All about DIGE: quantification technology for differential-display 2D-gel proteomics

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Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) has been the traditional workhorse of proteomics, allowing for the resolution of several thousand proteins in a single gel. Difference gel electrophoresis (DIGE) is an emerging technology that allows for accurate quantification with statistical confidence while controlling for non-biological variation, and also increases the dynamic range and sensitivity of traditional 2D PAGE. With inclusion of an internal standard formed from equal amounts of every sample in an experiment, DIGE technology also allows for repetitive measurements and multi-variable analyses to be quantitatively analyzed in one coordinated experiment, yielding statistically-significant changes in protein expression related to many disease states. This technique promises to be an important tool in clinical proteomics and the study of the mechanism of disease, investigating diagnostic biomarkers and pinpointing novel therapeutic targets.

The application of proteomics technology in clinical studies does not only center around the elucidation of the mechanism of disease, but also the search for new diagnostic biomarkers and potential therapeutic targets. This area of proteomics is rapidly developing and will have a major impact on the way diseases will be diagnosed, treated and managed in the future [1]. The breadth of techniques that support clinical proteomics is also expanding at a considerable rate.

Historically, 2D polyacrylamide gel electrophoresis (PAGE) has been the protein separation technique most commonly associated with proteomics. Although this is considered to be well established, it has undergone a rebirth over the past few years. Despite the development of alternative proteomic strategies, 2D-PAGE promises to remain one of the key methodologies in clinical proteomics. 2D-PAGE has been widely used over the past 40 years to resolve several thousand proteins in a single sample. Proteins first undergo isoelectric focusing (IEF), which separates species based on their net charge, or isoelectric point (pI). The orthogonal second dimension is then used to further separate proteins based on molecular weight in the presence of denaturing conditions. The excellent resolving power of this technique has facilitated the identification of major proteins in tissue or subcellular fractions by mass spectrometric methods. In addition, 2D-PAGE has been used to compare relative abundances of proteins in related samples, such as control and diseased, allowing the response of classes of proteins to be determined. To date, most comparative protein profiling studies have produced qualitative data, which have enabled the investigator to determine whether or not a particular protein shows an increase or decrease in expression. This provides no measure of the extent of this change in expression. Therefore, it is unsuitable for the clustered data analysis required for an insight into functionality and potential elucidation of protein expression, which is characteristic of a diseased cell or tissue. Quantitative proteomics allows coexpression patterns to be studied, and proteins showing similar expression trends may be assigned membership of the same functional groups; however, quantification from 2D-PAGE has been hampered by several factors. First, silver
staining, being more sensitive than Coomassie staining methods, has been widely used for high-sensitivity protein visualization on 2D-PAGE, but it is unsuitable for quantitative analysis, as it has a limited ($10^4$) dynamic range. The most sensitive silver staining methods are also incompatible with protein identification methods based on mass spectrometry (MS). More recently, the Sypro family of postelectrophoretic fluorescent stains (Molecular Probes Inc./Invitrogen, Inc.) have emerged as alternatives, offering an increase dynamic range ($10^3$) and ease of use compared with silver staining [2]. Another problem has been the unreproducibility of 2D gels, as there can be considerable gel-to-gel variation, and corresponding proteins between two gels must be carefully and often laboriously matched prior to quantification. Finally, normalization has proved challenging, especially in the case of silver staining where staining is protein dependent. A combination of these factors adds variability to the system, which makes it unsuitable for accurate quantification.

Difference gel electrophoresis (DIGE) circumvents many of the issues associated with traditional 2D-PAGE, such as reproducibility and limited dynamic range, and allows for more accurate and sensitive quantitative proteomics studies. This review details DIGE experimental methodology, provides an overview to its applications in the literature, and describes its strengths and limitations.

