Chapter

2D Gel Electrophoresis to Address Biological Issues

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Abstract

Two-dimensional (2D) gel electrophoresis is a high-resolution technique for the study of proteome. This chapter describes how it can be applied to characterize specific differences in the proteome profile of breast cancer cells following gene target interference. The proteome is the complete set of proteins encoded by a genome, and proteomic analysis consists in profiling the whole proteins expressed in a given cell, tissue, organ, or organism. Proteomic expression has the main purpose of qualitatively and quantitatively comparing proteins expressed under physiological and/or pathological conditions. Although it is not the unique approach used in modern proteomics, two-dimensional electrophoresis (2DE) is unrivaled allowing simultaneous separation of thousands of proteins and the detection of post-translational modification, not predictable through genome analysis. 2DE combines two physical principles to separate complex protein mixtures: the isoelectric point and the molecular weight. The result is a gel map in which each protein isoform present in the sample can be visualized as a spot, analyzed, quantified, and identified by mass spectrometry analysis. Here we outline features and advantages of the 2DE-based proteomic approach and we describe how 2DE meets biochemistry and molecular biology to address specific issues.

Keywords: 2D gel electrophoresis, proteomics, mass spectrometry, breast cancer, western blot, image analysis, silver staining, trypsin digestion

1. Introduction

Proteins are the effectors of almost all functions in living cells. The proteome is the complete set of proteins encoded by a genome [1, 2]. For a given organism or living cells, genome is a static entity, except if a peculiar mutation occurs. Proteome is dynamic and its composition differs under the influence of specific physiological and or pathological stimuli. Proteomics is the science that describes the proteome and provides identification and quantification of proteins, including co- and post-translational modifications, their localization, interactions, activities, and functions [3].

Resolution of thousands of proteins in a single experiment implies the employment of high-throughput analytical tools with high resolution, sensitivity, and reproducibility. Two-dimensional gel electrophoresis is a key separation technique for proteomic research, useful for qualitative and quantitative protein expression profiling. The technique has been initially applied for protein separation in 1975 by O′ Farrel [4]; it has been used for over three decades, and it is still the most widely utilized protein separation method for proteome or

sub-proteome analysis. The main advantage of 2DE is the capability of simultaneously resolving complex protein mixture, up to 10,000, allowing their quantitation as well as the analysis of all post-translational modifications [5]. 2DE has been adopted for a variety of applications: from drug discovery to diagnostics, therapy, and biochemistry [6–10].

In the last decade, 2DE has been significantly improved and currently it is a high-resolution, reliable, and reproducible method [11–13]. 2DE coupled together isoelectric focusing (IEF) with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), resolves proteins by two independent physic principles: the isoelectric point (pI) in the first dimension and molecular mass (Mr) in the second dimension. Therefore, any modifications that may change protonable groups of a protein, such as those due to phosphorylation, acetylation, and alkylation, would greatly affect the migration rate on the first dimension, likewise modifications of molecular mass as glycosylation, ubiquitination, farnesylation, for example, might affect the migration rate on second dimension. A combination of these ptms might result in the vectorial addition of the two perpendicular shifts.

The result is a 2D gel map of resolved protein isoforms that may be visualized with either visible colorimetric method (Coomassie blue, silver stain) [14, 15] or fluorescent die [16]. In theory, 2DE is capable of detecting and quantifying protein amounts of less than 1 ng per spot.

After 2DE separation, gel images may be acquired using densitometers, and quantified by means of specific software for image analysis. After that, spots of interest are excised, destained, and analyzed by LC-MS/MS [8, 13, 17].

Here we give an overview of the technique and provide protocol useful for the analysis of the proteome of specific cellular line.

2. Protocol text

2.1 Methods

2.1.1 Protein isolation

Cell lines were washed twice with PBS (1X), PBS was carefully removed, and cells were scraped after addition of 500 μl of lysis buffer prepared in accordance with **Table 1**. Cell suspension was collected, incubated at 0°C for 30 min, and sonicated using Ultrasonic Baths (VWR) at 4°C for 10 s. Cell lysate was centrifuged at 15000×*g* for 20 min. The supernatant was carefully removed and stored at −80°C for further analysis [9].

2.1.2 Protein concentration assay

The main step of proteomic analysis is the measurement of protein extract concentration. An accurate protein quantitation is essential to avoid bias in the determination of differentially expressed proteins.

Protein content from control and treated samples was measured using the Bradford method (Bio-Rad) [18].

Standard curve preparation (for sample with 1–18 μg ml^{-1} protein)

We prepare seven dilutions of a HSA protein standard in a range from 1 to 18 μg protein.

A total of 10 μl of each standard solution was diluted in 790 μl of Milli-Q water. A total of 200 μl of Bradford reagent was added to each tube; the solution was mixed thoroughly and incubated at RT for 15 min in the dark.

