

Proteomics Course

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



Dr. Sanjeeva Srivastava
IIT Bombay



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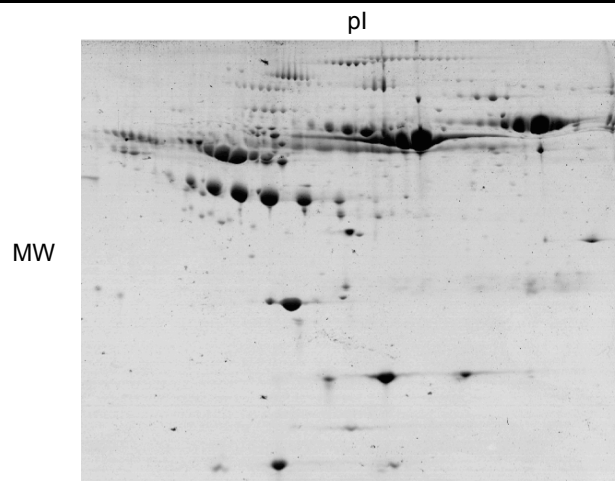
Previous lecture

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

Today's lecture

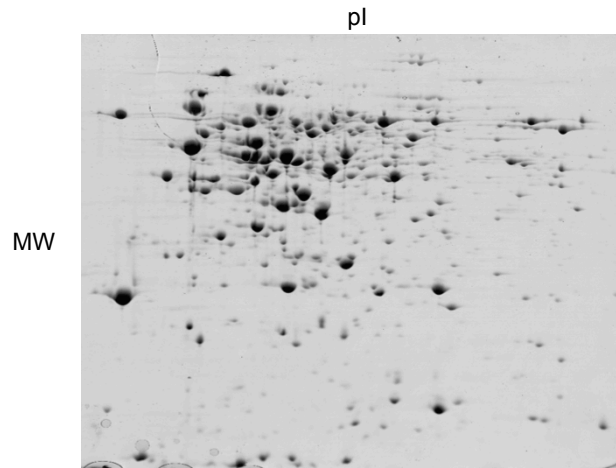
- Challenges of 2-DE
- 2-D Fluorescence Difference Gel Electrophoresis (DIGE)

A good 2-D gel: when everything goes well



Representative 2-DE gel - Human serum sample

A good 2-D gel: when everything goes well



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

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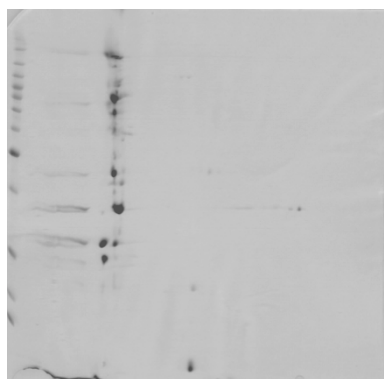
Challenges associated with 2-D gels

6

Major sources of variation in 2-D

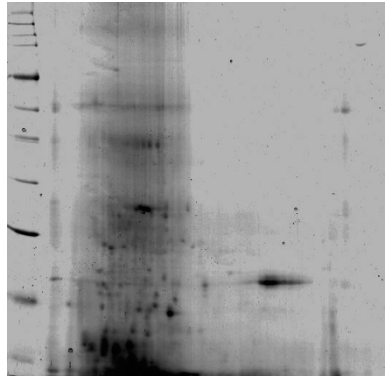
- Gel artifacts and gel-to-gel variation
 - samples, electrophoresis, staining
- Image analysis
 - defining spot boundaries
 - user bias in data analysis
- Biological and technical variations

Sample preparation issues



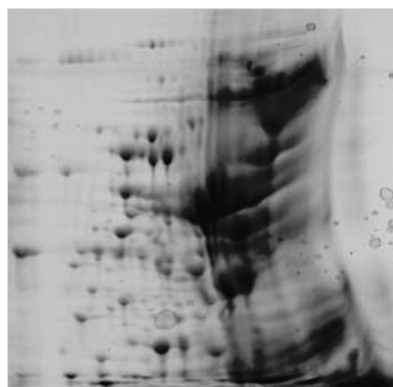
TCA-acetone precipitation,
TCA in pellet

Sample preparation issues (2)