**Overview of the technique**

The 2D-DIGE technique was first described by Jon Minden’s laboratory [3] and has subsequently been refined and marketed by Amersham Biosciences (now part of GE Healthcare). This technique relies on pre-electrophoretic labeling of samples with one of three spectrally-resolvable fluorescent CyDyes (Cy2, 3 and 5) allowing multiplexing of samples into the same gel. The scanned images from a typical 2D-DIGE gel are shown in **Figure 1**. There are currently two forms of CyDye labeling chemistries available: N-hydroxy succinimidyld ester reagents for low-stochiometry labeling of the ε-amine groups of lysine side chains (minimal labeling), and maleimide reagents for labeling cysteine sulfhydryls to saturation (saturation labeling).

**Minimal labeling**

The most established DIGE chemistry is the minimal labeling method, which has been commercially available since July 2002. CyDye DIGE fluoros are supplied as a N-hydroxy succinimidyld ester, which reacts with primary amino groups, typically the terminal amino group of lysine side chains. Labeling reactions are optimized such that only 2–5% of the total number of lysine residues are labeled. It is vital to maintain a low dye:protein ratio in labeling reactions to avoid multiple dye additions, which would result in multiple spots being resolved in the second dimension of the 2D-DIGE gel. The three fluoros are mass matched, each labeling event adding approximately 500 Da to the mass of the protein. The fluor carry an intrinsic charge of +1, such that the pI of the protein is maintained upon labeling. Labeling with CyDye DIGE fluoros is extremely sensitive. The detection limit is in the order of 150–500 pg of a single protein depending on the experiment, with a linear response in protein concentration over five orders of magnitude. In comparison, silver staining has a detection limit of 1 ng of protein with a dynamic range of less than two orders of magnitude [4,5].

The labeling system is compatible with the downstream processing commonly used to identify proteins via MS and database interrogation, which involves the generation of tryptic peptides within excised gel plugs. Trypsin cleaves the peptide bonds carboxyl-terminal of lysine and arginine residues; however, peptide generation is mostly unhindered as so few lysine residues are modified by dye labeling. Minimal labeling ensures that quantification is performed using protein molecules that have been labeled only once. However, both the mass and hydrophobicity

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**Figure 1.** Images from a pH 3–10 nonlinear 2D-DIGE gel of two protein samples isolated from mouse brain labeled with minimal CyDye DIGE fluoros. (A) The Cy3 image corresponding to sample 1 (false colored green). (C) The Cy5 image corresponding to sample 2 (false colored red). (B) The two images overlaid.

DIGE: Difference gel electrophoresis.
of the CyDyes influence protein migration during second-dimensional sodium dodecyl sulfate (SDS)-PAGE, such that the labeled portion of the protein may migrate at a higher apparent molecular mass than the majority of the unlabeled protein (exacerbated in lower molecular weight species). As a result of this fact, minimally labeled 2D-DIGE gels are often post-stained with a total protein stain such as SyproRuby to ensure that the maximum amount of protein is excised for subsequent in-gel digestion and MS.

**Saturation labeling**

A second labeling chemistry has been more recently released by Amersham Biosciences in 2003. It is designed for use in situations where sample abundance is limited. This saturation chemistry differs from the original minimal chemistry, in that CyDyes are supplied with a thiol-reactive maleimide group and carry no intrinsic charge. Utilization of these dyes results in the labeling of every cysteine residue within a protein. Saturation labeling is therefore much more sensitive than minimal labeling, as more fluorophor is incorporated into each protein species. Shaw and coworkers reported an order of magnitude increase in sensitivity over the original minimal dyes [6]. Whilst the added sensitivity that these dyes provide is attractive, their use is technically more challenging, and the small percentage of proteins that do not contain cysteine will be excluded from the analysis. Careful optimization of the labeling conditions must be carried out for each new sample set to ensure complete reduction of cysteine residues. An appropriate protein:dye ratio must be achieved to bring about stoichiometric labeling. Insufficient labeling will lead to multiple spots in the second dimension. Over-labeling leads to side reactions with lysine residues, resulting in the formation of charge trains in the first dimension. Comparison of the 2D spot maps between samples labeled with the two chemistries is impossible, as proteins containing multiple cysteine residues will appear as larger molecular weight species when labeled with the saturation dyes. When used with limited sample quantities, a preparative gel with increased protein loading is often required to produce mass spectrometric data suitable for database interrogation. Saturation dye-labeled proteins have also been reported to be compatible with MS [7]. Even in the event that additional sample cannot be produced to enable protein identification, this technique still provides valuable diagnostic information.

