Proteomics Course

LECTURE-16
Two-dimensional difference gel electrophoresis (2D DIGE)

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Previous lecture

- Gel-based proteomics
- 2-DE work-flow
- New methods for proteomics applications

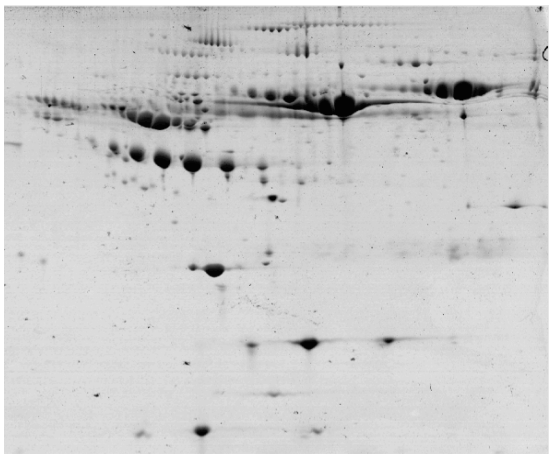
Today's lecture

- Challenges of 2-DE
- 2-D Fluorescence Difference Gel Electrophoresis (DIGE)

A good 2-D gel:
when everything goes well

pI

MW

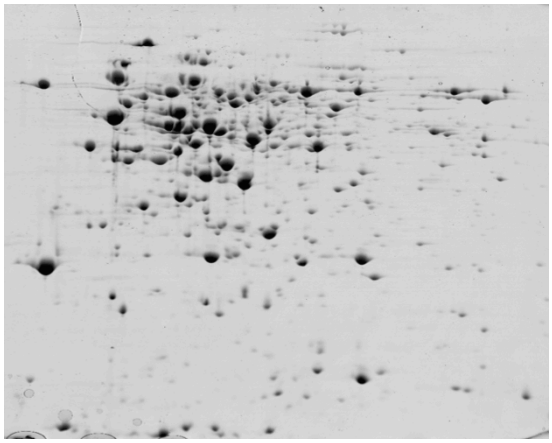


Representative 2-DE gel - Human serum sample

A good 2-D gel:
when everything goes well

pI

MW



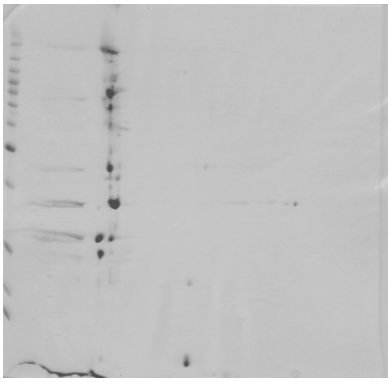
Representative 2-DE gel – Bacterial (*B. subtilis*) proteome

Challenges associated with 2-D gels

Major sources of variation in 2-D

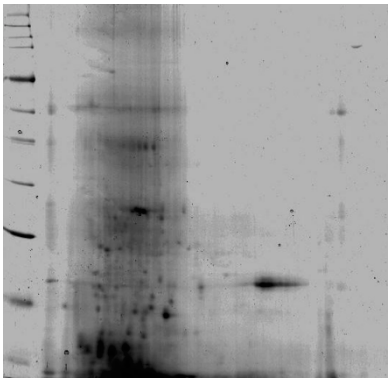
- Gel artifacts and gel-to-gel variation
  - samples, electrophoresis, staining
- Image analysis
  - defining spot boundaries
  - user bias in data analysis
- Biological and technical variations

Sample preparation issues



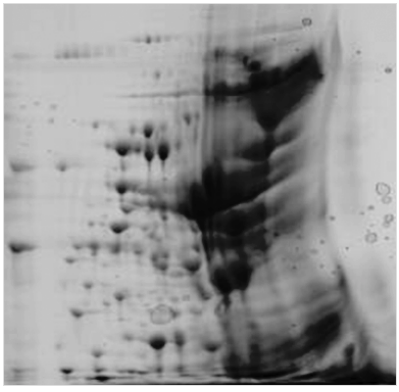
TCA-acetone precipitation,
TCA in pellet

Sample preparation issues (2)