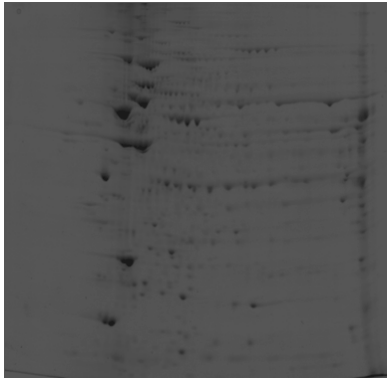
Plant protein extraction,
solubilization without precipitation

Sample preparation issues (3)



Crude sample

Chemical impurities (1)



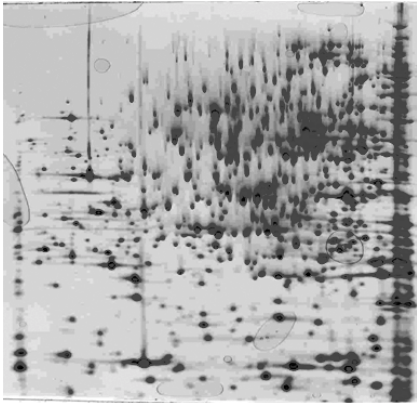
Carbamylation train
Impurities in urea

Chemical impurities (2)



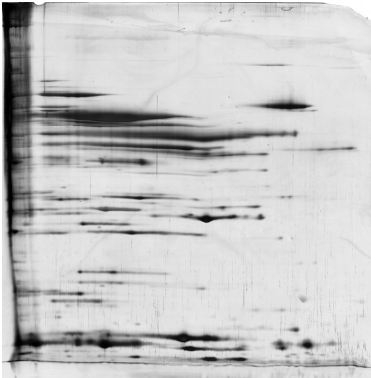
Old TEMED

Chemical impurities (3)

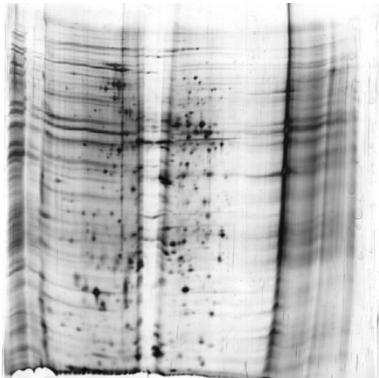


Tris impurity

Streaking

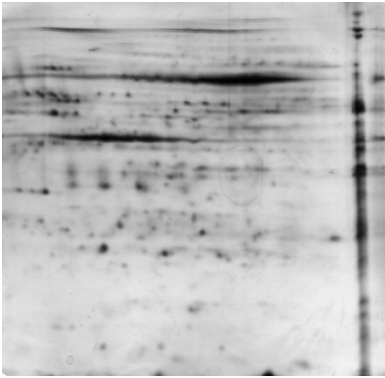


Streaking (2)



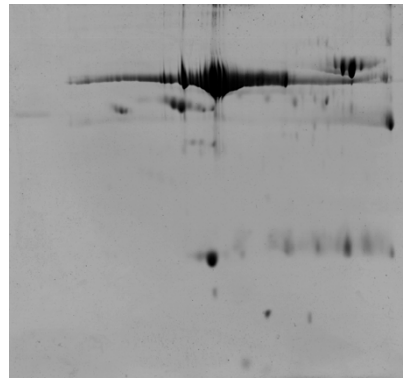
Salts

Streaking (3)



Vertical streaking (pH 4-7)

Abundant proteins



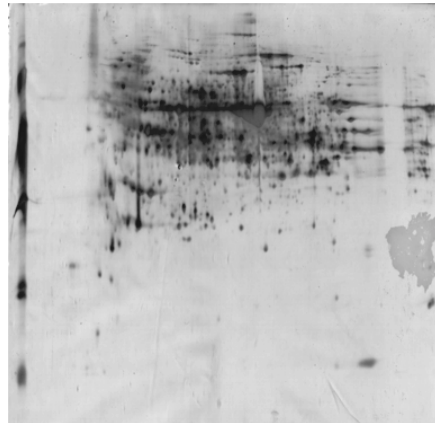
Overloading with highly abundant proteins in serum

Equilibration problem



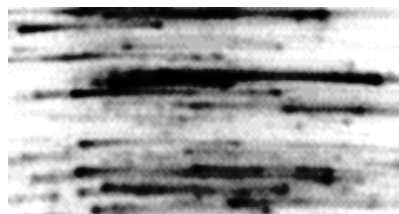
Only equilibration with DTT

Equilibration problem (2)



