Comparative Proteomics and Difference Gel Electrophoresis

Jonathan Minden
Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA, USA

The goal of comparative proteomics is to analyze proteome changes in response to development, disease, or environment. This is a two-step process in which proteins within cellular extracts are first fractionated to reduce sample complexity, and then the proteins are identified by mass spectrometry. Two-dimensional electrophoresis (2DE) is the long-time standard for protein separation, but it has suffered from poor reproducibility and limited sensitivity. Difference gel electrophoresis (DIGE), in which two protein samples are separately labeled with different fluorescent dyes and then co-electrophoresed on the same 2DE gel, was developed to overcome the reproducibility and sensitivity limitations. In this essay, I discuss the principles of comparative proteomics and the development of DIGE.

Introduction

What makes a stem cell distinct from its differentiated progeny? What makes a cancer cell behave differently from its normal neighbors? How do cells respond when exposed to different agents, such as drugs, viruses, or other cells? Whether these cellular changes are due to developmental, genetic, or environmental effects, they are ultimately mediated by changes in protein expression or modification. Mammalian cells contain thousands of different protein species, only a small number of which are changed under such circumstances. The primary goal of comparative, or differential, proteomics is to discover the protein changes that underlie these cellular changes.

Compared to genomics or transcript profiling, proteomics represents a much greater technological challenge, because the chemical complexity of the proteome is vastly greater than that of nucleic acids, and unlike nucleic acids, proteins cannot be amplified, which means that methods must be developed to handle minute amounts of protein. To appreciate the scale of chemical complexity of the proteome, let’s consider the chemical space that cellular proteins occupy. For the purposes of this discussion, this protein chemical space can be defined by three axes: protein mass, protein isoelectric point (pI), and protein abundance. Figure 1A shows 1000 hypothetical proteins populating this chemical space, where protein mass ranges from 10,000 to 1,000,000 Da, pI varies from 3 to 10, and abundance spans 1000 to 10,000,000 copies/cell. To avoid overcrowding, this plot underestimates the number of protein species by at least an order of magnitude and does not take alternative splicing and posttranslational modification into account. In reality, this plot would have much more densely distributed spots.

The challenge of comparative proteomics is to sift through this protein space and identify the few proteins that differ between the samples being compared. Proteome analysis has two essential components: protein fractionation and protein identification. Protein fractionation is necessary to reduce the protein complexity, so that the genes that encode the proteins of interest can be identified, and is typically done by column chromatography or gel electrophoresis. Protein identification is accomplished by mass spectrometry (MS). Although tremendous advances in MS technology have greatly improved our ability to identify protein fragments rapidly (1), fractionation is still necessary to reduce the complexity of the proteins, or their proteolytic fragments, prior to MS analysis.

Gel electrophoresis and column chromatography are the most common methods for protein fractionation. It is important to recognize that no fractionation scheme is capable of perfectly resolving all of the protein species found in cells or tissue—the chemical complexity is too great relative to the resolving power of these physical separation methods. Two

Figure 1. Simulation of protein chemical space. (A) Shows a simulation of 1000 proteins (black spots) distributed within a chemical space defined by protein isoelectric point (pI), molecular weight, and abundance. (B) Shows the addition of 50,000 tryptic peptide fragments (red spots) that result from the digestion of the protein spots. The spot size of the tryptic peptides are reduced relative to the protein spot so as not to overwhelm the image.
or more orthogonal separation schemes are typically used to increase resolution. Two-dimensional electrophoresis (2DE) relies on separating proteins based on pI and mass. Many technological improvements have made 2DE relatively inexpensive and accessible to most biomedical research labs. Column chromatography offers a wide range of matrices for protein separation. Still, 2DE and multidimensional chromatography are not sufficient to resolve all cellular proteins. Column chromatography offers the advantage of automation and direct feed into MS. However, directly feeding column fractions into the MS limits the duty time allotted to analyze each fraction. Each fraction, therefore, represents a moving target. 2DE allows separated protein samples to be maintained within the gel, thus allowing more time to analyze each position within this separation space. An added advantage of 2DE is that several samples can be run simultaneously in a single apparatus, while column chromatography is limited to a single sample per apparatus. Thus, the sample throughput is much higher for 2DE gels than column-based separation methods.

2DE and column chromatography subdivide the chemical space into small regions, thus lowering the complexity of the protein mixture within each fraction. It is important to recognize that these fractionation methods do not affect the protein concentration range within each fraction.

Difference Gel Electrophoresis

How does one find proteome differences between two or more samples? There are two general approaches to discover proteome differences: cataloging versus differential detection methods. Cataloging attempts to measure all proteins or peptide species within a single sample and then compares that catalog to another catalog. For cataloging to work, one must be able to reproducibly separate the samples to be compared into identical fractions, which is a thermodynamic impossibility. There are too many experimental variables, such as fluctuations in flow rate or voltage, temperature, sample preparation, etc. Thus, no two samples can be run identically, which means that comparing catalogs of proteins is inherently flawed. Indeed, early analysis of 2DE gels showed the need for computational methods to morph the image of one gel into another. These computational methods have been unsuccessful because the variation between gels is not predictable and thus cannot be sufficiently modeled to remove all gel-to-gel variation. Ideally, experiments should be run with internal controls, which would obviate the effects of experimental variation. The desire to generate a differential proteomic method that has an internal control prompted us to design matched fluorescent dyes that could be covalently linked to proteins without affecting their relative migration in 2DE gels. These efforts resulted in the development of difference gel electrophoresis (DIGE) (2).