**Experimental design**

The 2D-DIGE methodology is at its most beneficial in protein expression profiling studies involving multiple samples. When used in conjunction with well-considered experimental design, the result will be the accurate measurement of the relative abundance of proteins from across a set of samples. In the case of minimal labeling chemistry, samples to be compared are labeled with either Cy3 or Cy5 CyDye DIGE fluoros, whereas the Cy2 CyDye DIGE fluor is employed to label a pooled sample comprising equal amounts of each of the samples within the study, and acts as an internal standard (FIGURE 2). The internal standard ensures that all proteins present in the samples are represented, assisting both inter- and intra-gel matching. Variation in spot volumes due to gel-specific variation, such as sample entry and electrophoresis in either the first dimension immobilized pH gradient (IPG) strip or second dimension SDS-PAGE gel, will be the same for each sample within a single gel. Thus, the relative amount of a protein in a gel in one sample compared with another will be unaffected. The spot volumes are normalized using a method based on the assumption that the majority of

![Figure 2. Outline of a 2D difference gel electrophoresis study using an internal pooled standard constructed from equal amounts of all the samples in the study, labeled with Cy2.](image-url)
protein spots have not changed in expression level, and accounts for dye-related discrepancies arising from differences in laser intensities, intrinsic fluorescent properties of the gel matrix and filter transmittance [8]. Direct comparisons of spot volumes are made between the Cy3- or Cy5-labeled samples and the Cy2-labeled internal standard for that gel. These ratios are normalized and compared with those generated for that particular protein from the other gels. The presence of the same pooled standard present on each gel therefore allows for the application of Student’s t-test and analysis of variance (ANOVA) statistical analyses to replicate single- and multivariable samples despite having samples separated on different DIGE gels.

For the analysis, software developed for the DIGE system (DeCyder™, Amersham Biosciences) is typically used, although other software solutions are becoming available. The DeCyder software package has a triple codetection algorithm that allows simultaneous detection of labeled protein spots from images that arise from the same gel and increases accuracy in the quantification of standardized abundance [8]. The standardized abundances can then be compared across groups to detect changes in protein expression, regardless of whether the samples to be intercompared have been resolved on the same DIGE gel. In the case of the saturation dyes, where a Cy2 fluor is not available, the internal standard is labeled with one of the dyes and individual samples labeled with the other dye. This approach is equally as powerful, but doubles the number of DIGE gels within a comparative experiment [6].

The design of a protein profiling analysis experiment using DIGE is paramount to the amount of statistical significance that can be placed on the data. Consideration must be given to methods employed to assess both biologic and experimental noise within the system. Ideally, repetitive measurements should be made on independent experiments to best control for both biologic (normal variation) and technical (sample preparation) variation. Employing a dye-swapping approach will control for any dye-specific effects that may result from preferential labeling or different fluorescence characteristics of acrylamide at the different wavelengths of excitation for Cy2, 3 and 5, particularly at low protein spot volumes. This is easily incorporated into any DIGE analysis where repetitive samples are used (along with the internal standard to compare across multiple DIGE gels). For example, in a simple wild type versus mutant comparison (n = 3), Cy3 would be used to label the wild-type samples for the first two gels and Cy5 would be used for the third wild-type sample, and conversely for the three mutant samples (with Cy2 used to label the mixed-sample internal standard that is loaded onto all three gels).

