Large Gel Two-Dimensional Electrophoresis: Improving Recovery of Cellular Proteome

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Abstract: One of the goals of expression proteomics is to display and analyze all the proteins in a particular proteome. Cells are thought to comprise tens of thousands of proteins expressed in a dynamic range of 1-10^5 or 10^6. Low recovery of cellular proteome leads to a gross loss of important proteins. Thus, proteomics demands a powerful technology that separates complex mixture of proteins including low abundant ones. In the case of two-dimensional gel electrophoresis (2-DE), enlargement of the gel size appears a straightforward and effective strategy for improving the recovery of cellular proteins. Multiple narrow pH range immobilized pH gradients (nrIPGs) and long isoelectric focusing (IEF) gels afford improved separation of proteins in the first dimension according to isoelectric point. In addition, multiple long SDS-PAGE gels of different polyacrylamide concentrations provide a tool to improve the resolution of the second dimension according to molecular weight. Recent data suggest that 2-DE with large gels can display more than 11,000 protein spots expressed in a 1-10^5 dynamic range in cells.

Key Words: Proteome, Two-dimensional gel electrophoresis, Dynamic range, Low abundance, Resolution of separation, Recovery.

INTRODUCTION

Recent improvement of mass spectrometry (MS) together with accumulating genome information has opened up a field of proteomics. Identification of nanogram levels of proteins is now routinely performed by mass spectrometry combined with genome database searching (Aebersold and Mann, 2003; Roepstorff, 1997). Two-dimensional gel electrophoresis (2-DE) has been a core technology of proteomics that can separate complex protein mixtures in cells and tissues prior to MS analysis (Klose, 1975; O'Farrell, 1975; Dunn and Görg, 2001; Rabilloud, 2002). 2-DE-based proteomics not only enables identification of proteins expressed but also provides quantitative data of protein expression and post translational modification (Rabilloud, 2002), thereby affording a global analysis of protein behavior and a survey of novel diagnostic markers and drug targets of various diseases.

For these purposes, a challenge for proteomics is to display and analyze all the proteins in a particular proteome. At present, it is not clear how many proteins are expressed in individual cells. Recent genome studies have identified about 30,000-40,000 mammalian genes for coding proteins. In addition, a recent analysis of human platelet proteome suggested that single gene is on average represented by about 2.3 protein spots on 2-DE gels (O'Neil *et al.*, 2002). Therefore, if we postulate that a mammalian cell expresses 10,000 genes, we can estimate that it may have 23,000 or more modified proteins. On the other hand, a standard proteomic analyses with 2-DE usually detects only a few thousand of cellular proteins, which cover probably less than 10% of total proteome. Thus improvement of the coverage of cellular proteins is critical, in order not to miss a large number of important proteins. This paper presents an overview of recent advances in increasing the recovery of proteome by large gel 2-DE.

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CHALLENGE FOR 2-DE

Detection of low abundant, hydrophobic, high molecular weight, and basic proteins has been a challenging issue for increasing the recovery of proteome by 2-DE. However, recent progress in 2-DE technologies is gradually overcoming at least part of these problems. Improvements in the detection of hydrophobic and basic proteins are reported elsewhere (Görg *et al.*, 1997; Rabilloud, 1998; Dunn and Görg, 2001; Hoving *et al.*, 2002; Tastet *et al.*, 2003). Because the cellular expression levels of many important proteins, such as, signaling molecules and regulatory proteins are low, detection of low abundant proteins is of particular importance. A simple way for the detection of these proteins would be to load more protein samples to 2-DE gels or to increase the sensitivity of protein detection. However, resolutions of standard 2-DE gels are not enough for the separation of large number of proteins. Broad dynamic ranges of protein expression in cells are challenges to detect low abundant proteins by proteomics with 2-DE (Corthals *et al.*, 2000) and without 2-DE. The dynamic range of proteins expressed a cell is estimated to be 1-10^5 or 10^6, while that of standard 2-DE gels for protein detection is less than 1-10^4 (Rabilloud, 2002). When large amounts of protein samples are loaded, saturation and fusion of abundant protein spots occur in standard 2-DE gels while low abundant proteins are under the limit of detection.

