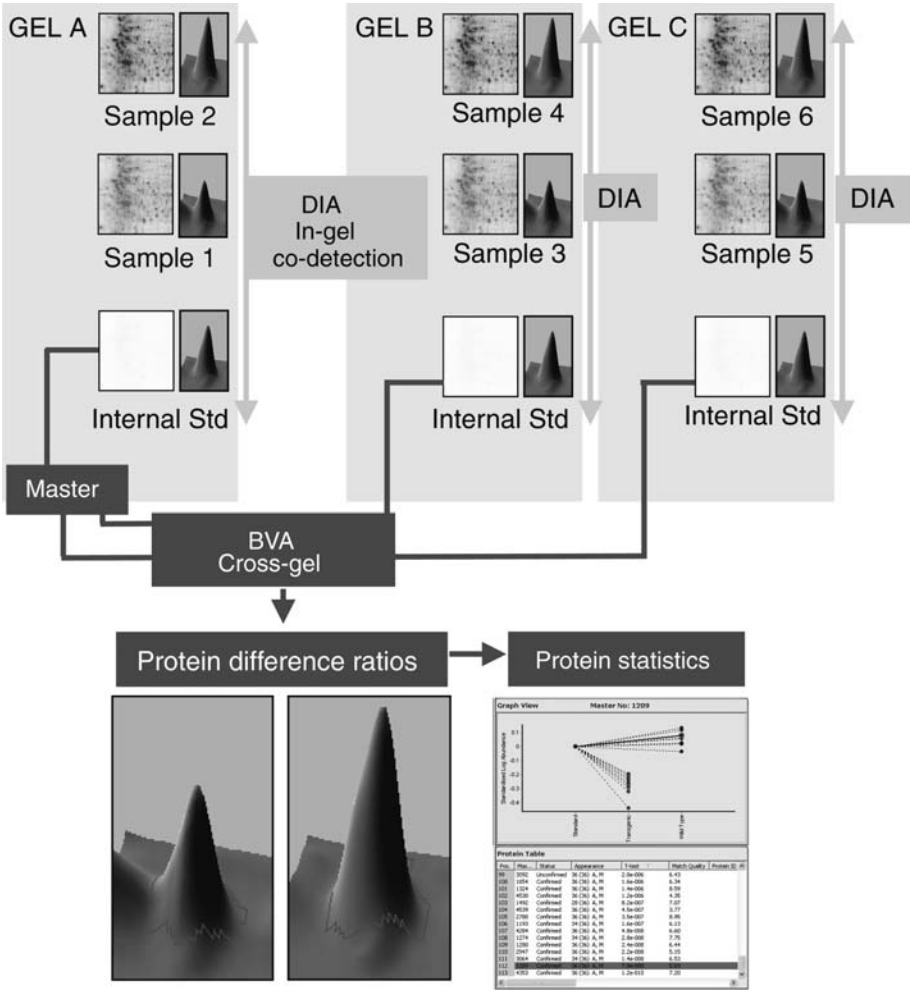

Fig. 6 Scheme showing spot co-detection on images from a single gel in the DeCyder DIA module and protein difference ratios and statistics between gels in the DeCyder BVA module

DIGE and cancer proteomics

The study of cancer using samples obtained by laser microdissection (LMD) has been coupled with many technologies including antibody array [41], reverse phase array [42], and 2D electrophoresis [43–48]. Although the combination of LMD and 2D electrophoresis is a promising combination (because of the ability of 2DE to visualize hundreds of proteins simultaneously) it is limited by the sensitivity of the silver staining method normally used for this purpose. In a typical experiment using this combination, approximately 25–50 μg protein is required for separation on a 2D gel, to obtain an image containing between 450–1000 protein spots. Obtaining this amount of protein by LMD could take 3 h or up to 4 days. In addition, the limited dynamic range of silver staining means that accurate quantitation of these small samples is very difficult.

Kondo et al. [12] used the combination of LMD and the CyDye DIGE fluor saturation dyes to compare the proteome of normal intestinal epithelium with that of adenoma in mice. Their study showed that only 2.7–6.6 μg total protein was sufficient to generate 2D images with 1,500 protein spots. In addition, the broad dynamic range of the fluorescent dyes meant that low and high-abundance spots could be accurately quantified. Of the 37 protein spots found to show reproducible differential expression, eight were identified by MS. Although the small number of spots identified raised the question of peptide ionization suppression (during MS) by the Cy-Dye DIGE fluor saturation dyes, previous work by Yan et al. [13] has shown that similar peptide coverage is obtained with MALDI-MS analysis of both labeled and unlabeled protein lysates. Three of the proteins identified in this study were not previously known to be associated with adenomas. This study concluded that fluorescence labeling of proteins from microdissected tissues, using the CyDye DIGE fluor saturation dyes, is a powerful cancer proteomic study tool.

