〔Full Paper〕

Standardization of protocol for Immobiline 2-D PAGE and construction of 2-D PAGE protein database on World Wide Web home page

Tosifusa Toda and Narimichi Kimura

SUMMARY

Organismic aging appears in the results of functional alteration of tissue-constituent cells. The replicative life span of normal human diploid fibroblasts in cell culture has been realized as a typical model of the research on mitotic cell aging. From the above premises, a new project has started in our institute for establishing my own age-related protein database of human diploid fibroblasts upon a 2-D PAGE technique. The protocol of 2-D PAGE with horizontal IPG-IEF and vertical SDS-PAGE was standardized including whole procedures from cell harvest through spot quantification, since the reproducibility of 2-D gel pattern depended on the procedure. The master 2-D gel pattern of human diploid fibroblasts was generated by merging skin fibroblast-specific spots into the standard 2-D gel pattern of lung fibroblasts in an image analyzing software, because fibroblasts showed tissue specificity. The master 2-D gel pattern with spot identification numbers and the database of age-dependent protein alteration were presented on my home page for free access through the Internet computer network.

Key words: two-dimensional electrophoresis, Immobiline, protein database, Internet, WWW home page.

INTRODUCTION

The first description of replicative senescence of human diploid fibroblasts was published by Hayflick and Moorhead[1] in 1961. The cellular senescence in culture was proposed to reflect processes that occur during organismic aging *in vivo*. However, the molecular mechanisms responsible for cellular aging are still poorly understood. The shortening of telomere has been proposed as the most possible mechanism of cellular senescence by Harley et al.,[2] but not all the phenotypes of senescent cells cannot be explained only in the term of telomere. Therefore, establishment of an age-related protein database is expected to promote researches on cellular aging. 2-D PAGE with IPG-IEF[3,4] is the most powerful technique to analyze the age-related protein alteration including post-translational

戸田年総，木村成道；Department of Molecular Biology, Tokyo Metropolitan Institute of Gerontology
Correspondence address: Tosifusa Toda, Department of Molecular Biology, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173, Japan

Abbreviations: 2-D PAGE, two-dimensional polyacrylamide gel electrophoresis; IPG, immobilized pH gradient; IEF, isoelectric focusing; SDS, sodium dodecyl sulfate; TMIG, Tokyo Metropolitan Institute of Gerontology; Mr, relative molecular mass; pI, isoelectric point; PBS, phosphate-buffered saline; WWW, world wide web; DMEM, Dulbecco's modified Eagle medium; BPB, bromophenol blue; IOD, integrated optical density.

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modifications. Thus in this paper, a standard protocol of the 2-D PAGE, a master 2-D gel pattern with spot identification numbers, and the main frame of 2-D PAGE protein database constructed on my home page in our WWW server are reported.

MATERIALS

Supporting media prepared as described in Table 1 were used. SDS-treatment solution and electrode buffer solution were prepared as shown in Table 2. Immobiline Dry Strip and Pharmalyte carrier ampholytes were purchased from Pharmacia (Uppsala, Sweden). Tris, Tricine, SDS, Triton X-100 were from Sigma (St. Louis, MO, USA). Marker proteins for Mr and pI calibration were obtained from Daiichi Pure Chemicals (Tokyo, Japan). Silicon oil KF-96L-5c/s was product of Shin-Etsu Chemical Co. (Tokyo, Japan). Silver Stain Kit 299-23841 and other chemicals of reagent grade were purchased from Wako Pure Chemicals (Osaka, Japan). Electrophoresis apparatus COOL PHOR Model 3600 and electric constant power supply Model 311S with a programmable controller were from Anatech (Tokyo, Japan). PDQUEST software package was from PDI (Huntington Station, NY, USA).