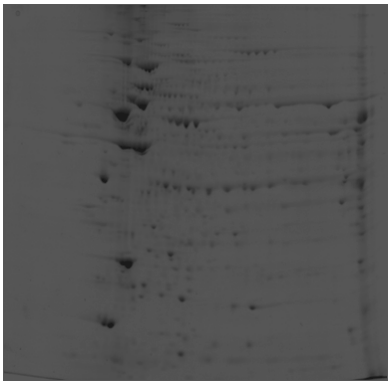
Plant protein extraction,
solubilization without precipitation

Sample preparation issues (3)



Crude sample

Chemical impurities (1)

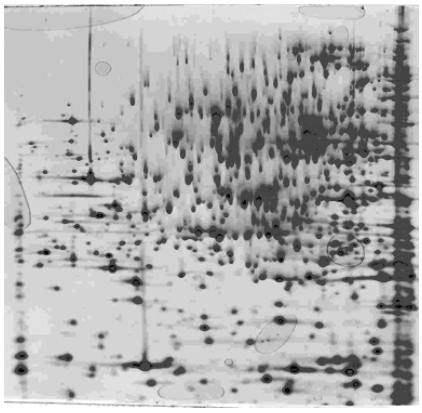


Carbamylation train
Impurities in urea

Chemical impurities (2)

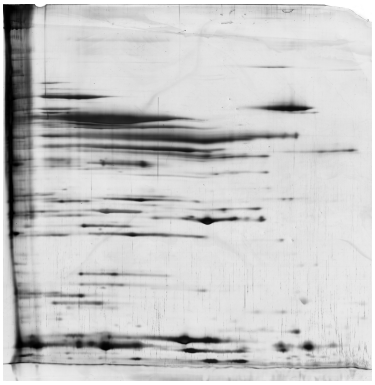
Old TEMED

Chemical impurities (3)

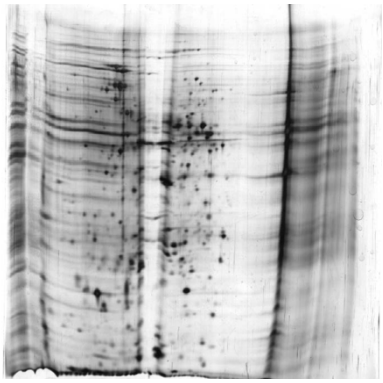


Tris impurity

Streaking

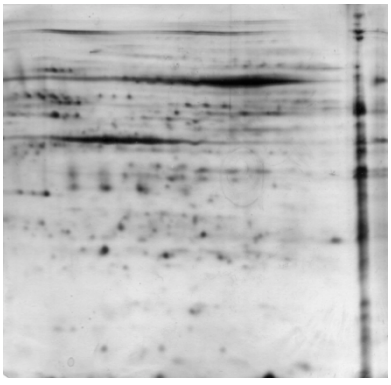


Streaking (2)



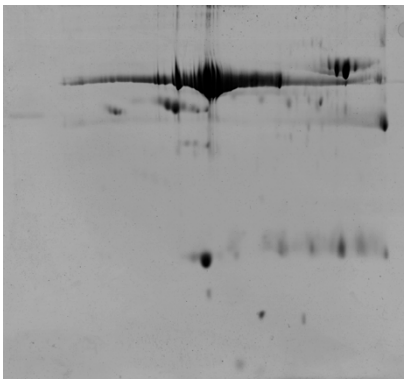
Salts

Streaking (3)



Vertical streaking (pH 4-7)

Abundant proteins

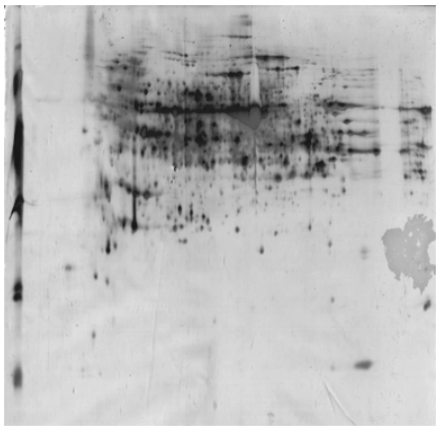


Overloading with highly abundant proteins in serum

Equilibration problem

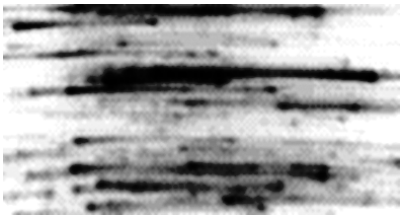
Only equilibration with DTT

Equilibration problem (2)