Gharbi et al. [5] used the minimal dyes and DeCyder to study ErbB-2-mediated transformation of luminal epithelial cells in a model breast cancer system. Over-expression of ErbB-2 receptor tyrosine kinase is known to be associated with 25–30% of breast cancers. The aim of this study was to identify those proteins in the ErbB-2 model breast cancer system that changed in their expression as these would be associated with ErbB-2 mediated transformation. Of the 35 proteins spots found

to show consistent differential expression between the two cell lines, 18 were identified by MALDI-MS. Mass spectrometric analysis showed that many of the proteins identified have a real biological role in ErbB-2-mediated transformation and hyperproliferation of the luminal epithelial cells studied. Using the 2D DIGE approach, several potential targets for breast cancer therapy were identified including heat shock protein 27 (showed the greatest increase in expression in DeCyder) which has been shown to be overexpressed in several human cancers. This study concluded that 2D DIGE is more rapid than conventional 2D analyses and is a highly reproducible technique for identifying statistically significant differences.

2D DIGE addresses shortcomings of 2D electrophoresis

Many of the disadvantages and shortcomings of 2D electrophoresis (2DE) have been overcome by 2D DIGE, making it an ideal technique for comparison of protein samples obtained under different physiological, developmental, or experimental conditions. Compared with Coomassie blue staining, 2D DIGE is very sensitive and, in contrast with silver staining, 2D DIGE has a high dynamic range resulting in more reliable quantification of protein spots than that obtained using standard colorimetric staining methods. Possibly the most important advantage of 2D DIGE is the ability to run multiple prelabeled samples on the same 2D gel. This eliminates the integral variability, common to standard 2D electrophoresis, thereby increasing the reproducibility and accuracy of results [6]. The high variability of standard 2DE also means there is a need to run several replicate gels for the generation of statistically confident results. The high accuracy and reproducibility of 2D DIGE means that biological (sample) replicates can replace gel replicates requiring fewer gels for more accurate results. In addition, the large amount of tissue normally required for running a 2D gel poses a real problem in the analysis of small samples, for example those from laser microdissection. This review has shown that this type of study can now be performed using the CyDye DIGE fluor saturation dyes. Two-dimensional DIGE is fully compatible with mass spectrometry. For identification of proteins of interest, two approaches can be applied. The first is not dependent on a prelabeling procedure and evidence of the second approach where prelabeling has been used with enough protein, shows that the labeling procedure does not interfere with the ionization of the protein during mass spectrometric analysis.

However, 2DE still has several limitations. One of the main disadvantages of 2DE is the inability to visualize low-copy-number proteins when highly abundant gene products are present. Although approximately 10,000–30,000 proteins are present in the proteome of a cell (even more in tissue), only between 1,000 and 2,000 of the most abundant proteins can be visualized on a single 2D gel. The development of several prefractionation and enrichment techniques, for example subcellular fractionation of organelles and protein fractionation based on different protein properties, enables more of these low-copy-number proteins to be visualized.

In addition to limitations in the number of proteins that can be visualized on a 2D gel, the types of protein which can be visualized are also limited. For example, the protein mixture obtained from a eukaryotic cell lysate is too complex to be completely resolved on a single 2D gel. Many large or hydrophobic proteins will not enter the gel during the first dimension, and proteins of extreme acidity or basicity are not well represented [30].

Limitations specific to DIGE

Limitations specific to 2D DIGE, are mainly associated with the labeling chemistry required for attaching the dye to the proteins. For example, with the minimal dyes, proteins with a high percentage of lysine residues could be labeled more efficiently compared with proteins containing few or no lysines. Therefore, a high abundance protein spot in a conventional gel system could be a medium or low abundance protein spot in the DIGE system because of its low lysine content. In addition, the technique is not applicable to those proteins without lysine (when labeling with the minimal dyes) or cysteine (when labeling with the saturation dyes) [4].

Conclusions

Though many have heralded the death of 2D electrophoresis, this technique continues to be one of the ''work horses'' of proteomics. The ability of this technique to deal with complex lysates and to simultaneously visualize large parts of the proteome under study remains unrivaled. For this reason, the use of new techniques recently developed continues to be complimentary rather than replacing 2DE.

The added advantages of reproducibility, sensitivity, and accuracy of quantitation provided by 2D DIGE has revolutionized the technique of 2D electrophoresis and, to some extent, increased the lifespan of this technique tool for proteome studies.

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