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Selective labeling of cell-surface proteins using CyDye DIGE Fluor minimal dyes

Key words: *cell-surface protein labeling ● Ettan DIGE ● CyDye DIGE Fluor minimal dyes ● cell-surface proteins*

Cell-surface proteins are relevant to almost all protein-protein interactions, for example as inducers of intra-cellular signaling, environment adaption, and drug treatment, and are often involved in disease pathogenesis and pathology (1). Therefore cell-surface proteins and receptors as potential drug targets are of great interest to the pharmaceutical industry (1).

Two-dimensional (2-D) electrophoresis is used extensively for visualization of biomarkers and targets. However, cell-surface proteins, partly due to their low abundance (1–2% of cellular proteins), can be difficult to detect in a 2-D gel without fractionation or some other type of enrichment. They are also often poorly represented in 2-D gels due to their hydrophobic nature and high molecular weight (2).

In this study, we present a new protocol using CyDye™ DIGE Fluor minimal dyes to visually enrich for and detect this important group of proteins. This protocol is rapid, simple to use, and all three CyDye DIGE Fluor minimal dyes (Cy™2, Cy3 and Cy5) can be used to label cell-surface proteins. These features allow for multiplexing according to 2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE) using Ettan™ DIGE technology and analysis of protein expression changes using DeCyder™ 2-D Differential Analysis Software. In this way, the level of cell-surface proteins can be studied in different disease states or when responding to different treatments. Small changes in abundance can be detected with high accuracy, and results are supported by defined statistical methods.

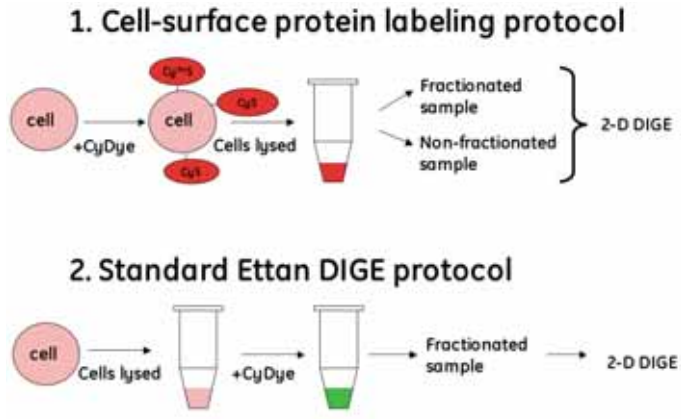

Fig 1. Overview of labeling workflow protocols

Materials

GE Healthcare products used

CyDye DIGE Fluor Cy2 minimal dye	RPK0272
CyDye DIGE Fluor Cy3 minimal dye	RPK0273
CyDye DIGE Fluor Cy5 minimal dye	RPK0275
2-D Quant Kit	80-6483-56
IGP Buffer pH 3–11NL	17-6004-40
Immobiline™ DryStrip pH 3–11NL, 24 cm	17-6003-77
Immobiline Reswelling Tray	80-6465-32
DeStreak™ Rehydration solution	17-6003-19
Immobiline DryStrip Cover Fluid	17-1335-01
Ettan IPGphor™ II IEF Unit	80-6505-03

Ettan IPGphor Manifold	80-6498-38
Ettan DALT*twelve* Gel Caster	80-6467-22
Ettan DALT*twelve* Large Vertical System	80-6466-27
Typhoon™ 9410 Variable Mode Imager	63-0038-56
DeCyder 2-D Differential Analysis Software Version 6.0	11-0010-95
ImageQuant™ Analysis Software	Inquire
Urea	17-1319-01
CHAPS	17-1314-01
Dithiothreitol (DTT)	17-1318-01
Bromophenol Blue	17-1329-01
Tris	17-1321-01
Sodium Dodecylsulfate (SDS)	17-1313-01
PlusOne™ Glycerol 87 %	17-1325-01
PlusOne ReadySol IEF 40% T/ 3% C	17-1310-01
PlusOne TEMED	17-1312-01
PlusOne Ammonium Persulfate	17-1311-01
PlusOne Glycine	17-1323-01

