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Application of the ProteomeLab™ PF2D protein fractionation system in proteomic analysis for human genetic diseases

Review Article

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Abstract: Proteomic analysis has been widely used in elucidating the mechanism of diseases. As a classical proteomic approach, two-dimensional gel electrophoresis (2DGE) has been commonly applied in finding differentially expressed proteins through a first dimension of separation by the isoelectric point (pI) of proteins and a second dimension of separation according to the molecular weight (MW) of proteins. Compared to 2DGE, a recently developed commercial system from Beckman Coulter, the two-dimensional protein fractionation (PF2D), separates proteins according to the pI of proteins in the first dimension followed by a second dimension of separation according to the degree of protein hydrophobicity. As a liquid-based fractionation system, PF2D could facilitate the extraction and separation of broader protein categories and improve reproducibility and quantification as well as be less labor-intensive, which are usually identified as limitations of a gel-based 2DGE platform. This review evaluates the applications of the PF2D system and discusses the perspectives and advantages of PF2D in the investigation of cancer and genetic disorders and in protein mapping in human biological fluids and cell cultures.

Keywords: *ProteomeLab™ PF2D Protein Fractionation System • Cancer research • Pediatric neurodegenerative disorder • Premature aging disease*

1. Introduction

As the basis of modern two-dimensional (2D) electrophoresis, 2D polyacrylamide gel electrophoresis (2D-PAGE), first introduced by O'Farrell in 1975 for separating cellular proteins under denaturing conditions, enabled the resolution of hundreds of proteins [1]. Since then, 2D gel electrophoresis (2DGE) has been widely accepted and greatly improved [2-6]. Generally, this technique separates proteins into two dimensions, according to two independent properties of proteins: the first dimension is isoelectric focusing (IEF), which separates proteins according to their isoelectric points (pI); the second dimension is a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their molecular weights (MWs). In this way, complex mixtures consisting of thousands of different proteins can be resolved into discrete spots, and the relative amount of each protein can be determined. Combined with mass spectrometry (MS) technology [7], resolved proteins could be further identified and characterized with an accurate MW.

2DGE is a well-established technique and is considered one of the most powerful methods for conducting proteomic studies, *i.e.*, the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples such as sera, plasma, amniotic fluid, ascites, saliva, urine, *etc.*, to look for novel

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biomarkers and investigate disease pathogenesis [8-13]. However, severe limitations have greatly restricted the application of 2DGE, such as lack of reproducibility, difficulty in automating, poor resolution for proteins of extreme hydrophobicity, and limited pI ranges [14,15]. In addition, proteins are embedded in the gel, requiring labor-intensive procedures to excise the spots for further analysis by mass spectrometry.

Multidimensional liquid chromatography (MDLC) coupled with MS has subsequently emerged as the technique of choice for large-scale protein studies due to its superior throughput and sensitivity [16]. Generally, MDLC proteomic methods have focused on separating, by high pressure liquid chromatography (HPLC), complex mixtures of peptides obtained following digestion of the proteome, which are then fragmented and sequenced through MS to identify the parent proteins. This peptide-based MDLC analysis, also called bottom-up proteomics, shows inherent selectivity and sensitivity, and now is widely used in most laboratories [16].

Peptide-based MDLC is also restricted by a few major drawbacks [17]. This proteomic analysis is performed at the peptide level and requires sample proteolysis with enzymes, which is time-consuming. Moreover, huge amounts of peptides generated from protein complexes make it difficult for the current separation methods to resolve so many components in a single analytical dimension prior to the MS analysis, which subsequently results in the reduced efficiency and accuracy of MS analysis. Shared peptides interference problem is another challenge in bottom-up proteomics and can lead to ambiguities in determining the identities of proteins in the sample [18]. The major fundamental drawback is that peptide-base MDLC cannot address the post-translational modifications (PTMs) of proteins, as protein information such as identification and quantification is all deduced from the detected peptides. PTMs of proteins such as glycosylation or phosphorylation may lead to functional modification. Therefore, fractionating complex protein mixtures while maintaining intact proteins in the liquid phase is a most desirable feature for use in further analyses. For these reasons, an alternative approach, top-down proteomics, has attracted attention in the last few years [19-22]. Top-down proteomics allows intact protein molecular ions to be separated directly by liquid chromatography (LC) and then analyzed by the mass spectrometer. The top-down strategy has the potential to identify a larger fraction of protein sequences and the ability to locate and characterize PTMs.