METHODS

Cell culture and protein extraction

Normal human diploid fibroblasts were cultured in DMEM with 10% fetal calf serum on 6-cm i.d. dishes at 37°C in a 7%-CO_2 incubator. Cells at 70–80% confluent on dishes were rinsed with PBS-three times, and harvested in 100μl of lysis buffer by scratching with a plastic scraper. The cell suspension was transferred to a microfuge tube and supplemented with urea to be 8M finally. Proteins were extracted in the supernatant by sonication and centrifugation.

Protein concentration

When the concentration of protein was not high enough to make 2-D PAGE analysis, protein in the sample was precipitated by mixing with 9 volumes of acetone. The precipitated protein was spun down, dried and resolubilized in a small volume of lysis buffer supplemented with 8M urea by sonication again.

Table 1. Supporting media for Immobiline 2-D PAGE.

[IEF] Immobiline Dry Strip, swelled overnight in the gel-swelling solution prepared as follows:	
	for 8 strips of 180 mm long Immobiline
urea	19.2 g
10 %(v/v) Triton X-100	2.0 ml
Pharmalyte 3-10	0.2 ml
DTT	80.0 mg
0.1 %(w/v) Orange G	1.0 ml
0.1 M acetic acid	1.0 ml
Pure water	up to 40.0 ml
[SDS-PAGE] 7.5 % T, 3 % C polyacrylamide gel slab prepared as follows:	
	for 8 slabs of 190×180×1 mm gel
35 %(w/v) acrylamide	100.0 ml
2 %(w/v) BIS	55.0 ml
1.5 M Tris-HCL, pH 8.8	117.0 ml
50 %(w/v) ' glycerol	80 ml
Pure water	up to 470.0 ml
----to be degassed in vacuo before adding reagents shown below----	
10 %(w/v) SDS	4.8 ml
10 %(w/v) ammonium persulfate	0.5 ml
TEMED	0.3 ml

Table 2. Buffer solutions for Immobiline 2-D PAGE.

SDS-treatment solution prepared as follows:	
	(for 8 strips of 180 mm long Immobiline)
urea	29.0 g
DTT	0.4 g
0.5 M Tris-HCl, pH 6.8	8.0 ml
10 % (w/v) SDS	16.0 ml
0.1 % (w/v) BPB	2.0 ml
50 % (v/v) glycerol	40.0 ml
Anodic electrode buffer for SDS-PAGE prepared as follows:	
Tris	24.2 g
pure water	ca. 800 ml
----- to be adjusted the pH to 8.8 with HCl -----	
pure water	up to 1,000 ml
Cathodic electrode buffer for SDS-PAGE prepared as follows:	
Tris	12.1 g
Tricine	17.9 g
SDS	1.0 g
pure water	up to 1,000 ml

Two-dimensional gel electrophoresis

Immobiline Drystrip was hydrated in a slim tube with the gel-swelling solution overnight (Table 1). IPG-IEF was carried out at 20°C in a chamber of horizontal electrophoresis apparatus COOL PHOR Model 3600. Ten microliters of the cell extract was mixed with 1 μl of Daiichi 2D Marker solution when pI calibration was required. After removing the excess of the gel-swelling solution from the gel strip by gentle blotting with filterpaper, the strip was laid on the filterpaper gel-side-up. A sample application piece (Pharmacia, Uppsala, Sweden) was put on the gel 1-cm inside from the cathodic end. Ten microliters of sample solution was applied to the sample application piece, and the gel strip was placed on each lane of the gel supporting plate in the electrophoresis chamber. Electrode pads wetted with pure water were put on both ends of the gel strips. A couple of platinum electrodes were made contacted to the pads. All of the gel strips and electrode pads were covered with silicon oil to be isolated from atmospheric CO_2. The chamber was chilled at 20°C by circulation of coolant, and isoelectric focusing was carried out under increasing constant voltage in seven steps, i. e. 500 V for 180 min, 700 V for 60 min, 1,000 V for 60 min, 1,500 V for 60 min, 2,000 V for 60 min, 2,500 V for 60 min and 3,000 V for 720 min.