Buffer	Components	Notes
Lysis buffer (LB)	15 mM Tris HCl pH 7.5, 120 mM sodium chloride, 30 mM potassium chloride, dithiothreitol 0.1%, and Triton X-100, 0.5%, supplemented with protease and phosphatase inhibitor cocktail (Halt Protease Inhibitor Cocktail/Halt Phosphatase Inhibitor Cocktail, Thermo Fisher Scientific Inc.).	Add protease inhibitors just prior to use. The 2X lysis buffer can be stored at 4°C up to 1 month.
Rehydration buffer (RB)	8 M Urea, 4% CHAPS, 0.8% (v/v) carrier ampholytes (pH 3–10 NL), traces of bromophenol blue, and 70 mM DTE (add as powder).	Add DTE and carrier ampholytes just prior to use. RB buffer can be stored at −20°C up to 6 months.
Equilibration buffer A	6 M Urea, 2% (w/v/) SDS, 50 mM Tris–HCl, pH 8.8, 30% (v/v), and 1% (w/v) dithiothreitol (DTT).	Dissolve by shaking, but not by heating the solution. All chemical used must be of ultrapure quality.
Equilibration buffer A	6 M Urea, 2% (w/v/) SDS, 50 mM Tris–HCl, pH 8.8, 30% (v/v), 2.5% (w/v) iodoacetamide (IAA).	Dissolve by shaking, but not by heating the solution. All chemical used must be of ultrapure quality.
Acrylamide stock	30% acrylamide–bis solution (29:1).	
SDS stock	20% SDS in MQ-water.	
Tris stock	1.5 M Tris–HCl, pH 8.8.	
APS stock	10% ammonium persulfate.	Prepare fresh by dissolving 0.1 g APS in 1 mL MQ-water.
SDS running buffer (lower chamber)	25 mM Tris base, 192 mM glycine, and 0.1% SDS.	
SDS running buffer (upper chamber)	25 mM Tris base, 192 mM glycine, and 0.2% SDS.	
Agarose sealing solution	1% Agarose in 1X SDS running buffer with trace of bromophenol blue.	
Gel solution	10% acrylamide–bis solution (29:1), 37.5 M Tris–HCl, pH 8.8, $Na_2S_2O_3$ 0.025% , APS 0.1%, and TEMED 0.04%; adjust volume using MQ-water.	Add TEMED and APS just before use.
Fixing solution (silver staining)	40% (w/v) Ethanol and 10% (w/v) acetic acid.	Prepare the solution using Milli-Q water.
Sensiting solution (silver staining)	0.02% Sodium thiosulfate pentahydrate.	Prepare the solution using Milli-Q water.
Silver nitrate solution (silver staining)	0.2% Silver nitrate and 0.02% formaldehyde (37%).	Prepare the solution using Milli-Q water.
Developing solution (silver staining)	3% Sodium carbonate, 0.05% formaldehyde (37%), and 0.005% sodium thiosulfate pentahydrate.	Prepare the solution using Milli-Q water. Solution must be prepared just before use.
Stop solution (silver staining)	2% Acetic acid.	Prepare the solution using Milli-Q water.

Buffer	Components	Notes
Destaining solution (Spot excision)	30 mM Potassium ferricyanide and 100 mM sodium thiosulfate.	Prepare the solution using HPLC grade water.
Gel washing solution (spot excision)	50% acetonitrile, 50 mM ammonium bicarbonate.	Prepare the solution using HPLC grade water.
Digestion buffer (spot excision)	Resuspend lyophilized trypsin (20 μg/vial) in 100 μL of the 1 mM HCl, yielding a 0.2 μg/μL stock solution. Aliquot into 10 × 10 μl and place at −20°C. To obtain the working solution, take 10 μl of this trypsin solution and add 500 μl of 50 mM NH_4HCO_3.	Prepare the solution using HPLC grade water. Avoid repeated freeze-thaw of trypsin stock solutions more than once.
Extraction solution	50% ACN–0.2% trifluoroacetic acid (TFA).	

Components and notes for buffers described and used in this chapter are summarized.

Table 1.
Buffer components.

The absorbance was measured at 595 nm using a BioPhotometer (Eppendorf). The assay was done in triplicate using a specific clear cuvette (UV-Vis transparent between 220 and 1600 nm). The BioPhotometer was calibrated on the basis of standard quantitative data.

About 2 μl of each protein extract was suspended in 798 μl of Milli-Q water, 200 μl of Bradford reagent was added to the mixture, vortexed, and incubated at RT for 15 min in the dark. The assay was performed in triplicate. Sample absorbance was measured at 595 nm using a calibrated BioPhotometer (Eppendorf).

2.1.3 First dimension: isoelectric focusing (IEF)

During isoelectric focusing, proteins are resolved in function of their isoelectric point (pI). The pI of a protein is the value of pH at which the electrophoretic mobility is nil. The majority of proteins have a pI within pH 4–7, although some proteins show an extreme value of pI as histone proteins. IEF is carried out using a precast strip of polyacrylamide with the most appropriate pH gradient range. The use of immobilized pH gradient (IPG) strips improves reproducibility, resolution, and protein load capability.

The pH gradient is created by dissolving in the IPG a mixture of aliphatic polyamino-polycarboxylic acids (carrier ampholines) that under the influence of an electric field create a stable pH gradient.

Proteins are resolved, in addition to their amino and carboxyl group, in function of a protonable group in their side chains. When the pH < pI, a protein has a net positive charge and at pH > pI, it becomes negatively charged. Therefore, under the influence of an electric field, proteins with acidic pI migrate toward the cathode and those with basic pI move toward the anode, until it reaches the strip region in which pH = pI. The method allows the separation of all protein isoforms [19].

In this experiment, we loaded 130 μg of each sample diluted into RB buffer (**Table 1**) during the rehydration step. When IEF is carried out for comparative experiments, IPG strips must have the same length and identical pH range; samples must be handled in parallel.

The rehydration volume is a function of strip length [19], for a strip 24 cm long, the appropriate volume is 480 μl. The rehydration tray was cleaned, dried, and leveled to ensure uniform reswelling.

A total of 480 μl of RB buffer, containing the sample, was distributed along the reswelling slot. The strips were deprived of protective cover and carefully flat on the fluid with the gel side down. The strips were checked for the presence of bubbles below and covered with mineral oil to prevent sample evaporation and urea crystallization. The tray was closed with the lid and rehydration was carried out for 16 h at RT.

IEF conditions may vary accordingly with sample amount, sample composition, IPG strip length, and pH range.

After the rehydration step, the IPG strips were removed from the slot, rinsed with Milli-Q water, and transferred on the Ettan IPGphor ceramic tray. During IEF, the gel strip is sided up. The strips were overlaid with mineral oil and the movable electrodes were assembled properly at the cathodic and the anodic end of the strip. A bit piece of filter paper was positioned between the strip and the electrode to eliminate ion excess.

IEF was carried out on a GE Healthcare IPGphor unit at 20°C, applying 50 uA/Strip, until a total of 70,000 Vh was reached. The following IPGphor program was used: (1) 300 V 3 h step, (2) 1000 V 6 h gradient, (3) 8000 V 3 h gradient, and (4) 8000 V 3 h step.