Recently, a newcomer in top-down proteomics platforms and also two-dimensional LC (2D-LC) from Beckman Coulter, the Proteome-Lab™ PF2D platform (PF2D), has shown advantages in circumventing some limitations of fractionation using 2DGE [23,24] and MDLC.

As shown in Fig. 1, PF2D is an automated fractionation platform that separates intact proteins in the first dimension according to their pI using chromatofocusing based on charge, followed by fractionation according to hydrophobicity, using nonporous reversed-phase chromatography in the second dimension. In combination with ProteoVue and DeltaVue software, a highly detailed pI *versus* hydrophobicity protein expression map can be established in a 2-D "lane and band" format. Both dimensions of analysis use standard HPLC equipment designed to reproducibly handle large numbers of samples in the liquid phase. This equipment also uses UV absorption for detection so that quantitative comparisons of protein expression can be performed between samples. The signal of the first dimension was recorded at 280 nm, and the signal of the second dimension was recorded at 214 nm. The pH gradient was generated using start buffer (pH 8.5) and elution buffer (pH 4), which meant that the general pH range of PF2D is from 4 to 8.5 [23]. Lee *et al.* established an efficient PF2D system for analysis and rapid semi-quantification of membrane proteins present in a wide pH range (4.0-10.5) by adjusting the pH of start buffer to 10.5 and adjusting the elution buffer to pH 7.0 [25]. In addition, this system also allows the separation of particular proteins with 9-40 kDa MW [26].

PF2D facilitates the extraction and separation of membrane-associated proteins, allows broader pI (4.0-10.5) and MW ranges, and improves run-run reproducibility. PF2D also provides a precise quantification of protein and is highly automated and seamless in association with mass spectrometry (MS) for further identification. Thus, PF2D enhances the precise detection of isoforms or PTMs that can alter the pI or hydrophobicity of a protein. Also, by resolving protein complexes on the basis of their intact characteristics prior to mass spectrometry analysis, PF2D allows a high degree of sequence coverage for more accurate identification in the case of PTMs. This is in contrast to peptide-based MDLC, in which protein identifications can be made on a single observed peptide with no information about the nature of the parent protein. Several recent proteomic studies have shown that a 2D-LC platform allows more efficient identification of proteins than 2D gels and may be more applicable for novel protein discovery [27,28]. Coupled with direct online MS analysis, PF2D facilitates the effective implementation of a 2D-LC system and has been used increasingly in proteomic studies, including human diseases, especially in research on cancer and genetic disorders. This review consists of three sections that

