

Detection and mass spectrometry identification of protein changes in low-abundance tissue using CyDye DIGE Fluor saturation dyes

Key words:

Ettan DIGE system • CyDye DIGE Fluor saturation dyes • laser capture microdissection • mass spectrometry

Traditional two-dimensional electrophoresis (2-DE) is a well-established technique for protein analysis, but its use in identifying changes in protein abundance in tissue samples has been limited by system variability and low sensitivity. These limitations have largely precluded its use to identify protein differences in scarce materials such as those recovered from laser-capture microdissection (LCM).

This study demonstrates the application of 2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE) to detect changes in protein abundance in samples recovered from LCM. The analysis uses novel fluorescent dyes, CyDye™ DIGE Fluor saturation dyes, to label the proteins and provide the sensitivity required for quantitation of minute protein abundance differences. Combined with an internal standard and using Ettan™ DIGE system, proteins from the hippocampus of rats expressing human amyloid precursor protein (APP) were compared with those from normal animals. This work complements a more comprehensive study by Kondo *et al* (1).

Introduction

The variability between gels in traditional 2-DE makes it difficult to distinguish between system and induced biological change, which means that differences in protein expression can rarely be predicted with confidence. Moreover, the technique is not well-suited for the analysis of low-abundance proteins. Two-Dimensional Fluorescence Difference Gel Electrophoresis (2-D DIGE) offers significant benefits over the traditional method. In 2-D DIGE using Ettan DIGE system, samples are prelabelled with one of the CyDye DIGE fluors and can be multiplexed within the same gel along with an internal standard.

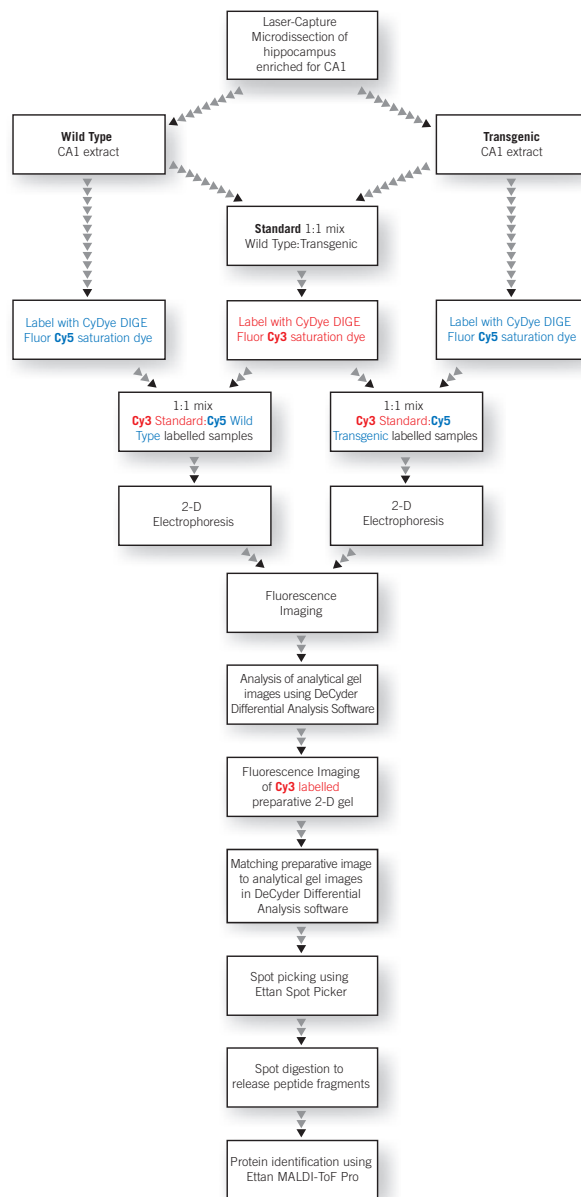


Fig 1. Schematic representation of Ettan DIGE system experimental workflow.

To demonstrate the application of CyDye DIGE Fluor saturation dyes to the proteomic study of scarce, low-abundance samples, we chose the study of the neurodegenerative disorder Alzheimer's disease (AD) as our model system. Based on normal and transgenic rats carrying the human APP gene, the system utilizes LCM to harvest a region from rat hippocampus enriched for the CA1 area for further analysis. Prior to electrophoresis, samples are differentially labelled with fluorescent, spectrally distinct CyDye DIGE Fluor saturation dyes then co-electrophoresed with an internal standard, in the same 2-D gel.