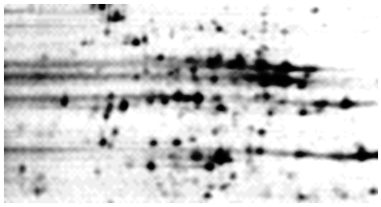
20 min DTT / 30 min IAA

Focusing problem



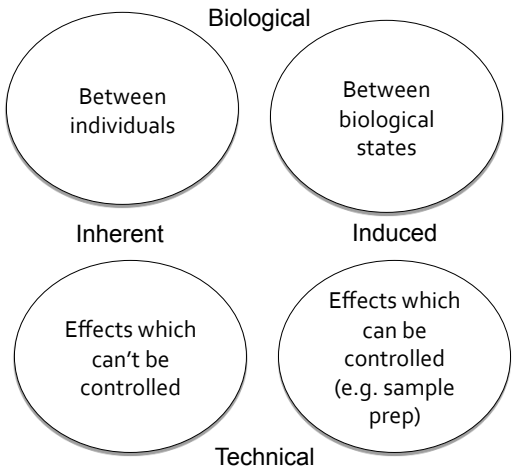
Under-focusing

Focusing problem (2)



Over-focusing

Source of variation



Two-dimensional difference gel electrophoresis (2D DIGE)

Two-dimensional difference gel electrophoresis (2D DIGE)

- To overcome intrinsic gel-to-gel variability of 2-DE, the DIGE multiplexing technology emerged
- This method was reported by Unlu et al. 1997
  - Electrophoresis 1997, 18, 2071–2077
- A protein labelling and separation technique
- Optical detection of proteins with a fluorescent tag
- Linear detection in wide range of protein abundance

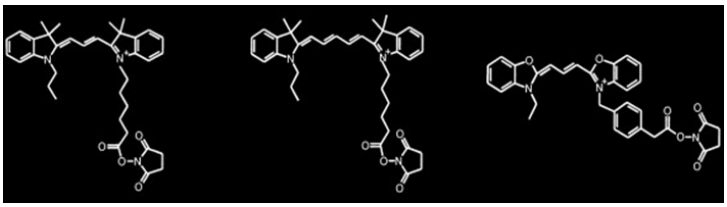
DIGE: basic principle

- Commonly used fluorescent protein labelling reagents for DIGE
- N-hydroxysuccinimidyl (NHS) ester derivatives of cyanine dyes Cy2, Cy3 and Cy5
- Size and charge matched - labeled samples co-migrate within gel

DIGE: labeling methods

Cyanine dyes

NHS-Cy3 NHS-Cy5 NHS-Cy2



MW - 582.8 Da
Excitation – 540 nm
Emission – 590 nm

MW - 580.7 Da
Excitation – 620 nm
Emission – 680 nm

MW - 550.6 Da
Excitation – 480 nm
Emission – 530 nm

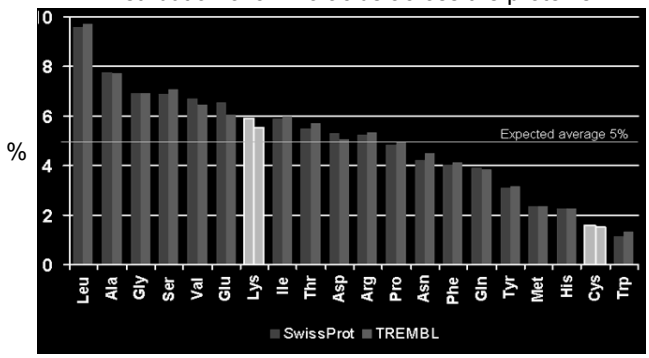
- Photostable
- pH insensitive
- Spectrally distinct

DIGE: two labeling methods

- Minimum labeling
  - Lysine labeling
  - Charge and size matched, no multiple labels
  - labelling of 3% of all proteins, Cy2, Cy3, Cy5
- Saturation labeling
  - Cysteine labeling
  - Charge neutral and size matched, multiple labels
  - Very sensitive, only Cy3 and Cy5

Why Lysine labeling?