SDS-treatment of gel strip and performance of SDS-PAGE

After removing silicon oil by brief rinsing in pure water, the gel strip was equilibrated to the SDS-treatment solution (Table 2) for 40 min at room temperature with gentle shaking. If SDS-PAGE is not performed in the same day, the gel strip was wrapped in Saran Wrap and stored in a deep freezer. Prior to proceed to the second-dimensional SDS-PAGE, the Immobiline gel strip was thawed and incubated in the SDS-treatment solution for 5 min at room temperature again. The top space of the gel slab was filled with the cathodic electrode solution. The gel strip was put on the top of a gel slab, taking care not to trap air bubbles between the gel slab and the gel strip. The strip was gently pressed with a shark-tooth-shaped plastic comb. Molecular mass marker proteins absorbed in small pieces of cellulose acetate membrane were put beside the gel strip on the gel slab, if necessary for Mr calibration. SDS-PAGE was carried out under a constant current (5 mA per gel) until the dye front of BPB came near to the gel bottom.

Protein staining and 2-D densitometry

Protein on the 2-D gel slab was visualized by silver staining using Silver Stain Kit of Wako (Osaka, Japan), which showed the highest linearity in spot quantification in comparison with other kits

in preliminary experiments. Most of other 2-D database are prepared on autoradiogram of isotope-labeled protein pattern, however post-translational modification cannot be detected in newly synthesized protein spots. Thus I used silver staining for spot detection, because post-translational modification of protein may be included in aging mechanism. The 2-D gel image was acquired through a CCD scanner into a hard disk of SUN SPARC Station installed with PDQUEST 2-D image processing software. Protein spots were detected and quantitated automatically. Each corresponding spots on different gels were matched for the following quantitative comparison of polypeptides whose relative abundance in aged fibroblasts may differ from that in young fibroblasts.

RESULTS

Quantitative reproducibility of 2-D gel pattern

To assess the quantitative reproducibility of protein spots, whole procedure of the standard protocol was performs in duplicate. IOD of corresponding spots on a couple of 2-D gel images were compared by PDQUEST software and the quantitative re-

