Proteomics

Sheila Sadeghi

Proteomics 2016-17 (Aula A, DBIOS)

	31-Oct	01-Nov	02-Nov	03-Nov	04-Nov
9-10					
10-11					
11-12					Intro & 2D gels
12-13					
	07-Nov	**08-Nov**	**09-Nov**	**10-Nov**	**11-Nov**
9-10					
10-11					
11-12	Mass Spec				Protein Digest
12-13					
14-15			Surface Plasmo Resonance		
15-16					
16-17					
17-18					
	14-Nov	**15-Nov**	**16-Nov**	**17-Nov**	**18-Nov**
9-10					
10-11					
11-12	LC-MS equipmen				
12-13					
14-15			Protein Microarra		
15-16					
16-17					
17-18					
	21-Nov	**22-Nov**	**23-Nov**	**24-Nov**	**25-Nov**
9-10		Seminars A			
10-11					
11-12		Seminars B			
12-13					
14-15					
15-16					
16-17					
17-18					

Exam dates

- Written exam dates for academic year 2016-17:
  - 2 & 23 Feb 2017
  - 22 June
  - 13 July
  - 7 Sept
- Short lab practical and LC-MS visit:
  - List of students working in pairs

3

Final Exam Mark

- Practical and seminar (20% of final Mark):
  - Presentation 10 min
  - Scientific paper published in the last 2 years
- Written exam 1 hr (80% of final Mark):
  - 3-4 short questions regarding the subjects covered in the lectures

Proteomics is the study of the overall state of an organism's temporal protein composition

The biological state of the proteome is encoded in:

- The relative abundance of currently expressed proteins (and their isoform)
- Their localisation relative to cellular (or extracellular) structures
- Their interaction partner molecules and substrates
- Their current post-translational modification state
- Their folded structures

Why Proteomics?

- Orthogonal verification of gene activity.
- Observe biological state after more levels of regulation and control – closer to phenotypic outcome.

Genome ➔ Transcriptome ➔ *Proteome* ➔…➔ *Phenotype*

- Observe proteomes of extracellular locations – blood plasma/serum, urine etc.

Proteomics

- Classical biochemistry
- Two-dimensional gels (2DGE)
- Mass spectrometry
- Computational analysis

Methods in Proteomics

- Separation
  - Gels
  - Immunochemistry
  - Chromatography
- Identification
  - Immunochemistry
  - Mass spectrometry
- Quantitation
  - All of the above

Two dimensional electrophoresis

- "Proteomics" is the large-scale screening of the proteins of a cell, organism or biological fluid, a process which requires stringently controlled steps of sample preparation, 2-D electrophoresis, image detection and analysis, spot identification, and database searches.
- The core technology of proteomics is 2-DE
- At present, there is no other technique that is capable of simultaneously resolving thousands of proteins in one separation procedure. (sited in 2000)

Evolution of 2-DE methodology

Traditional IEF procedure:

- IEF is run in thin polyacrylamide gel rods in glass or plastic tubes.
- Gel rods containing: 1. urea, 2. detergent, 3. reductant, and 4. carrier ampholytes (form pH gradient).
- Problems:
- 1. tedious.
- 2. not reproducible.
- 3. Works well for native protein, not good for denaturing proteins

Evolution of 2-DE methodology

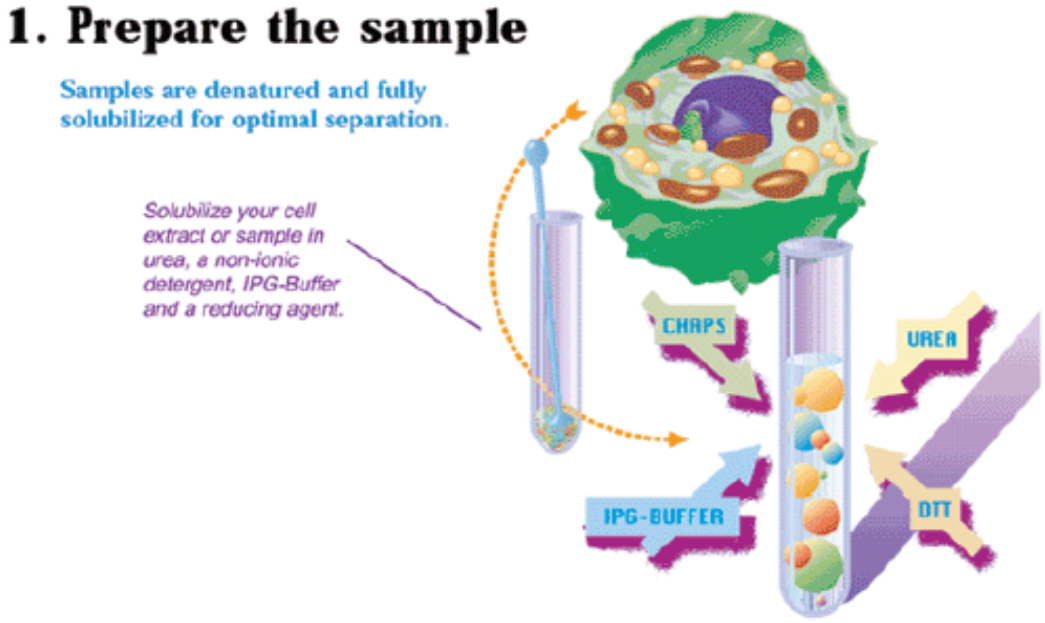
Resolution for IEF: Immobilized pH gradients.

