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Prototype for integrated two-dimensional gel electrophoresis for protein separation

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Abstract

Two-dimensional gel electrophoresis practitioners have long waited for a fully automated system. This article presents an integrated platform that is capable of complete automation from sample introduction to spots detection. The strip gel for the first dimensional separation is fixed on the edge of a discrete planar stage before separation. A pair of platinum pin electrodes for isoelectric focusing (IEF) makes contact from underneath the stage. IEF is performed directly after rehydration and protein loading. After the first dimensional separation, sodium dodecyl sulfate (SDS) equilibration is done on the same stage without moving the gel. The IEF stage is then moved horizontally to couple with a precast second dimensional gel. The <0.5 mm gap between the two gels is filled with poly (ethylene oxide) solution. After SDS-polyacrylamide gel electrohporesis separation, a charge-coupled device camera is used to detect spots via protein native fluorescence excited by a Hg (Xe) lamp with the gel inside the running cell. Potential for full automation is demonstrated with 0.5 μg of *Escherichia coli* proteins on this miniaturized platform. More than 240 spots are detected in a total experiment time of <2.5 h.
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1. Introduction

Proteins are directly responsible for cellular structure and function. Proteomics, focusing on the large-scale identification and quantification of proteins, is an important area for bioanalytical chemistry [1,2]. To date, most separations of complex protein mixtures are carried out using two-dimensional gel electrophoresis (2D-GE) [3–7]. In O'Farrell's original paper [8], in vivo ^{14}C- or ^{35}S-labeled proteins from *Esherichia coli* were first separated in a glass tube according to their isoelectric points (p*I*) by isoelectric focusing (IEF) with carrier ampholytes. The IEF gel was extruded after protein focusing and equilibrated with sodium dodecyl sulfate (SDS) buffer. The cylindrical gel was then laid on top of the second dimensional slab gel and kept in place by 1% agarose to separate proteins according to molecular weight. Slab gels were dried after separation and detected by autoradiography. About 1100 protein spots were detected. This high resolution, however, comes at the expense of intensive labor, long operation time and low reproducibility. The experiment requires meticulous handling of the delicate gels, and takes days to finish. Sample handling, gradient drifting over prolonged focusing, and gel deformation during extrusion all contribute to run-to-run variation.

Technological improvements over the last three decades have greatly simplified 2D-GE separation and protein detection. Tube gels were replaced with immobilized pH gradient-gel strips bonded on a plastic film [9]. This greatly facilitated gel handling. Radioactive labeling, plagued by biohazard concerns and days of exposure time [10,11], is gradually replaced by more environmentally-friendly and fast-staining or fluorescence-labeling methods. With specially designed instruments, separation and detection can be completed in a day on a mini size (6–8 cm) gel.

However, the labor-intensive and time-consuming nature of traditional 2D-GE has not been improved. Protein laboratories are still transferring gels with tweezers in and out of buffers and holders. Gels of smaller size and thickness,

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which offer even faster separation due to improved heat dissipation [12,13], are seldom used not only because of the lack of detection systems sensitive enough for the low-abundance proteins, but also because of the lack of automation and thus insufficient precision [12].

Increasingly, liquid-based multidimensional liquid chromatography (LC), capillary electrophoresis (CE), and LC-CE are used in peptide and protein separation [14–17] because of the availability of automated instrumentation. Different combinations of LC modes have been used for proteins separation, such as ion-exchange and reversed-phase (IEC-RPLC) [18,19], size-exclusion and reversed-phase (SEC-RPLC) [20,21], and ion-exchange and size-exclusion (IEC-SEC) [22]. A prime demonstration published recently is the multidimensional protein identification technique (MudPIT), developed in the Yates' group [23]. Using a step-gradient, trypsin digested proteins are separated by strong cation exchanger (SCX) and reversed-phase stationary phases that are packed in a single fused-silica capillary in series, and directly analyzed by mass spectrometry (MS) or tandem MS via electrospray ionization. Thousands of proteins can be identified within a few hours by database searching. RPLC are also coupled with electrophoresis for peptides and protein separation [24–27]. Multidimensional CE protein separation, based on principles other than the combination of IEF and SDS-GE, have also been performed [28–32]. Moreover, although most of the methods have peak capacity comparable with or even higher than traditional 2D-GE, the 2D-in-time configurations generally involve some resolution compromise because of the low sampling frequency of the second dimension comparing to the peak widths of the first dimension [33].