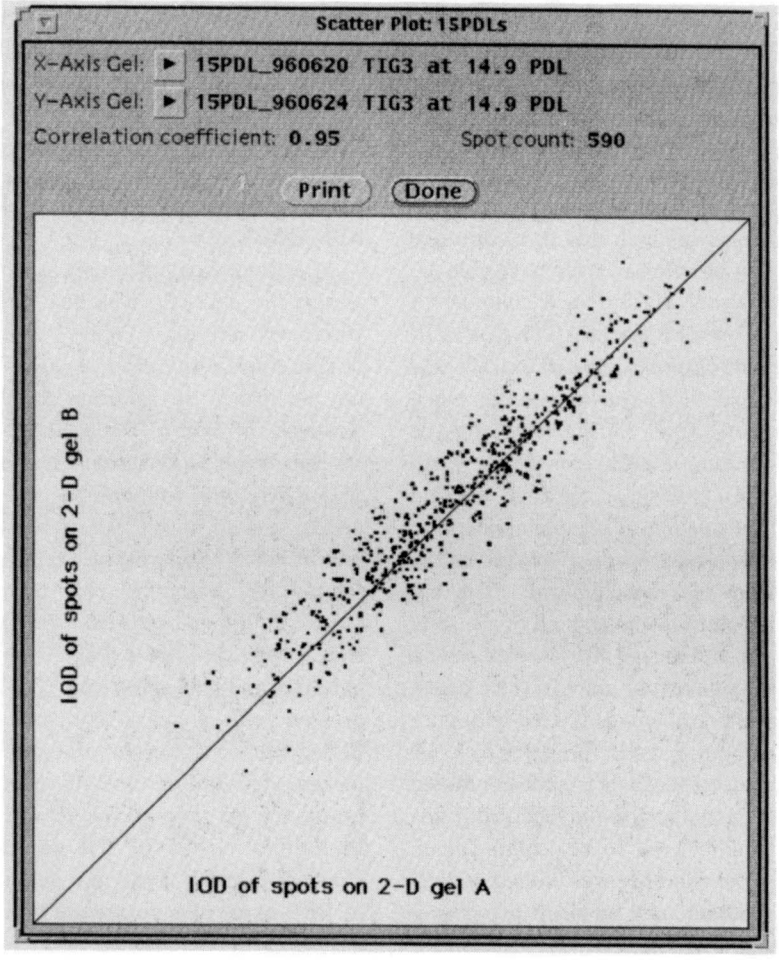

Fig. 1. Scatter plot of IOD of corresponding spots on a couple of 2-D gel patterns.

The whole procedure from sample preparation through 2-D densitometry was performed according to the standard protocol described in METHODS. Each dot on the plot indicates corresponding IODs obtained in matched spots on 2-D gel A and B.

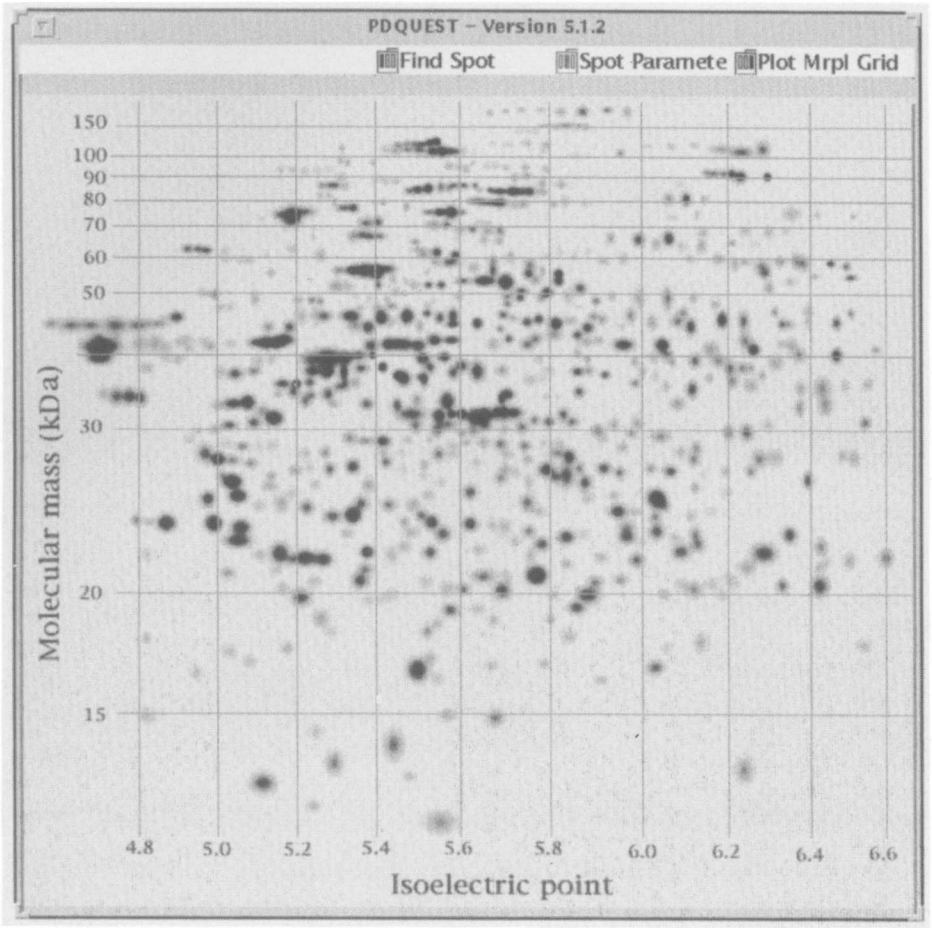

Fig. 2. Master 2-D gel protein pattern of human diploid fibroblasts.