Distribution of amino acids across the proteins

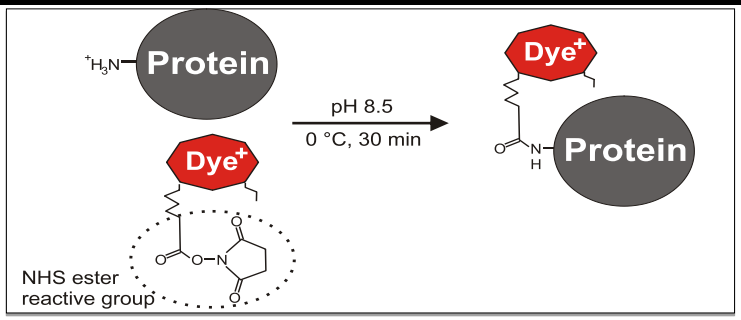


Lysine is preferred amino acid to label, it's frequency is >5%; statistically almost every protein has at least one lysine residue

DIGE: Lysine labelling

- Cyanine dyes react with primary amine groups of target proteins
  - N-terminal α-amino and lysine ε-amino groups
  - Nucleophilic substitution
- All available lysine labelling would create very hydrophobic proteins
  - Minimal labelling - only 3% of whole proteome is labeled

DIGE: Labeling chemistry



- Structure of CyDye Fluor comprises
  - positive charged CyDye fluorophore, linker, NHS ester reactive group
- At pH 8.5 NHS ester couples with amine group of lysine in proteins, forms covalent amide bond

DIGE: Lysine labelling

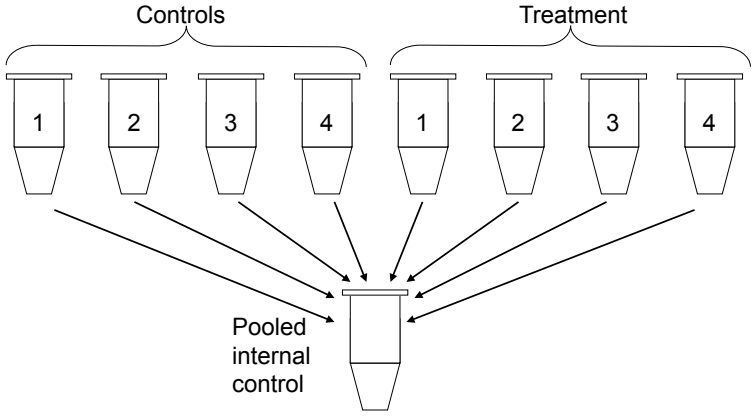
- The amine group of a lysine residue is positively charged at neutral or acidic pH's.
- Dye is also positively charged, so the net charge pI is effectively unchanged
- Cy3 and Cy5 are two spectrally resolvable dyes matched for mass and charge

DIGE: sample preparation

- CyDyes - reconstitute in Dimethyformamide (DMF)
- Lysis buffer:
  - 30 mM Tris, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, pH 8.5
  - No IPG buffers or carrier ampholytes; no reductant
- 50 μg protein label with 400 pmoles of dye on ice-water for 30 min
- Reactions are quenched with excess of primary amine (free lysine - 1 μL of 10 mM lysine)

Internal Standard

Internal standard



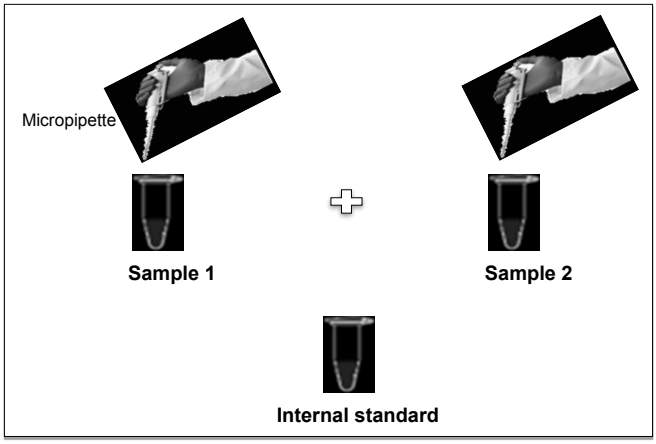
- Pooled internal standard preparation by combining aliquot of equal amounts of protein from each samples in experiment