CyDye DIGE Fluor saturation dye molecules have molecular weights of approximately 680 and are matched in mass to give equivalent migration of the labelled proteins. The dyes react with all available cysteine residues in the protein sample, giving a high labelling concentration. Because they have a net zero charge, there is no charge alteration to the labelled protein due to the conjugation of the dye to cysteine thiol groups. CyDye DIGE Fluor saturation dyes enable visualization of scarce/limited amounts of sample (typically 5 µg of protein per labelling reaction), due to their high emission intensity and the sensitivity of the Typhoon™ 9400 Series Variable Mode Imager that is used for scanning the gels. CyDye DIGE Fluor saturation dyes are more sensitive than Coomassie™, silver, or Sypro™ dye staining methods, with a wider dynamic range.

The results highlight the suitability of this 2-D DIGE labelling technique for the analysis of extremely low amounts of complex samples and the interface to high-throughput protein identification employing MALDI-ToF mass spectrometry with database searching.

The Ettan design proteomics platform has also been extensively applied to the study of other model systems (2–4).

Products used

Amersham Biosciences products used:

CyDye DIGE Fluor Labelling Kit for Scarce Samples	25-8009-83
CyDye DIGE Fluor Labelling Kit for Scarce Samples and Preparative Gel Labelling	25-8009-84
Pharmalyte pH 3–10	17-0456-01
PlusOne Dithiothreitol (DTT)	17-1318-02
Immobiline DryStrip pH 4–7, 18 cm,	17-6002-46
Immobiline DryStrip Reswelling Tray	80-6465-32
DeStreak Reagent	17-6003-18
Ettan IPGphor Manifold	80-6498-38
PlusOne Bind-Silane	17-1330-01
Ettan IPGphor IEF System	80-6414-02
Ettan DALT <i>twelve</i> Large Vertical System	80-6466-27
DALT Gel Caster	80-6467-22
Low fluorescence glass plates with integral spacers for Ettan DALT	80-6475-58
Ettan DIGE Gel Alignment Guides for Ettan DALT	80-6496-10
Gel Orientation Guide	80-6496-67
Ettan Spot Picker	18-1145-28
Reference Markers	18-1143-34
Typhoon 9410 Variable Mode Imager	60-0055-81
DeCyder Differential Analysis Software, v5.0	18-1163-05
Ettan MALDI-ToF Pro	18-1142-33

Methods

Sample preparation

Nine male, wild type rats (Fischer F344 strain) and nine male transgenic rats of the same strain carrying a human APP 751 isoform containing the “Swedish” double mutation under the control of an HMGCoA reductase promoter were selected when weighing 250–300 g (approximately three months old). Entire brains were removed and frozen in the vapor phase of liquid nitrogen (5, 6). To prepare samples for analytical 2-D gels, brains were mounted onto aluminum chucks with 2% w/v carboxymethylcellulose, and 30 µm-thick tissue sections were cut at –12 °C using a Brights cryostat and thaw-mounted onto membrane slides (Leica,

UK). The sections were air-dried for 30 min and stored frozen at -70°C until use. Large tissue areas (approximately $280\,000\ \mu\text{m}^2$) surrounding the hippocampus CA1 region were selected and cut by microdissection using a Leica AS LMD (Leica, UK), multiple tissue areas being cut and pooled from each hippocampus (Fig 2). Tissue sections were not counter-stained prior to LCM. Cut sections were collected in a microcentrifuge tube lid (Eppendorf, Germany) placed under the slide. The lid contained $40\ \mu\text{l}$ of sample lysis buffer containing 30 mM Tris, pH 8.0, 5 mM magnesium acetate, 7 M urea, 2 M thiourea, and 4% (w/v) CHAPS to lyse the sample. Each sample in lysis buffer was transferred into the tube then placed on ice in an ultrasonic waterbath and sonicated on ice for $3 \times 30\ \text{s}$ intervals. Following sonication, the tubes were centrifuged using a benchtop microcentrifuge at $9000 \times g$ for 30 s prior to collection of the final supernatant. To prepare samples for preparative 2-DE, hippocampi, dissected free-hand, were homogenized in ice-cold lysis buffer prior to sonication, as described above. The protein concentration of each lysate was determined using a detergent-compatible protein assay (Protein Determination Reagent, USB).