The master image was prepared by mixing 2-D gel protein spots of fetal lung and adult skin fibroblasts using PDQUEST software. SSP numbers were automatically assigned to each corresponding spots by the software. Numerical data of each spot were saved in the EXCEL format. The Mr-pI grid was generated by giving satndard values of marker proteins using "Enter MrpI Data" operation of PDQUEST software.

producibility was confirmed in the scatterplot (Fig. 1). The result indicates that the quantitative reproducibility of 2-D gel pattern is satisfactory.

Master 2-D gel pattern of human diploid fibroblast protein

The silver-stained 2-D gel protein patterns of adult skin fibroblasts and fetal lung fibroblast were very similar but not the same (data is not shown). Therefore the master 2-D gel image of human diploid fibroblast protein for constructing database was prepared by merging skin fibroblast-specific spots to the normalized 2-D gel image of lung fibroblasts by "High-Level Matchset" operation of PDQUEST software (Fig. 2). The Mr-pI grid was automatically generated by giving standard values of marker proteins in the software.

TMIG 2D-PAGE protein database on WWW home page

The 2D-PAGE protein database was prepared on a SUN SPARC Station-20 installed with NCSA httpd software. The master gel pattern and information of each protein spot are now open to access on my home page in our WWW server at the URL address "http://www.tmig.or.jp/2D/2D_Home.