2.1.4 Second dimension

During SDS PAGE, IPG-focused proteins were separated according to their molecular weights.

For gel casting, a single gel for each IPG strip was prepared. Gel casting was done simultaneously. Gel caster was assembled with glass plates, separator sheets, and blank cassette inserts. Each component was previously cleaned and dried. Monomer solution was prepared according to the protocol (**Table 1**: gel solution) and carefully poured in the funnel casting.

After 2 min, the surface of each gel was layered with 2 ml of isopropanol to minimize exposure to oxygen. After 10 min, isopropanol was replaced with Milli-Q water and the gel was allowed to polymerize for at least 5 h. The gels were prepared concomitantly with the start of IEF and stored at 4°C until use.

Equilibration step: the equilibration step is essential to saturate focused proteins with SDS and allow their separation by SDS PAGE.

After IEF, IPG strips were equilibrated using 15 ml of equilibration buffer A (**Table 1**) followed by treatment with 15 ml of equilibration buffer B (**Table 1**) [19, 20]. The equilibrated IPG strip was rinsed with running buffer 1X and positioned with acidic end to the left and the plastic back facing one the higher glass plates, avoiding the formation of bubbles between the strip and the top of the gel.

A small piece of filter paper soaked with 10 μL of protein MW marker was positioned at the right side using forceps.

The strips were sealed using agarose solution. The second dimension was done on 10% SDS-polyacrylamide gels.

The electrophoretic unit was filled with running buffer 1X and connected with the MultiTemp III Thermostatic Circulator. When the temperature of the buffer reached the seat value of 25°C, the gels were positioned in the chamber; the empty slots were filled with blank cassettes. The upper chamber was positioned in the electrophoretic unit and filled with running buffer 1X, SDS 0.02%. The lid was closed and attached to the power supply. Run was done at constant voltage (2 W/gel, 25°C) until the bromophenol blue dye front reached the end of the gels [20, 21].

After electrophoresis, gels were carefully removed from their gel cassettes and placed on a glass tray for staining.

2.1.5 Staining with MS-compatible silver staining

Gels were stained with the MS-compatible silver staining procedure [14, 21]. The methods used for staining are summarized in **Table 2**.

2.1.6 Gel image analysis

The main aim of proteomics consists in the identification of differentially expressed proteins. To proceed with the 2DE analysis, the first step is the digitalization of gel images. It has been done by Image Scanner II (GE healthcare).

The gels were scanned at 600 dpi and saved as tiff.

Subsequent analysis was performed using the Image Master 2D-Platinum software, version 6.0 (GE Healthcare) according to the software manual. Briefly, raw images were imported into the software and processed for protein detection. The spot auto-detect function was used for all group comparisons applying the following parameters: smooth 2, min area 5, saliency 1.00000. Digital images were checked to ensure that all the proteins present in the gel were correctly detected. Differences in protein expression were assessed using the relative volume (%Vol) option of the software. Using this option, the data becomes independent variables caused by differences in loading or staining [22].

Analysis was done using three independent replicates. All data were presented as mean ± SEM (N), where SEM is the standard error of the mean and N represents the number of replicates. Protein levels in every data set were compared to control group using unpaired t-test. We considered statistically significant a two-sided p-value <0.05. Excel spreadsheet (Microsoft) was used for data plotting [23].

2.1.7 Spot excision and digestion

A main issue during spot excision and digestion procedure is avoiding contamination with keratins that can affect subsequent mass spectrometry analysis. Proper lab-equipment and dedicated reagents mast be used for protein spot

Step	Solution	Volume	Time
Fixing	Fixing solution	500 ml	2 h
Washing	Wash with 30% ethanol	500 ml	30 min
Sensiting	Sensiting solution	500 ml	1 min
Washing	Milli-Q water	500 ml	2 × 2 min
Staining	Silver nitrate solution	500 ml	30 min
Rinsing	Milli-Q water	500 ml	2 × 2 min
Developing	Developing solution	500 ml	2 min
Developing	Developing solution	500 ml	Develop for the time necessary to clearly visualize the spot.
Stop solution	Stop solution	500 ml	20 min
Washing	Milli-Q water	500 ml	3 × 10 min

The table summarizes the steps used for silver staining.

Table 2.
Silver staining steps.

digestion. All instruments, as well as the benchtop, were meticulously cleaned with 70% ethanol.

Protein spots, with a differential expression profile, were manually excised from silver stained gel using OneTouch Plus Spotpicker (Gel company).

Gel pieces were washed with Milli-Q water and destained incubating with a destaining solution (**Table 1**) for 15 min.

The destaning solution was removed and gel spot washed three times with 200 μl of 200 mM NH_4HCO_3 pH 7.8 for 15 min; the incubation was done in a tube shaker at 37°C. Spots were incubated with ACN for 10 min at 37°C. A total of 70 μl of trypsin working solution (**Table 1**) was added to gel pieces prior to incubation at 37°C overnight. Trypsin solution was carefully recovered and placed in a fresh tube (t).

Additional peptides were recovered by incubating the gel piece with extraction solution at 37°C for 10 min. The solution was recuperated and pooled in the fresh tube (t). Peptide solution was concentrated using a speedy vac [24, 25].

The resulting tryptic peptides were purified by Pierce® C18 Spin Columns (Thermo Fisher Scientific Inc.) according to the manufacturer's procedure, eluted with 40 μL of 70% acetonitrile, and dehydrated in a vacuum evaporator [26].

2.1.7.1 Nanoscale LC-MS/MS analysis

Tryptic peptides were analyzed using Nanoscale LC-MS/MS. The analysis was performed as service in our mass spectrometry facility.