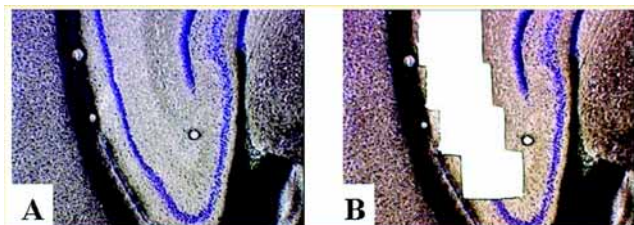


Fig 2. Images of hippocampal sections (A) before and (B) after LCM. Note that the samples used for the proteomic analysis were not counter-stained. The stained images are for illustrative purposes only.

Sample labelling

To derive statistically valid data on differences between the wild type and transgenic rats, equal amounts ($5\ \mu\text{g}$) of each wild type and transgenic protein sample were mixed and designated as the “pooled internal standard.” The standard sample was bulk labelled with CyDye DIGE Fluor CyTM3 saturation dye in sufficient quantity to include a standard on every gel. Proteins ($5\ \mu\text{g}$) from each wild type or transgenic rat were labelled separately with CyDye DIGE Fluor Cy5 saturation dye.

A $5\ \mu\text{g}$ aliquot of each rat hippocampus CA1-enriched protein lysate was reduced with 2 nmol of TCEP for 1 h at 37°C in the dark. Four nanomoles of each CyDye DIGE Fluor Cy5 saturation dye were dissolved in anhydrous dimethyl formamide and added to each sample (Fig 1). The labelling reaction was performed at 37°C in the dark for 30 min. Equal volumes of $2\times$ sample buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2 mg/ml DTT, 2% (v/v) PharmalyteTM 3–10 were added to each of the labelled protein samples.

Electrophoretic separation

Prior to electrophoretic separation, labelled samples were mixed as described in Table 1. Each gel contained $5\ \mu\text{g}$ of Cy3 labelled standard and $5\ \mu\text{g}$ of an individual sample labelled with Cy5.

Table 1. 2-D DIGE experimental design for analysis of biological variation

Gel number	Sample labelled with CyDye DIGE Fluor Cy3 saturation dye	Sample labelled with CyDye DIGE Fluor Cy5 saturation dye
1–9	Pooled standard	Wild type
10–18	Pooled standard	Transgenic

Immobiline™ DryStrip gels, pH 4–7 (linear), 24 cm, were rehydrated in DeStreak™ Rehydration Solution containing 1% (v/v) Pharmalyte. The first-dimension separation, isoelectric focusing (IEF) using the cup-loading technique, was carried out on an Ettan IPGphor™ IEF System with Ettan IPGphor Manifold. The separation protocol consisted of five phases of graduated voltages from 300 to 8000 V with a total focusing of 50 000 Vh. Prior to second-dimension SDS-PAGE, strips were equilibrated in a single buffer containing 6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, 100 mM Tris, pH 8.0, reduced with 30 mM DTT. The strips were immediately applied to a 12.5% T SDS polyacrylamide gel, cast using DALT Gel Caster with low-fluorescence glass plates (26 × 20 cm) and 1-mm spacers. A 0.5% agarose overlay containing 0.001% bromophenol blue was applied. Second-dimension electrophoresis was carried out using Ettan DALT*twelve* Electrophoresis System. Initially, gels were run at 2 W/gel overnight, and the following morning the power was increased to 10 W/gel until the bromophenol blue dye front migrated off the bottom of the gel.

Imaging and analysis

Gel images for analysis were obtained using a Typhoon 9400 Series Variable Mode Imager, designed to optimally detect CyDye DIGE Fluor saturation dyes using the following settings: For Cy3, 532 nm excitation laser and 580 BP 30 emission filter; and for Cy5, 633 nm excitation laser and 670 BP 30 emission filter.