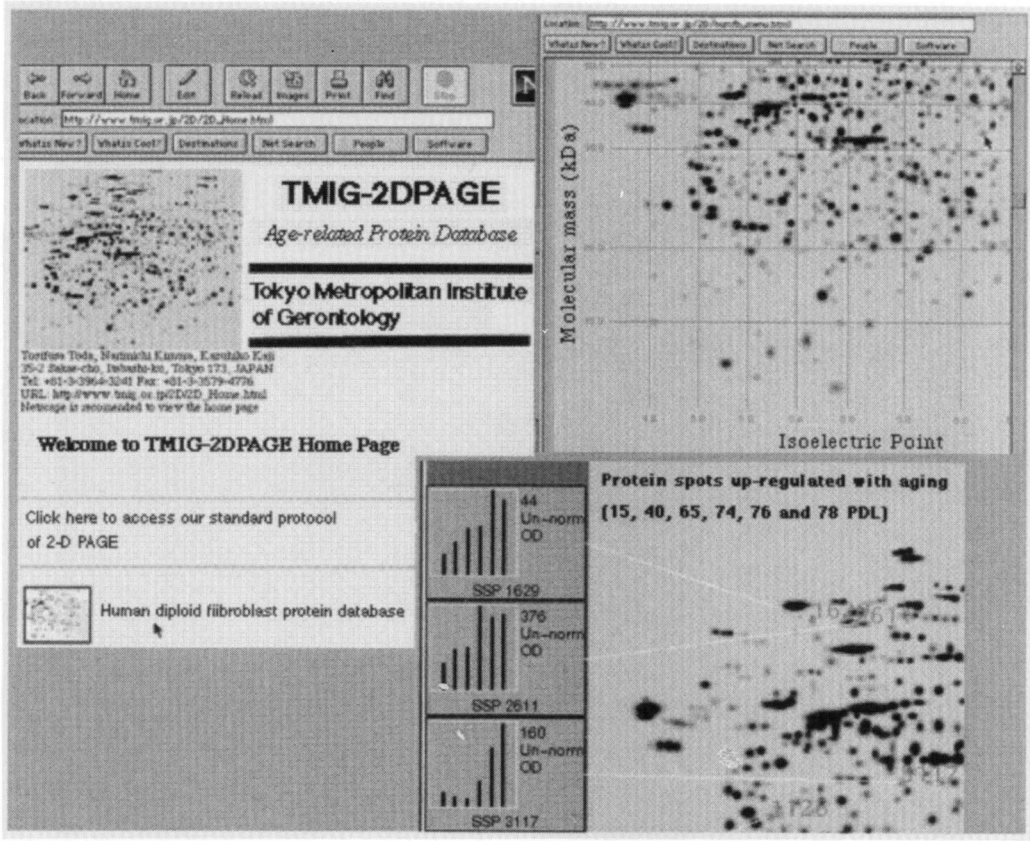

Fig. 3. View of TMIG-2DPAGE protein database on my home page in our World Wide Web server.
The database is accessible using a web viewer such as Netscape Navigator or NCSA Mosaic at the URL address: http://www.tmig.or.jp/2D/2D_Home from Macintosh or Windows 95-compatible computer connected to the Internet.

html" in the Internet computer network (Fig. 3).

DISCUSSION

Celis et al.[5] have already constructed their 2-D PAGE protein database of human diploid fibroblast upon the original method of 2-D PAGE reported by O'Farrell[6] using a mixture of carrier-ampholytes, pH 5-7 and 3.5-10 (IEF) or pH 7-9 and 8-9 (NEPHGE) in controlled proportions to reproduce pH gradient. But in fact, the quality control of the mixture of carrier-ampholytes is not so easy for me to keep the reproducibility. Furthermore, they did not described the procedure of sample preparation for silver staining. We tried to establish my standard protocol from sample preparation through spot quantification to reproduce the high-resolution 2-D gel pattern as described in METHODS. Since crystal of urea appeared during scraping when the cell harvest was performed using the extraction buffer containing both SDS and urea, I decided to add urea after transferring the cell suspension to a microfuge tube. The improved method of protein extraction offered reproducible 2-D gel patterns. Utilization of the ready-made gel strip of immobilized pH gradient for IEF shot the trouble in reproduction of the same pH gradient on every gel. However the limited choice in pH range of Immobiline Dry Strip was another problem for database construction. The resolution of spots on the strip of narrow pH range (pH4-7) was very high, though polypeptides of basic pI were excluded from the pH range. Consequently, I decided to use the narrow-range Im-

mobiline Dry Strip in my standard protocol for establishing the main frame of protein database. Data of basic protein will be supplemented using another wide pH range (3.5-10 non-linear) Immobiline Dry Strip, since no strip of narrow range in basic pH is commercially available as of now. The master 2-D gel pattern of human diploid fibroblast protein, which is primarily consists of about 590 silver-stained spots with spot identification numbers, is open to supplement of immunochemically detected minor spots. Age-related alteration of cellular protein on the 2-D gel pattern was analyzed, and the resultant data were presented in the database on my home page in our WWW server. The age-related protein database is expected to offer new clues to the mystery of cellular aging mechanisms in the future.

REFERENCES

1) Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Exp Cell Res 1961; 25: 585-621.
2) Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature 1990; 345: 458-60.
3) Gianazza E, Dossi G, Celentano F, Righetti PG. Isoelectric focusing in immobilized pH gradients: generation and optimization of wide pH intervals with two-chamber mixers. J Biochem Biophys Meth 1983; 8: 109-33.
4) Westermeier R, Postel W, Weser J, Gorg A. High-resolution two-dimensional electrophoresis with isoelectric focusing in immobilized pH gradients. J Biochem Biophys Meth 1983; 8: 321-30.
5) Celis JE, Dejgaard K, Madsen P, Leffers H, Gesser B, Honore B, Rasmussen HH, Olsen E, Lauridsen JB, Ratz G, Mouritzen S, Basse B, Hellerup M, Celis A, Puype M, Van Damme J, Vandekerckhove J. The MRC-5 human embryonal lung fibroblast two-dimensional gel cellular protein database: Quantitative identification of polypeptides whose relative abundance differs between quiescent, proliferating and SV 40 transformed cells. Electrophoresis 1990; 11: 1072-113.
6) O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. J Biol Chem 1975; 250: 4007-21.