Chromatography analysis was done on Easy LC 1000 nanoscale liquid chromatography (nanoLC) system (Thermo Fisher Scientific, Odense, Denmark) as previously reported by Scumaci et al. [23]. Purified peptides were re-suspended with 0.1% formic acid and injected at 500 nL/min on the analytical column. A binary gradient was used for the elution of peptides. Mass analysis was done by means of a quadrupole-orbitrap mass spectrometer Q-Exactive (Thermo Fisher Scientific, Bremen, Germany) working in positive ion mode, using nanoelectrospray (nESI) potential at 1800 V. Acquisition of data was achieved with a top-5 method using resolution (FWHM), AGC target, and maximum injection time (ms) for full MS and MS/MS of, respectively, 70,000/17,500, 1e6/5e5, and 50/400. Precursor ion isolation was done using a mass window of 2.0 m/z, while normalized collision energy was 30. Ion threshold for generating MS/MS events was 2e4. Dynamic exclusion was set at 15 s. Data were processed using Proteome Discoverer 1.3 (Thermo Fisher Scientific, Bremen, Germany), search engine was Sequest, and the HUMAN-refprot-isoforms.fasta was used as a sequence database. Searching parameters were: MS tolerance 15 ppm; MS/MS tolerance 0.02 Da; fixed modifications: cysteine carbamidomethylation; variable modification: methionine oxidation, serine, threonine, and tyrosine phosphorylation; enzyme: trypsin; max. missed cleavages 2; taxonomy Human. We consider only protein hits generated with two successful peptide identifications valid and acceptable [27].

2.1.8 Western blot validation

Western blot analysis of prohibitin and gelsolin was done to verify 2D gel electrophoresis data. Analysis was performed on cell extracts of MCF7wt and BRCA1 interfered MCF7.

About 50 μg of each protein extract were resolved on a 4–15% SDS PAGE precast gel (Bio-Rad), and blotted to a nitrocellulose membrane followed by immunoblotting. The working concentration of rabbit monoclonal antibody against BRCA1 (clone D-20, Santa Cruz was 1 μg/ml) [23].

Anti-PHB1 (Prohibitin 1) from cell signaling technology was used at 1:1000 dilution and Anti-Gelsolin (Santa Cruz) was used at 1:1000 dilution. The signal was detected using the specific horseradish peroxidase-conjugate secondary antibodies; blots were developed using the SuperSignal West Femto ECL substrate (Pierce). Densitometric software (Alliance 2.7 1D fully automated software) determined the percent distribution of blotted proteins after image acquisition by Alliance 2.7 (UVITEC, Eppendorf, Milan, Italy) [28].

Data were analyzed and plotted using Excel spreadsheet (Microsoft), and expressed as mean ± SEM (N), where SEM represents the standard error of the mean and N indicates the number of experimental repeats. Unpaired t-test was used to compare protein levels in each data set. A two-sided p-value <0.05 was considered statistically significant.

3. Results

2D gel electrophoresis was applied to analyze the whole proteome of breast cancer cell expressing or not BRCA1. BRCA1 is a tumor suppressor gene, often mutated in hereditary breast and ovarian cancers. The gene encodes for a large protein involved in several cellular pathways that include DNA damage-induced cell cycle checkpoint activation, maintenance of genomic stability, DNA damage repair, as well as chromatin remodeling, protein ubiquitination, transcriptional regulation, and apoptosis [29]. Recently, it has been also reported that BRCA1 is able to induce reprogramming of metabolism toward aerobic glycolysis in breast cancer cells [30]. Here we induce the transient inactivation of BRCA1 in MCF-7 cell line for 48 h by transfection with Oligo siRNA/Brca1 [20, 31]. It has been done by Lipofectamine 2000 reagent (Invitrogen, Paisley, UK), according to the manufacturer's instructions. Oligo siRNA/Brca1 duplex was purchased from Sigma Aldrich and used at a final concentration of 100 nM. The silencing of BRCA1 was checked by western blot analysis (**Figure 1**). Transient knock down of BRCA1 was done at 48 h according to our previous results [20]. The analysis workflow is summarized in **Figure 2**. The analysis was performed using three biological replicates.

Using 2D gel electrophoresis, we obtained a gel map of about 500 protein spot for each replicate (**Figure 3**). Image analysis allowed to identify 25 differentially

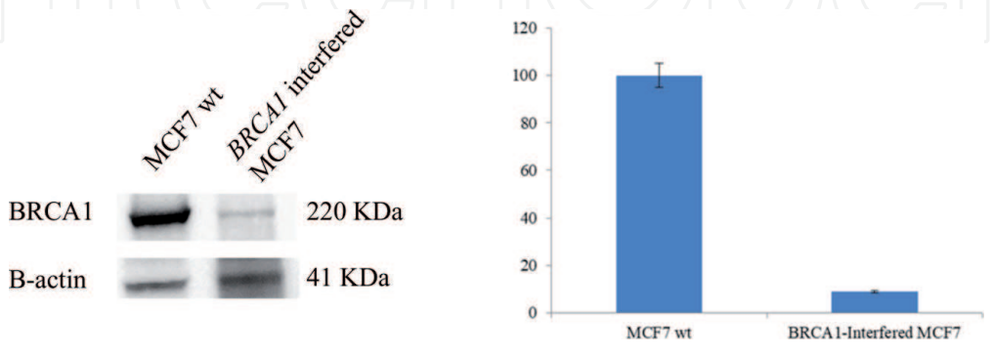

Figure 1.
Gene interference. Western blot analysis confirmed BRCA1 switch off. Equal amounts of MCF7 protein extracts were separated on a 4–15% SDS PAGE precast gel (Bio-Rad) and transferred to a nitrocellulose membrane followed by immunoblotting. In each panel, the left panel is representative data of western blot analysis; the right panel shows densitometric analysis. Analysis was performed using three independent experiments. Data are mean ± SEM (N = 3). p < 0.05. In each panel, β-actin blot shows equal amounts of protein loading.

expressed proteins. Of these, 15 protein spots were down-expressed, while 10 were up-regulated.

Differentially expressed proteins were manually excised, destained, trypsin digested, and identified using LC-MS/MS analysis. A list of identified protein is provided in **Table 3**.

2DE data were confirmed using western blot analysis for two selected candidates (**Figure 4**).

Differentially expressed protein was analyzed using Ingenuity software PA tool.