Other materials required

General cell culture equipment and suitable cell culture medium

Cell dissociation media, enzyme free PBS-based (Invitrogen)

2-D Sample Prep for Membrane proteins (Pierce)

Methods

Cell culture

Grow Chinese Hamster Ovary cells (CHO-K1) using standard cell culture procedures in F-12 Ham medium with GlutaMAX™ I containing 10% fetal calf serum, 50 U/ml penicillin, and 50 µg/ml streptomycin sulfate (Invitrogen). To perform an Ettan DIGE experiment, exchange the culture medium to a serum-free medium. Label the cell-surface proteins at different time points with CyDye DIGE Fluor Cy3 or Cy5 minimal dyes (see cell surface labeling section). Pool equal fractions from each time point and label with CyDye DIGE Fluor Cy2 and use as an internal standard for each 2-D gel.

The majority of the experiments were performed with CHO-K1 cells, but mouse embryo fibroblasts (3T3 L1) and mouse ascites lymphoma lymphoblasts (EL4) were also used. The two latter cell types were grown in DMEM medium with GlutaMAX II, but otherwise with identical conditions as for the CHO-K1 cells.

Cell surface labeling

Carefully detach adherent cells non-enzymatically, count cells and divide into aliquots of 5–10 × 10^6 cells. For cells growing in suspension, omit the detaching step. Centrifuge the cells at about 800 × g for 5 min and remove the supernatant containing the medium. Wash the pellets by resuspension in 1 ml ice cold Hank's Balanced Salt Solution (HBSS) pH 8.5 and centrifuge at 800 × g at 4 °C for 2 min. Remove the supernatant and resuspend the cell pellet in 200 µl ice cold labeling buffer (HBSS pH 8.5, 1 M urea). Label the intact cells with 600 pmol CyDye DIGE Fluor minimal dyes for 20 min on ice in the dark. Quench the reaction by adding 20 µl 10 mM lysine and incubate for 10 min. Wash the surface-labeled cells twice by resuspension in 500 µl HBSS pH 7.4 followed by centrifuging at 800 × g at 4 °C for 2 min.

For comparison with the standard Ettan DIGE protocol see Figure 1.

Cell lysis and fractionation

Lyse the surface-labeled cells in 150 µl cold lysis buffer directly (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, 5 mM magnesium acetate pH 8.5) and leave on ice for at least 1 h with occasional vortexing. Centrifuge the lysates at 12 000 × g at 4 °C for 10 min. Transfer the supernatant, which is the cell surface protein–labeled, non-fractionated sample (containing proteins from internal and cell surface membranes, as well as cytosol), to a new tube. Wash the pellets as above and then fractionate (using a membrane fractionation kit, Pierce) into membrane and cytosolic fractions prior to 2-D gel electrophoresis. The membrane fraction contains proteins from internal and cell-surface membranes. For whole-cell labeling (Fig 1, protocol 2), follow the standard Ettan DIGE procedure, and label the lysed cells before fractionation (3).

Protein quantitation

Quantitate the proteins from the different fractions using the 2-D Quant kit.

1-D and 2-D electrophoresis

Rehydrate Immobiline DryStrip gels, pH 3–11NL (24 cm) using Immobiline DryStrip Reswelling Tray, 24 cm in 450 µl DeStreak Rehydration solution (0.5% IPG Buffer) overnight. Apply the CyDye labeled samples onto Immobiline DryStrip gels by anodic cup loading in the Manifold and perform isoelectric focusing (IEF) using Ettan IPGphor II IEF System according to instructions (3). After IEF, equilibrate the strips in two steps and place on top of large (26 x 20 cm) 12.5% polyacrylamide gels (SDS-PAGE) and overlay with 0.5% agarose (in running buffer containing bromophenol blue). Perform 2-D electrophoresis using Ettan DALT*twelve* Large Vertical System at 5 W/gel for 30 min, and then at 15 W/gel until the dye front reaches the bottom of the gel.