- Developed by Bjellqvist (1982, Biochem. Biophys Methods, vol 6, p317)
- pH gradient are prepared by co-polymerizing acrylamide monomers with acrylamide derivatives containing carboxylic and tertiary amino groups.

R = amino or carboxylic groups Acrylamide

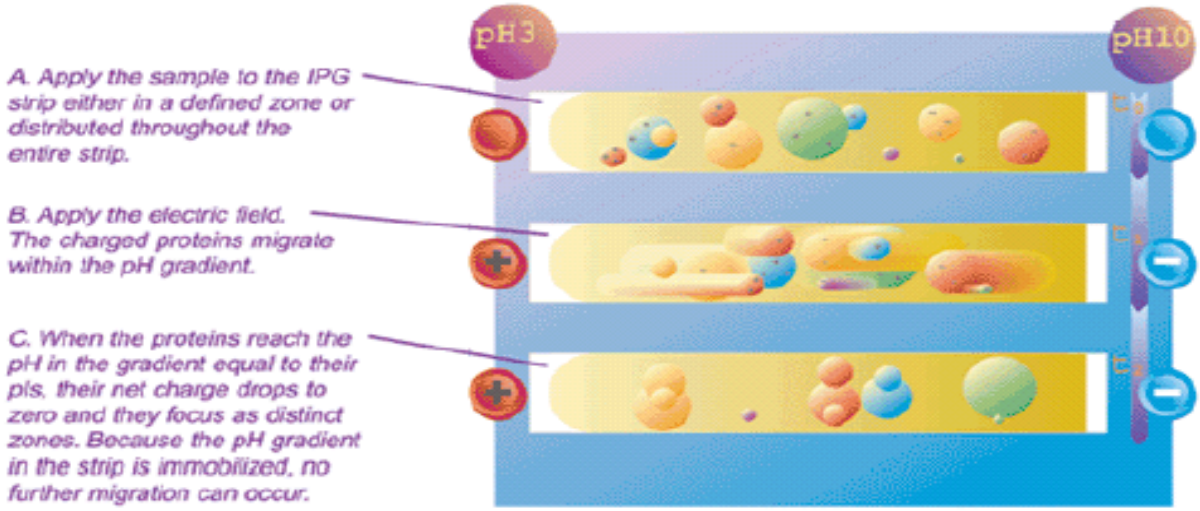
1. The pH gradient is fixed, not affected by sample composition.
2. Reproducible data are presented.
3. Modified by Angelika Gorg by using thin film to support the thin polyacrylamide IEF gel, named Strips. (1988, Electrophoresis, vol 9, p 531)

Run 2-DE, step by step



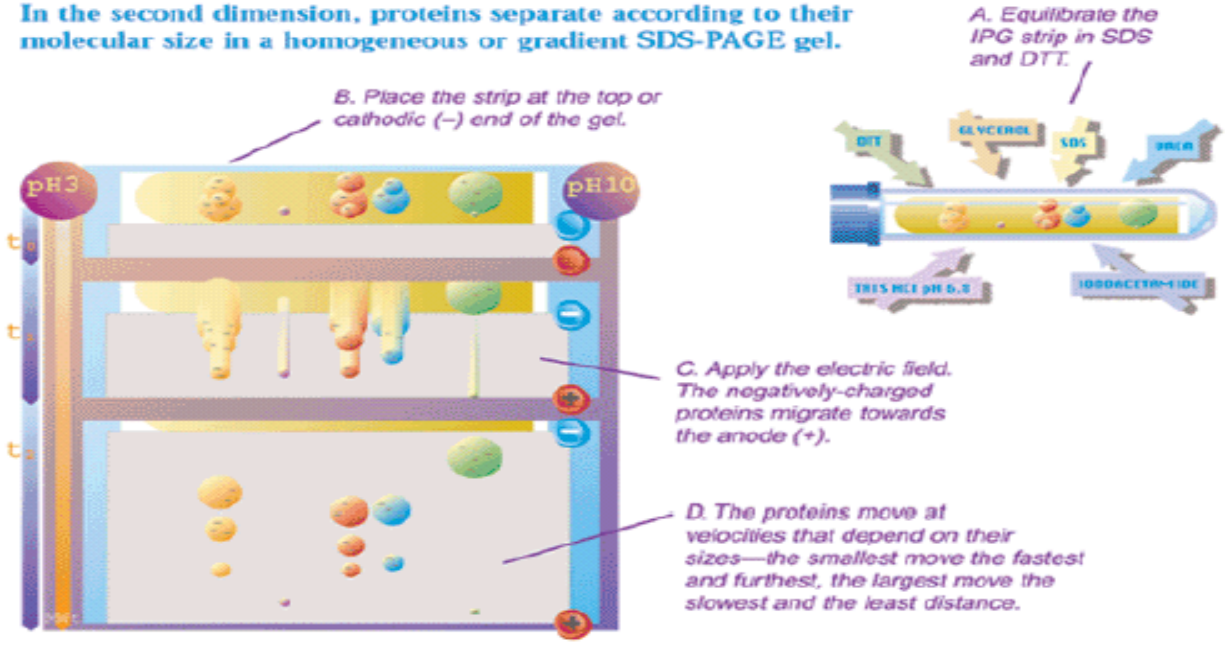
2. Run the first dimension – IEF

In the first dimension, proteins separate by isoelectric point (pI) in the immobilized pH gradient (IPG) of the Immobiline DryStrip gel.