Despite fast separation and automation, the above methods do not provide p*I* or molecular weight information that can be directly related to databases familiar to biologists. The lack of databases for methods other than 2D-GE makes MS the necessary detector for protein identification. Also, although the common digestion-before-separation approach in these methods is potentially helpful for fast protein mapping and/or for biological marker identification when combined with MS, important protein information, exemplified by protein quantity and post-translational modifications (especially multiple modifications on the same tryptic peptide), tends to be missed. 2D polyacrylamide gel electrophoresis (PAGE), however, is known to work well if the goal is to look for protein modification and/or quantitative change (up down regulation) [34].

To take advantage of both the large database of 2D-GE and the fast, automatable separation of CE, traditional 2D-GE has been converted to the capillary format with polymer solutions as the anti-convection and sieving matrix [35,36]. Some schemes even use parallel separation in the second dimension to further increase the throughput [37,38]. However, band broadening due to either heterogeneity caused by labeling or distorted electric field distribution during protein transfer are observed.

The direct PI and molecular weight information, the large database available, and the low operational cost have greatly favored 2D-GE over other protein separation and detection methods. It is the low degree of automation that hindered traditional 2D-GE's application in modern day protein analysis. Full automation will be a major advance in 2D-GE development and the inherent high throughput of a fully automated system will definitely further consolidate its role in proteomics. Here, we demonstrate an integrated 2D-GE system that is ready to for full automation. Sample application and first dimensional separation, IEF, are performed on a discrete stage without special holders. Coupling this to the second dimensional gels is accomplished by moving the IEF strip linearly and filling the gap with poly(ethylene oxide) (PEO) solution. The whole running cell is directly put under a UV lamp and spots are detected with a charge-coupled device (CCD) camera via protein native fluorescence [39–41].

2. Experimental

2.1. Chemicals and samples

Sample proteins from *E. coli*, carrier ampholytes (Bio-Lyte 3/10, Bio-Lyte 5/7), urea, dithiothreitol (DTT), 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), acrylamide and bisacrylamide mixture (3.3% crosslinker), 1.5 M Tris–HCl solution (pH 8.8), 0.5 M Tris–HCl solution (pH 6.8), and SDS-PAGE running buffer were obtained from Bio-Rad (Hercules, CA, USA). Dry IEF gels and agarose were purchased from Amersham Biosciences (Piscataway, NJ, USA). SDS, ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), PEO (M_r 8,000,000), and glycerol were purchased from Sigma (St. Louis, MO, USA). SDS running buffer is obtained from Bio-Rad and diluted according to the manufacturer's instructions. All electrodes were made of platinum wire (0.25 mm diameter) from Surepure Chemetals (Florham Park, NJ, USA).

2.2. Apparatus

2.2.1. 2D-GE cell design

The designed platform for 2D-GE adopts a horizontal format, as shown in Fig. 1. Part I (Fig. 1A) includes the 3-mm wide IEF stage and the cathodic buffer well for the second (SDS-PAGE) dimension. The embedded IEF electrodes, 10 mm apart, are wired from the bottom and protrude only 0.1 mm on the stage. IEF strip, 1 mm × 19 mm with only 11 mm dry gel in the center, is placed facing down along the edge of the stage before the experiment. Both bare plastic ends of the strip are clamped on the 0.4 mm high steps at the two sides of the IEF stage. Part II (Fig. 1B) includes the 20 mm × 20 mm slab gel cassette stage, extension pocket, and the anodic buffer well for SDS-PAGE. The

