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Difference gel electrophoresis: A single gel method for detecting changes in protein extracts

We describe a modification of two-dimensional (2-D) polyacrylamide gel electrophoresis that requires only a single gel to reproducibly detect differences between two protein samples. This was accomplished by fluorescently tagging the two samples with two different dyes, running them on the same 2-D gel, post-run fluorescence imaging of the gel into two images, and superimposing the images. The amine reactive dyes were designed to insure that proteins common to both samples have the same relative mobility regardless of the dye used to tag them. Thus, this technique, called difference gel electrophoresis (DIGE), circumvents the need to compare several 2-D gels. DIGE is reproducible, sensitive, and can detect an exogenous difference between two *Drosophila* embryo extracts at nanogram levels. Moreover, an inducible protein from *E. coli* was detected after 15 min of induction and identified using DIGE preparatively.

1 Introduction

Two-dimensional polyacrylamide gel electrophoresis (2-DE) has become the primary tool for analysis of complex protein mixtures due to its high resolution and sensitivity. Since its introduction in 1975 [1], there have been many modifications of the technique (reviewed in [2,4]. Nevertheless, the basic premise has remained the same: 2-DE separates a complex protein mixture into its components first by isoelectric focusing (IEF), which separates proteins according to their p*I*, and then by SDS/Tris polyacrylamide gel electrophoresis (SDS-PAGE), which separates according to size. Detection is generally by post-run Coomassie blue or silver staining or by prerun radiolabeling of proteins followed by post-run fluorography or autoradiography. 2-DE provides a global view of the state of the proteins from a sample, since thousands of proteins can be visualized at once. Changes in the protein composition of cell extracts from many organisms and cell types, for example, protein differences between nerve and glial cells [5], and different *Drosophila* imaginal discs [6], have been examined using 2-DE. Also, efforts are underway to establish protein maps of several organisms and cell lines; most of these 2-D gel databases are accessible on the World Wide Web*. Identification of the isolated protein(s) by integration of 2-DE-based protein detection techniques with mass spectrometry-based database searches, together with the exponentially increasing sequence information available in the databases due to the various genome projects, promises to allow 2-DE to become an even more powerful discovery tool. The ultimate aim of these efforts is to find out how events such as cell transformation, development, and differentiation impinge on aggregate protein expression patterns, and to identify the affected proteins.

To detect protein differences between two samples, conventional 2-DE methodology relies on the comparison of at least two different gels. Unfortunately, no two gels are identical due to inhomogeneities in the polyacrylamide gels, electric and pH fields, and thermal fluctuations. Reproducible spot matching requires immaculate technique and spot-matching software. Even with such attention to detail, exact matching of spots can still be a difficult task. Thus, it is often necessary to run numerous 2-D gels to analyze one sample. The number of gels that need to be run is increased when one additionally desires to obtain sufficient material for sequence analysis from the different proteins that have been discovered.

We have developed a modified 2-DE technique, called difference gel electrophoresis (DIGE), that circumvents these problems. Two protein samples are prelabeled with two cyanine dyes (Fig. 1), thus enabling one to run two different samples on the same gel in both dimensions. Hence, the two samples are subjected to the same procedure and environment throughout the experiment. Protein spots can be detected by fluorescence imaging immediately after electrophoresis with a sensitivity equal to silver staining. Differences in protein composition between the two samples due to differences in gene expression or protein modification can be identified quickly without any post-processing of the gel. As an adjunct to the analytical detection scheme described above, we have also developed a preparative method that requires a small number of preparative steps to obtain quantities of protein necessary for partial sequence determination by taking advantage of the fact that the protein is fluorescently labeled. We report initial experiments determining the limit of protein detection with labeled bovine serum albumin (BSA) in *Drosophila* extract, and the identification, isolation, and microsequencing of an inducible, cloned protein from *E. coli* extracts.