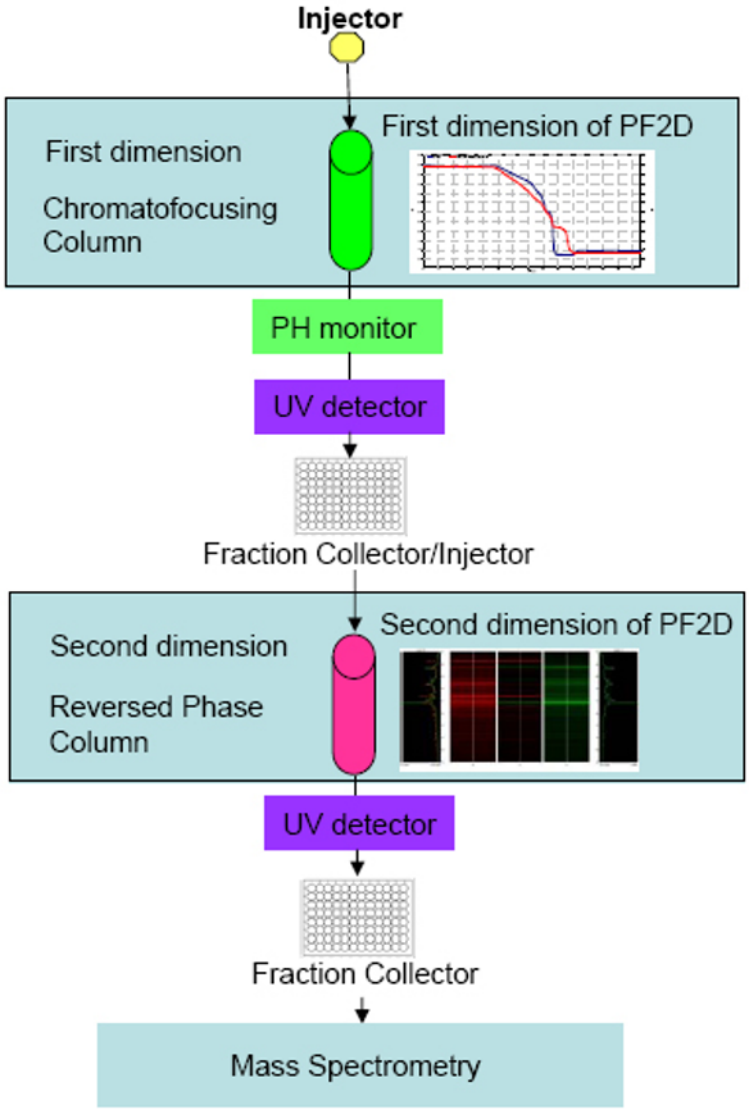

Figure 1. Two-dimensional LC (2D-LC) from Beckman Coulter, the Proteome-LabTM PF2D platform (PF2D).

describe the application of PF2D in cancer research, genetic disorders, and protein mapping in human biological fluids and cell cultures.

2. Application of PF2D in cancer research

Cancer biomarkers would provide earlier and better diagnosis and treatment of patients [29]. Proteomics has distinct advantages over genomic and ribonucleic acid expression studies because it is the proteins that are ultimately responsible for the malignant phenotype. Proteomics also can provide an "expression profile," which refers to the expression of thousands of individual proteins simultaneously in a given tissue sample [30]. Application of proteomic methods provides a systematic overview of expressed protein profiles, which can ultimately improve the diagnosis, prognosis, and management of patients by revealing the protein interactions affecting overall tumor progression [30,31]. Furthermore, analysis of differentially expressed proteins can be used to compare tumors with normal tissues and indicate a range of protein markers that can provide earlier and better diagnosis of patients in clinical settings and initiate proper treatment in the early stages of disease [30-32].

As a newly developed non-gel–based proteomic technique, PF2D systems have been widely used in tumor research. The research studies include investigations of the mechanisms of carcinogenesis and the identification of cancer biomarkers. Hurst *et*

al. identified differentially expressed proteins [33], which were unique in the extracellular matrix facilitating growth of bladder cancer cells, compared to that of matrix derived from normal basement membrane. Their results showed strong evidence for post-translational modification of the malignant phenotype of bladder cancer cells. In addition, the author showed 10-fold higher sensitivity when the samples were fractioned by PF2D compared to gel-based separations [33]. A similar analysis was also applied to map the protein content of ovarian surface epithelial cells and an ovarian carcinoma-derived cell line [34]. Quantitative changes in protein expression were measured within the cell lysates of these two cell lines. Highly expressed as well as down-regulated proteins were both seen in cancer cells compared to those in normal cells. Zhao *et al*. compared the protein profile in whole cell lysates from an estrogen-treated and untreated premalignant, estrogen-responsive cell line, MCF10AT1 [35]. They found that approximately 40 proteins were up-regulated and 17 were down-regulated in treated cells. Many altered proteins correspond to the development of a malignant phenotype and may play an important role in signal pathways [35]. Using human breast cancer cells, another group analyzed the changes in the phosphorylation profile resulting from treatment of the cancer cells with a tyrosine kinase inhibitor. They identified a large group of proteins that show post-translational modification of phosphorylation upon treatment with the inhibitor, of which some were potentially involved in signaling events [36]. PTMs of proteins are very important in the mechanism of carcinogenesis. For example, aberrant phosphorylation has been observed in many cancers, and this phosphorylation can abnormally activate signal pathways, thereby initiating cancer or accelerating the progression of cancer. Mapping differentially expressed proteins, including differentially modified proteins, between cancer cells and normal cells has been a promising platform for the research of carcinogenesis. PF2D as a top-down proteomics platform can not only differentiate proteins with higher sensitivity compared to gel-based separations, but also differentiate PTMs of proteins compared to peptide-based MDLC.