The resulting images were processed using DeCyder Differential Analysis Software v5.0. The spots on the gel were co-detected automatically as 2-D DIGE image pairs, which intrinsically link a sample to its in-gel standard. Matching between gels was performed using the in-gel standard from each image pair. The experimental setup and relationship between samples was assigned in DeCyder Differential Analysis Software. Each Cy5 gel image was assigned an experimental condition as either wild type or transgenic, and all Cy3 images were assigned as the standard. The gel with the highest spot count was automatically assigned as the master gel. Student's T-test was performed for every matched spot set, comparing the average and standard deviation of protein abundance for a given spot between the wild type and transgenic groups. The protein spots were filtered to include only proteins that demonstrated a significant change ($p \leq 0.0005$) in abundance.

Mass spectrometry and protein identification

For mass spectrometry analysis, preparative gels were run using a pool containing 250 µg each of wild type and transgenic rat lysates obtained from the whole hippocampus (500 µg total). The protein sample was reduced with 200 nmol of TCEP for 1 h at 37 °C in the dark. After reduction, 400 nmol of Cy3 CyDye DIGE Fluor saturation dye, freshly dissolved in anhydrous dimethyl formamide, was added to each sample. The labelling reaction was performed at 37 °C in the dark for 30 min. Prior to gel casting, two reference markers were attached to a glass plate treated with PlusOne™ Bind-Silane. Immobiline DryStrip gels (see Electrophoretic separation section) were used with in-gel rehydration, due to the higher protein load. When using the saturation labelling technique, all available protein was labelled, therefore prelabelling the sample with CyDye DIGE Fluor Cy3 saturation dye meant that poststaining was not necessary. The preparative gel image was automatically matched to the master gel image in the analytical gel match-set using DeCyder Differential Analysis Software. A spot-picking list was generated from DeCyder Differential Analysis Software and exported to Ettan Spot Picker. The gel spots were excised as 2 mm-diameter plugs and delivered into 96-well microplates.

In-gel digestion was performed after washing the gel plugs twice with 50 mM ammonium bicarbonate, then 50% (v/v) acetonitrile in 50 mM ammonium bicarbonate followed by 100% (v/v) acetonitrile for dehydration. Following overnight digestion with 500 ng of trypsin (Promega, USA) in 50 mM ammonium bicarbonate (pH 8.0) at room temperature, peptides were extracted using sequential steps of 1% (v/v) aqueous trifluoroacetic acid (TFA), followed by 50% (v/v) acetonitrile in 0.2% TFA. The peptides were then resuspended in 10 µl of 0.1% TFA before being cleaned using ZipTip™ pipette tips (Millipore). The tips were wet with three washes in 50% acetonitrile and equilibrated with three washes in 0.1% TFA, then the peptides were aspirated 10 times to ensure binding to the column. The column and peptides were washed three times in 0.1% TFA before being eluted in 3 µl of 50% acetonitrile/0.2% TFA.

Peptide-mass fingerprinting was performed using the Ettan MALDI-ToF Pro mass spectrometer. The procedure for protein identification with Ettan MALDI-ToF Pro is described elsewhere (7).

MALDI-generated mass spectra were internally calibrated using trypsin peaks. The peptide masses were searched against the National Center for Biotechnology Information nonredundant mammalian database (8) using ProFound™ (9) and confirmed using a Mascot™ search from Matrixscience (10) and the SwissProt™ database from MS-Fit (11). One missed cleavage per peptide was allowed, and an initial mass tolerance of 100 ppm was used in all searches. Partial oxidation for methionine was assumed.

Results and discussion

The experimental design outlined in Table 1 was applied to this study to enable accurate statistical analysis of protein abundance taking into account the biological variation between animals. The internal standard consisted of pooled aliquots of all the biological samples within the experiment. This ensured that the standard was representative of every

protein within the experiment. Normalization between gels is therefore permitted by determination of the relative abundance of fluorescent signal between the internal standard across gels. Each sample can then be compared with its in-gel internal standard, thereby reducing gel-to-gel variation and increasing statistical confidence (12).

A set of gels, containing CyDye DIGE Fluor Cy3 saturation dye-labelled internal standard versus CyDye DIGE Fluor Cy5 saturation dye-labelled wild type or transgenic rat samples, was run using the experimental design outlined in Table 1. The gels were batch-processed using DeCyder Differential Analysis Software. The gel images labelled with Cy3 were classified as “Standard” while Cy5 gel images from gels 1–9 were classified as “Wild Type,” and Cy5 gel images from gels 10–18 were classified as “Transgenic.” The greatest number of features was detected on gel 7, thereby automatically designating gel 7 as the master gel (Fig 3).