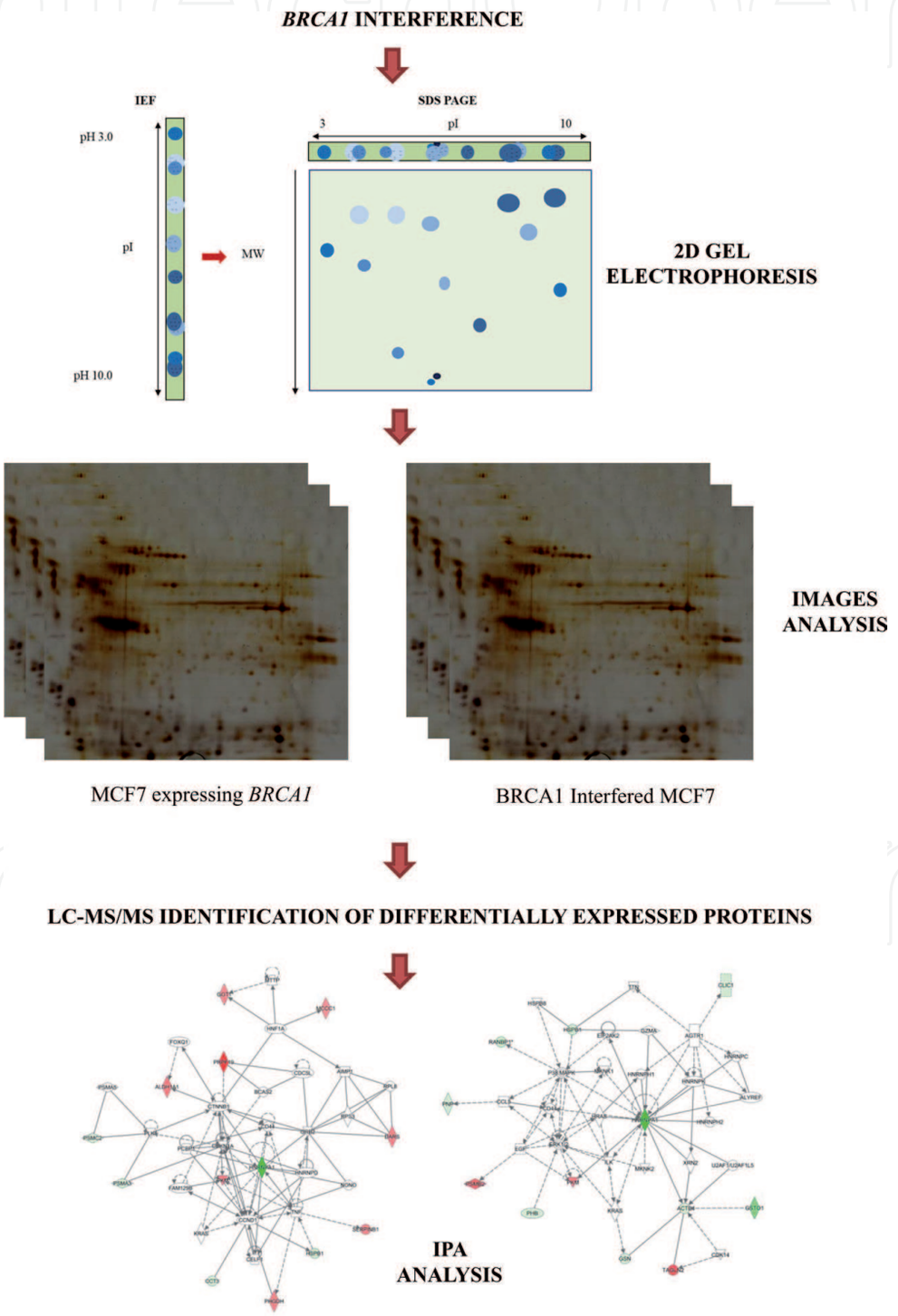

Figure 2.
Proteomic approach work flow.

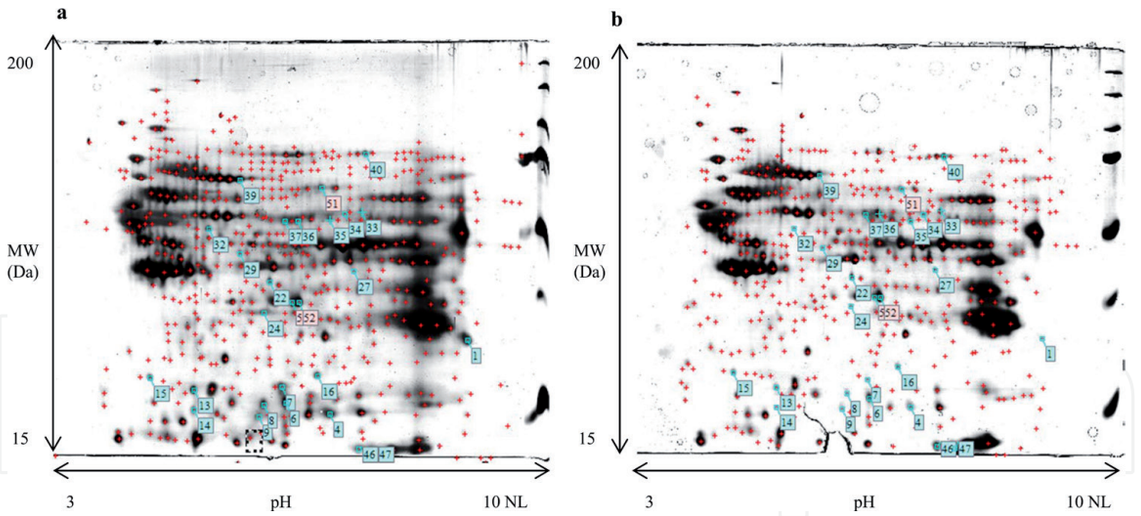

Figure 3.
2D gel electrophoresis maps. (a) Representative 2D gel electrophoresis maps of MCF7 wt protein extracts and (b) representative 2D gel electrophoresis maps of BRCA1 interfered MCF7 protein extracts. Isoelectrofocusing was carried out on 3–10 NL IPGstrip, 24 cm length. The second dimension was performed on 10% SDS-PAGE. Gel images were analyzed using Decider software. Numbered spots indicate proteins that have statistically significant differential expression between samples according to Image master 2D Platinum 7.0 software (GE Healthcare).