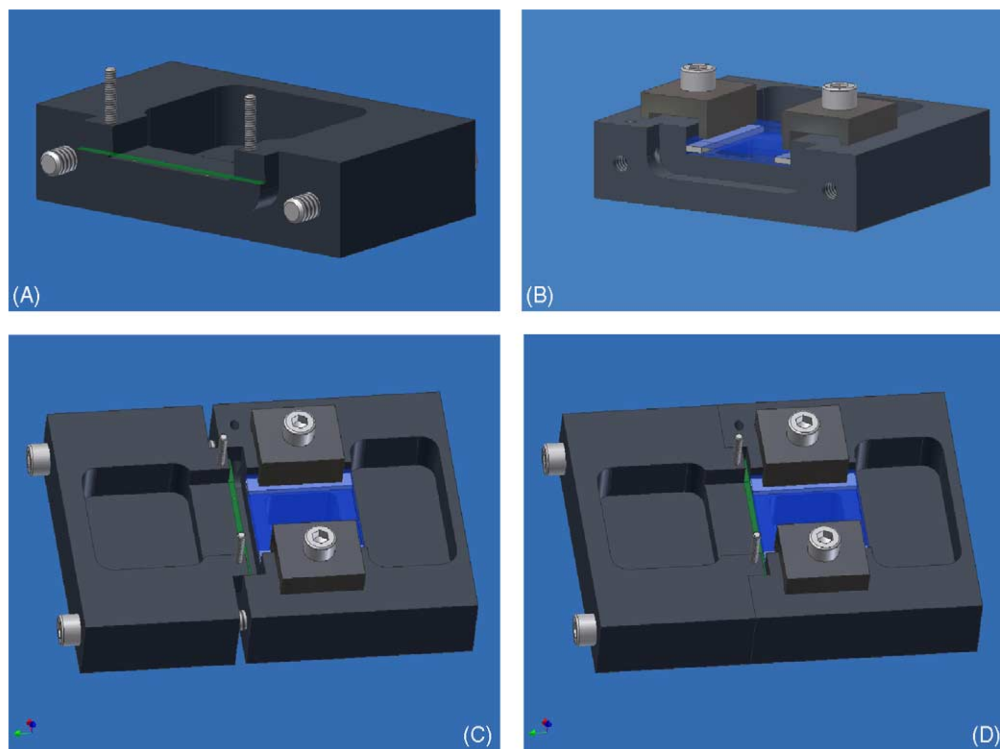

Fig. 1. Schematic diagram of integrated 2D-GE cassette. (A) Part I block for IEF with dry strip in green; (B) Part II block for SDS-PAGE with gel in blue; (C) assembly with 2-mm gap and buffer wells on both sides empty for first dimensional separation; and (D) assembly fitted together and buffer wells filled with SDS buffer for second dimensional separation.

gel cassette is made of low UV fluorescence bottom glass, 20 mm × 4 mm × 0.75 mm thick spacers, and fused-silica cover glass. Gels are cast before experiments. The slab gel consists of a 3-mm stacking gel (4%) and a 12-mm resolving gel (12%) buffered at the same pH and ionic strength (pH 8.8 and 0.375 M Tris–HCl). The cassette glass choice is necessary for direct native fluorescence excitation and detection. A 14 mm × 14 mm opening is cut through the center of the stage to reduce the background fluorescence. The gel cassette is held tightly against the stage by clamps on the sides. Glue or rubber gasket can be used between the stage and gel cassette to prevent leaking. The extension pocket fits the IEF stage of Part I. The two parts are aligned 2 mm apart, creating a temporary void before experiments (Fig. 1C). Both parts are made of Delrin.

2.2.2. Detection system

Details of the native fluorescence detection setup are described elsewhere [13]. Native fluorescence detection eliminates the staining and destaining steps. It can be implemented by direct excitation through quartz plates. Briefly, collimated light from a Hg (Xe) lamp is selected by a set of filters to give 270–320 nm excitation band at about 1 mW/cm^2. The fluorescence signal passing through emission filter set (>320 nm), is collected by a UV camera lens and detected by a cooled CCD camera. We have shown previously that this system is capable of detecting a protein spot of 0.04 ng.

2.3. Experimental protocol

The integrated operation can be divided into three steps.