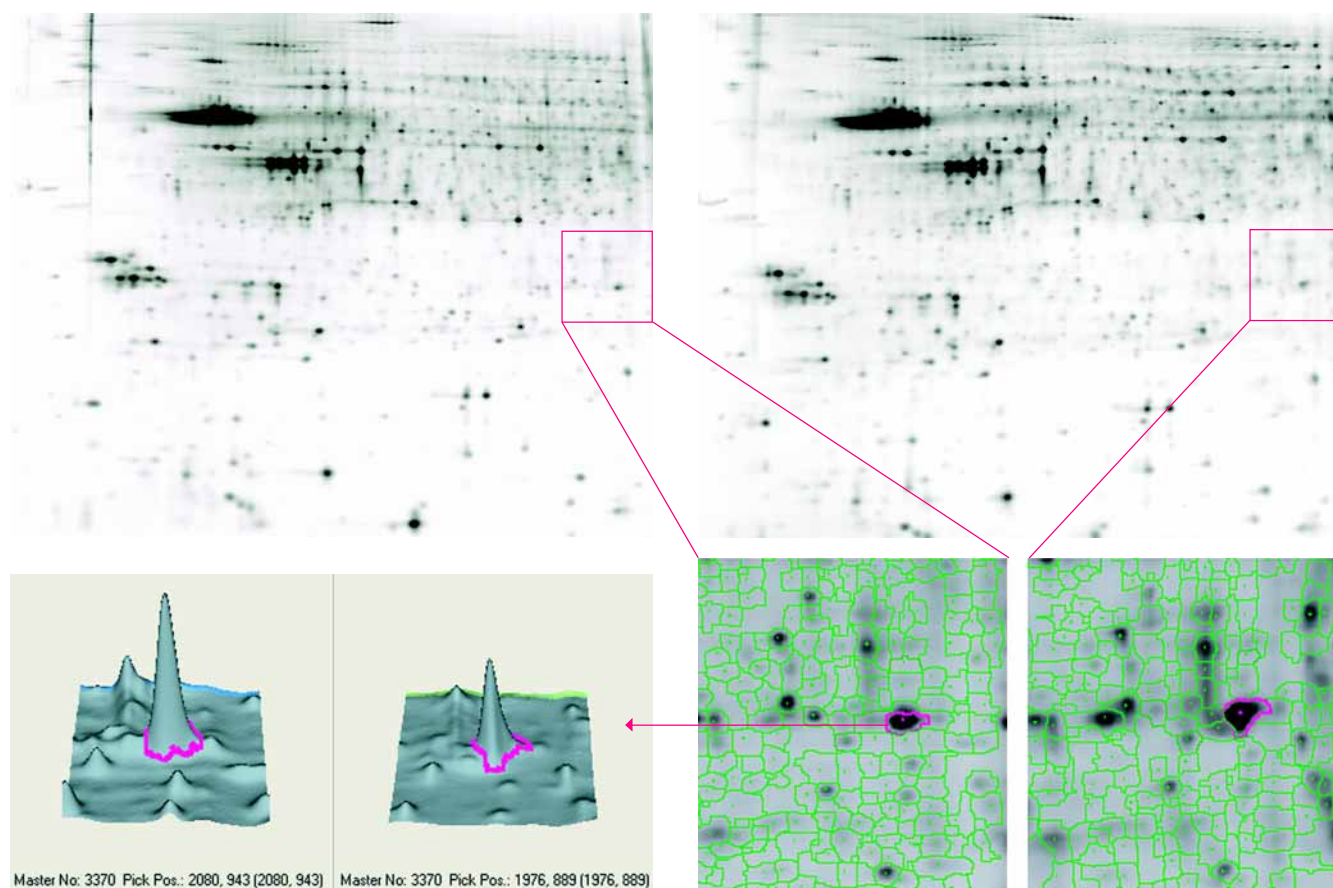


Fig 3. DeCyder Differential Analysis Software output showing the master gel image with CyDye DIGE Fluor Cy3 saturation dye-labelled standard (above left) and CyDye DIGE Fluor Cy5 saturation dye-labelled wild type (above right). The magnified box regions are shown (below right), as well as 3-D plots in pink (below left).