Figure 2. Structure of difference gel electrophoresis (DIGE) dyes. Minimal labeling dyes coupled to primary amines via a N-hydroxy succinimadyl (NHS) ester. Saturation labeling dyes are covalently linked to cysteine residues by forming a maleimide adduct. Minimal labeling dyes have a net positive charge to compensate for the charge on primary amines. Saturation labeling dyes are zwitterionic to match the zero charge on cysteine.

Four rules were applied when designing fluorescent dyes for DIGE: (i) the dyes have the same charge; (ii) the charge on the dye molecules match the charge of the protein residue to which the dye is covalently attached; (iii) the dyes have similar molecular weight; and (iv) the dyes have sufficiently different fluorescent properties so that they can be discriminated using appropriate optical filters. Cyanine dyes were used because of their versatility in derivative synthesis, stability, and fluorescent characteristics. The original pair of DIGE dyes (propyl-Cy3-NHS and methyl-Cy5-NHS) have a single positive charge, are targeted to primary amines such as the N terminus and lysine residues, differ in mass by 2 Da (464 Da versus 466 Da for propyl-Cy3-NHS and methyl-Cy5-NHS, respectively), and are excited by 550 and 650 nm light, respectively (Figure 2). A third amine-reactive dye has also been created, Cy2. These dyes allow the comparison of proteins within two or three cellular extracts by separately labeling the extracts with either Cy2, Cy3, or C5 and then co-electrophoresing the labeled samples on the same 2DE gel. The optimal extent of labeling is dictated by the frequency of occurrence of the residue to which the dyes are target-ed, which in this case is lysine. An average size protein of 50 kDa has about 30 lysine residues. Labeling all of these lysines with the DIGE dyes causes large mass shifts in the proteins and compromises their solubility. Anything less than 100% labeling causes the proteins to form smears along the molecular mass [sodium dodecyl sulfate (SDS)] dimension. To avoid this heterogeneity, we use a minimal labeling strategy where 1%–2% of all lysines are labeled, which results in only a fraction of each protein species carrying a single dye molecule, while the rest are unlabeled.

The next generation of DIGE dyes target cysteine residues. Cysteine occurs much less frequently than lysine, thus allowing for saturation labeling of all cysteines. These saturation labeling dyes are also based on the cyanine framework (Figure 2). They have no net charge and have similar molecular masses. Thus, both the minimal labeling
amine-reactive and saturation labeling cysteine-reactive dyes satisfy the four basic requirements of DIGE dyes. In practice, the minimal labeling dyes are used when one has >100 µg protein/sample. Saturation dyes are typically used for low abundance, precious samples.

The typical workflow for a DIGE experiment is to isolate protein extracts in a buffer that is amenable to the particular DIGE dyes (pH 8.0 amine-free buffer, such as HEPES, for minimal labeling dyes or tris(2-carboxyethyl)phosphine (TCEP)-reduced samples for saturation labeling dyes). The labeling reactions typically require a 30- to 60-min incubation, followed by a quenching step to neutralize unincorporated dye. The samples are then combined and run on the same 2DE gel according to standard protocols. The resultant gels are then imaged on a fluorescence imager capable of detecting the DIGE dyes. This produces two or three images that are in perfect register for comparing protein differences. Once protein differences are discerned, the protein spots are excised from the gel, and the difference proteins are identified by MS (3).

DIGE labeling is fast, only adding about an hour to normal sample preparation time. One can run 10 or more 2DE gels over a 2-day period, depending on the electrophoresis equipment. It is sensitive, detecting <0.5 fmol protein with minimal labeling and <25 amol with saturation labeling. A key advantage of DIGE is that it allows the detection of isoform changes, such as posttranslational modification or alternative splicing.

There are MS-based difference methods based on the introduction of stable, heavy isotope tags or amino acids (1). These methods rely on measuring the abundance ratio of differently tagged peptides. These methods are high-throughput with respect to peptide analysis, but each comparison takes 1 to 2 days per mass spectrometer. Thus multiple comparisons and their replicates can be time consuming. These methods are not well suited for detecting posttranslational modification, unless a scheme is used to isolate specifically modified peptides. In terms of chemical space, tryptic digestion increases the mixture complexity 50- to 60-fold, since an average size 50-kDa protein has 50–60 tryptic cleavage sites. These peptides are restricted to a smaller mass and pI range, but the abundance profile is unaffected (Figure 1B).