2 Materials and methods

2.1 Synthesis of the dyes

Cy3 and Cy5 were synthesized using a modification of the previously described protocol [7]: (i) Indole deriva-

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Nonstandard abbreviations: DIGE, difference gel electrophoresis; **IPTG,** isopropylthiogalacto pyranoside

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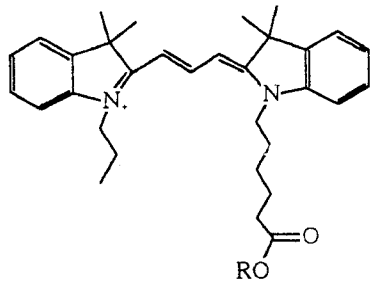
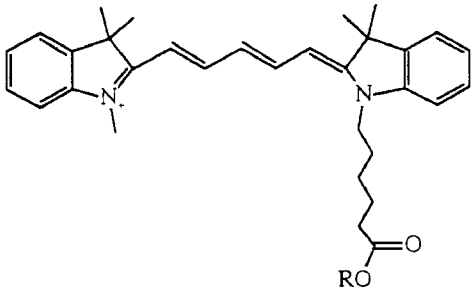
* A list of known 2-D gel databases is available at the following URL: http://www-lmmb.ncifcrf.gov/EP/EPemail.html

0173-0835/97/1111-2071 $17.50+.50/0



Propyl-Cy3, Absorption(max): 550 nm



Methyl-Cy5, Absorption(max): 650 nm.

R = N-hydroxy succinimidyl ester

Figure 1. The structures of the cyanine dyes used in DIGE.

tive synthesis: 4.8 g (30 mmoles) of 2,3,3, trimethyl-(3H)-indole (Kodak) and 35 mmoles of the desired bromoalkyl reagent (6-bromohexanoic acid or 1-bromopropane; Kodak) in 40 mL of 1,2-dichlorobenzene were heated to 110°C under N_2 and refluxed with stirring overnight. The product of the reaction with 6-bromohexanoic acid or 1-bromopropane will be referred to as acid indole and propyl indole, respectively. (ii) Cy5 linker synthesis: A solution containing 280 mL double distilled H_2O, 21 mL HCl, and 14.8 mL of aniline was added dropwise to a solution containing 342 mL double distilled H_2O, 17 mL HCl, and 21 mL malonaldehyde bis(dimethyl) acetal (Aldrich, St. Louis, MO) while stirring at 50°C. The precipitate, malonaldehyde dianil, was isolated by filtration. (iii) Cy3 intermediate synthesis: 1.5 g (7.5 mmoles) of propyl indole (from a) was added to 1.6 g (7.6 mmoles) of *N,N'* diphenyl formamidine (Aldrich) in 20 mL of glacial acetic acid and refluxed for 4 h. (iv) Cy5 intermediate synthesis: This was the same as above except than 2-methylene-1,3,3,-trimethyl indolene (Aldrich) was used instead of propyl indole, and malonaldehyde dianil, the product from (ii) was used instead of *N,N'* diphenyl formamidine. (v) Synthesis of propyl Cy3: 5 mL of triethylamine and 1.8 mL of anhydrous Ac_2O were added to the intermediate from step (iii) and refluxed for 5 min; 1.70 g (5.0 mmoles) of acid indole (from i) was then added and the mixture was refluxed for 2 h. The solvent was removed under vacuum and the products were dissolved in 10 mL CH_2Cl_2. (vi) Synthesis of methyl Cy5: This step is the same as above, except that the intermediate from (iv) was used instead of the intermediate from step (iii).

2.2 Purification

The unsymmetrical forms of the dyes (propyl Cy3 and methyl Cy5, hereafter referred to as Cy3 and Cy5, respectively (see Fig. 1), were isolated from the symmetrical side products by flash chromatography on silica. The mobile phase was 40% MeOH in CH_2Cl_2. The final product was contaminated by the dialkyl form of the dye, which represented less than 40% of total dye. Since the dialkyl form of the dye has no carboxylic acid, it could not be covalently linked to lysine residues of proteins and thus had no effect on the labeling.

2.3 Active ester formation

The carboxylic acid moiety was converted into an *N*-hydroxysuccinimidyl ester (OSu) as described previously [8]. A quantity of purified dye, 1.5 equivalents of *N,N'* disuccinimidyl carbonate (Aldrich), and 0.1 mL dry pyridine per 100 mg dye was dissolved in 5 mL of dry dimethylformamide (DMF). The reaction was carried out under N_2 with refluxing at 60°C for 90 min. Cy3-OSu and Cy5-OSu were then stored in 200 µL aliquots at −80°C.

2.4 Protein sample preparation for IEF

Two cultures of *E. coli* were grown in parallel. To one, 1 mM isopropylthiogalacto-pyranoside (IPTG) was added; samples were taken from both cultures after 15 min. All subsequent steps were performed at 4°C. The bacteria were isolated by centrifugation, resuspended in minimal sonication buffer (5 mM HEPES-NaOH, 5 mM Mg $(OAc)_2$, 50 µg/mL RNase, pH 8.0), and sonicated. DNase was added to 5 µg/mL and the solution was incubated on ice for 30 min before adding urea and CHAPS to give final concentrations of 2.5% CHAPS, 8 M urea. *Drosophila melanogaster* embryos were collected for 10 h on agar plates. They were then dechorionated before homogenizing directly in lysis buffer (8 M urea, 2.5% CHAPS, 5 mM HEPES-NaOH, 5 mM DTT, pH 8.0).