2.3.1. Sample application and IEF

Five microliters of rehydration solution (containing 0.1 μg/μL sample protein, 8 M urea, 1.5% CHAPS, 1.8% Bio-Lyte 5/7, 1.2% Bio-Lyte 3/10, 0.3% freshly added DTT) is pipetted along the inner edge of the dry gel strip onto the IEF stage. The solution is readily drawn under the strip by capillary action. Then, the cassette is placed on top of a thermoelectric cooler (Advanced Thermoelectric, Nashua, NH, USA). The temperature is set at 18 °C. The electrophoretic cell is put under a polycarbonate cover with an open container filled with water alongside. Both temperature and humidity are maintained for the 1 h gel rehydration and the subsequent IEF. IEF is performed directly after rehydration at 75 V for 4 min, 200 V for 4 min, 300 V for 10 min, and 400 V for 30 min. Total IEF is no more than 280 V h. Current decreases steadily for the period of constant voltage except for the last 5–10 min, where current stays at around 20 μA.

2.3.2. Coupling of the two-dimensional gels

Sixty microliters equilibration solution (containing 2% SDS, 60 mM Tris–HCl at pH 6.8, 10% (v/v) glycerol, 1% freshly prepared DTT) is added on the IEF stage along the gel strip. The SDS and protein are allowed to interact for

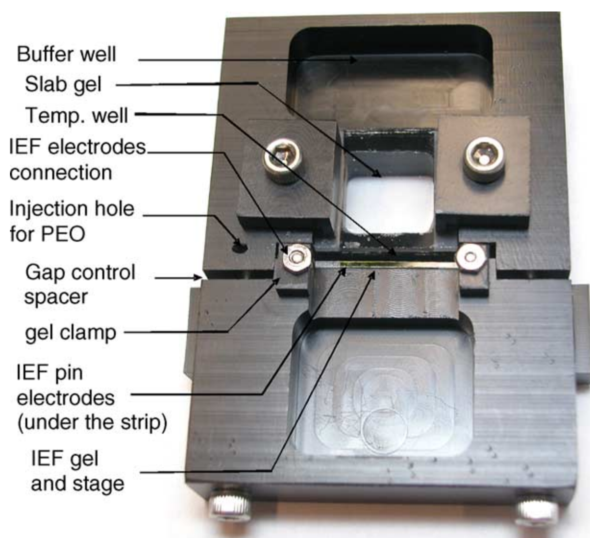

Fig. 2. 2D-GE platform. The overall dimension is 5 cm × 7 cm. Two millimeters spacers are used to isolate the two parts during first dimensional separation and are removed before mating the two parts together for the second dimensional separation.

15 min. Then, 0.2 mL of 2% (w/v) PEO solution [containing 0.05% (w/v) bromophenol blue] is added into the temporary well from the side channel with a syringe. Tightening the alignment screws couples the two blocks together and gradually reduces the volume of the well, squeezing the PEO solution up to connect the two gels (Fig. 1D). A rubber gasket is added between the two parts to prevent possible leaking during SDS-PAGE. A photograph of the entire assembly is shown in Fig. 2.

2.3.3. SDS-PAGE and protein detection

About 2 mL of 1× Tris/glycine/SDS is added to each buffer well to merge over the IEF strip but not over the cover glass. Twenty millimeters long electrode pairs are dipped into the buffer wells parallel to the IEF gel. SDS-PAGE is carried out at 100 V for about 7 min till the bromophenol blue marker line migrates out of the gel. Buffer solution is drained and the cover glass is flushed with DI water and air-dried before the whole unit is put under the detection setup for imaging. The exposure time is set to 2 min.

3. Results and discussion

3.1. Performance of the device

An image of the second dimensional gel is shown in Fig. 3. Two hundred and forty-four spots are detected with the 2D image analysis software PDquest from Bio-Rad. This represents better detection sensitivity for native fluorescence detection than previously reported [13]. The reason might be that protein loss due to gel rinsing after separation is eliminated since the gel is directly detected inside the glass cassette. However, the spot number is still a bit lower than conventional mini gel (7 cm IEF and 6 cm SDS-PAGE), where ∼300 spots are detected [13]. This is mainly because the loading amount is 40 times lower in the small gel than the conventional one. Low loading can be useful since the biological samples are usually quite limited.