The multigel approach allows many data points to be collected for each group to be compared. Spots of interest can be selected by looking for significant change across the groups. Typically, a univariate statistical test such as Student's t-test or ANOVA is employed. These give a probability score (p) for each spot, indicating the probability that the observed change occurred due to stochastic, random events (null hypothesis). Probability values less than 0.05 are traditionally used to determine a statistically-significant difference from the null hypothesis. As this represents 50 potential false positives for 1000 resolved proteins, confidence intervals within the 99th percentile (p < 0.01) are arguably more valid, and can be attained using DIGE [9-13]. The number of replicates required in a study depends on the amount of variation in the system being investigated. Increasing the number of replicates will increase confidence in smaller changes in expression. In this regard, it is also beneficial to measure experimental variation in the DIGE process for any new set of samples. This will provide an indication of the inherent error of the system and suggest the threshold of significance or a fold change above which true changes in expression can be measured. This can be achieved by running sets of gels where the same sample is labeled with all dyes to be used, loaded onto one gel and fully analyzed. Subsequent classification and hierarchical clustering analysis can also be performed on the results, as in Seike and coworkers [14] and Yokoo and coworkers [15]. Such analyses are beneficial in extending DIGE applications for diagnostic and prognostic uses.

Applications of DIGE

2D-DIGE technology has been used very effectively to study a variety of systems and has enabled the detection of more subtle changes in protein expression than conventional 2D-PAGE [16]. Such studies include human breast cancer cells [17], human esophageal cancer cells [18], human colon cancer [10], yeast [19,20], Drosophila [13,21], murine mitochondria [22], mouse brain [23] and rat heart [24]. Many published studies utilized DIGE technology to test a single variable using a single DIGE gel. In these studies, statistical significance of the protein abundance changes was determined relative to the variation between the samples being compared. For example, proteins that exhibit an abundance change within the top 5% of the population would fall into the 95th-percentile confidence interval, which is roughly equivalent to two standard deviations of the mean abundance change for all of the proteins in the analysis (assuming a normal distribution of abundance changes).

More subtle abundance changes can be demonstrated with statistical confidence using the pooled internal standard methodology. This is because replicate samples can be intercompared across multiple DIGE gels, and the statistical analysis is performed on the variance of the mean change for a given protein across several measurements, irrespective of the overall variation that may exist in the global comparison. Elegant experiments can be designed whereby multiple variables (each measured repetitively for statistical confidence) can be intercompared across a set of DIGE gels that are all co-ordinated with the same pooled internal standard. Examples can range from single-variable comparisons (experimental vs. control) measured in triplicate and run on three co-ordinated DIGE gels, to complex multivariable comparisons and/or time course studies (using a concomitantly larger number of gels).

One such example using the Cy2-labeled internal standard approach compares the relative abundance of proteins in cerebral cortex from wild-type mice and neurokinin 1 receptor knockout mice, to elucidate molecular pathways involving...
this protein [9]. The authors compared relative abundance and significance values for differentially expressed proteins derived from gels incorporating the pooled Cy2-labeled standard, with values derived from the same gels but without normalizing protein spot volumes to the corresponding pooled standard. They demonstrated that virtually all differentially expressed proteins gave lower significance levels and a higher incidence of false positives when derived without using the pooled standard for normalization. The authors reported being able to measure as little as 10% change in abundance with 95% confidence (p < 0.05).