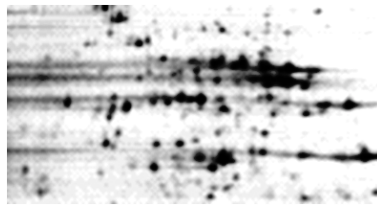
20 min DTT / 30 min IAA

Focusing problem



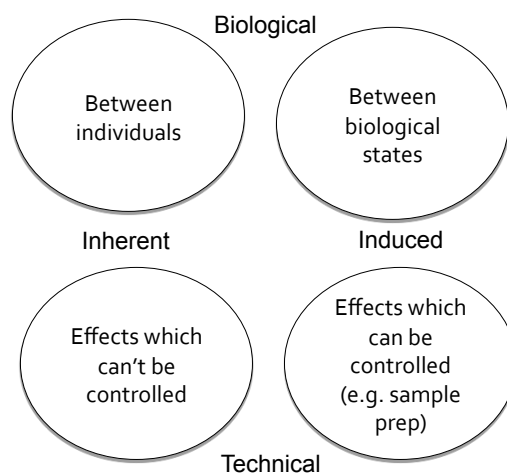
Under-focusing

Focusing problem (2)



Over-focusing

Source of variation



Two-dimensional difference gel electrophoresis (2D DIGE)

Two-dimensional difference gel electrophoresis (2D DIGE)

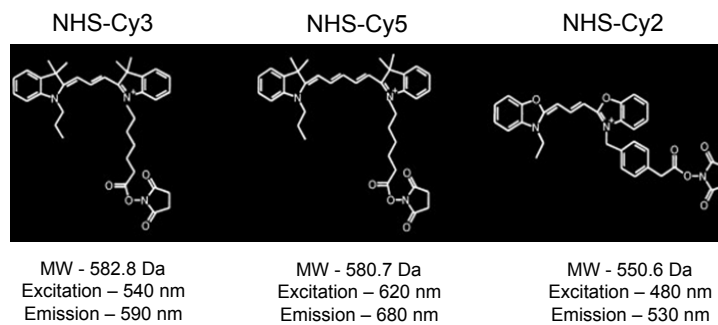
- To overcome intrinsic gel-to-gel variability of 2-DE, the DIGE multiplexing technology emerged
- This method was reported by Unlu et al. 1997
 - Electrophoresis 1997, 18, 2071–2077
- A protein labelling and separation technique
- Optical detection of proteins with a fluorescent tag
- Linear detection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

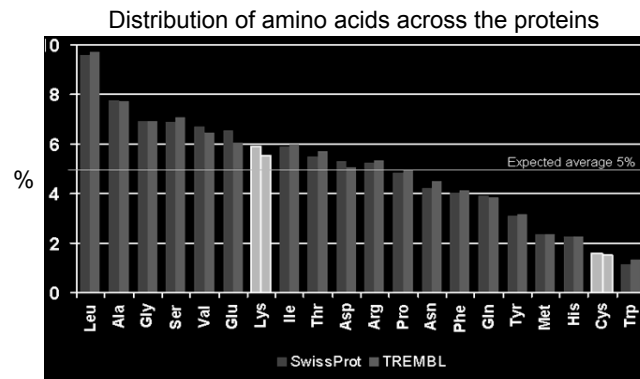


- Photostable
- pH insensitive
- Spectrally distinct

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?

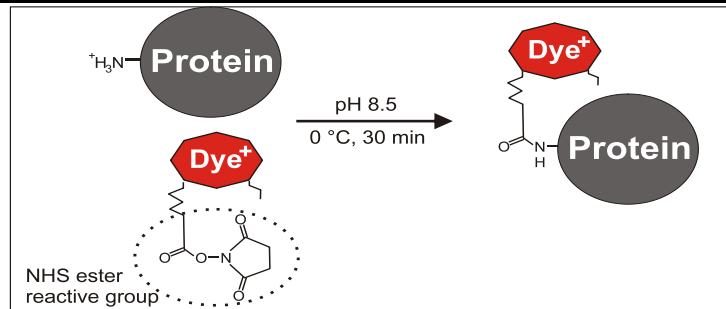


Lysine is preferred amino acid to label, it's frequency is >5%; statistically almost every protein has at least one lysine residue