Figure (**1**) shows an example. Protein sample (50 μg) of cultured rat hippocampal neurons was separated by a

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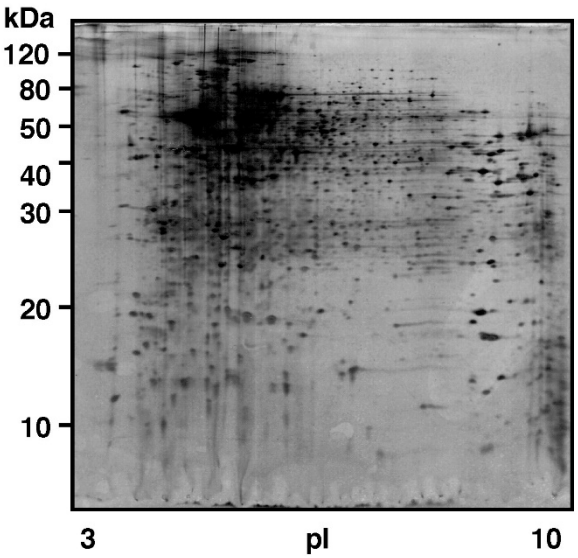

Fig. (1). A silver stained standard 2-DE gel (pH 3-10 / 18 cm x 20 cm/13.5% (w/v) acrylamide) detected 853 spots from 50 μg samples of primary cultured rat hippocampal neurons (Oguri *et al.,* 2002).

standard 2-DE gel (18 cm x 20 cm). Silver staining detected 853 protein spots in the gel (Oguri *et al.*, 2002). The protein concentrations of the most abundant ones, tubulin and actin, were about 2 μg in this sample. On the other hand, the detection limit of protein spots was about 2 ng. Therefore, the dynamic range of the protein detection was about 1-10^3 using this gel. Importantly saturation and fusion of protein spots are noted in the crowded region of the gel (Fig. **1**), thereby showing the limitation of the resolution of the gel. Therefore, further processes are needed for improving the separation of protein mixtures.

SAMPLE PREFRACTIONATION

Sample prefractionation is a possible approach to increase the recovery of proteome by 2-DE. Readers are referred also to the recent review on this matter by Corthals *et al.* (2000). Briefly, complex mixtures of cellular proteins are fractionated prior to 2-DE by various approaches, such as, isoelectric focusing (IEF)-based technology (Weber *et al.*, 2000; Görg *et al.*, 2002; Locke *et al.*, 2002; Zuo and Speicher, 2002), reverse phase chromatography (Badock *et al.*, 2001; Van Den Bergh *et al.*, 2003), protein extraction (Klose, 1999a; Molloy *et al.*, 1999) and subcellular fractionation (Krapfenbauer *et al.*, 2003). These procedures efficiently simplify and enrich mixtures of proteins including low abundant ones, therefore, are useful to improve the recovery of proteome by 2-DE.

MULTIPLE nrIPGs AND LONG IEF GELS IMPROVE PROTEIN SEPARATIONS IN THE FIRST DIMENSION OF 2-DE

Although various procedures of sample prefractionation enable to detect increased number of cellular proteins by 2-DE, they appear not very useful for quantitative proteomics.