3. Run the second dimension – SDS-PAGE

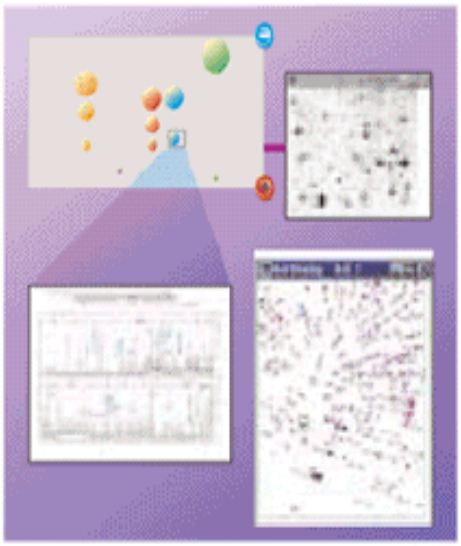
In the second dimension, proteins separate according to their molecular size in a homogeneous or gradient SDS-PAGE gel.



4. Visualize and analyze

Detect separated proteins by autoradiography, staining, or immunodetection after blotting onto a membrane. An array of powerful tools and techniques is available to compare samples and identify proteins of interest:

- Image analysis software to determine spot position and abundance, compare gel images, create databases, and search for patterns;
- Mass spectrometry to determine masses with high precision, peptide fragment fingerprints, amino acid sequences, and nature and site of post-translational modifications.



Challenges for 2-DE

- 1. Spot number:
  - 10,000-150,000 gene products in a cell.
  - PTM makes it difficult to predict real number..
  - It's impossible to display all proteins in one single gel.
- 2. Isoelectric point spectrum:
  - pI of proteins: range from pH 3-13. (by in vitro translated ORF)
  - PTM would not alter the pI outside this range.
  - pH gradient from 3-13 dose not exist.
  - For proteins with pI > 11.5
    - need to be handled separately.

Challenges for 2-DE

- 3. molecular weights:
  - Small proteins or peptides can be analysed by modifying the gel and buffer condition of SDS-PAGE.
- Protein > 250 kDa do not enter 2nd SDS-PAGE properly. 4. hydrophobic proteins:
  - Some very hydrophobic proteins do not go in solution.
- 5. Sensitivity of detection:
  - Low copy number proteins are very difficult to detect, even employing most sensitive staining methods.

Sample preparation

Some important concepts for sample preparation

1. A good sample preparation is the key to good result.
2. The protein composition of the cell lysate or tissue must be reflected in the patterns of 2-DE.
3. Co-analytical modification must be avoided. (pre-purification sometimes leads to CAM)
6. Treatment of sample must be kept to a minimum to avoid sample loss.
    6. Keep sample as cold as possible.
    7. Shorten processing time as short as possible.
    8. Removal of salts
    9. Minimized the unwanted processing, e.g. proteolytic degradation, chemical modification.

Frequently applied treatments

1. Cell washing
2. Cell disruption
3. Removal of contaminant
4. Microdialysis
5. Electrophretic desalting
6. Precipitation methods
7. Treatments for very hydrophobic protein

2. Cell disruption

- Gentle lysis method
  1. Osmotic lysis (cultured cells)
     - Suspend cells in hypo-osmotic solution.
  2. Repeated freezing and thawing (bacteria)
     - Freeze using liquid nitrogen
  3. Detergent lysis (yeast and fungi)
     - Lysis buffer (containing urea and detergent)

  4. Enzymatic lysis (plant, bacteria, fungi)
     - Lysozyme (bacteria)
     - Cellulose and pectinase (plant)
     - Lyticase (yeast)

2. Cell disruption

- Vigorous lysis method
  1. Sonication probe (cell suspension)
     - Avoid overheating
     - French pressure (microorganism with cell wall)
     - Cells are lysed by shear force.
  3. Mortar and pestle (solid tissue, microorganism)
     - Grind solid tissue to fine powder with liquid nitrogen.

  5. Glass bead (cell suspension, microorganism)
     - Using abrasive vortexed bead to break cell walls.

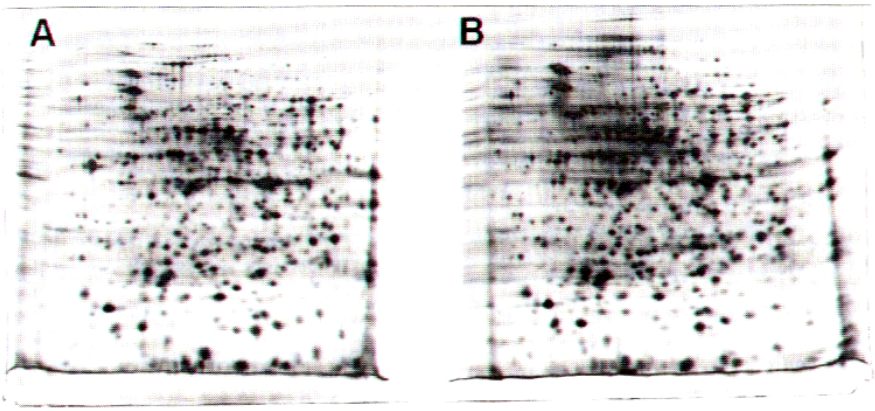
3. Removal of contaminants

- Major type of contaminants:

1. DNA/RNA
2. Lipids
3. polysaccharides
4. Solid material
5. Salt

Precipitation methods.