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

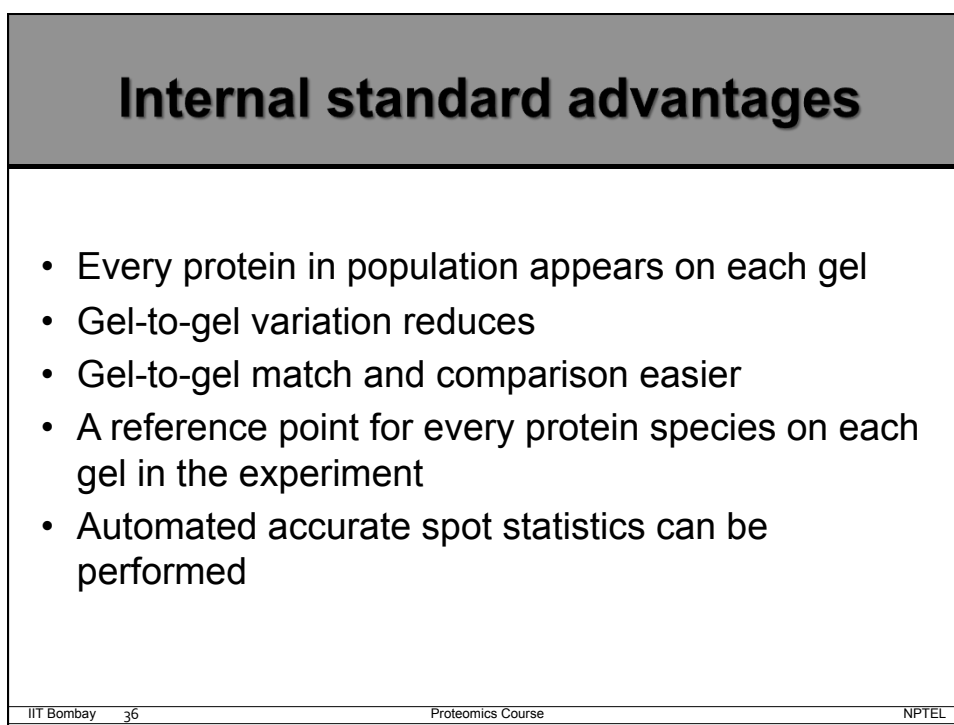
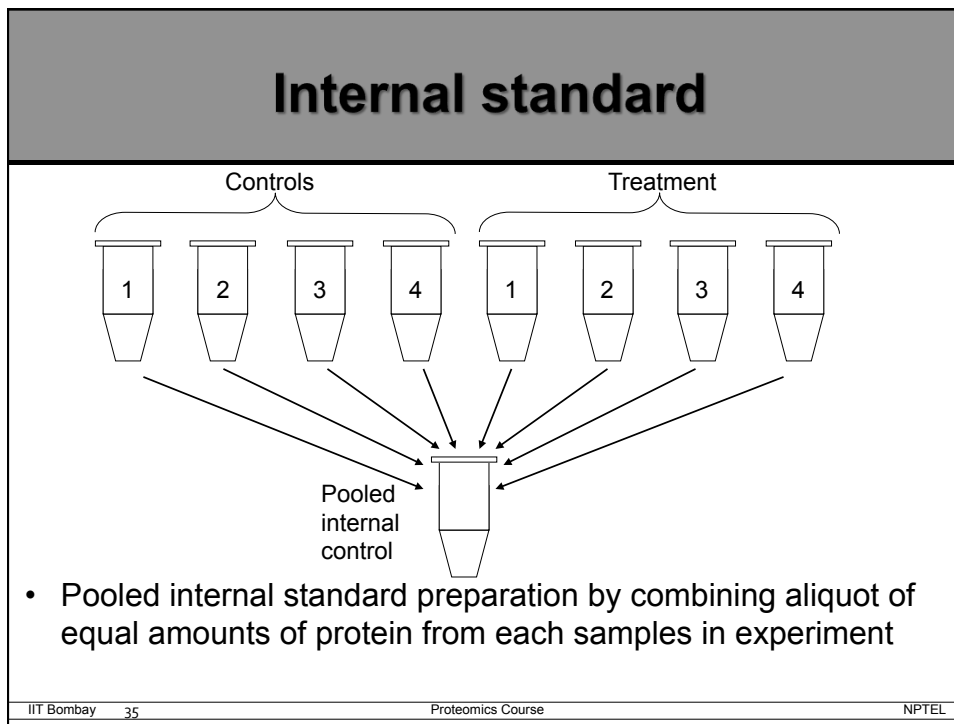
DIGE: Lysine labelling