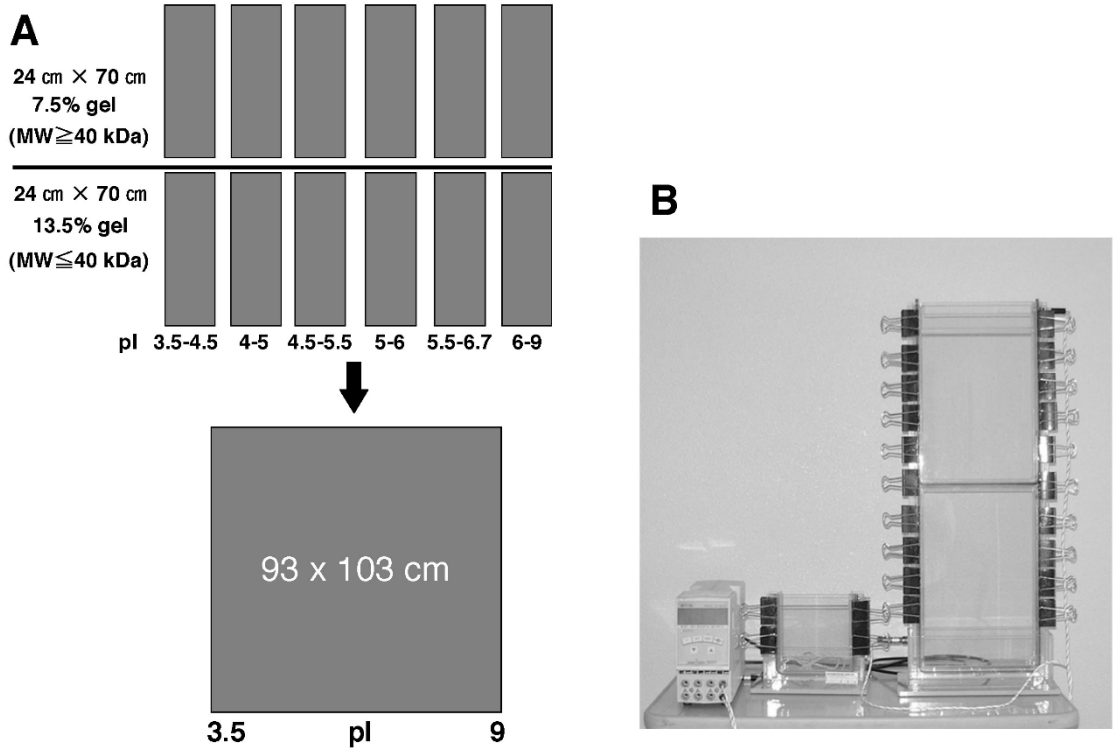

Fig. (2). (**A**) The strategy to make a 93 cm x 103 cm cyber gel from multiple long gels. (**B**) The long gel (gel size: 24 cm x 70 cm x 1 mm) vertical electrophoresis unit (right) and a standard medium sized (gel size: 16 cm x 16 cm x 1 mm) vertical electrophoresis unit (center).

Additional procedures prior to the electrophoresis reduce quantitative capability, a distinctive advantage of 2-DE over 2-DE free proteomic approaches. Another strategy for improving protein recovery without reducing the quantitative capability is enlargement of the 2-DE gel size. Recent studies showed improved resolving power of the first dimensional electrophoresis by introducing long IEF gels (Klose, 1999b; Poland *et al.*, 2003) or multiple narrow pH range immobilized pH gradients (nrIPGs) (Görg *et al.*, 2000; Hoving *et al.*, 2000; Cordwell *et al.*, 2000; Westbrook *et al.*, 2001; Tonella *et al.*, 2001; O'Neil *et al.*, 2002). In particular, commercially available nrIPGs provided increased reproducibility to the results, thereby making 2-DE techniques easier. As successful examples, Gauss *et al.* (1999) detected 8767 protein spots from mouse brain supernatant by using 40 cm x 30 cm gels. Combinations of multiple nrIPGs displayed 4950 protein spots from *Escherichia coli* proteome (Tonella *et al.*, 2001) and 2300 protein spots from human platelet samples (O'Neil *et al.*, 2002).

MULTIPLE AND LONG SDS-PAGE GELS FOR IMPROVED PROTEIN SEPARATIONS IN THE SECOND DIMENSION OF 2-DE

In addition, multiple and long SDS-PAGE gels of different polyacrylamide concentrations increases the resolution in the second dimension of 2-DE (Oguri *et al.*, 2002). We consider that improvement of the resolving power