2.5 Labeling proteins, determination of protein concentration and dye-to-protein ratio

Since commercially available methods of measuring protein concentration were not reproducible in the buffer system employed, we determined the protein concentration in our samples as follows: 2–200 ng of BSA (Fischer, Pittsburgh, PA) in lysis buffer was labeled to saturation with Cy3, and spotted on a silica High Performance TLC plate (Whatman). The unbound dye was separated from the labeled protein with 1:1 MeOH/CH_2Cl_2. Imaging (see below) the TLC plate and summing the fluorescence from each protein spot enabled the precise quantitation of the protein concentration. Plotting these values gave a linear standard curve that was then used to measure the amount of protein in samples of unknown protein concentration. The advantage of this technique was that it required a small amount of protein and gave excellent reproducibility despite having urea, detergent, and DTT in the protein sample buffer. For 2-DE, ~ 0.4 mmole dye was added per 25 µg of protein prior to

electrophoresis. After 30 min on ice, any unreacted dye was deactivated by adding 5 nmoles of 1,3-diamino-2-hydroxy-propane for 15 min. To determine dye-to-protein ratio, free dye was removed by overnight adsorption to SM-2 beads (Bio-Rad, Hercules, CA). After removal of the beads, protein concentration was measured by $A_{260/280}$ and dye concentration by A_{548} for Cy3 and A_{650} for Cy5. Additionally, 1 μg of purified lysozyme (Boehringer Mannheim, Indianapolis, IN) was labeled with either dye and directly subjected to electrospray ionization mass spectrometry (ESI-MS) using a Fisons (Beverly, MA) Quattro 2 triple quadrupole instrument at the University of Pittsburgh Biotechnology Center Mass Spectrometry Facility.

2.6 Analytical electrophoresis

IEF was carried out on 18 cm long pH 3–10 nonlinear precast Immobiline gels (Pharmacia, Piscataway, NJ) according to the manufacturer's instructions, except that the reswelling buffer contained 2.5% CHAPS, 8 M urea, 1% 3–10 Pharmalytes (Pharmacia), 2 mM acetic acid, and 5 mM DTT. IEF was also carried out on 16 cm long pH 4–9 nonlinear gel strips, poured as described [9]. Typical run conditions were initially 1000–1500 V for 1–2 h, followed by 10–16 h at 3500 V, for a total of 30–50 kVh per run; 10–15% gradient gels (16 × 24 cm) were used for SDS-PAGE.

2.7 Preparative electrophoresis

This was carried out as above, with the following differences: Once a difference protein had been identified using analytical electrophoresis, between five and ten IEF strips were loaded with Cy3-labeled sample containing the desired protein. After IEF, a small (usually 1–2 cm long) region containing the difference was cut from each strip. These fragments were run in tandem on one or two second-dimension gels. Immediately after the second-dimension run, gels were imaged wet and a slice containing only the spot of interest was excised without fixing or staining the sample. The slices were placed serially on a horizontal electrophoresis apparatus (Pharmacia) and electrophoresed into a rectangular, 7% stacking gel. The protein was prevented from leaving the gel by placing a dialysis membrane between the gel and the electrode wick. This enabled the protein in each slice to stack into one band under the anode. This band was then blotted onto a Trans-Blot PVDF membrane (Bio-Rad) and visualized by Coomassie stain prior to being microsequenced.

2.8 Gel imaging

The imager was constructed from a 30 × 20 × 25 cabinet (IKEA) with the interior painted flat black to minimize background scatter. Two 100 W halogen lamps (Oriel), mounted on top of the cabinet at ± 60° incident angles to the bottom, were used for illumination. Each lamp was fitted with 2.5 cm diameter bandpass filters (Chroma Technology, Brattleboro, VT), housed in a manual filter wheel: 545 ± 10 nm and 635 ± 15 nm for Cy3 and Cy5, respectively. A scientific-grade, cooled, charge-coupled device (CCD) camera (Photometrics model CH250, Tucson, AZ), fitted with a double-wavelength bandpass emission filter (Chroma Technology), 587.5 ± 17.5 nm and 695 ± 30 nm, was used for fluorescent imaging. Gels were placed flat on a black plexiglass surface at the bottom of the cabinet and were placed under destain solution (10% acetic acid, 40% MeOH in distilled H_2O) to minimize dust accumulation. In a typical experiment, two sequential images of the gel were acquired, changing only the excitation filters between the acquisitions. CCD exposure time varied from 1 to 30 min, depending on the experiment.