- 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

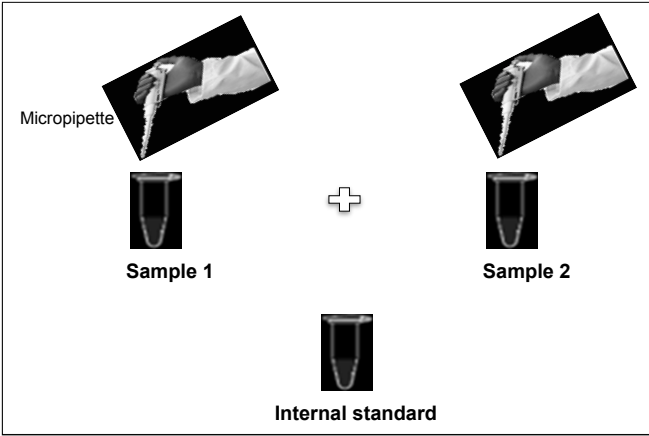
- CyDyes - reconstitute in Dimethylformamide (DMF)
- Lysis buffer:
 - 30 mM Tris, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, pH 8.5
 - No IPG buffers or carrier ampholytes; no reductant
- 50 μg protein label with 400 pmoles of dye on ice-water for 30 min
- Reactions are quenched with excess of primary amine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

Internal Standard



DIGE: Step-by-step procedure

Internal standard preparation



Dye Labeling

The diagram illustrates the dye labeling process for three samples: Sample 1, Sample 2, and Internal standard. Each sample is combined with a specific dye (Cy3, Cy5, or Cy2) and then processed.

Sample 1 + Cy3 → [Sample 1 + Cy3] → [Labeled Sample 1]

Sample 2 + Cy5 → [Sample 2 + Cy5] → [Labeled Sample 2]

Internal standard + Cy2 → [Internal standard + Cy2] → [Labeled Internal standard]

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Sample mixing

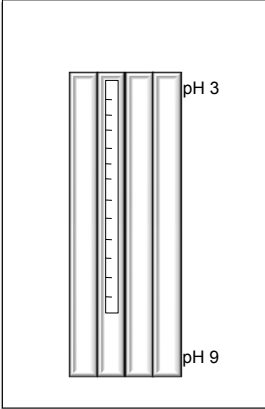
The diagram illustrates the sample mixing process. Three vials labeled Sample 1, Sample 2, and Internal standard are shown. Lines from each vial converge on a central point labeled "Mix" with a plus sign. Below this, a micropipette is shown mixing the samples in a vial.

Sample 1 + Sample 2 + Internal standard → Mix → [Mixed Sample]

Micropipette

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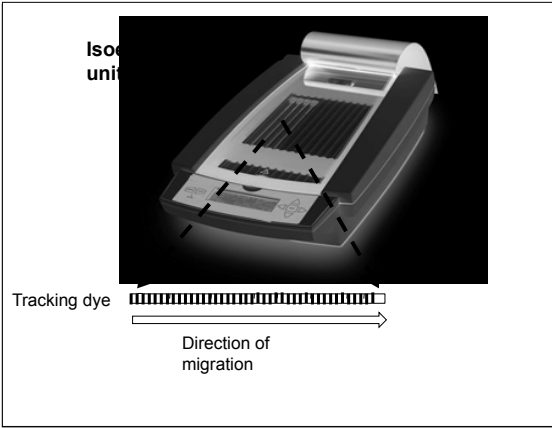
Rehydration



A diagram of a vertical gel strip used for rehydration. The strip is shown with a central channel and two side channels. On the right side, there are two pH markers: 'pH 3' at the top and 'pH 9' at the bottom. The strip is enclosed in a rectangular frame.

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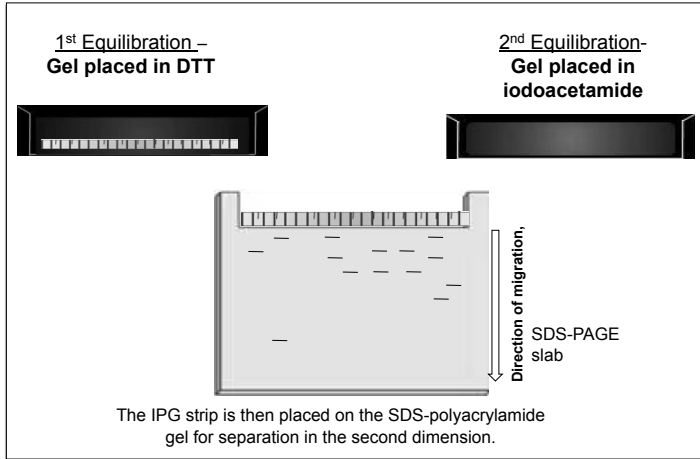
IEF