Spot ID	ID protein	Description	Score	Coverage	Unique peptides	Molecular weight	pI	BRCA1 interfered MCF7/ MCF7 wt
1	P09651-3	Isoform 2 of Heterogeneous nuclear ribonucleoprotein A1 OS=*Homo sapiens* GN=HNRNPA1 – [ROA1_HUMAN]	76.57	43.07	9.00	29.37	9.14	–24.63
4	Q15102	Platelet-activating factor acetylhydrolase IB subunit gamma OS=*Homo sapiens* GN=PAFAH1B3 PE=1 SV=1 – [PA1B3_HUMAN]	5.59	11.26	3.00	25.72	6.84	–2.60
6	P25788-2	Isoform 2 of Proteasome subunit alpha type-3 OS=*Homo sapiens* GN=PSMA3 – [PSA3_HUMAN]	18.38	23.79	7.00	27.63	5.33	–1.93
7	P78417	Glutathione S-transferase omega-1 OS=*Homo sapiens* GN=GSTO1 PE=1 SV=2 – [GSTO1_ HUMAN]	14.83	21.99	6.00	27.55	6.60	–13.10

Spot ID	ID protein	Description	Score	Coverage	Unique peptides	Molecular weight	pI	BRCA1 interfered MCF7/ MCF7 wt
8	P43487	Ran-specific GTPase-activating protein OS=*Homo sapiens* GN=RANBP1 PE=1 SV=1 – [RANG_HUMAN]	0.00	8.96	2.00	23.30	5.29	–3.84
9	P04792	Heat shock protein beta-1 OS=*Homo sapiens* GN=HSPB1 PE=1 SV=2 – [HSPB1_HUMAN]	22.60	30.73	6.00	22.77	6.40	–4.42
13	P35232	Prohibitin OS=*Homo sapiens* GN=PHB PE=1 SV=1 – [PHB_HUMAN]	46.62	45.22	11.00	29.79	5.76	–1.94
14	P43487	Ran-specific GTPase-activating protein OS=*Homo sapiens* GN=RANBP1 PE=1 SV=1 – [RANG_HUMAN]	4.76	8.96	2.00	23.30	5.29	–4.99
15	O00299	Chloride intracellular channel protein 1 OS=*Homo sapiens* GN=CLIC1 PE=1 SV=4 – [CLIC1_HUMAN]	55.50	57.68	9.00	26.91	5.17	–1.15
16	P00491	Purine nucleoside phosphorylase OS=*Homo sapiens* GN=PNP PE=1 SV=2 – [PNPH_HUMAN]	10.39	16.96	4.00	32.10	6.95	–2.77
22	P30740	Leukocyte elastase inhibitor OS=*Homo sapiens* GN=SERPINB1 PE=1 SV=1 – [ILEU_HUMAN]	25.33	27.70	10.00	42.71	6.28	2.21
24	Q9NP79	Vacuolar protein sorting-associated protein VTA1 homolog OS=*Homo sapiens* GN=VTA1 PE=1 SV=1 – [VTA1_HUMAN]	4.65	9.45	2.00	33.86	6.29	–4.32

Spot ID	ID protein	Description	Score	Coverage	Unique peptides	Molecular weight	pI	BRCA1 interfered MCF7/ MCF7 wt
27	B7Z7E9	Aspartate aminotransferase OS=*Homo sapiens* GN=GOT1 PE=2 SV=1 – [B7Z7E9_HUMAN]	3.86	8.67	3.00	44.12	8.07	2.02
29	P35998	26S protease regulatory subunit 7 OS=*Homo sapiens* GN=PSMC2 PE=1 SV=3 –[PRS7_HUMAN]	93.17	60.28	26.00	48.60	5.95	−1.45
32	F8VPV9	ATP synthase subunit beta OS=*Homo sapiens* GN=ATP5B PE=2 SV=1 – [F8VPV9_HUMAN]	20.60	22.39	8.00	55.27	5.40	−3.96
33	P14618	Pyruvate kinase isozymes M1/M2 OS=*Homo sapiens* GN=PKM PE=1 SV=4 – [KPYM_HUMAN]	29.08	32.02	13.00	57.90	7.84	2.36
34	P00352	Retinal dehydrogenase 1 OS=*Homo sapiens* GN=ALDH1A1 PE=1 SV=2 – [AL1A1_HUMAN]	21.35	24.35	10.00	54.83	6.73	2.31
35	P14868	Aspartate--tRNA ligase, cytoplasmic OS=*Homo sapiens* GN=DARS PE=1 SV=2 – [SYDC_HUMAN]	7.94	8.18	4.00	57.10	6.55	2.15
36	Q9UMS4	Pre-mRNA-processing factor 19 OS=*Homo sapiens* GN=PRPF19 PE=1 SV=1 – [PRP19_HUMAN]	7.76	8.93	4.00	55.15	6.61	3.80
37	O43175	D-3-phosphoglycerate dehydrogenase OS=*Homo sapiens* GN=PHGDH PE=1 SV=4 – [SERA_HUMAN]	40.04	29.08	12.00	56.61	6.71	2.16
39	B4DUR8	T-complex protein 1 subunit gamma OS=*Homo sapiens* GN=CCT3 PE=2 SV=1 – [B4DUR8_HUMAN]	11.04	15.20	6.00	55.64	5.64	−1.85