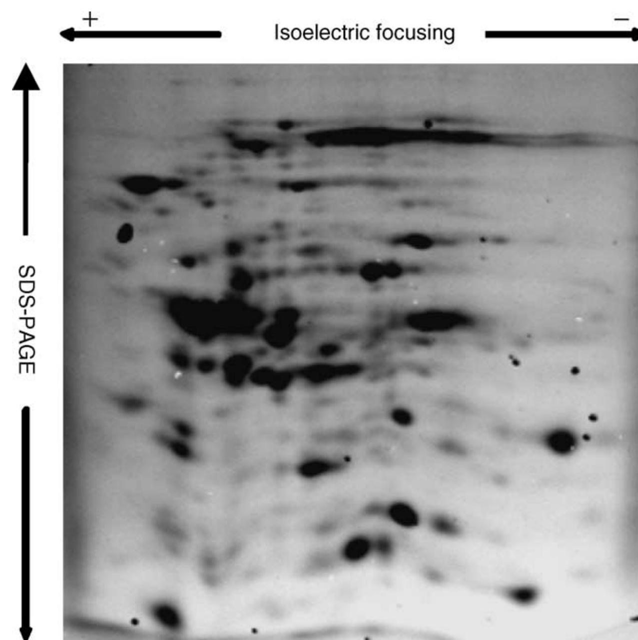

Fig. 3. Native fluorescence gel image of 0.5 μg *E. coli* proteins. The entire gel is 11 mm × 11 mm.

3.2. Automation potential of the device

The experimental design outlined is based on the potential for full automation. The flexible nature of polyacrylamide gels poses the biggest challenge for automating the separation. Running both dimensions on a single gel would be the best approach. However, unless there is an effective way to confine the proteins from spreading perpendicularly to the IEF electrical field, a 2D-on-one-gel configuration is not feasible. To ensure proper focusing of protein, the two gels have to be separated during the IEF step. We first tried to set the IEF gel at a small distance (∼1 mm) from the slab gel during IEF and sealed the gap afterwards with agarose solution (before SDS-PAGE). It works well if the sample loading step is performed off-line. Rehydration and loading in situ often causes current leakage due to sample solution diffusion. Protein focusing is thus not reproducible. Even if there is no current leakage and the proteins are focused well, the slab gel fluorescence image tends to have higher background, presumably because the portion of the sample proteins that are not absorbed by the IEF gel and are thus not focused also migrated into the second dimensional gel. Severe horizontal streaking is observed in the case of cup loading, where the dry IEF gel is rehydrated off-line. This also means handling of the hydrated gels. We thus decide to physically isolate the gel strip from the slab gel during isoelectric focusing and couple

the two gels by moving the two parts of the rigid running cell instead of the two flexible gels.

To automate the step in Section 2.3.1, i.e. sample loading and first dimensional electrophoresis, the strip gel is designed to be loaded onto the IEF stage before sample application. Mounting the strip with the gel side facing down exploits capillary action to drive the sample solution underneath the gel for proper gel rehydration and protein loading. Rehydration and loading now becomes analogous to sample injection and can be performed by a computer-controlled syringe through a channel in the cassette.

Integrating the IEF electrodes into the IEF stage helps to avoid the hassle of positioning them before IEF and in the removal afterwards. However, the traditional parallel IEF electrodes generate a considerable amount of bubbles during the second dimensional electrophoresis, thereby interrupting the separation. Here, a pair of tiny pin electrodes are used. These do not generate noticeable bubbles under the experimental conditions used due to the reduced surface area. To facilitate the coupling of the two gels, IEF is performed on one edge of an open, flat surface instead of inside a traditional holder, where rehydration solution and individual IEF strip gel are isolated and cover by a layer of mineral oil. Humidity control, however, becomes crucial to ensure proper gel rehydration and protein focusing since a very small volume of rehydration solution is applied. We found that air saturated with water vapor is as good as cover oil for preventing evaporation.