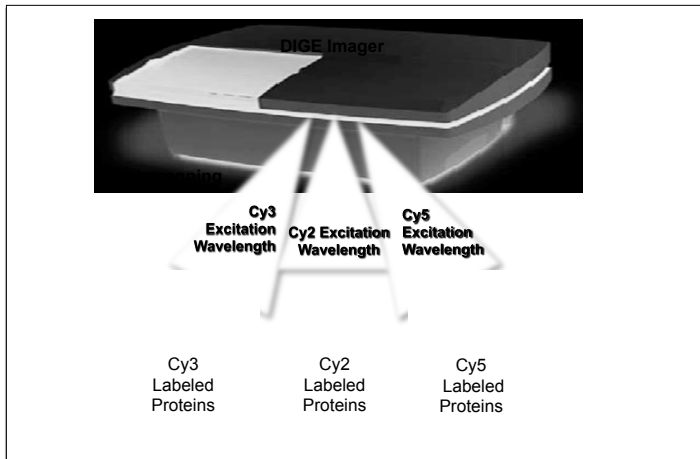
A photograph of an isoelectric focusing (IEF) unit. The unit is a rectangular device with a lid. Below the unit, there is a diagram of a tracking dye strip. The strip is labeled 'Tracking dye' and has a series of vertical bars representing the dye. Below the strip, there is a horizontal arrow pointing to the right, labeled 'Direction of migration'.

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Equilibration and SDS-PAGE



DIGE image scanning



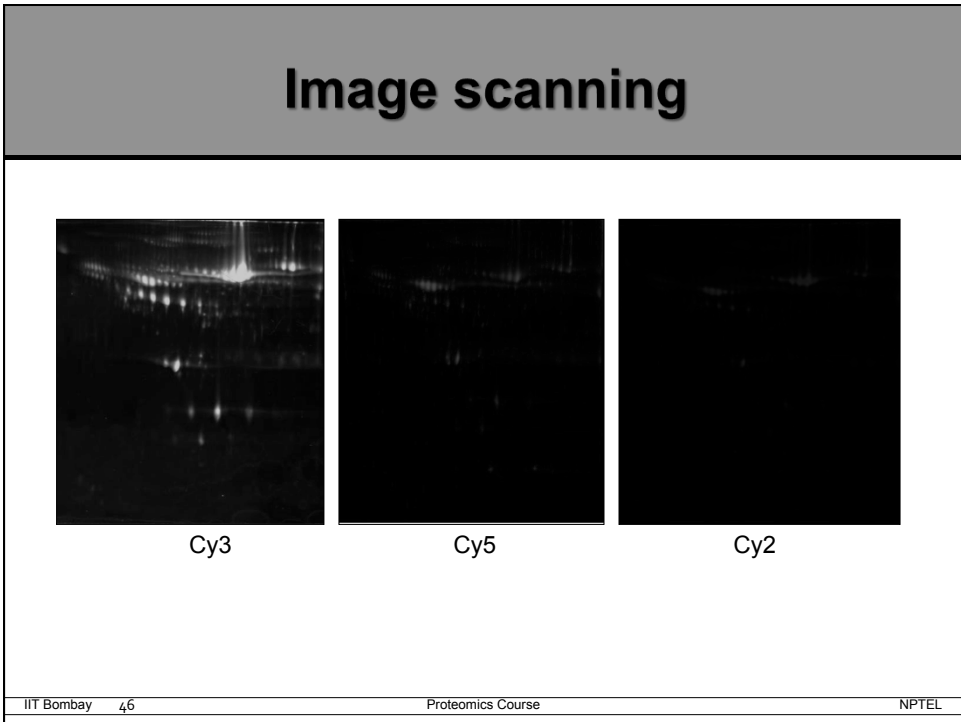
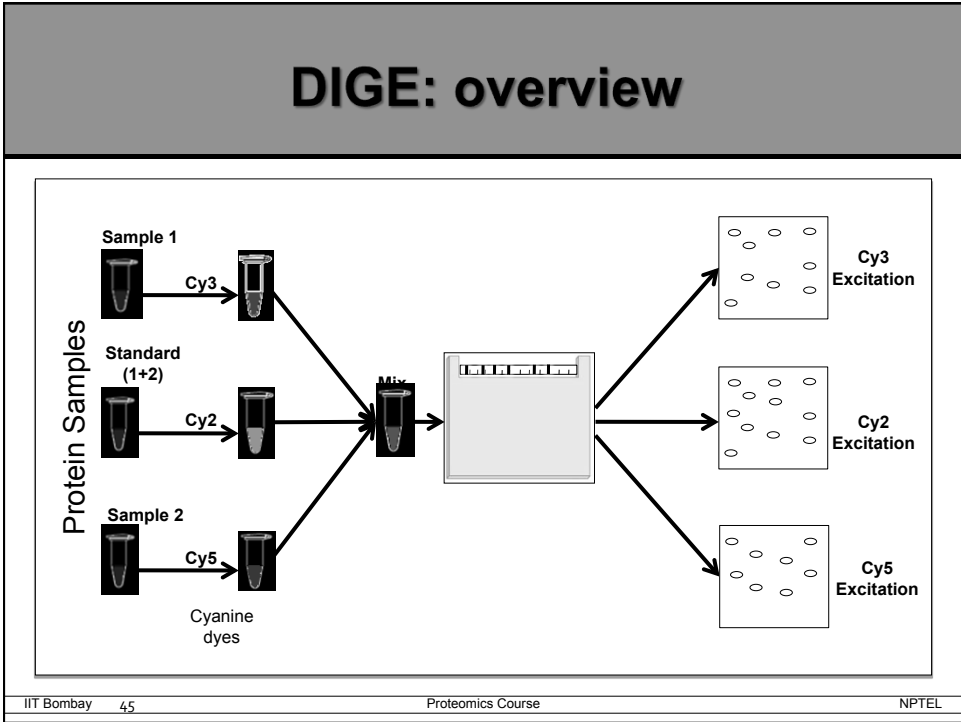
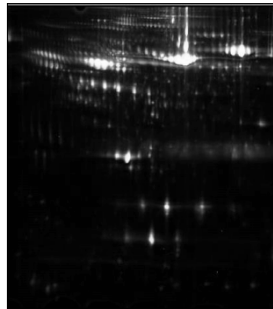
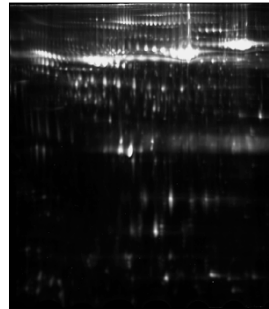