- Ammonium sulfate precipitation
- TCA precipitation
- Acetone precipitation
- TCA/Acetone precipitation
- Ammonium acetate/method following phenol extraction

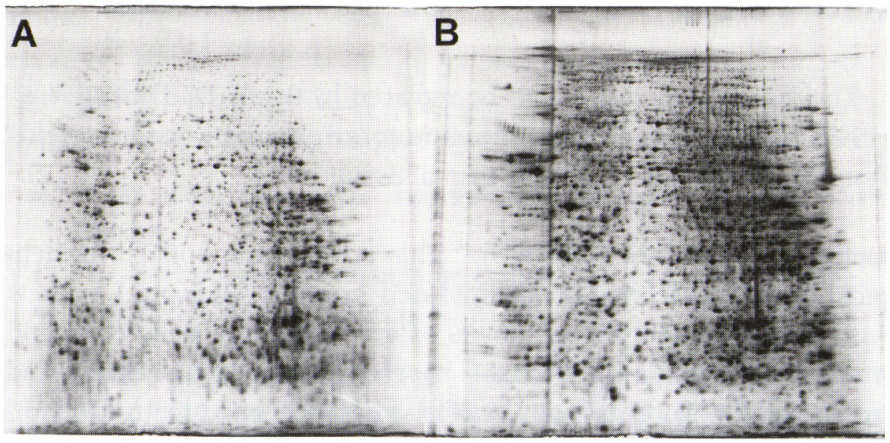


With Acetone precipitation Crude extract by lysis buffer

For very hydrophobic proteins

Membrane proteins do not easily go into solution. A lot of optimization work is required.

1. Thiourea procedure (7M urea + 2M thiourea (Rabilloud, 1998)
2. SDS procedure
3. New zwitterionic detergent and sulfobetains



Lysis buffer, 8M urea

Lysis buffer, 7M urea+ 2M thiourea

New zwitterionic detergent and sulfobetains

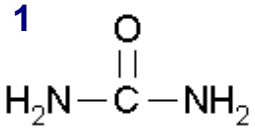
Three major types of detergent

1. Non ionic detergent
   - Triton x-100, Tween 20, Brij-35
2. Ionic detergent
   - SDS, CTAB, Digitonin
3. Zwittergent
   - CHAPS, CHAPSO, Zwittergent 3-08, 3-10, 3-12…

Composition of standard lysis buffer (for IEF)

1. 9M urea
2. 4% CHAPS
3. 1% DTT
4. 0.8% carrier ampholyte
5. 0.02% bromophenol blue.

1



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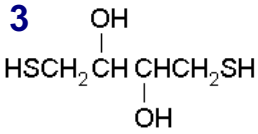

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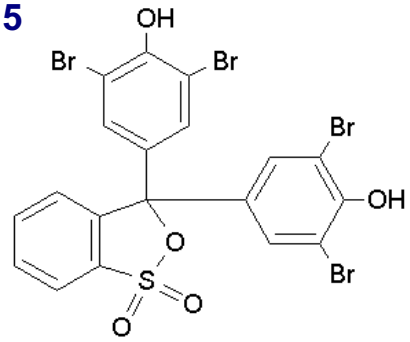

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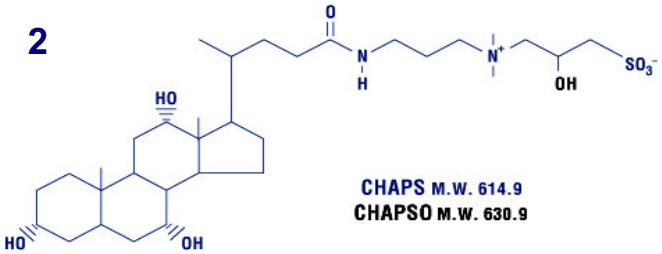
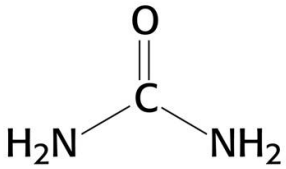
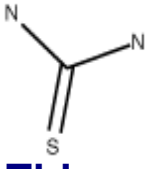


Functions of denaturant (Urea)

1. To convert proteins into single conformation by canceling 2nd and 3rd structure.
2. To keep hydrophobic proteins into solution.
3. To avoid protein-protein interaction.
4. Thio urea: for very hydrophobic proteins only.