Statistical analysis was performed on spots matched across all the gels. The average ratio (i.e. the degree of difference between the abundance of a protein in two different groups—wild type and transgenic) was automatically calculated and displayed. A Student's T-test value for each spot was also calculated to establish the statistical significance of the abundance differences between wild type and transgenic samples. The majority of spots showed small changes in protein abundance (less than 2.0-fold) between control and treated samples. However, differences showing a decrease of protein abundance over 4.0-fold were also detected in the transgenic rat CA1-enriched hippocampus region compared with wild type.

The data was filtered to include only protein spots that displayed a Student's T-test value of $p \leq 0.0005$. In the transgenic samples compared with wild type, 61 spots displayed standardized volumes that increased by more than 10%, and 67 spots had volumes that decreased by more than 10%.

To confirm that mass spectrometry IDs could be generated from samples labelled with CyDye DIGE Fluor saturation dyes, 10 gel plugs covering a range of isoelectric points and molecular weights were picked from the preparative gel and excised for tryptic digestion. The proteins were identified from their peptide-mass fingerprint (PMF) after a database search (data shown in Table 2). Work on this model system is continuing, hence protein function substitutes for identification of individual proteins.

DeCyder Differential Analysis Software data and PMF for one of the cytoskeletal proteins is shown in more detail in Figure 4. Using DeCyder Differential Analysis Software, a significant decrease in abundance of this protein was detected in the transgenic CA1-enriched hippocampus samples relative to wild type (Student's T-test 1.6×10^{-5}).

The proteins identified here are involved in normal cellular functions such as energy metabolism and synaptic function, as well as pathological events such as oxidative stress and protein degradation pathways, all processes known to be affected in the brains of patients with AD (13). Two of the proteins identified are concerned with metabolism of reactive oxygen species produced during normal oxidative metabolism. Oxidative stress resulting from the generation of such oxidants is a common feature in neurodegenerative diseases including AD (14), and the altered expression found in APP transgenics is consistent with this. Another protein identified is concerned with the cellular degradation of proteins, a system known to be affected in AD and that may give rise to the β -amyloid deposits characteristic of this disease. Programmed cell death has recently been suggested to play a role in AD and other neurodegenerative diseases, and several proteins are known to be induced by β -amyloid-mediated cell death. The finding of such proteins by proteomic analysis in this instance is therefore consistent with such changes (15).

Table 2. Summary of results from a selection of spots selected based on data from DeCyder Differential Analysis Software. The proteins were subsequently identified from PMF spectra generated by Ettan MALDI-ToF Pro

Protein No.	Protein Function	Avg. Ratio	T-test
3356	Metabolism	1.31	2.7×10^{-5}
2947	Apoptosis-related	1.37	4.5×10^{-8}
3504	Oxidative stress	1.26	2.9×10^{-5}
3526	Oxidative stress	1.27	1.9×10^{-5}
999	Protein metabolism	-1.15	5.0×10^{-5}
3370	Synaptic activity	1.25	3.9×10^{-4}
3804	Synaptic activity	1.16	2.5×10^{-4}
1476	Cytoskeletal	-1.39	1.6×10^{-5}
1492	Cytoskeletal	-1.49	4.5×10^{-7}
1347	Synaptic activity	-1.44	2.7×10^{-4}