Imaging and data analysis

Directly after completing 2-D electrophoresis, scan the gels using Typhoon 9410 Variable Mode Imager set for Cy2, Cy3, and Cy5 fluorescence. Compare spot maps from the membrane fractions, cytosolic fractions, and non-fractionated samples using DeCyder 2-D Differential Analysis Software (4).

Post-staining

After imaging, silver stain the gels according to standard procedure (5), or any post-stain such as Deep Purple™ Total Protein Stain.

Results and discussion

Protein concentration

An overview of the two labeling workflows is shown in Figure 1. Since the cells are still intact when labeled, according to the cell-surface protein labeling protocol, only the proteins on the cell surface are exposed to the dye (Fig 1). In the standard Ettan DIGE protocol, the cells are lysed first and proteins inside as well as outside the cell are labeled (Fig 1). The relative amount of dye to protein in the cell-surface protein labeling protocol is not known, since cell-surface proteins can not be specifically quantitated. However, it is known that cell-surface proteins constitute a very low proportion of the cellular proteins (2). Approximately 5–10 × 10^6 cells to 600 pmol of dye were used. It may be possible to use fewer cells since only 12.5–25% of the non-fractionated sample was used for 2-D electrophoresis. Protein concentrations in the different fractions were determined using the 2-D Quant Kit. The total protein amount derived from 10 × 10^6 CHO-K1 cells was 920 µg in the non-fractionated sample, 225 µg in the membrane/hydrophobic fraction and 770 µg in the cytosolic/hydrophilic fraction. These amounts will most likely vary depending on cell type and cell size. The proportion of proteins that are labeled in the cell-surface protocol is probably higher than the standard Ettan DIGE minimal labeling which is 2–3% of total protein.

It seems that only one dye is attached per protein molecule since the spot shape is rounded and there is no vertical streaking for the low molecular proteins on the gels (Fig 2 and 3). Two and more dye molecules per protein would cause vertical streaking due to increased molecular weight of the labeled protein. A range of molecular weight shifts would be seen on the gel depending on the number of additional dye molecules resulting in vertical streaking. This effect would best be seen for the low molecular weight proteins, since a change in molecular weight results in a larger shift on the gel compared to high molecular weight proteins.

Cell-surface protein specific labeling

Two identical samples of cells were surface labeled with CyDye DIGE Fluor Cy3. One was lysed and used directly for 2-D electrophoresis. The other sample was lysed and fractionated into membrane and cytosolic fractions. All the