Several recent studies with clinical relevance have also employed the incorporation of the internal standard to quantify small but relevant changes in protein expression to great success. In the first study of Friedman and coworkers [10], the DIGE technique was employed to investigate the tumor-specific protein expression changes in the protein of human colorectal cancer compared with normal mucosa. Paired tumor and neighboring normal mucosa samples were taken from six patients presenting with different stages of colon cancer and run on six separate DIGE gels co-ordinated by a Cy2-labeled mixture of all 12 samples for the internal standard. Statistically significant quantitative comparisons resulted in the identification of 52 unique proteins whose change in abundance between the normal and diseased state was consistent across all patients. The authors note that the same data analyzed without the inclusion of the internal standard within the experimental design would only have revealed a small subset of consistent changes, despite having direct normal versus tumor Cy3:Cy5 comparisons present on each DIGE gel. This dramatic difference was largely due to the high degree of variation inherent between normal and tumor samples for a given patient. Within each single-gel comparison, significance levels for each protein change are dictated only by the distributions of abundance changes and correspondingly higher cutoffs for the 95th-percentile confidence interval. A second study of Prabakaran and coworkers utilized the DIGE technology to test the hypothesis that a subset of proteins are translationally regulated by a novel component of the yeast ribosome, Asc1p/RACK1 [20]. The authors compared protein expression from wild type, asc1Δnull and null strains complemented with plasmids expressing either the yeast Asc1p or the mammalian homolog RACK1 (which functionally complements the asc1Δnull allele). Protein extracts were prepared from each of the four genotypes on three independent occasions to control for sample preparation variation and to provide replicate samples for statistical analysis. The resulting 12 samples were inter-compared across six DIGE gels that were co-ordinated by the same Cy2-labeled mixed-sample internal standard (comprising an equal mixture of all 12 samples), producing 18 DIGE proteome maps (FIGURE 3A). Of the approximately 1500 resolved proteins between pl 4–7, only 31 proteins were found to exhibit greater than 1.5-fold difference between wild type and asc1Δnull (p < 0.05, Student’s t-test). Remarkably, 27 of these proteins were upregulated in the asc1Δnull strain and complemented back to wild-type levels with either the yeast protein or mammalian homolog (FIGURE 3B). This phenotype was as predicted for a regulator of protein translation, and the results were further supported by the finding that transcript levels of the misregulated proteins were statistically indistinguishable amongst the different genotypes.

Most of the aforementioned studies utilized DIGE technology as a hypothesis-generating experiment, and as such, the technology has been the driving-force for the experimental design. However, as the technology becomes more established and commonplace, the technique will be increasingly utilized for hypothesis-testing experiments as part of a larger study. In a recent example, Gerbasi and coworkers utilized DIGE technology to test the hypothesis that a subset of proteins are translationally regulated by a novel component of the yeast ribosome, Asc1p/RACK1 [20]. The authors compared protein expression from wild type, asc1Δnull and null strains complemented with plasmids expressing either the yeast Asc1p or the mammalian homolog RACK1 (which functionally complements the asc1Δnull allele). Protein extracts were prepared from each of the four genotypes on three independent occasions to control for sample preparation variation and to provide replicate samples for statistical analysis. The resulting 12 samples were inter-compared across six DIGE gels that were co-ordinated by the same Cy2-labeled mixed-sample internal standard (comprising an equal mixture of all 12 samples), producing 18 DIGE proteome maps (FIGURE 3A). Of the approximately 1500 resolved proteins between pl 4–7, only 31 proteins were found to exhibit greater than 1.5-fold difference between wild type and asc1Δnull (p < 0.05, Student’s t-test). Remarkably, 27 of these proteins were upregulated in the asc1Δnull strain and complemented back to wild-type levels with either the yeast protein or mammalian homolog (FIGURE 3B). This phenotype was as predicted for a regulator of protein translation, and the results were further supported by the finding that transcript levels of the misregulated proteins were statistically indistinguishable amongst the different genotypes.

The recent studies of Prabakaran and coworkers [12] and Gerbasi and coworkers [20] are also of note in that DIGE is used as a key component to the study, but is not the driving force of the study. As such, less DIGE information is presented in these studies, with the main focus being on the results of the DIGE experiments and how they complement the other experimental tools used. This expected trend is another indicator that DIGE technology is gaining acceptance in the field of 2D-gel-based proteomics.
Alternative technologies

Historically, the most widely used technology platform for protein expression profiling has been 2D-PAGE, of which the 2D-DIGE refinement provides a robust manner in which statistically significant data can be obtained. Non-2D gel-based technologies are becoming increasingly more routine. One such technique involves the differential incorporation of stable isotopes at either the protein or peptide level. There are several ways in which this can be achieved. One method involves the growth of cultures in the presence of a defined medium containing a heavy isotope, typically $\text{^{15}N}$ [32,36–38]. Samples grown in the presence of the natural and heavy isotope can be pooled, reduced to peptides, and the peptides separated by multidimensional liquid chromatography before application to MS. The relative abundance of a peptide generated from a protein within cultures being compared is then calculated by measuring ion intensities of the light and heavy versions of the same peptide within the mass spectrometer. The use of tandem MS in this approach leads to the relative quantification of the peptides, as well as generating data for protein identification via database interrogation. This approach has limited applications within clinical studies due to the necessity of growing samples in defined media.