2.9 Image processing

Image processing was performed on either a UNIX workstation Personal Iris 4D/35 or Indy (Silicon Graphics, Inc., Mountain View, CA) or a PowerMac 8500/120 (Apple, Cupertine, CA). A number of software packages were used to process the images; these were: Delta Vision (Applied Precision, Issaquah, WA) and additional custom-written software on the UNIX workstations, IP Lab Spectrum (Signal Analytics, Vienna, VA) on the Apple computer. The following operations were performed with a combination of these programs. Each image was made up of a 256 × 256 (binned) array of pixels, with each pixel value indicating the fluorescence intensity at that position in the image field (binning refers to a procedure for summing the values of a two-by-two pixel area into a single pixel value). Each pixel represented a 0.4 mm^2 area of the gel. To detect differences between the two samples, the images were subjected to the following manipulations: First, each image was processed with a local background subtracting program. Then, each pixel intensity in one image was divided by the corresponding pixel intensity in the other image. Since samples could not be balanced perfectly for overall fluorescence *a priori*, the dimmer of the images was multiplied by a balancing constant that was determined empirically such that the mean pixel intensity after the division was close to one. The resultant ratio image represented the differences between the two samples. Alternatively, two images, each corresponding to one of the protein samples, were placed into a movie file. Viewing this two-frame movie played in a continuous loop allowed for the visual detection of even minute differences.

2.10 Microsequencing

N-terminal and internal fragment microsequencing of GAL4VP16 by automated Edman degradation was carried out by Ariad Pharmaceuticals (Cambridge, MA) and also at the Memorial Sloan Kettering Cancer Center microchemistry facility. The equipment used at Ariad Pharmaceuticals was an Applied Biosystems (Foster City, CA) Procise protein sequencer Model 494 with a Model 140C microgradient delivery system.

3 Results

3.1 Labeling proteins

The primary prerequisite for DIGE is that the two fluorescent dyes must not perturb the relative electropho-

retic mobility of proteins during 2-DE. This was accomplished by designing dyes which satisfied the following criteria: (i) The dyes must match the charge of the protein residues that they modified. (ii) The dyes must have similar M_r and charge. (iii) The dyes must possess distinctive fluorescence spectra in order to be discriminated. The dyes used in DIGE satisfied all these criteria (Fig. 1). However, it was also observed that, due to the hydrophobicity of the dyes, labeling could cause perturbations in the solubility of the proteins during electrophoresis. Proteins precipitated prior to entering the gel in a manner directly related to the extent of labeling (data not shown). This was probably caused by the replacement of primary amino groups with the hydrophobic cyanine dye. Labeling only 1–2% of all the lysines in a whole cell extract was found to be optimal as there was no detectable effect on protein solubility. ESI-MS was used to confirm the extent of labeling using labeled lysozyme. A single new peak at the expected higher M_r, which differed from the unlabeled peak by the M_r of a single dye molecule, was detected in the labeled samples with an abundance that was approximately 5% of the unlabeled peak for both Cy3 and Cy5. No other peaks were seen at higher M_r (data not shown).

3.2 Establishing the limit of detection of DIGE

The sensitivity of the imaging system was compared to silver staining using Cy3 labeled BSA (Fig. 2). The gel was first imaged for fluorescence, and then silver stained [10]. The limit of detection of the fluorescence imager