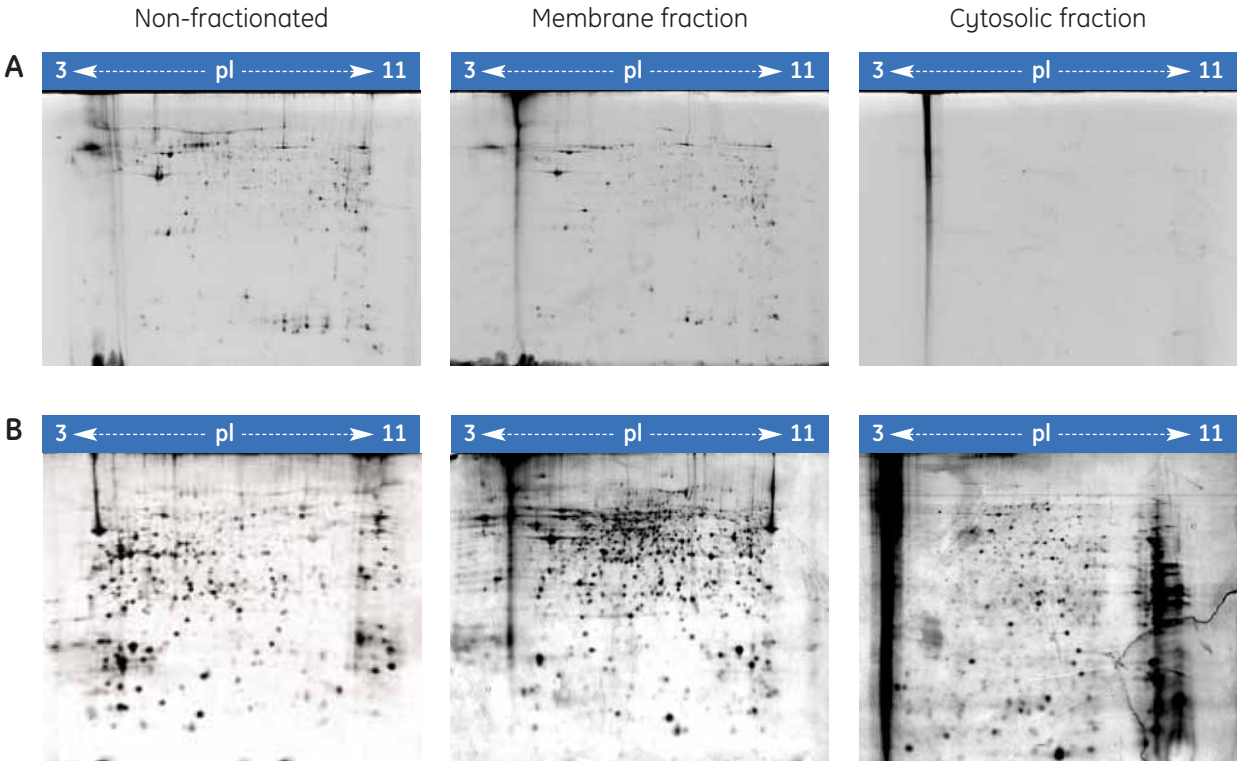

Fig 2. Specificity of cell-surface protein labeling. The cell-surface proteins of CHO-K1 cells were labeled with Cy3 and fractionated. The different fractions were separated by 2-D electrophoresis and scanned for Cy3 fluorescence (A). The same gels were then silver stained (B).

label appeared in the membrane fraction; the cytosolic fraction was devoid of any labeled proteins (Fig 2). The same gel was silver stained and the result showed that there were proteins in the gel, but they were not labeled using the cell-surface protein labeling protocol. These results suggest that this new labeling protocol is cell-surface protein specific. The CyDye DIGE Fluor minimal dye does not appear to enter the cell or pass through the cell membrane. The cells are kept on ice prior to the labeling and this may reduce any active transport across the membrane. The time for CyDye DIGE Fluor minimal dye exposure is also relatively short (20 min). Another probable explanation for the lack of labeling inside the cell is that even if the dye passes across the membrane, the pH inside the cell is too low (< pH 7.4) for an efficient labeling reaction to occur (optimal pH 8.5). The labeling reaction is quenched followed by washing of the cells, which further prevents any protein labeling after the cells have been lysed.

Fractionation

There are only minor differences in the spot pattern for the membrane fractionated sample compared with the non-fractionated sample (Fig 2). The two spot maps were compared using DeCyder 2-D Differential Analysis Software and all the spots detected in the membrane fraction were also present in the non-fractionated sample. Fractionation, therefore, is not necessary to improve detection of cell-surface proteins but can be used to verify lack of labeling of proteins inside the cells.

Comparison between protocols

To be able to evaluate the advantages with the new cell-surface protein labeling protocol, a comparison with the standard Ettan DIGE protocol was performed. Two identical samples from CHO-K1 cells grown in the same flask were labeled in parallel with the two different protocols, respectively (Fig 1). A cell-surface Cy5 labeled sample was run on the same gel as a Cy3 labeled cell lysate. The green spots on the gel (Fig 3A) represent the proteins labeled using the standard Ettan DIGE procedure followed by membrane fractionation. These spots are presumably membrane proteins including cell-surface proteins as well as proteins from membranes inside the cell (ER, Golgi, mitochondrion, and nucleus). Standard Ettan DIGE labeling procedure followed by a membrane fractionation step was chosen for comparison, since it should give the highest probability for detecting the low-abundant cell-surface proteins. The red spots on the gel (Fig 3A) are cell-surface specific proteins labeled according to the new cell-surface protocol that are not visible with the standard labeling procedure (green spots). Furthermore, the yellow spots represent overlapping proteins that occur in both samples using either procedure (Fig 3A).