A more widely applicable variation of this method is to label extracted protein with tags, which can be produced in more than one isotopic form, such as the isotope-coded affinity tag reagent that was pioneered by Aebersold in 1999 and has been further developed and marketed by Applied Biosystems [33,39]. This method involves the labeling of cysteine residues within a protein with a biotinylated tag that can be purchased in a light and heavy format. The heavy tag has nine $\text{^{13}C}$ atoms and hence, after labeling, pooling of the light and heavy labeled samples and trypsinolysis, the same peptide present in both starting samples will be present in two forms, one of which will be 9 Da heavier than the other. Simplification of the peptide mixture is brought about by applying the peptides to a monomeric avidin column that will bind only those peptides containing the biotin tag. Similar strategies involving labeling the amino-termini of every digested peptide from a complex mixture with isotopically labeled phenyl isocyanate [35] or with new isotope tagging reagents (e.g., iTRAQ™, Applied Biosystems [40]), or performing the trypsin digestion in the presence of $\text{^{16}O}$ versus $\text{^{18}O}$ water [34], are also available. These approaches are not limited to cysteine-containing peptides and therefore provide a more comprehensive analysis, as
well as increasing the likelihood of analyzing post-translational modifications. The increased complexity of the resulting peptide mixture becomes problematic during the MS stage, but this limitation is being overcome by newer generation mass spectrometers that provide increased scan speed and sensitivity.

There are additional methodologies for profiling protein expression that focus on intact protein expression. Particularly noteworthy are newer methodologies involving the profiling and imaging of intact proteins and peptides directly from tissue biopsies using MS [41–43]. With this approach, linear matrix-assisted laser desorption/ionization time-of-flight MS is used to acquire information on the relative abundance of intact proteins (mass-to-charge ratio \([m/z]\)) that can be tracked across intact tissue sections (or within subregions for a lower resolution profile), creating an image of the tissue section based on the distribution of several hundred \([m/z]\) signals. This technology has recently been applied to correlate drug delivery with protein abundance changes [44]. In some cases, it can even create patient-specific signatures that can be used as diagnostic or prognostic biomarkers for important diseases or traits [45]. Another completely different method of profiling protein expression is through the use of protein arrays. The most promising tool within this field for the application to clinical studies is the antibody array. In this system, antibodies raised against proteins of interest are covalently attached to a solid support. Extracted proteins are incubated with the array and the extent of binding of protein ligands to the array is assessed by a fluorescent tag. This approach has the potential to be very sensitive; however, for real-world applications it depends on the manufacture of a comprehensive array. It may be the case that such arrays will only ever contain a subset of proteins of interest, since producing an array that covers every isoform of every protein in the cell would be challenging to say the least [46].

**Expert opinion**

Comprehensive proteomic analyses almost always benefit from the complementary nature of multiple technology platforms, and DIGE is no exception. For global protein expression analysis, every protein should be resolved as a discrete detectable spot on a 2D gel. Proteins with extreme pI or molecular weight, hydrophobic proteins and lower abundance proteins are poorly represented on a 2D gel. Furthermore, the dynamic range of protein concentration in a cell is much greater than can be handled by any current proteomics technology, and low-abundance proteins are less tractable in 2D gels owing to total protein load limitations [27]. Comigration is also an issue, as proteins of similar pI and denatured molecular weight resolve at the same position of the gel, thus making it impossible to accurately determine the relative abundance of an individual protein within a mixed spot. However, improvements to the 2D-PAGE technique have been made over the last few years. Enhanced resolution of protein species can be achieved by the use of narrow range IPG strips and large-format 2D gels [28]; 24 cm width is commercially available, but see also Klose and coworkers [29]. Prefractionation of the sample is also useful in this regard [30]; however, it subjects the experiment to additional nonbiologic variation (although this can be controlled using repetition and the internal standard methodology). Membrane proteins are still problematic, even when more rigorous detergents such as amidosulphobetaine 14 are used [31]. Integral membrane proteins, which make up a mechanistically important subset of proteins, have a tendency to precipitate during IEF and their study is best carried out using alternative quantitative methodologies.