**Comparative Proteomics Limitations**

No comparative proteomic method is a panacea. DIGE is limited by the separation capabilities of 2DE, which does not efficiently resolve large proteins or integral membrane proteins. Minimal labeling produces populations of labeled and unlabeled proteins, with the majority being unlabeled. The addition of a single dye molecule causes a slight retardation of proteins in the molecular mass dimension (not the isoelectric focusing dimension). This shift is negligible for proteins >30 kDa. Proteins <30 kDa are generally shifted by less than one spot diameter. DIGE gels can be stained post-electrophoresis to assure that the correct spot for MS analysis is picked. Saturation labeling dyes do not have this shift problem, since all proteins are maximally labeled.

A major issue for any proteome analysis is contending with the wide range of protein abundance. Standard protein fractionation methods subdivide complex mixtures along specific axes of the protein-chemical space, such as: molecular mass, protein charge, which is a function of pI, or hydrophobicity. Unfortunately, there are no direct methods for fractionating along the abundance axis. Subcellular fractionation is often suggested as a means to reduce protein complexity. However, subcellular fractionation may not reduce the abundance range of protein in the mixture. For example, nuclei are composed of highly abundant histones and very low abundance transcription factors, representing a four order of magnitude abundance range. Different membrane fractions also contain highly abundant proteins and very low abundance proteins. This is made even more acute when considering rare transient modification states, such as phosphorylation and ubiquitination. An alternate approach for reducing the complexity of protein samples is affinity chromatography, in which specific chemical attributes, such as phosphorylation, are used to isolate

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**Figure 3. Visualization of fluorescent protein spot from a two-dimensional electrophoresis (2DE) difference gel electrophoresis (DIGE) gel.** (A–E) The maximum pixel intensity values are halved from 32,000 to 2000 to demonstrate the presence of very low abundance proteins. The images are scaled between: (A) 700–32,000 counts, (B) 700–16,000 counts, (C) 700–8,000 counts, (D) 700–4,000 counts, and (E) 700–2,000 counts. (F) The result of subjecting the image to analysis by SExtractor astronomical software (7). The difference in intensity of the brightness protein (red arrowhead) and the dimmest protein (yellow arrowhead) is about 20,000-fold. Notice that this image is displayed on a logarithmic intensity scale, allowing one to see dim and bright spots simultaneously.
certain populations of proteins. However, there are high abundance phosphoproteins as well as low abundance phosphoproteins. Thus, it is important to develop methods that can accommodate protein abundance variation over four or more orders of magnitude.

Several reports have claimed that 2DE is unable to detect low abundance proteins (4–6). This limitation has more to do with the detection method and the MS analysis. These reports use silver-stained gels, which have a detection range of about 40-fold. Digital imaging of 2DE-DIGE gels with a scientific-grade, cooled charge-coupled device (CCD) camera is capable of detecting proteins over a 20,000-fold concentration range. This large concentration range is impossible to appreciate with unaided eyes, which have a detection range of about 30-fold under constant illumination. Figure 3 shows a fragment of a DIGE gel at various intensity settings. The first frame shows the gel with the pixel intensity scaled linearly between the dimmest and brightest pixels, 700 and 32,000 counts, respectively. This scaling does not reveal many protein spots. Subsequent images reduce the maximum pixel setting by half each frame. Pixel values above this maximum setting appear white. As the maximum pixel setting is lowered, one can see more and more protein spots. However, as this value is lowered to 2000 counts, the image blooms, and most of the spots appear to fuse together. Notice that new protein spots appear in areas of the gel that appear blank at higher maximum pixel settings. Astronomers have had to contend with sky images that have very broad intensity ranges. Applying their methods to DIGE gel images has allowed us to display proteins over a much wider intensity range, as well as automatically detect proteins and quantify them (7,8). Comparing the brightest protein spot to the dimmest shows a 20,000-fold concentration range. Thus, the limitation in protein identification from 2DE gels may have more to do with efficient handling of very low quantities of protein and MS detection limits.

There are several issues to bear in mind when embarking on a comparative proteomics study: (i) experimental design, (ii) sample preparation, and (iii) difference protein validation. The most crucial aspect of DIGE is sound experimental design. It will always take more than one comparison to get sufficient information. Endeavor to maximize for cells of the desired phenotype and avoid introducing sampling artifacts. DIGE is very sensitive, and variation in tissue dissection or sample fractionation can create sampling errors and incorrect conclusions. Sample preparation is critical for ensuring the removal of compounds that may interfere with labeling reactions and 2DE. Once differences are identified, it is essential to validate the role the difference proteins play in the biological process being investigated or as indicators of the process-of-interest. This requires the development of reliable high-throughput assays for the functional assessment of candidate proteins.

Prior to DIGE, 2DE had two critical limitations: reproducibility and sensitivity. DIGE has been very effective at solving the reproducibility problem by allowing one to compare multiple samples within a single gel. The sensitivity problem of the past had more to do with the detection systems than the theoretic limits of the electrophoresis method. Fluorescent labeling, high-end imaging devices, and analysis allow us to peer more deeply into the proteome than ever before. In fact, the fluorescent detection methods challenge our technical ability to move these very low abundance proteins out of the gel and into the mass spectrometer in sufficient amounts for identification. Thus DIGE provides a reliable and sensitive platform for discovering proteome changes in a boundless variety of circumstances.

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References