Internal standard advantages

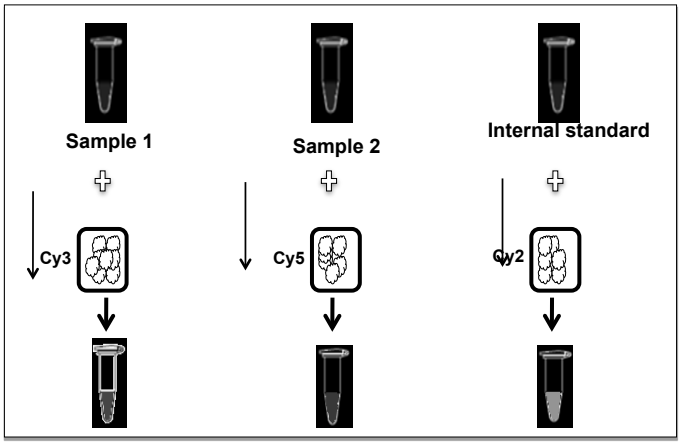
- Every protein in population appears on each gel
- Gel-to-gel variation reduces
- Gel-to-gel match and comparison easier
- A reference point for every protein species on each gel in the experiment
- Automated accurate spot statistics can be performed

DIGE: Step-by-step procedure

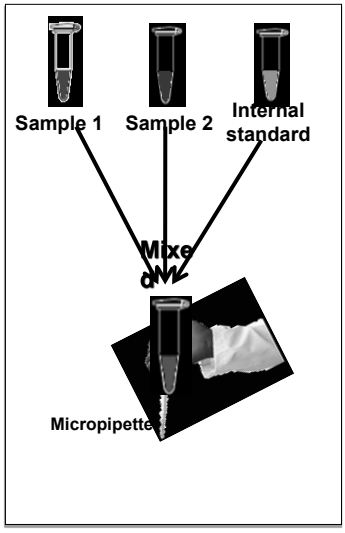
Internal standard preparation



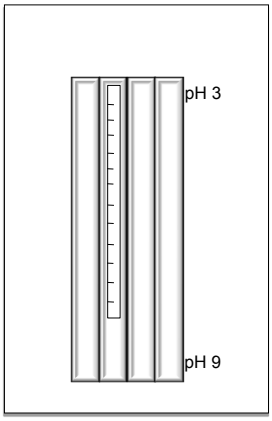