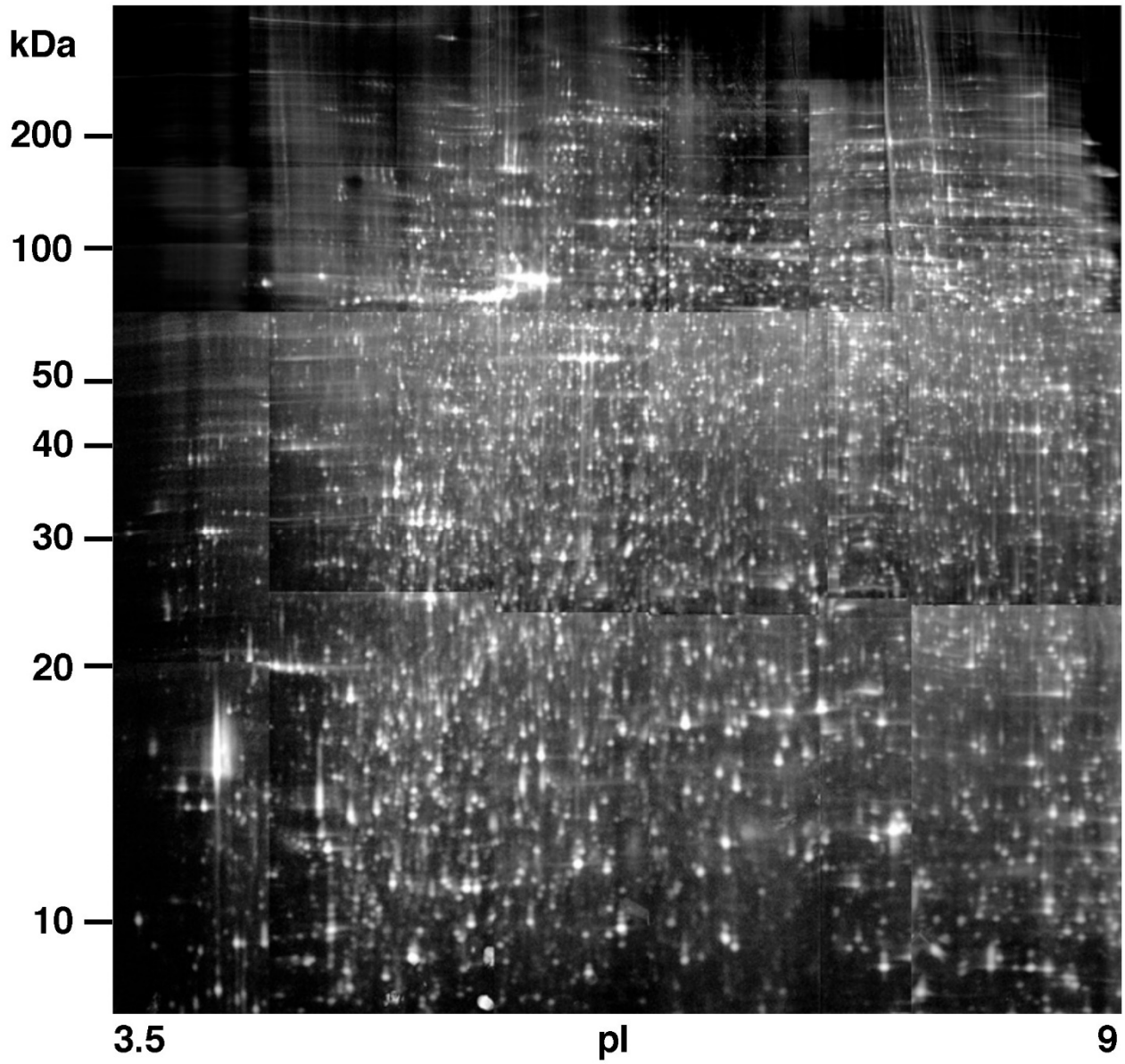

Fig. (3). The 93 cm x 103 cm cybergel detects about 11,000 protein spots of primary cultured rat hippocampal neurons by [^{35}S] autoradiography.

of the second dimension is also required for satisfactory separation of mammalian proteome. Followings are our current strategy to increase the first and second dimensional resolution (Katsuta *et al.*, 2003). We utilized six different 24 cm nrIPGs (pH 3.5-4.5, pH 4.0-5.0, pH 4.5-5.5, pH 5.0-6.0, pH 5.5-6.7, pH 6-9) in the first dimension (Fig. **2A**). In the second dimension, double 70 cm height gels (Fig. **2B**) were run for each nrIPG, in order to increase the resolution of second SDS page. High MW proteins (≧ 40 kDa) were separated by a 7.5% (w/v) acrylamide / 70 cm height gel, while low MW proteins (≦ 40 kDa) were analyzed by a 13.5% (w/v) acrylamide / 70 cm height gel. Then all the 12 gel images were assembled into a 93 cm x 103 cm cybergel (Fig. **2A**).