Direct coupling in Section 2.3.2 can be very simple if no SDS equilibration is required. Although IEF gels can be coupled directly with the second dimensional slab gel without a SDS equilibration step, it usually causes severe vertical streaking due to incomplete SDS–protein binding [42]. With the gel strip poised on the edge of IEF stage, SDS buffer can be directly added onto the stage. Handling the gel strip in and out of solution is avoided. The SDS buffer does not need to be removed because the composition is similar to the second dimensional running buffer. After SDS equilibration, the IEF stage is brought to close contact with the slab gel stage. However, due to the casting process employed, there is still a ∼0.5 mm gap between the two gels. 2% PEO solution is used instead of the traditional agarose gel to avoid the prior melting step. Because of its high viscosity, the PEO solution cannot only be held in the make-shift well temporarily without leaking out, but also connects the two gels without being diluted by the equilibration solution or the running buffer before protein transfer is completed. Thus, with two computer-controlled syringes to dispense SDS equilibration buffer and PEO solution, and one motorized stage attached to either part of the cassette, the 2D coupling step can be automated.

The second dimensional electrodes can be also integrated into the buffer wells since they do not interfere with the first dimensional separation. The liquid level in both of the wells can be regulated by a pair of pressure valves. Water flushing and air drying of the cover-glass surface can also be done in situ before detection. Direct spot detection by protein native fluorescence with the gel inside the electrophoresis cell represents the simplest detection mode. Due to ubiquitous UV fluorescence of the cassette, it is essential to create an opening at the center of the slab gel cassette stage to reduce the background noise for detecting the low signal from proteins. This also benefits heat dissipation during the second dimensional electrophoresis because the cooling unit can be in direct contact with the bottom glass. Moreover, the distinct noise spots in the gel images [13] are greatly reduced because dust particles, the presumed major source responsible for the spots, attach more easily to the exposed gel than to the glass surface.

4. Conclusions

The simple design demonstrated above is capable of automated 2D-GE separation and detection without complicated robotic operations. The most expensive instrumental component required would be a CCD camera, but that is not much more costly than a high resolution scanner typically used for gel scanning. In a commercial version of this system, one would have the two disposable blocks of the cassette manufactured with the two gels precast onto them. We note that prepared IEF strips and precast SDS-PAGE gels are already commercially available in sealed packages in larger formats.

Traditional 2D-GE has been used both for differential expression profiling in analytical scale and isolating pure proteins in preparative scale. Miniaturized automatic 2D-GE can thus be used in differential expression experiments, such as disease diagnosis or drug response assay, as a complementary technique to protein microarrays. Horizontal expansion of the current design will allow processing several gels in parallel. For example, the hydration steps can be done in parallel off-line to achieve high throughput preparation. Robotics can then be employed to mate the focused (IEF) gel blocks to the SDS-PAGE gel block sequentially for size separation and detection at the optical module. Although the suitability for automation of this design is demonstrated in a miniaturized format, it can easily be scaled up for higher loading capacity when low abundance proteins are of interest and the detection sensitivity is limited, or if proteins are to be isolated.

Despite these advantages, an automated system solves only part of the problems facing 2D-GE. Improvement of cell proteome coverage, especially proteins with high molecular weight, high hydrophobicity, and extreme p*I* values, will need further development. Detection of low abundance proteins, particularly with limited sample amounts, is another critical issue for both 2D-GE and other proteomics methods. It is also important to combine automated separation and detection with new techniques that link gel proteins with MS when protein identification is desired. When new protein spots are detected, MS or MS/MS is usually performed to identify these spots. The current gel-protein MS identification method involves multiple operation steps, typically including spot excision, proteolytic digestion, peptide extrac-

tion/concentration, and repeated washing and drying. Simple but efficient protein transfer from gel to MS, such as the integration of electronic protein transfer and membrane proteolytic digestion [43], are challenges that require additional development of the integrated system reported here.

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