Spot ID	ID protein	Description	Score	Coverage	Unique peptides	Molecular weight	pI	BRCA1 interfered MCF7/ MCF7 wt
40	Q96RQ3	Methylcrotonyl-CoA carboxylase subunit alpha, mitochondrial OS=*Homo sapiens* GN=MCCC1 PE=1 SV=3 – [MCCA_HUMAN]	36.71	24.69	13.00	80.42	7.78	1.84
46	P49721	Proteasome subunit beta type-2 OS=*Homo sapiens* GN=PSMB2 PE=1 SV=1 – [PSB2_HUMAN]	14.09	27.86	5.00	22.82	7.02	2.90
47	P37802	Transgelin-2 OS=*Homo sapiens* GN=TAGLN2 PE=1 SV=3 – [TAGL2_HUMAN]	12.17	34.17	6.00	22.38	8.25	3.23
51	P13020-2	Isoform 2 of Gelsolin OS=*Mus musculus* GN=Gsn – [GELS_MOUSE]	10.66	4.10	3.00	80.71	5.72	−2.03
52	P60709	Actin, cytoplasmic 1 OS=*Homo sapiens* GN=ACTB PE=1 SV=1 – [ACTB_HUMAN]	13.39	17.07	2.00	41.71	5.48	−1.15

In the table are reported mass spectrometry identifications for protein spots that resulted differentially expressed following 2DE analysis. For each spot are reported: spot id, gene name, identification score, number of unique peptides, isolectric point (pI), and molecular weight (MW). In the last columns are reported quantitative data. Data are reported as fold change between BRCA1-interfered breast cancer cells (MCF7) versus breast cancer cell (MCF7) wt. (p value < 0.05).

Table 3.
Densitometric data of 2D gel electrophoresis and protein identification by LC-MS/MS analysis.

3.1 Pathway and network analysis

Differentially expressed proteins were connected using ingenuity pathway analysis (Ingenuity Systems, www.ingenuity.com). Data sets with protein identification and quantitative data were loaded into the application that creates hypothetical networks of protein interaction based on ingenuity pathway knowledge base. IPA software generates a list of networks in function on their connectivity, allowing to associate specific biological functions to genes here included [23, 27, 29, 32].

Proteins were mapped onto three networks (**Figure 5**). The most representative, with a score of 28 and 13 focus molecules, had function associated with cancer, cellular movement, and connective tissue disorders. The second network, including 12 focus molecules with a score of 25, exhibited functions connected with free radical scavenging, protein synthesis, DNA replication, recombination, and repair. Finally, the last network grouped proteins involved in cancer, cell death and survival, and cell morphology.

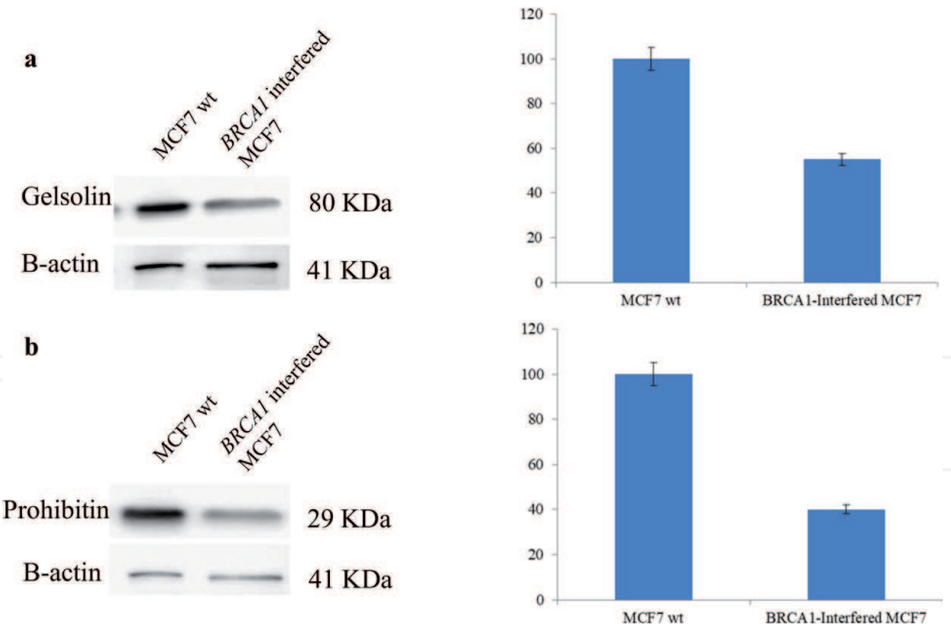

Figure 4.
Western blot analysis confirming 2DE data for gelsolin (a) and prohibitin (b). Analysis was performed on protein extracts from MCF7 expressing or not BRCA1. Equal amounts of MCF7 protein extracts were separated on a 4–15% SDS PAGE precast gel (Bio-Rad) and electrotransferred to a nitrocellulose membrane with a Trans-blot turbo system (Biorad). Images were acquired using the Alliance 2.7 system (UVITEC, Eppendorf, Milan, Italy) and analyzed by Excel spreadsheet. In each panel, the left panel is representative of western blot analysis; the right panel shows densitometric analysis. Analysis was performed using three independent experiments. Data are mean ± SEM (N = 3). $p < 0.05$. In each panel, β-actin blot shows equal amounts of protein loading.

a

ID	Molecules in Network	Score	Focus Molecules	Top Diseases and Functions
1	AIMP1,ALDH1A1,BCAS2,CCND1,CCT3,CD44,CDC5L,CDKN1A,CELF1,CTNNB1,DARS,FAM129B,FOXQ1,GOT1,GRB2,HNF1A,HNRNPA1,HNRNPD,HSPB1,KRAS,MCCC1,MTTP,NONO,PCBP1,PHGDH,PKM,PLK1,PRPF19,PSMA3,PSMA5,PSMC2,RPL8,RPS3,SERPINB1,TNF	28	13	Cancer, Cellular Movement, Connective Tissue Disorders
2	ACTB,AGTR1,ALYREF,CCL5,CD44,CDK14,CLIC1,EGF,EIF2AK2,ERK1/2,GSN,GSTO1,GZMA,HNRNPA1,HNRNPC,HNRNPH1,HNRNPH2,HNRNPK,HRAS,HSPB1,HSPB8,ILK,KRAS,MKNK1,MKNK2,P38 MAPK,PHB,PKM,PNP,PSMB2,RANBP1,TAGLN2,TTN,U2AF1/U2AF1L5,XRN2	25	12	Free Radical Scavenging, Protein Synthesis, DNA Replication, Recombination, and Repair
3	SATB1,SUZ12,VTA1	2	1	Cancer, Cell Death and Survival, Cell Morphology