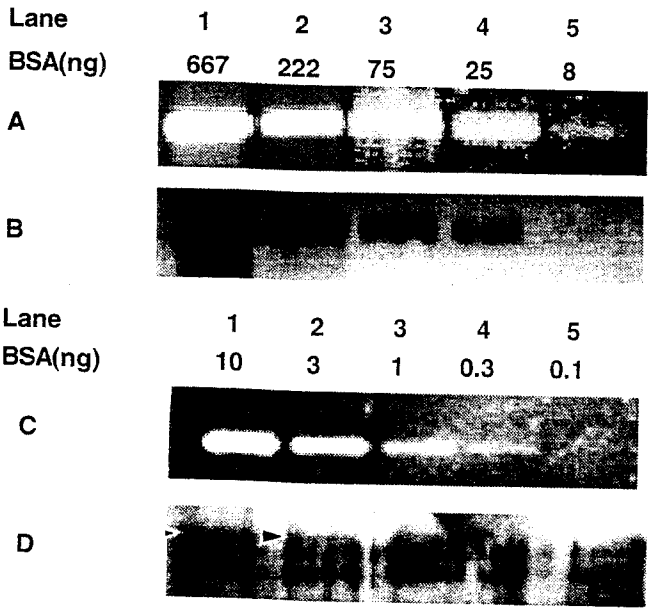


Figure 2. Comparison of fluorescence detection *versus* silver staining. (A) Serial dilutions of Cy3-labeled BSA were run on a 10% SDS-PAGE minigel and detected using fluorescence imaging. The first two lanes were exposed for 3 min, whereas lanes (3)–(5) were exposed for 15 min, since the first two lanes saturated the detector after 15 min of exposure. (B) Same gel as in (A), imaged after silver staining. (C) Same as (A), except less BSA was used and CCD exposure was lengthened to 30 min. (D) Same gel as in (C), imaged after silver staining. The arrowheads point to the BSA band. The doublet immediately underneath the BSA band is a commonly seen contaminant in the sample buffer that appears after "staining to maximum sensitivity" [12].

was better than that by silver staining by a factor of three. However, this required a 30 min exposure time. Silver staining using a different protocol gave similar results [11]. Fluorescent labeling had no effect on silver staining intensity (data not shown). In order to determine the sensitivity for detecting a single protein difference between two complex protein samples using DIGE, *Drosophila* embryo extract was "spiked" with BSA (Fig. 3). The BSA spiked extract was labeled with Cy3, and compared to the same amount of Cy5-labeled, unspiked extract. The ratio image of Cy3/Cy5 with a 0.1% w/v BSA spike indicated multiple spots at the expected position of BSA (Fig. 3C). In order to show that this difference was not an artifact of labeling with two different dyes, the experiment was repeated, with the BSA spiked sample labeled with Cy5 and the unspiked sample with Cy3. The same difference was detected in the ratio image of Cy5/Cy3. To determine the lower limit for detection, lower amounts of BSA were spiked into *Drosophila* extracts as above. Three levels of spike were used: 0.05% (w/w), 0.02% and 0.005%. The 0.05% and 0.02% spikes were clearly detectable (Fig. 3D-E), whereas 0.005% (Fig. 3F) was not. Fourteen ng of BSA was used in the 0.02% spike. Identical results were obtained with purified bovine carbonic anhydrase (Bio-Rad, data not shown).

3.3 Identification and microsequencing of an overexpressed protein

To demonstrate that a partial peptide sequence of differentially detected proteins can also be determined with DIGE, whole cell extracts from bacteria expressing GALaVP16 under *lac* control were prepared. Proteins from bacteria IPTG-induced for 15 min were compared to uninduced proteins using analytical DIGE (Fig. 4). In order to prove that the difference seen was indeed GAL4VP16, microgram quantities of protein was isolated using preparative DIGE. A ten amino acid long sequence was generated by *N*-terminal microsequencing; this protein was found to be 100% identical to the Gal4 sequence, positively identifying it as GAL4VP16. This result was repeated with a different protein preparation, processed at a different sequencing facility where two internal peptide fragments were microsequenced.

4 Discussion

DIGE was developed to facilitate a direct and reproducible comparison between mixtures of proteins. Its main advantage over current 2-DE techniques is the elimination of the need for detecting protein differences on separate gels. By running two samples on the same gel, an internal control is provided for the experiment, thus allowing for a faster and reproducible identification of differences in protein composition. Furthermore, once protein differences have been detected, DIGE also provides a means to scale up to preparative levels for microsequencing. We have detected a single protein difference between two protein samples at the low nanogram level (Fig. 3). This was accomplished by pre-labeling the two samples with two fluorescent dyes so that they could be differentiated from each other after 2-DE. Labeling pro-

teins with fluorescent molecules either prior to IEF [12] or between the IEF and SDS-PAGE steps [13, 14] is not a novel idea. However, previously, only a single fluorophore was used for nonspecific protein labeling. Furthermore, others have differentially labeled two samples, but using two different radionuclides [13]. The main diffi-