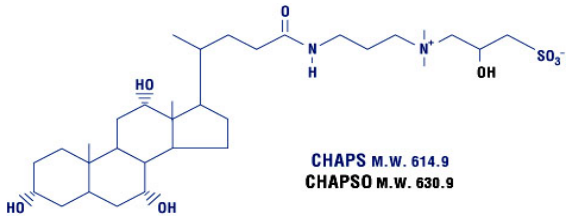
Urea



Thiourea

Functions of detergent (CHAPS)

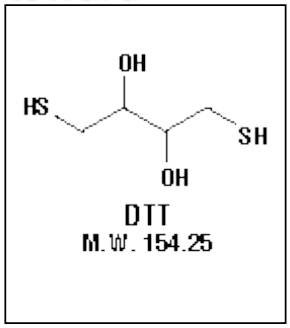
- To Combine all the advantages of polar, sulfobetaine-containing detergents and hydrophobic, bile salt, anionic detergents into a single molecule with superior membrane protein solubilization properties
- Non-denaturing
- Able to disrupt nonspecific protein interactions
- Less protein aggregation than non-ionic detergents
- Electrically neutral
- Easily removed by dialysis



CHAPS M.W. 614.9
CHAPSO M.W. 630.9

Functions of reductant

- To prevent different oxidation steps of proteins.
- 2-mercaptoethanol should not be used because its buffering effect above pH 8.
- Keratin contamination might from 2-mercaptoethanol.
- DTT (dithiothreitol) or DTE (dithioerythritol) are used widely.
- DTT and DTE ionized above pH8. They move toward anode during IEF in basic pH gradient.
- It leads to horizontal streaking at basic area.



DTT
M.W. 154.25

Before running IEF, you should…

- Measure the protein conc. in your samples.

  – Widely used protein assay methods

  1. Biuret
  2. Lowry methods.
  3. Bradford methods.
  4. UV methods.
  5. Other commercial methods.
     1. BCA assay (bicinchoninic acid assay, Pierce)
     2. DC protein assay (detergent compatible, Bio-rad)
     3. DC/RC protein assay (detergent/reducing agent compatible, Bio-rad)

2. Lowry method

– Principle: The reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteay phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids (Try, Try).

– Sensitivity: > 0.1 mg

3. Bradford method

- Principle: The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The Coomassie® dye binds primarily with basic and aromatic side chains. The interaction with arginine is very strong and less strong with histidine, lysine, tyrosine, tryptophan, and phenylalanine. About 1.5 to 3 molecules of dye bind per positive charge on the protein.

- Sensitivity: >10 -100 ug

4. UV methods

- Principle: The aromatic groups (Phe, Tyr, Trp) and the peptide bonds have maximum UV absorbance around 280nm and 200nm. 280nm was used most frequently.

- Interfering substance: anything containing

- Sensitivity: >mg

5. Commercial methods

A. BCA assay (bicinchoninic acid assay, Pierce)

This process is a two-step reaction.

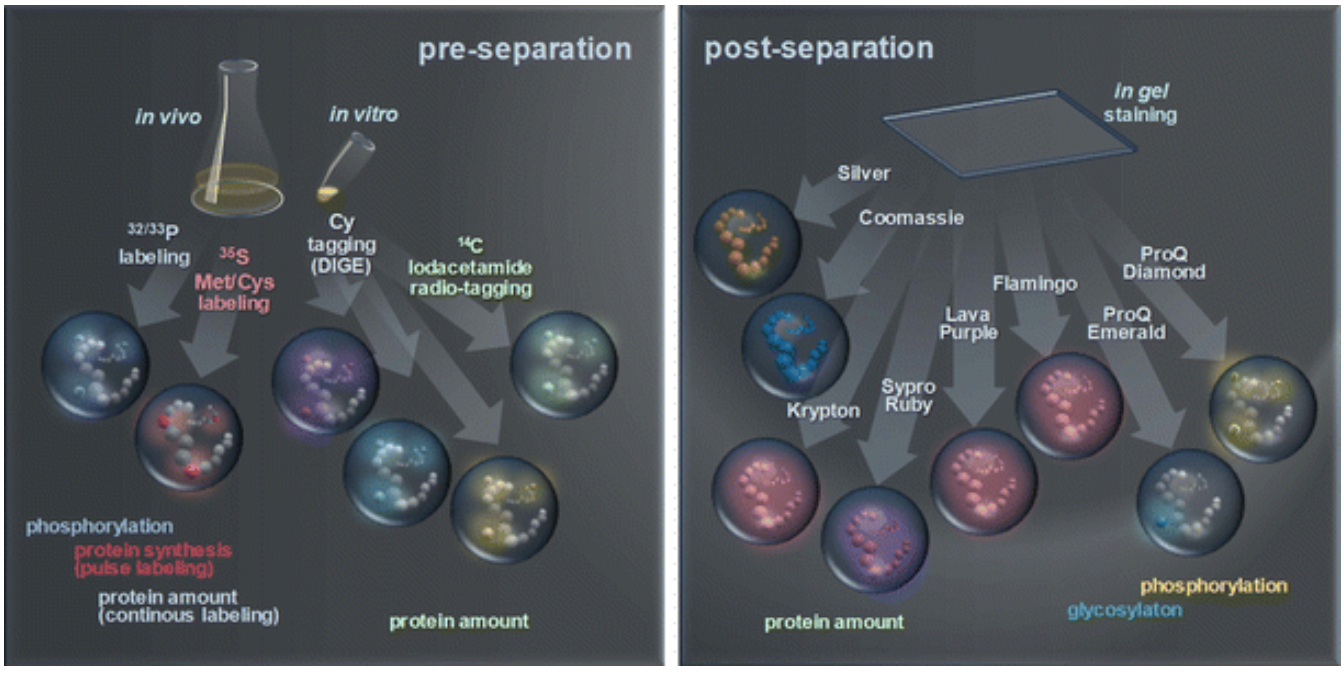
Protein + Cu^{2+} + OH^- ——— Cu^{1+}

Cu^{1+} + 2 BCA ——— Cu^{1+}/BCA chromophore (562 nm).