A comparison of these results using DeCyder 2-D software, revealed over 80 novel proteins spots present only in the cell-surface protein labeled fraction (Fig 3B). The cell surface protein labeling protocol improved the detection of many proteins when compared with the standard method. Cy5 cell-surface labeled samples and Cy3 labeled lysates from 3T3 fibroblasts and EL4 lymphoblasts (growing in suspension) were also compared using DeCyder 2-D software. Similar results (50 to 100 new spots) were obtained as for the CHO-K1 cells (data not shown).

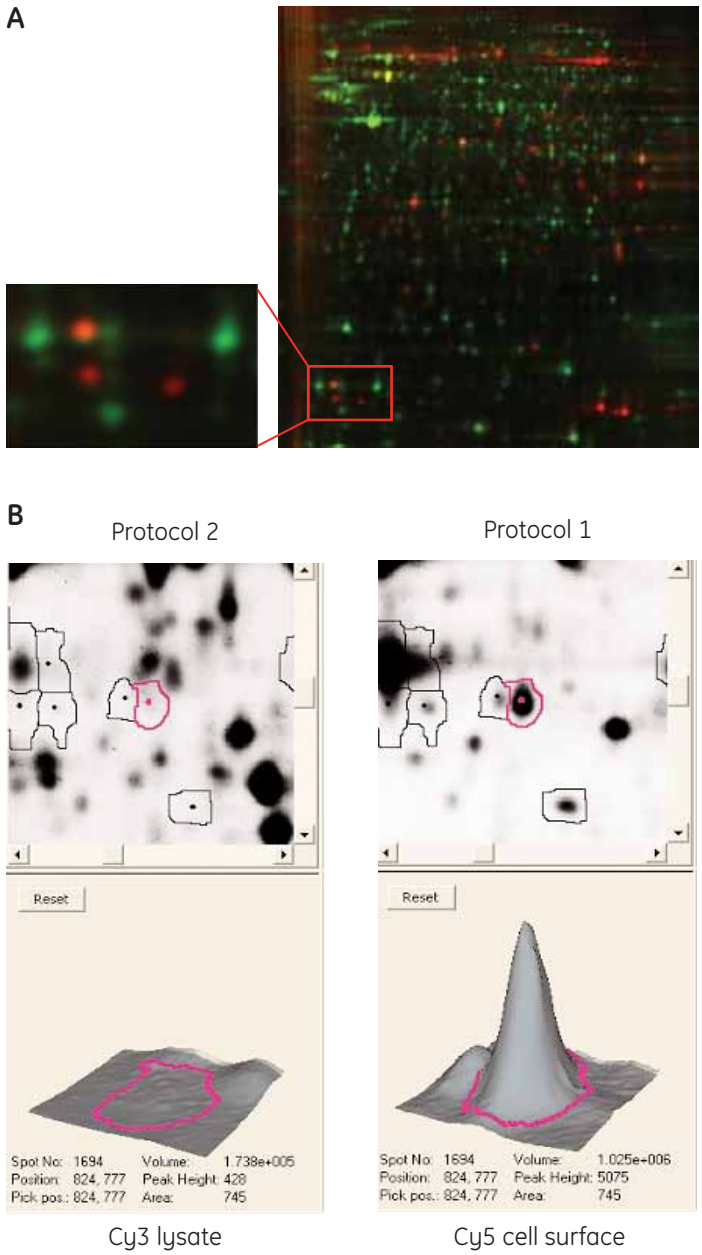

Fig 3. (A) 2-D gel images of a CHO-K1 Cy5 cell-surface labeled sample (red spots, see protocol 1, Fig 1) and a membrane fractionated Cy3 sample (green spots, see protocol 2, Fig 1) labeled according to standard Ettan DIGE protocol run in the same 2-D gel. (B) DeCyder 2-D Differential Analysis Software views from the 2-D gel showing a cell-surface labeled protein not visible using the standard Ettan DIGE protocol.