PF2D systems also have been widely used in the identification of cancer biomarkers. As a promising prostate cancer biomarker that is suppressed in a majority of early-stage prostate cancers, annexin I has been identified previously by 2D-PAGE coupled with mass spectrometry. PF2D improves the sensitivity of this measurement [37] and facilitates hope for future clinical diagnosis. The protein profiles of eight ovarian serous carcinoma cell lines and three ovarian surface epithelial cell lines were investigated by Wang *et al*. using PF2D [38]. Hierarchical clustering analysis was used to classify the different samples according to their protein expression profiles, showing that specific types of serous carcinoma cell lines tend to cluster together. Several other cell lines, such as ovarian clear cell carcinoma cell line and endometrioid carcinoma cell lines and breast epithelial cell lines, were also fractionated and mapped by the 2D liquid method, and cluster analysis was performed on a total of 18 samples. The proteins in cell lysates could be mapped and clustered closely between cancer cell lines, and *vice versa* for normal ovarian surface epithelial cell lines, indicating that these differentially expressed proteins may serve as markers of ovarian serous carcinomas [38].

Protein biomarkers, indicating the anti-cancer effects of clinical or experimental treatments, are also of interest to researchers. Skalnikova *et al*. mapped the total protein expression of CEM lymphoblastic leukemia cells following treatment of bohemine, a cyclin-dependent kinase inhibitor [15]. Four proteins were found to be up-regulated, which may represent candidate biomarkers of cancer cell response to the bohemine treatment. These findings shed light on pathways that are associated with the anticancer activity of the cyclin-dependent kinase inhibitor. Yan *et al*. also compared protein expression between Peninsularinone-treated and -untreated HCT-116 human colon adenocarcinoma cells by means of PF2D, and identified multiple differentials expressed following drug-treatment [39]. More specifically, in the pH 5.9-5.7 fraction, the proteins annexin I, heterogeneous nuclear ribonuclear protein H, and cold shock domain protein A are strongly upregulated in the untreated colon cancer sample versus the drug-treated sample. In the pH 6.87-6.67 fraction, histone, DBPB, and RIG protein are strongly upregulated in the untreated sample versus the drug-treated sample [39]. Identification of cancer biomarkers is important in the improvement of cancer detection and treatment. Application of PF2D provides a great opportunity to identify new cancer biomarkers to improve this effort.

3. Application of PF2D in pediatric neurodegenerative disorder

A genetic disorder is a disease that is caused by an abnormality in an individual's genome. Gene mutations can result in abnormally expressed proteins that can be separated and characterized by proteomic methods. PF2D uses UV absorption for detection so that quantitative comparisons of protein expression can be performed between samples. The neuronal ceroid lipofuscinoses (NCLs), commonly referred to as

"Batten disease," are a group of neuronal degenerative diseases primarily affecting children. They comprise one dominant and nine recessive variants as well as several rare and atypical unclassified subtypes. Eight genes have been identified for the NCLs. They are *CLN1-3, CLN5-8,* and *CLN10* [40-42]. PF2D has been applied to finding differential-expressed proteins in variant NCL cell lines in order to detect common alterations or common pathways in the different subtypes of NCLs [43]. Twenty-four proteins were identified, and functional analysis showed that these differentially expressed proteins could be grouped into several functional categories and pathways, including intermediate filaments, cell motility, apoptosis, cytoskeleton, membrane trafficking, calcium binding, neucleosome assembly, pigment granule, and cell development. These findings may facilitate the discovery of NCL-specific biomarkers and help further elucidate the pathogenic mechanisms underlying the NCLs.