B. DC protein assay (detergent compatible, Bio-rad)

C. DC/RC protein assay (detergent/reducing agent compatible, Bio-rad)

Labelling of sample



Ref:Berth et al.,Appl Microbiol Biotechnol. 2007;76: 1223

Quantitation Experiment - Labelling

- Label samples in such a way as to not affect subsequent processing but allow differentiation in final analysis. Examples:
  - Fluorescent dyes (2DGE)
  - SILAC amino acid labels (MS)
  - Isobaric mass tags (MS/ MS)

- Process multiple samples simultaneously, differentiate only in final analysis on basis of label.
  - Avoid some proportion of technical variance
  - Best to worst (for avoiding technical variance):
    - Labelling in vivo
    - Labelling protein mixture
    - Labelling peptide digestion mixture

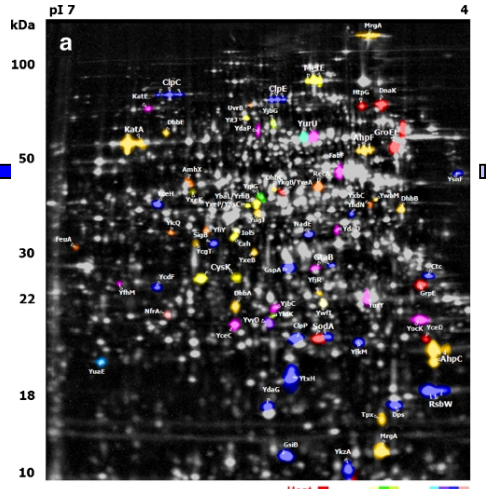
Table 2
The most commonly used dyes in 2-D gels

Ref:Berth et al.,Appl Microbiol Biotechnol. 2007;76: 1223

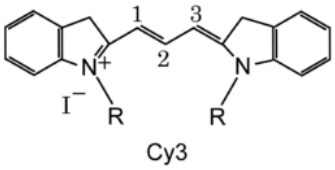
Dye	Principle	Sensitivity	Quantitation	Amount/signal
Coomassie Brilliant Blue	Absorption	Very low	After calibration	Nonlinear
Colloidal Coomassie Blue	Absorption	(very) high	After calibration	Nonlinear
Silver Staining	Absorption	Very high	Impossible	Logistic
Sypro Ruby	Fluorescence	High	Yes	Linear
Ruthenium II tris (bathophenanthroline disulfonate)	Fluorescence	High	Yes	Linear
Flamingo	Fluorescence	High	Yes	Linear
Lava Purple	Fluorescence	High	Yes	Linear
Krypton	Fluorescence	Very high	Yes	Linear

2DGE

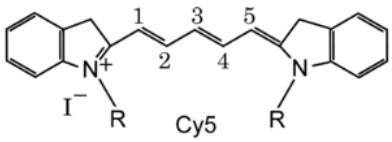
- Separate proteins by isoelectric point, then by mass
- Visualise with silver staining or coomassie
- Use CyDyes to label samples so they can run together on the same gel