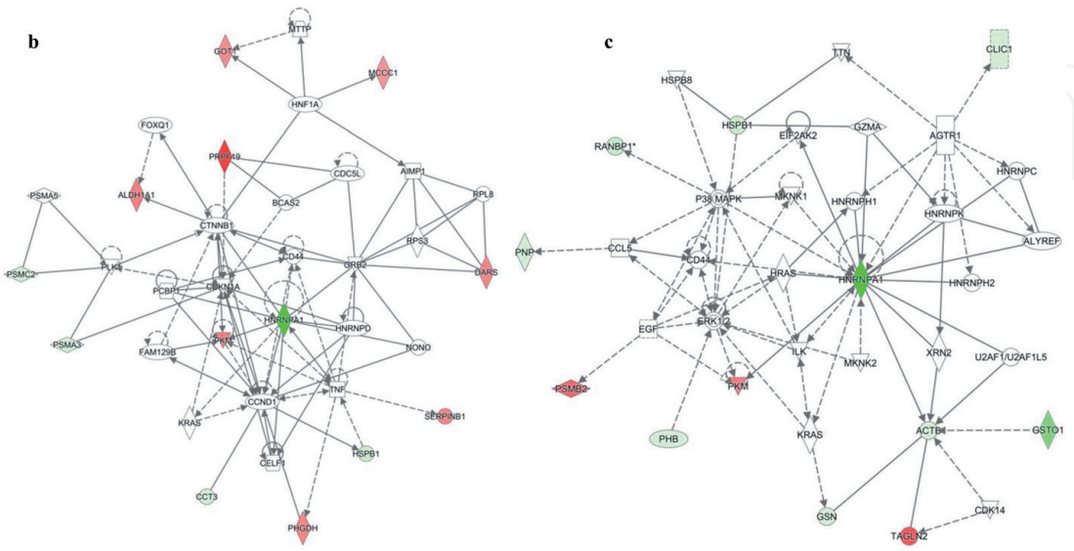

Figure 5.
IPA analysis. (a) the list of networks generated from IPA analysis and significantly modulated (log p-value) in BRCA1-interferd MCF7 versus MCF7 wt.; the top 2 signaling networks; (b) cancer, cellular movement, and connective tissue disorders; (c) free radical scavenging, protein synthesis, DNA replication, recombination, and repair.

4. Discussion

The aim of this work is to describe how 2D gel electrophoresis might be effectively applied to provide a comprehensive, quantitative description of protein expression and its change under the influence of a specific biological perturbation. Here we provide an overview of the 2DE methodologies with special focus on the technical procedure.

To provide an example of the technique application, we describe the proteome profiling of breast cancer cells following BRCA1 interference.

Using 2D gel electrophoresis, we found that BRCA1 silencing affected the expression of 25 proteins belonging to different cellular compartments and implicated in a variety of cellular pathways. We focused our attention on the downregulation of prohibitin and gelsolin as well as the upregulation of pyruvate kinase.

Upon BRCA1 interference, we found a reduced level of prohibitin, coherently with literature data that highlighted the ability of prohibitin in inhibiting breast cancer cell proliferation and promoting apoptosis [33]. In addition, we found a reduced level of gelsolin, fully in agreement with what we previously published [20]. Otherwise, the expression of pyruvate kinase, together with D-3-phosphoglycerate dehydrogenase, was found up-regulated, according with a metabolic shift toward glucose metabolism [34].

Although interesting, these findings deserve validations and further investigations that largely exceed the scope of this chapter. However, the approach provided the opportunity to highlight the capability of 2D gel electrophoresis in proteome profiling breast cancer cells and in detecting the specific phenotypic changes induced by the interference of the key gene BRCA1.

The fundamental step of our proteomic analysis was the preparation of the samples. Protein extracts for 2DE were done directly on a plate with a minimum amount of lysis buffer, to avoid contaminations with 2DE interfering substances, but enough to ensure the complete extraction, solubilization, and disaggregation of all cellular proteins avoiding proteolysis and poor recovery. This was a critical step because experimentally induced protein changes may be challenging to distinguish from biological differences under investigations. Sample protein content was carefully quantized and loaded using the "in-gel rehydration" procedure. The second dimension was performed at constant voltage and temperature. Silver staining was done in glass tray with accuracy, avoiding the formation of gray precipitates as well as the formation of the dark background caused by an extra developing.

The reproducibility of the spot pattern in gel images of different samples reflects the accurateness of the procedures. Moreover, the presence of spot well distributed on the gel with finely defined borders as well as the lack of streak and background staining proved the quality of 2D separation.

5. Conclusions

In this chapter, we point out the key aspects of 2D gel electrophoresis describing in practice the use of the technique for the proteomic profiling of breast cancer cells expressing or not BRCA1.

We offer an overview of methodological peculiarities, undelaying that, in spite of some technical limitations, 2D gel electrophoresis is still one of the major current analytical technologies useful to investigate specific change in the profiles of proteome in different cells, tissues, or organism [35].

The proteome of higher eukaryotes holds a huge complexity due to dynamic changes in protein expression, modification, and interactions. It's obvious that a

single proteomic approach is not enough to address complex biological questions, but we are confident that the methods, here deepened, represents a gold standard for the analysis of intact proteins and all relative post-translational modifications responsible for a peculiar phenotype or function [36–40].

2DE is unrivaled by other proteomic approach because it consents the simultaneous detection and quantification of thousands of proteins isoforms, not predictable through other techniques including genome analysis [41, 42].

We are confident that protocols and methodologies we described are going to be useful for all scientists interested in 2DE separation to address their own biological questions.

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Conflict of interest

The authors declare that they have no competing financial interests.

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