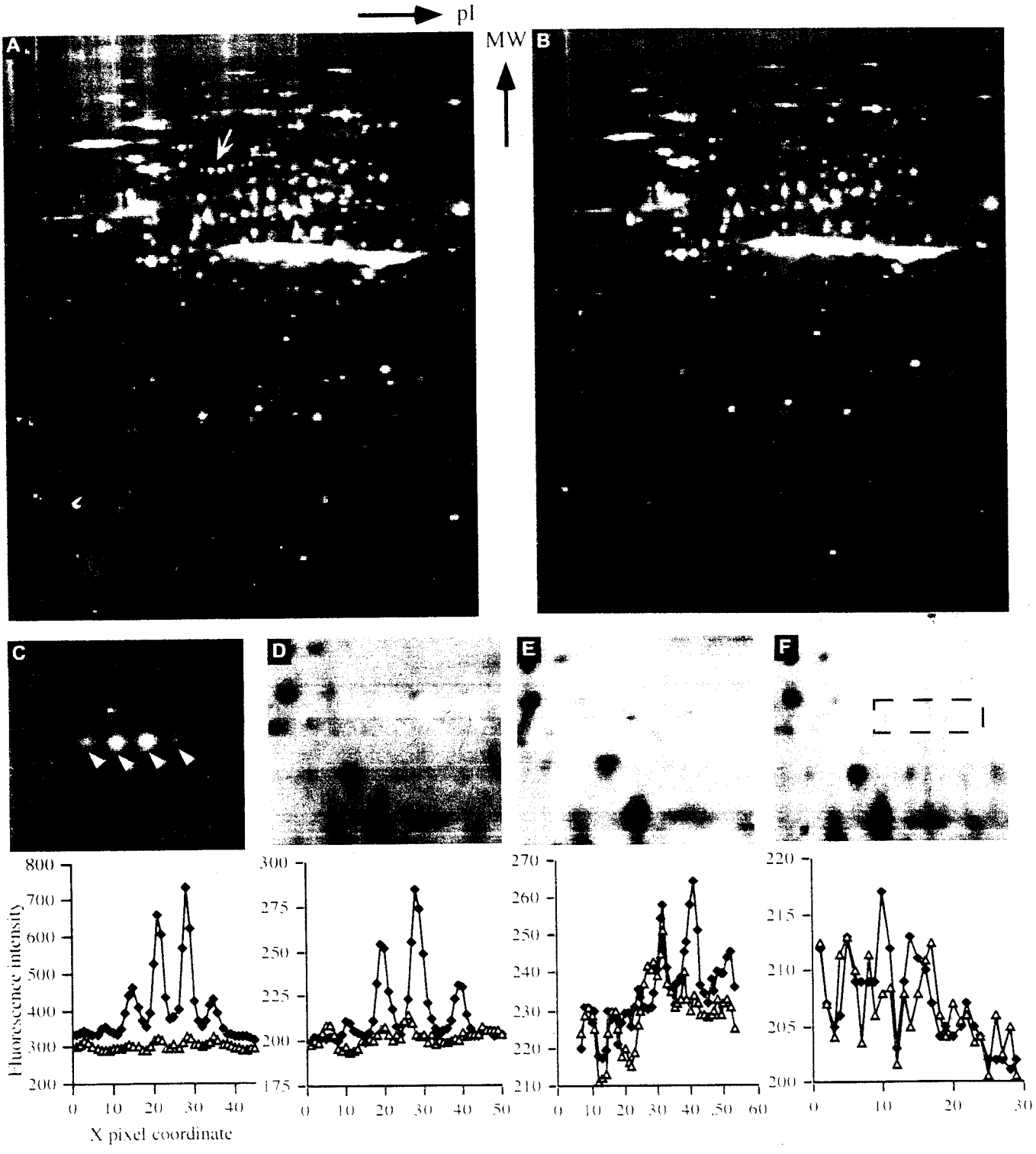


Figure 3. DIGE of BSA-spiked *Drosphila* embryo extract. (A) Image of *Drosphila* extract spiked with BSA to a final concentration of 0.1% w/w of total protein and labeled with Cy3. Axis arrows indicate the direction of increasing p*I* and M_r. The white arrow points to the BSA spots. CCD-exposure time was 3 min. (B) Image of the unspiked extract, labeled with Cy5. (C) Top: ratio image (Cy3/Cy5) of the BSA region from the gel in (A) and (B). The arrowheads point to individual BSA spots. Bottom: line plot showing the fluorescence intensity from a row of pixels bisecting the horizontal center of the BSA spots in the Cy3 (—●—) and Cy5 (—△—) images. The values were first normalized to equalize total fluorescence intensity. (D)–(F) As in (C), but from ratio images of gels with a range of BSA spikes and CCD exposure times, as follows: (D) BSA, 0.05% w/w; CCD exposure, 10 min; (E) BSA spike, 0.02% w/w; CCD exposure, 25 min; (F) BSA spike, 0.005% w/w; CCD exposure, 30 min. The dashed rectangle encompasses the area in which the BSA spike can be detected in the other images; it was not detectable in this experiment. Notice that since BSA resolves into multiple spots in both (D) and (E), the limit of detection for a single spot is 0.01% w/w.