Ref:Berth et al.,Appl Microbiol Biotechnol. 2007;76: 1223



Cy3



Cy5

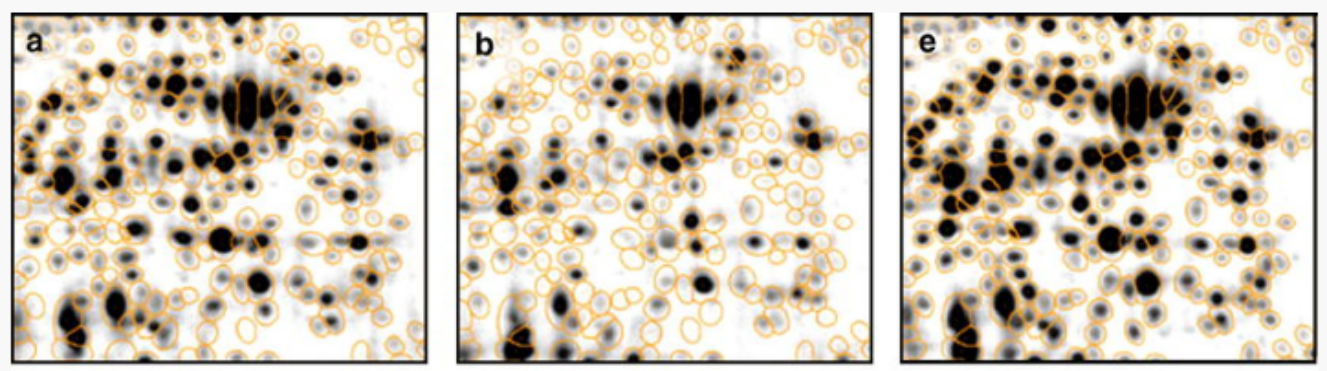
Finally, the overall workflow

Typical workflow of a 2-D-gel-based proteomics analysis

- Performing a biological experiment. The first sample preparation step is freezing the sample in the current state.
- Performing 2-D separation
- A variety of staining techniques can be applied before or after separation to enable spot detection.
- Capturing the gel images by using scanners, charge-coupled device (CCD) camera-based, or laser imaging devices (depending on the protein labelling or staining techniques). The capturing process results in one or more digitized computer images per gel that can be displayed with common image analysis software. The image capture step transforms the quantitative information of the gel into computer-readable data.

Ref: Berth et al.,Appl Microbiol Biotechnol. 2007;76: 1223

- Correction of positional spot variations by image warping. 2-DG results in spot patterns with variations in spot positions between gels.
- gel images are positionally corrected by a combination of global and local image transforms (image warping).



Consensus spot pattern applied to four gel images (a–b), before remodeling of spot shapes. The consensus spot pattern is generated by spot detection on the synthetic fusion image (e) which was computed from the original images

Ref: Berth et al.,Appl Microbiol Biotechnol. 2007;76: 1223

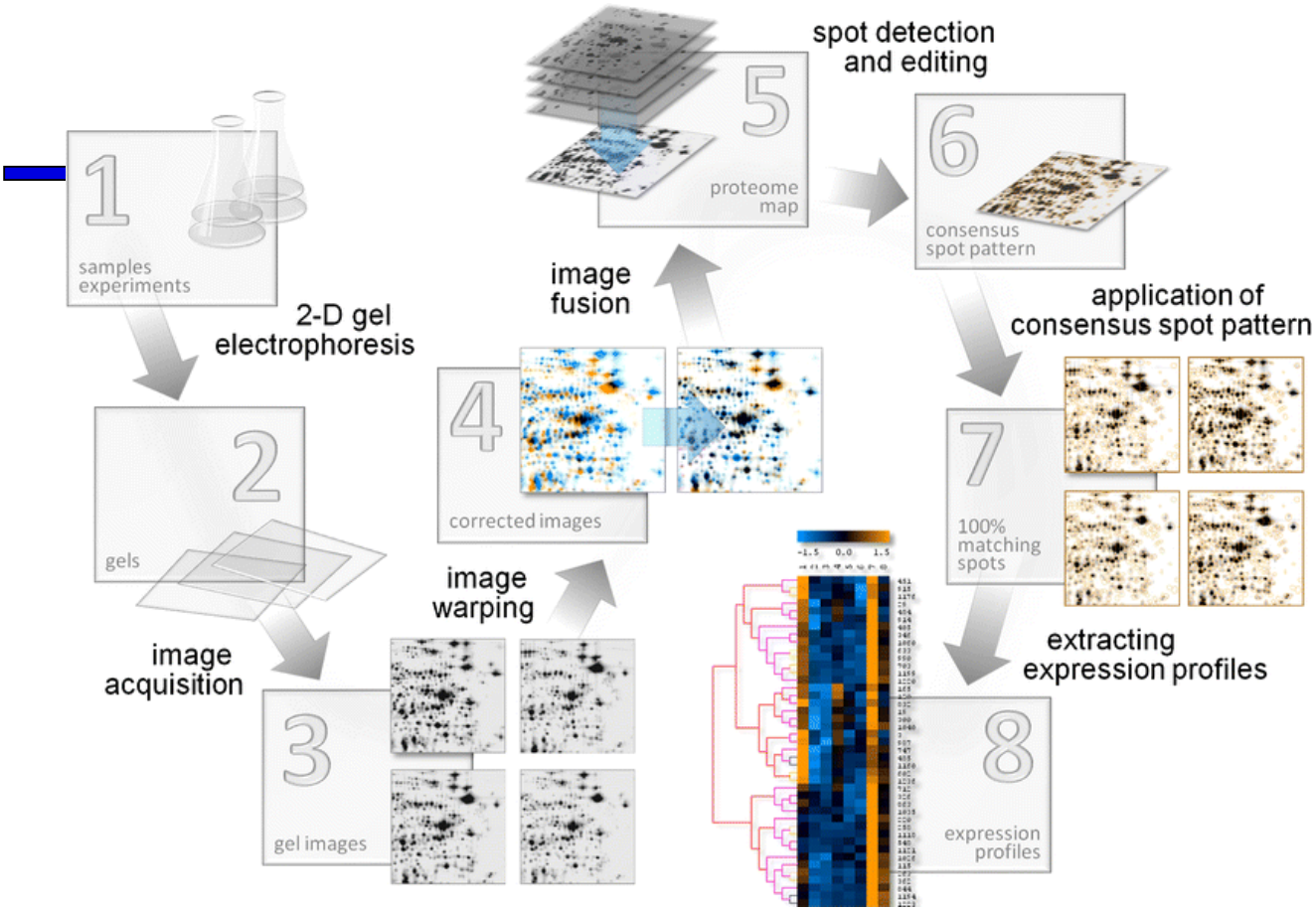
- Image fusion and proteome maps condense the image information of the whole experiment into one fusion image, also called a proteome map.
- Spot detection is performed on the proteome map. As a result, a consensus spot pattern is generated, which is valid for all gels in the experiment. It describes the position and the general shape of all protein spots from the experiment.
- For spot quantitation and building expression profiles, the consensus spot pattern is applied to all gel images of the experiment.
- Expression profile analysis identifies interesting spots which will be marked for further analysis, protein identification, and interpretation.