Dye Labeling



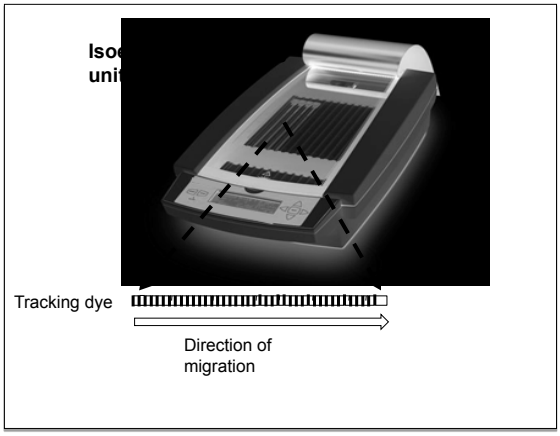
Sample mixing



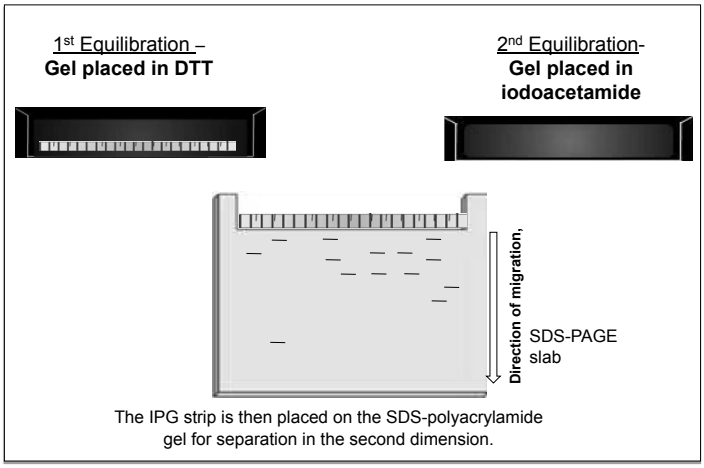
Rehydration



IEF

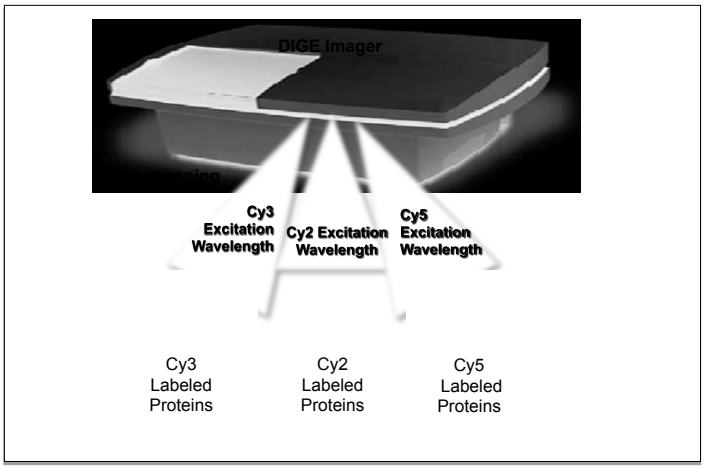


Equilibration and SDS-PAGE



The IPG strip is then placed on the SDS-polyacrylamide gel for separation in the second dimension.

DIGE image scanning



DIGE: overview

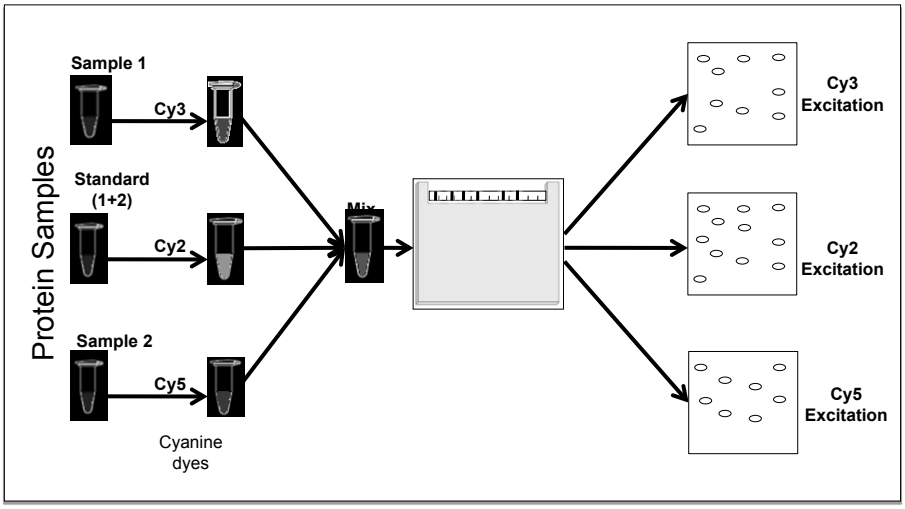
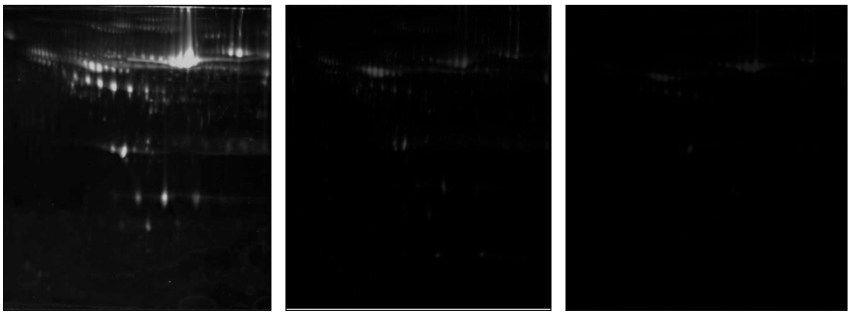
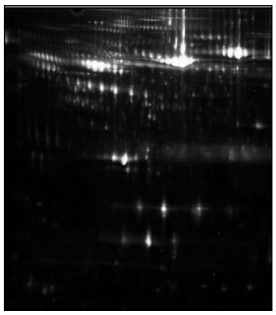