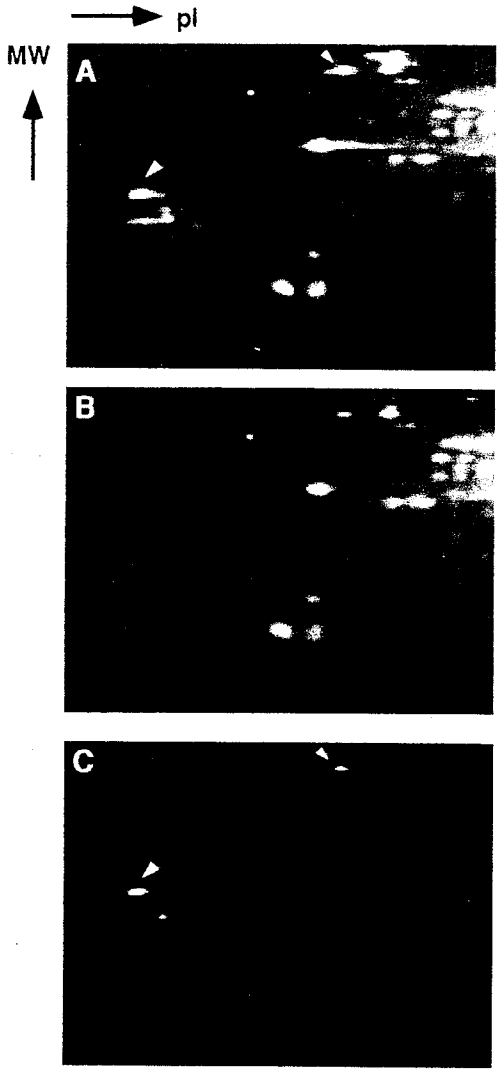


Figure 4. DIGE of induced GAL4VP16 protein. *E. coli* transformed with the chimeric protein GAL4VP16 [15] were induced for 15 min with IPTG. Protein extract from the induced culture was compared to proteins from uninduced bacteria using analytical DIGE. (A) The induced extract, labeled with Cy3. The large arrowhead indicates the GAL4VP16 protein. A smaller arrowhead indicates another difference, which may be induced β-glactosidase, given its M_r. (B) Uninduced extract labeled with Cy5. (C) The ratio of Cy3/Cy5. CCD exposure was for 3 min. Axis arrows point in the direction of increasing M_r and p*I*.

culty inherent in the latter approach is the fact that radioactivity was used. Our approach was to design a matched-pair of amine reactive fluorescent dyes (Fig. 1) for differential labeling. The cyanine dyes were designed with an inherent positive charge, which preserved the overall charge on the protein after dye coupling. By changing the linker length between the two indole moieties, dyes with sufficiently different fluorescent spectra were generated. The difference in M_r introduced by the different length linkers was compensated for by synthesizing unsymmetrical dyes with different length alkyl moieties opposite the linker moiety.

The initial approach was to label all lysines to completion with excess dye, since intermediate amounts of labeling could be expected to give rise to heterogeneities in electrophoretic mobility. However, carrying out the labeling reaction to completion was found to have deleterious effects on protein solubility. As an alternative approach, 1–2% of all the lysines were labeled, which resulted in minimal labeling where, on the average, a minority of proteins was modified with a single dye and the rest were unlabeled. Using ESI-MS, this was found to be the case for minimally labeled lysozyme. Minimal labeling still caused slight M_r differences between labeled and unlabeled protein, which was observed when fluorescent images were compared to silver-stained images. The effect had a different magnitude for each protein and ranged between no detectable difference in migration for some proteins to as much as one spot diameter for others, and in general was seen to be greater for lower M_r proteins. This M_r shift does not pose a problem for analytical DIGE where both sets of proteins are labeled to the same extent. For preparative DIGE, we compensated for the shift by cutting a 50% larger region of interest, biased to lower M_r.