4. Protein mapping in human biological fluids and cell cultures

4.1. Human serum proteome

In the last few years, the interest in exploring the proteome of biological fluids has expanded through the search for new biomarkers of diseases. The serum proteome is the most useful and also the most complicated part of the human proteome. There have been several attempts at proteomic analysis of serum to look for disease-related biomarkers [44-46]. Because low-abundance proteins are very important in biomarker discovery, it is necessary to optimize the methods of protein separation and selective depletion of high-abundance proteins such as immunoglobulin (*e.g.* IgG) and albumin, in human serum. Sheng *et al*. identified 150 non-redundant serum proteins, 81 of which have not been identified previously in serum using PF2D the system [47]. The addition of 20% isopropanol to the first dimension buffer enhances the elution of proteins at or near their theoretical pI. Numerous cellular proteins were identified to be specifically the skeletal muscle isoform in serum from healthy individuals. The detection of these specific skeletal muscle protein isoforms reflects the physical turnover that occurs in skeletal muscle, which can be used as biomarkers without further characterization of the precise isoforms or post-translational modifications [47]. In order to validate the reliability and characteristics of the PF2D system's fractionation of human serum proteins, highly standardized 2D gels were used to analyze different fractions obtained from the first dimension of PF2D separation. A total of 36 human serum proteins were identified in different PD2D-generated fractions. This study proved that the PF2D approach can reliably separate a wide range of proteins in the serum proteome, and in the first dimension, the vast majority of immunoglobulins were eluted, which can reduce the number of proteins of little interest in human plasma proteomics [48]. In combination with the detection capacity of both high- and low-abundance proteins and a highly automated platform, the PF2D platform could become a reliable system for the routine analysis of human serum specimens to find disease-related biomarkers.

4.2. Human urine proteome

Urine is another commonly examined clinical specimen of human biological fluid. As a promising tool for understanding kidney physiology and identifying potential biomarkers for the diagnosis and/or monitoring of renal diseases [49], human urinary proteome has also been preliminarily investigated by PF2D. Soldi *et al.* analyzed the urine specimens from human donors using the PF2D platform to focus on evaluation of the reproducibility and sensitivity of this system in protein resolution [26]. They analyzed consecutively 10 aliquots of urine pool on 10 different days using the same column lot. Their data showed that this platform can provide high reproducibility of 2-D protein maps and allow a seamless connection with subsequent MS analysis for further identification, without further need for extraction or solubilization of samples, as required for spots excised from the two-dimensional electrophoresis (2-DE) gels. In addition, this system also exhibited the advantage of sensitivity in separating proteins particularly with 9-40 kDa molecular weight, whereas it was difficult to efficiently separate small molecular weight proteins using 2-DE gels [26]. Compared to traditional 2-DE gels, PF2D shows higher reproducibility, ease of handling, and higher sensitivity, which makes PF2D a promising platform for urine analysis to perform both physiological and pathological investigations.