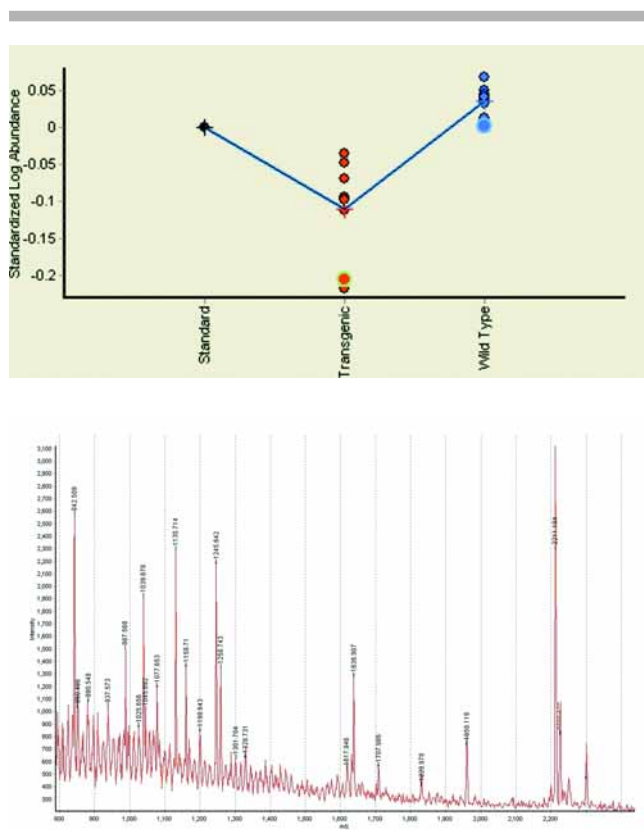


Fig 4. DeCyder Differential Analysis Software statistical output (top) and Ettan MALDI-ToF Pro PMF spectrum (bottom) of protein 1476, which was successfully identified after database searching. DeCyder Differential Analysis Software statistical output shows the graph of standardized log abundance (y-axis) against the three groups (standard, wild type, and transgenic [x-axis]) for protein 1476. The graphical data clearly indicates that the abundance of this protein is reduced in the transgenic samples (red circles) relative to wild type (blue circles). The thick blue line represents the average standardized log abundance value across the three groups.

Conclusions

Two-dimensional fluorescence difference Gel Electrophoresis (2-D DIGE) using CyDye DIGE Fluor saturation dyes has been applied to enable the proteomic study of small amounts of material. This study demonstrates the value of the CyDye DIGE Fluor saturation dyes and DeCyder Differential Analysis Software for the complete 2-D analysis and identification of protein differences from samples that are available in very limited amounts, for example LCM samples. Data showing the compatibility of samples labelled with CyDye DIGE Fluor saturation dyes with subsequent analysis by mass spectrometry have also been described.

Using this system, proteins potentially important in the development of neurodegenerative disease involving the human APP have been detected with a high level of statistical confidence. The use of DIGE in combination with LCM therefore allows proteomic studies on small samples to be achieved with relative ease and will allow analysis of other scarce samples in various fields.

References

1. Kondo, T. *et al.* Application of sensitive fluorescent dyes in a linkage of laser microdissection and two-dimensional gel electrophoresis as a cancer proteomic study tool. *Proteomics*. **3**, 1758–1766 (2003).
2. Gharbi, S. *et al.* Evaluation of two-dimensional differential gel electrophoresis for proteomic expression analysis of a model breast cancer cell system. *Mol. Cell. Proteomics*. **1**, 91–98 (2002).
3. Zhou, G. *et al.* 2D differential in-gel electrophoresis for the identification of esophageal squamous cell cancer-specific protein markers. *Mol. Cell. Proteomics* **1**, 117–123 (2002).
4. Skynner, H. *et al.* Alterations of stress related proteins in genetically altered mice revealed by two-dimensional differential in-gel electrophoresis analysis. *Proteomics* **2**, 1018–1025 (2002).
5. Close, B. *et al.* Recommendations for euthanasia of experimental animals: Part 1. DGXI of the European Commission. *Lab Anim*. **30**:293–316 (1996).
6. Close, B. *et al.* Recommendations for euthanasia of experimental animals: Part 2. DGXT of the European Commission. *Lab Anim*. **31**:1–32 (1997).
7. Ettan MALDI-ToF user manual, Amersham Biosciences, 18-1144-01, Edition BB (2002).
8. <http://www.ncbi.nlm.nih.gov/>
9. http://129.85.19.192/profound_bin/WebProFound.exe
10. http://www.matrixscience.com/cgi/index.pl?page=search_form_select.html
11. <http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm>
12. Alban, A. *et al.* A novel experimental design for comparative two-dimensional gel analysis: Two-dimensional difference gel electrophoresis incorporating a pooled internal standard. *Proteomics* **3**, 36–44 (2003).
13. Annaert, W. and De Strooper, B. A cell biological perspective on Alzheimer's disease. *Annu. Rev. Cell Dev. Biol.* **18**, 25–51 (2002).
14. Perry, G. *et al.* Comparative biology and pathology of oxidative stress in Alzheimer and other neurodegenerative diseases: beyond damage and response. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **133**, 507–13 (2002).
15. Vila, M. and Przedborski, S. Targeting programmed cell death in neurodegenerative diseases. *Nat. Rev. Neurosci.* **4**, 365–75 (2003).

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