Image scanning

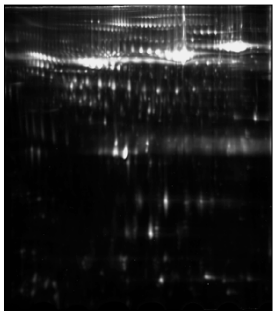


Cy3 Cy5 Cy2

Image scanning



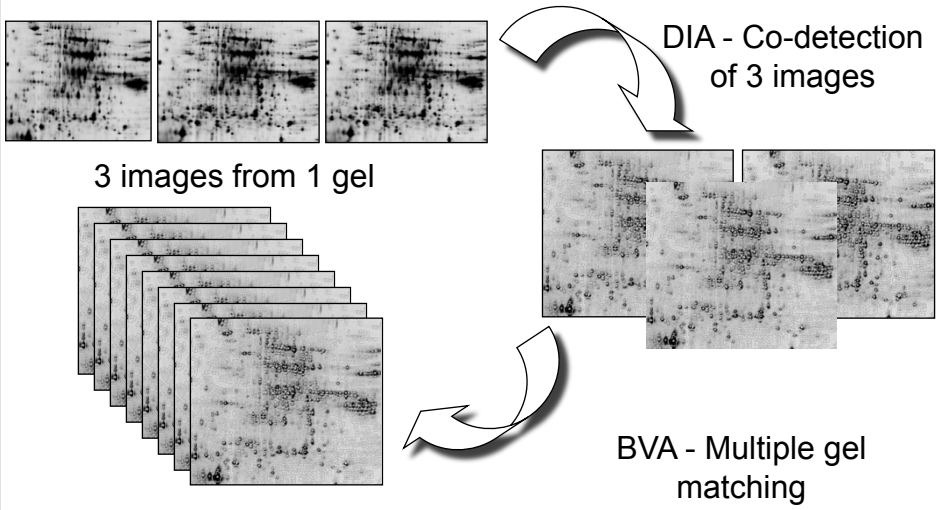
Combined

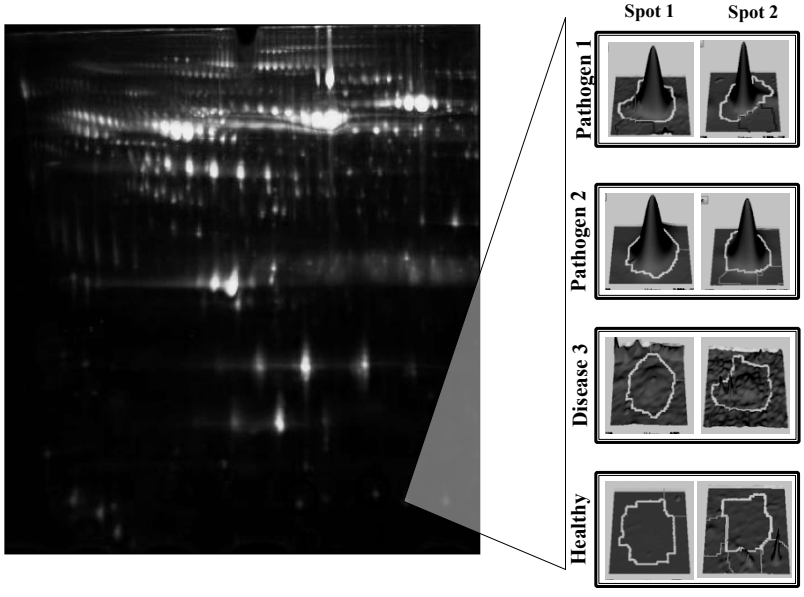


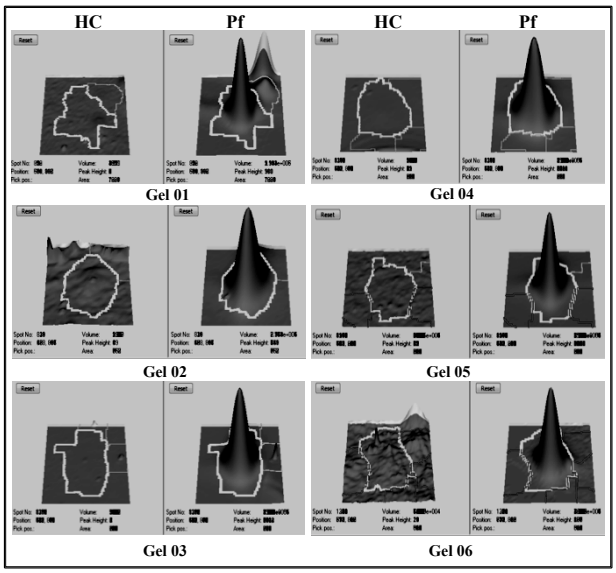
Overlapped

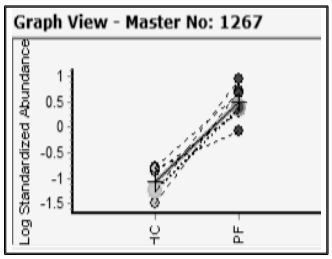
DIGE: Image Analysis

DIGE Image Analysis





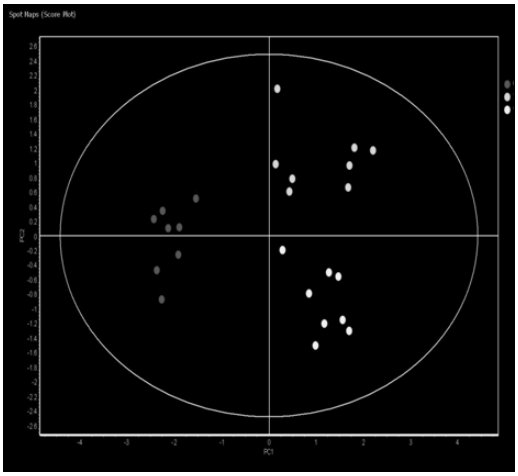




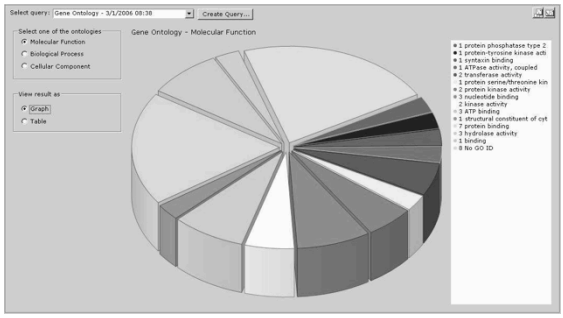
EDA analysis: Principle Component Analysis (PCA)

- Principal component - a linear combination of optimally-weighted observed variables
- PCA analysis to test, whether:
  - Protein expression is uniform in multiple sample from same experimental group
- If results are consistent, spot map from same experimental group located in same area

Principle Component Analysis (PCA)

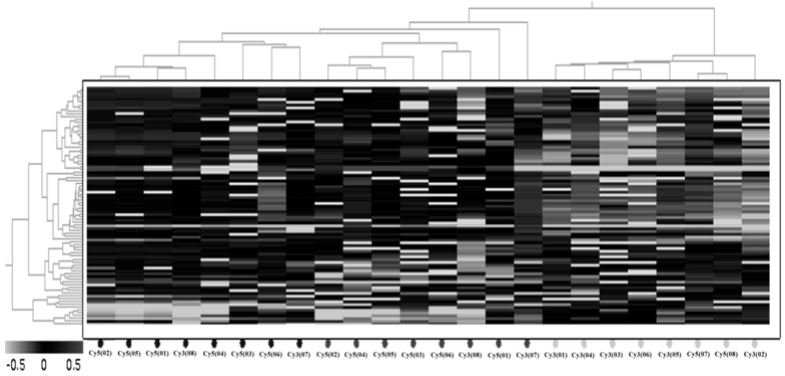


EDA analysis: Discriminant Analysis



- Discriminant analysis - To classify unknown data to known classes

EDA analysis: Hierarchical Clustering



Items (proteins, spot maps, experimental groups) are grouped together in an hierarchical way

DIGE interactivity

- Which of these two would be the better technique to separate serum protein samples obtained from 250 patients in a clinical trial?

2-D gel electrophoresis (2-DE)

Difference gel electrophoresis (DIGE)

2DE vs. DIGE

- Gel to gel variation is overcome by separating proteins on single gel
- Large sample variations can be reduced by internal standard
- User bias in data analysis can be eliminated by co-detection method

Summary

- Challenges associated with 2-DE
- Fluorescence Difference Gel Electrophoresis
- Labeling, Sample preparation, step-by-step procedure
- Comparison of 2-DE vs. DIGE

REFERENCES

- Unlü M., Morgan EM, Minden JS: Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 1997, 18, 2071-2077
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