Multiplexing

To test the cell-surface protein labeling protocol in an Ettan DIGE experiment using all three dyes (6), a series of samples from serum depleted CHO-K1 cells were collected and cell-surface proteins labeled at different time points (Table 1). Samples were separated by 2-D electrophoresis. All three CyDye DIGE Fluor minimal dyes labeled cell-surface proteins similarly (data not shown). Changes in expression during serum starvation for many of cell-surface proteins were detected using DeCyder 2-D software (Fig 4).

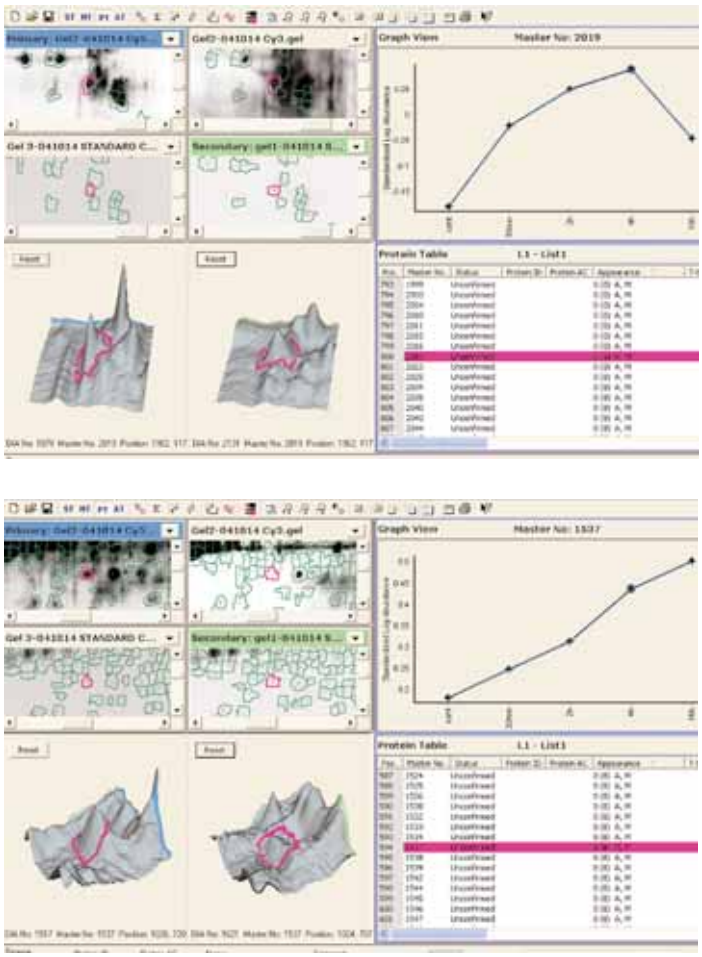

Fig 4. Change in expression of two cell-surface proteins during starvation of CHO-K1 cells. Spot maps were analyzed using DeCyder 2-D Differential Analysis software.

Table 1. An Ettan DIGE experiment was performed using samples from serum depleted cells labeled according to the cell-surface protein labeling protocol in figure 1.

Sample	Time of serum depletion	Labeled with CyDye	Gel number
1	-	Cy3, Cy2	1
2	30 min	Cy5, Cy2	1
3	2 h	Cy3, Cy2	2
4	4 h	Cy5, Cy2	2
5	16 h	Cy5, Cy2	3

Conclusions

The new Ettan DIGE protocol for cell-surface protein labeling is rapid, simple to use and a specific method for labeling cell-surface proteins. Many novel cell-surface proteins are only visible using the cell-surface protein labeling protocol. Over 80 new cell-surface proteins for CHO-K1 cells were detected using DeCyder 2-D Differential Analysis Software. Multiplexing is achieved using the three CyDye DIGE Fluor minimal dyes, and in combination with DeCyder 2-D software, this new protocol is a powerful tool for studying cell-surface proteins.

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