The effect of the dyes on p*I* is more difficult to estimate because the ionizable positive charges of the primary amino groups are being replaced by the nonionizable quaternary amino group of the dye. Theoretically, this causes the pK_a of each labeled residue to change from ~9 to 14. However, at the range of pHs in an IEF gel (typically 3–10), this change should have a negligible effect. In fact, we were not able to detect any p*I* changes between either differently labeled or between labeled and unlabeled proteins. As a final test, *E. coli* extracts were labeled with BODIPY-FLIA™ (Molecular Probes, Eugene, OR), a sulfhydryl-reactive, uncharged fluorescent dye, and coelectrophoresed with the same sample labeled with Cy5. Comparison of the BODIPY pattern to the Cy5 pattern showed no p*I* differences (data not shown). However, there were a number of proteins that were shifted in the molecular weight dimension, indicating that labeling with BODIPY-FLIA caused some proteins to have an increased apparent M_r compared to the same proteins labeled with Cy5. These results show that preserving the charge on the modified amino acid residue maintains the p*I*, and that labeling to the same extent maintains the relative M_r mobility of a protein. This was shown to be the case in the BSA spiking experiment where all Cy3- and Cy5-labeled spots, aside from BSA, comigrated to the exact same position (Fig. 3).

The fluorescence imaging system is capable of detecting proteins at low levels – with a sensitivity at least equal to silver staining. One problem arose from the fact that in cell extracts protein concentrations differ over a 10000-fold range. This protein concentration range outstripped the CCD camera's dynamic range of ~4000. The signal from abundant proteins saturated the detector before the lower abundance proteins could be discerned above the background noise. One solution to this problem may be to use a scanning mechanism or a CCD camera with a higher dynamic range. In order to be an effective technique, DIGE should be able to detect and also identify differences in protein composition. We have also shown the preparative capability of the system by isolating and identifying a known difference protein (Fig. 4). This was accomplished by taking advantage of

the fact that the sample was already labeled, which minimized the number of steps, and therefore losses, involved in the preparative stage.

While many proteins are known to exist at lower concentrations in the cell than can be detected with DIGE, we imagine that future development of the technique will allow one to detect the very-low-abundance class of proteins. One area of potential improvement is dye design. The synthesis of more hydrophilic dyes may allow for the modification of a higher percentage of lysines. Improved dyes coupled to a detector with a greater dynamic range will lower the detection limit to a range that is closer to the level of very low abundance proteins. Development of MS-based database search techniques [14] will expedite the identification of difference protein sequences and thus will allow one to immediately obtain genetic sequence from an identified difference protein spot.

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5 References

[1] O'Farrell, P. H., *J. Biol. Chem.* 1975, *250*, 4007–4021.
[2] Chambrach, A., Dunn, M. J., Radola, B. J. (Eds.), *Advances in Electrophoresis*, VCH, New York 1987, Vol. 1.
[3] Jorgenson, J. W., Phillips, M. (Eds.), *New Directions in Electrophoretic Methods*, American Chemical Society, Washington DC 1987, Vol. 335.
[4] Anderson, N. G., Anderson, N. L., *Electrophoresis* 1996, *17*, 443–453.
[5] Garrels, J. I., *J. Biol. Chem.* 1979, *254*, 7961–7977.
[6] Santaren, J. F., Damme, J. V., Puype, M., Vandekerckhove, J., Garcia-Bellido, A., *Exp. Cell. Res.* 1993, *206*, 220–226.
[7] Mujumdar, R. B., Ernsst, L. A., Mujumdar, S. R., Waggoner, A. S., *Cytometry* 1989, *10*, 11–19.
[8] Ogura, H., Takanori, K., Keiko, S., Kazumasa, K., Kazuyoshi, T., *Tetrahedron Lett.* 1979, 4745–4746.
[9] Righetti, P. G., *Immobilized pH Gradients: Theory and Methodology*, Elsevier, Amsterdam 1990.
[10] Oakley, B. R., Kirsch, D. R., Morris, R. N., *Anal. Biochem.* 1980, *105*, 361–363.
[11] Morrissey, J. H., *Anal. Biochem.* 1981, *117*, 307–310.
[12] Urwin, V. E., Jackson, P., *Anal. Biochem.* 1993, *209*, 57–62.
[13] Goldman, C., Trus, L., Leive, L., *Eur. J. Biochem.* 1983, *131*, 473–480.
[14] Patterson, S. D., Aebersold, R., *Electrophoresis* 1995, *16*, 1791–1814.
[15] Chasman, D. I., Leatherwood, J., Carey, M., Ptashne, M., Kornberg, R. D., *Mol. Cell. Biol.* 1989, *9*, 4746–4749.