Image scanning



Combined



Overlapped

DIGE: Image Analysis

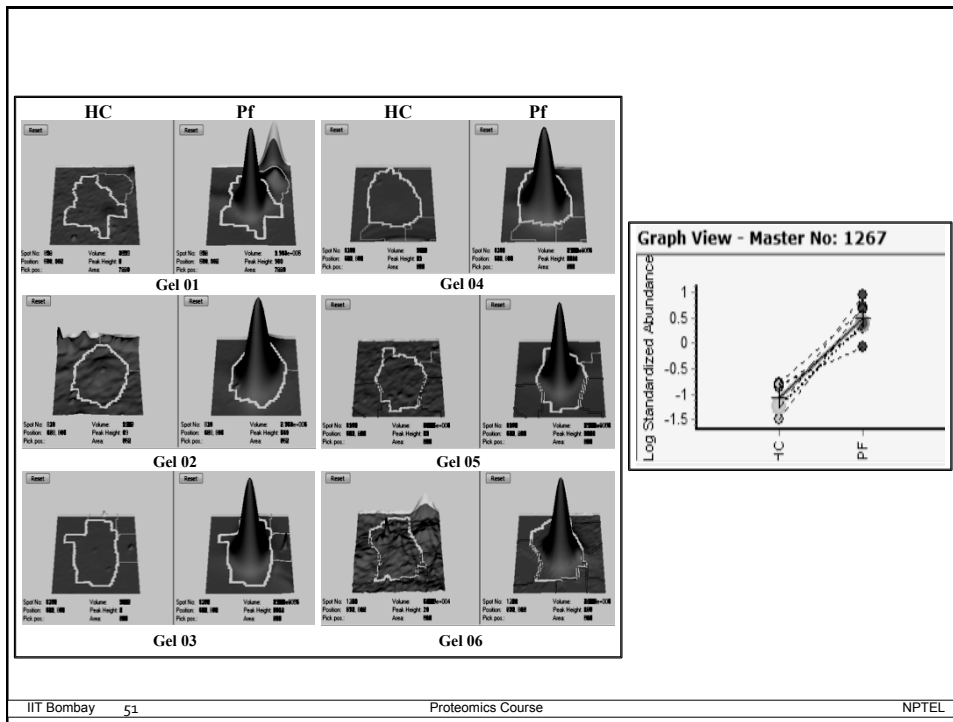
DIGE Image Analysis

The diagram illustrates the workflow for DIGE image analysis. It starts with '3 images from 1 gel', shown as three separate grayscale images of gel spots. An arrow labeled 'DIA - Co-detection of 3 images' points to a single, larger grayscale image where the spots from the three original images are overlaid. A second arrow labeled 'BVA - Multiple gel matching' points from this overlaid image to a stack of several similar images, representing the process of matching spots across multiple gels.