**Five-year view**

2D-DIGE is the most powerful 2D-PAGE-based approach for widespread protein profiling studies by virtue of its ability to multiplex and link samples across numerous different gels in a study using an internal standard. This approach also provides information about more subtle changes in protein expression than conventional 2D-PAGE. The advent of the saturation dyes has increased the sensitivity of this system. No single proteomics technology platform is capable of a true global analysis of the entire proteome, although several platforms often provide complementary information. 2D gel-based proteomics have an advantage in visualizing changes in the intact molecular weight and pI of a protein, (reflective of biologically significant processing and charge-altering post-translational modifications such as phosphorylation, sulfation and acetylation/deacetylation). However, even with the increase dynamic range and detection sensitivity afforded using DIGE, hydrophobic and low-abundant proteins are difficult to identify owing to inherent limitations of the 2D system and subsequent recovery of in-gel-digested peptides. Other complementary techniques, most notably MS-based approaches using differentially labeled stable isotopes, offer a greater sensitivity in protein detection and larger dynamic range as the protein signals are detected directly by the mass spectrometer, which has sensitivity in the low- to subfemtomole range [32–35]. Quantification is performed at the peptide level using MS after proteolytic digestion of a starting protein mixture. However, as protein mixtures become more complex, it becomes more challenging to resolve peptides to acquire mass spectral information on enough peptides from a given protein to distinguish between proteolytic products and/or post-translational modifications that are readily available through the visualization of protein migration in a 2D gel.

Specific limitations of both gel- and MS-based proteomic technology platforms are not insurmountable. The past several years have seen many limitations overcome by advances in instrumentation and quantification. As such, one can expect both 2D-DIGE and isotope labeling MS approaches to be used on a complementary basis to study expression profiling of proteins.

**Information resources**

- An Amersham Biosciences Ettan DIGE user manual: www4.amershambiosciences.com
  (Viewed November 2004)
Key issues

• 2D difference gel electrophoresis (DIGE) involves the pre-electrophoretic labeling of complex protein samples using cyanine based fluorescent tags prior to carrying out separation by 2D polyacrylamide gel electrophoresis.

• There are currently two labeling chemistries available within this technique: minimal labeling of 2–5% of lysine residues via N-hydroxy succinimidyl ester-derivatized Cy2, 3 and 5 CyDye DIGE fluoros; and saturation labeling of all cysteine residues via maleimide-derivatized Cy3 and Cy5CyDye DIGE fluoros. This latter type of labeling is particularly useful in cases where sample amounts are scarce.

• In both cases, labeling does not interfere with subsequent identification by mass spectrometric techniques of proteins excised from 2D-DIGE gels, since most peptides will not contain a label.

• Multiplexing of samples on a single 2D gel is possible as Cy2, 3 and 5 are spectrally resolvable, allowing separate images pertaining to each sample to be detected.

• The relative abundance of a protein between two or more samples can be measured by comparing the amount of fluorescence associated with each dye for a given spot.

• Experimental design is crucial for the success of the 2D-DIGE technique. In cases where individual samples are compared with an internal standard, made up of an equal amount of protein from each sample in an experiment, small amplitudes in the changes in expression for given protein can be reliably measured because repetitive measurements can be made.

• The mixed-sample internal standard technique also allows for multivariable analyses, as samples that are to be intercompared can be separated on several DIGE gels that are all co-ordinated by the internal standard.

References


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