Ref: Berth et al.,Appl Microbiol Biotechnol. 2007;76: 1223

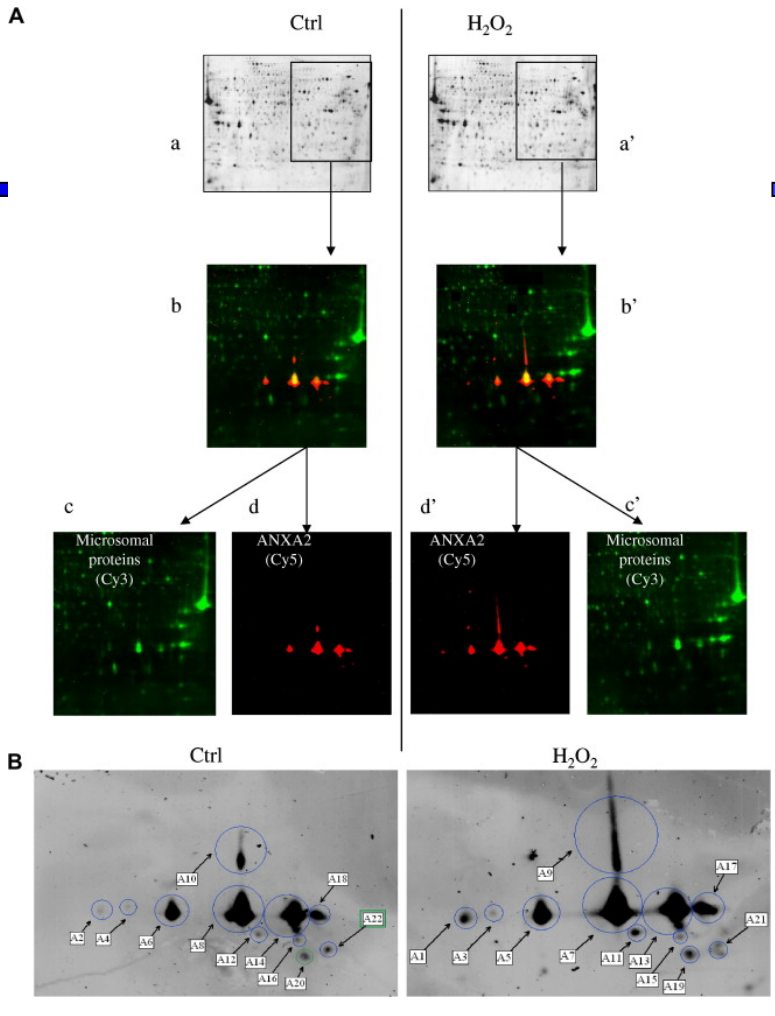
Table 1
Current commercial software products for 2-D gel image analysis

Company	Products
Bio-Rad, Hercules, CA, USA, www.biorad.com	PDQuest, ProteomWeaver
Compugen, Tel Aviv, Israel, www.compugen.com	Z3 (discontinued)
DECODON, Greifswald, Germany, www.decodon.com	Delta2D
GE Healthcare, www.gelifesciences.com	Decyder 2D, ImageMaster Platinum*
Genebio, Geneva, Switzerland, www.genebio.com	*Melanie (ImageMaster Platinum)
Nonlinear Dynamics, Newcastle, UK, www.nonlinear.com	Progenesis, SameSpots
Syngene, Cambridge, UK, www.syngene.com	Dymension

Ref: Berth et al.,Appl Microbiol Biotechnol. 2007;76: 1223



Ref: Berth et al.,Appl Microbiol Biotechnol. 2007;76: 1223



Chrétien et al., Upregulation of annexin A2 in H2O2-induced premature senescence as evidenced by 2D-DIGE proteome analysis Experimental Gerontology, 2008, 43:353

Learning outcome

- From this lecture you should understand:
  - Reasons behind studying proteomics
  - The principle of 2D gel
    - Different steps in sample preparation
    - Examples of detergents and reducing agents

References

- Angelika Gorg 1988, Electrophoresis, vol 9, p 531
- Bjellqvist 1982, Biochem. Biophys. Methods, vol 6, p317
- Chrétien et al. Upregulation of annexin A_2 in H_2O_2-induced premature senescence as evidenced by 2D-DIGE proteome analysis. Experimental Gerontology, 2008, 43:353
- Rabilloud and Lelong. Two-dimentional gel electrophoresis in proteomics: A tutorial. 2011, J. Proteomics 74:1829
- Berth et al., The state of the art in the analysis of 2D gel electrophoresis images. Appl Microbiol Biotechnol. 2007;76: 1223