4.3. Cell culture and tissue protein

By comparing the cell proteome from normal cells and tumor cells or cells separated from abnormal tissues, differentially expressed proteins can be found and validated to create a differentially expressed protein profile. By analyzing this profile, the possible pathogenesis of diseases and/or biomarkers can be found. There have been a lot of attempts to find differentially expressed protein profiles using 2-DGE, but there are a lot of limitations. For example, in 2-DE separation and profiling of membrane proteins, there were limitations of reproducibility, quantification,

and resolution that were not suitable for large-scale analysis. Hydrophobic proteins may not fully migrate into the gel because the conformation of these proteins can sometimes be restored during the sample loading process, and during the denaturation process, these hydrophobic proteins cannot be fully denatured, which also can influence the migration of protein into gels [50,51]. Although 2-DE has been used for separation of soluble proteins, it is still difficult for the separation of membrane protein because of their poor solubility. The PF2D system has been widely used for separating many kinds of proteins including membrane proteins from cultured cells or body fluids [26,27,43,47,48,52-54]. Bacterial membrane antigens and rat liver mitochondrial proteins have been identified and characterized by an immuno-PF2D approach [27,53,54]. Using the PF2D system, rat liver microsomal proteins were isolated in two different pH ranges (4.0-8.5 and 7.0-10.5), and this system produced 182 proteins with more than two transmembrane domains (TMD) [25].

5. Applying PF2D in studying Hutchinson-Gilford progeria syndrome

Hutchinson–Gilford progeria syndrome (HGPS, or progeria) is a rare genetic disease characterized by segmental premature aging. Applying PF2D, Wang *et al*. identified 30 differentially expressed proteins in cultured HGPS fibroblasts [55]. The differentially expressed proteins were categorized into five groups: methylation, calcium ion binding, cytoskeleton, duplication, and regulation of apoptosis. Among these 30 proteins, 23 were down-regulated, while seven were up-regulated in HGPS fibroblasts as compared to normal fibroblasts. Three differentially expressed cytoskeleton proteins, vimentin, actin, and tubulin, were validated and characterized by immunostaining, which revealed densely thickened bundles and irregular structures in the HGPS cells. Expression of vimentin in senescent fibroblasts is also up-regulated, and the vimentin network in senescent cells is also densely organized [56]. This suggests that the development of HGPS shares similar mechanisms with normal aging. Furthermore, the cell cycle G1 phase was elongated, and the concentration of free cytosolic calcium was increased, which suggests intracellular retention of calcium in HGPS cells. The higher concentration of intracellular calcium can increase the activity of calcium-dependent enzymes such as NO synthase, phosphatidase, proteolytic enzyme, and nuclease. The increased enzyme activity induces uncoupling of oxidative phosphorylation in mitochondria, inhibits cell respiration, and influences cell metabolism [57]. These results may have implications for understanding the aging process.

6. Conclusion

In summary, PF2D was found to be a promising automated proteomic platform for protein profiling and a means for biomarker discovery. PF2D offers an alternative approach for the LC system to address issues of complexity and utilization of fractions after analysis. This approach can capture the alternations of proteins caused by PTMs. Also, this platform can be used for profiling basic and hydrophobic proteins that are hard to analyze by 2-DE [58,59]. Additionally, fractionated proteins are maintained in a liquid phase, making them available and flexible for various assays without loss of material, such as mass spectrometer analysis, enzymatic digests, additional fractionation, Western blot, or a combination of analytical tests.

PF2D also inevitably bears some weaknesses. One of them is the low throughput, which only allows 2–3 samples per week per instrument. Another limitation is the relatively large amount of sample required for analysis. Large sample size is not an issue when serum, plasma, or cell culture samples are analyzed, but it is usually unavailable for some precious clinical material such as body tissues. In some cases, the low quantities of differential protein in a given fraction may also compromise the identification of potential biomarkers [60].

Although most of the current MDLC systems were developed for peptide separation, with the rapid development of top-down proteomics, development of MDLC systems for protein separation, such as the PF2D platform, may still attract more attention. The promising applications of PF2D in discovery of biomarkers would facilitate early diagnosis and intervention, as well as treatment. In addition, PF2D also benefits the investigation of pathogenesis *via* quick automatic recovery of lower-abundant expressed proteins, and therefore sheds light on prophylaxis and therapy of human diseases. Refinement of this system, for instance, improvement of throughput, lower requirement of sample size, as well as improvement of sensitivity for identification of proteins in low profile, is necessary and will play a role in future proteomics studies.

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