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This slide shows a 2D gel electrophoresis image with numerous spots. A magnified view of a specific spot is shown on the right, divided into two columns: 'Spot 1' and 'Spot 2'. The rows represent different conditions: 'Pathogen 1', 'Pathogen 2', 'Disease 3', and 'Healthy'. Each cell in this grid contains a 3D surface plot of the spot's intensity, with a white outline indicating the spot's boundary. The 'Healthy' row shows a flat surface, while the 'Pathogen' and 'Disease' rows show distinct peaks, indicating protein expression changes.

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EDA analysis: Principle Component Analysis (PCA)

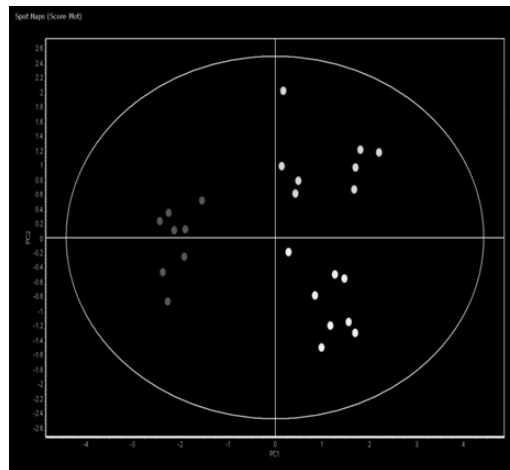
- Principal component - a linear combination of optimally-weighted observed variables
- PCA analysis to test, whether:
 - Protein expression is uniform in multiple sample from same experimental group
- If results are consistent, spot map from same experimental group located in same area

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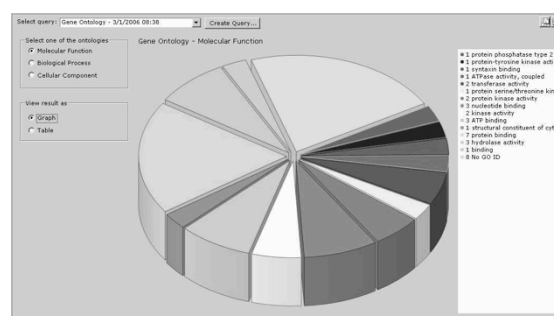
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Principle Component Analysis (PCA)

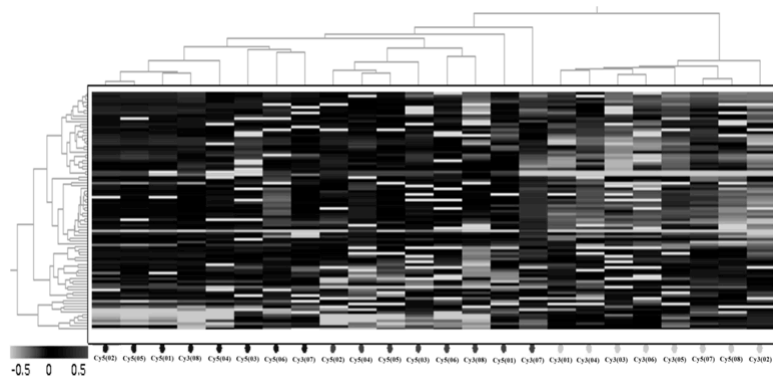


EDA analysis: Discriminant Analysis



- 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
- Van den Bergh, G., Arckens, L.: Fluorescent 2-D DIGE unveils the potential of gel-based proteomics. *Current Opinion in Biotechnology*, 2004, 15, 1-6
- Patton, W.F.: Detection technologies in proteome analysis. *J Chromatogr B*, 2002, 771, 3- 31
- Yuguang Wang, Haiying Li, Sixue Chen. Advances in quantitative proteomics. *Frontiers in Biology*. June 2010, Volume 5, Issue 3, pp 195-203.

Acknowledgment

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