Figure (**3**) shows an example of data with the cybergel. Primary cultured rat hippocampal neurons were labeled by L-[^{35}S]methionine and L-[^{35}S]cysteine. Protein samples were resolved by the multiple large 2-DE gels and visualized by autoradiography. The assembled 93 cm x 103 cm cybergel could detect about 11,000 spots by autoradiography, thereby indicating that the improvement of the resolution in the first and second dimensions led to a 1,200% increase in the number of detected spots in comparison to a standard procedure we did (Fig. **1**). The most prominent protein spots in the cyber gels, tubulin and actin, showed radioactivity of about 2 x 10^5 cpm while those of the faintest spots were about 2 cpm. Therefore, we consider that the present gel can display protein spots expressed in a 1-10^5 dynamic range in cells, about 100 times broader than a standard gel shown in (Fig. **1**). The increased resolution by the cybergel system is further shown in (Fig. **4**). When proteins of rat hippocampal neurons were separated by a standard 2-DE gel, protein spots of the growth associated protein-43 (GAP-43) comigrated and fused in the gel (Fig. **4A**). On the other hand, the 93 cm x 103 cm cybergel resolved them into a series of 4 prominent spots, which we identified as GAP-43 by MS and immuno-blot analysis (Fig. **4B**). Furthermore, a series of 8 additional spots, probably modified forms of the main spots, were detected (Fig. **4B**, arrows). These results clearly show that large gel 2-DE also provides a powerful tool to resolve complicated forms of protein modifications.

CONCLUDING REMARKS

Enlargement of the 2-DE gel size is an effective strategy for detection and analysis of global proteome including low abundant proteins. 2-DE with large gels detects a large number of proteins expressed in a broad dynamic range. Furthermore, it is suitable for quantitative proteomics and analysis of post-translational modifications. Further high recovery of cellular proteome will be achieved when large gel 2-DE is combined with sample prefractionation. Although 2-DE currently is not a high throughput technology, it has a powerful potential to resolve the complex nature of cellular proteomes.

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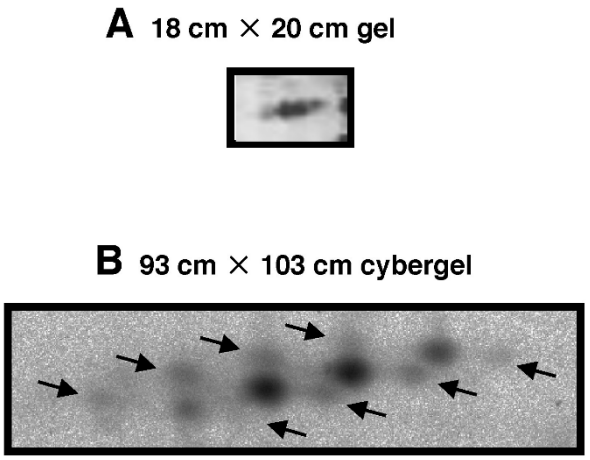

Fig. (4). The increased separation of GAP-43 achieved by the 93 cm x 103 cm cybergel. Samples from primary cultured rat hippocampal neurons were resolved by a standard 18 cm x 20 cm 2-DE gel (**A**) and the 93 cm x 103 cm cybergel (**B**), then visualized by silver staining and [^{35}S] autoradiography, respectively.

LIST OF ABBREVIATIONS

2-DE	=	Two-dimensional gel electrophoresis
IEF	=	Isoelectric focusing
MS	=	Mass spectrometry
nrIPG	=	Narrow pH range immobilized pH gradient

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