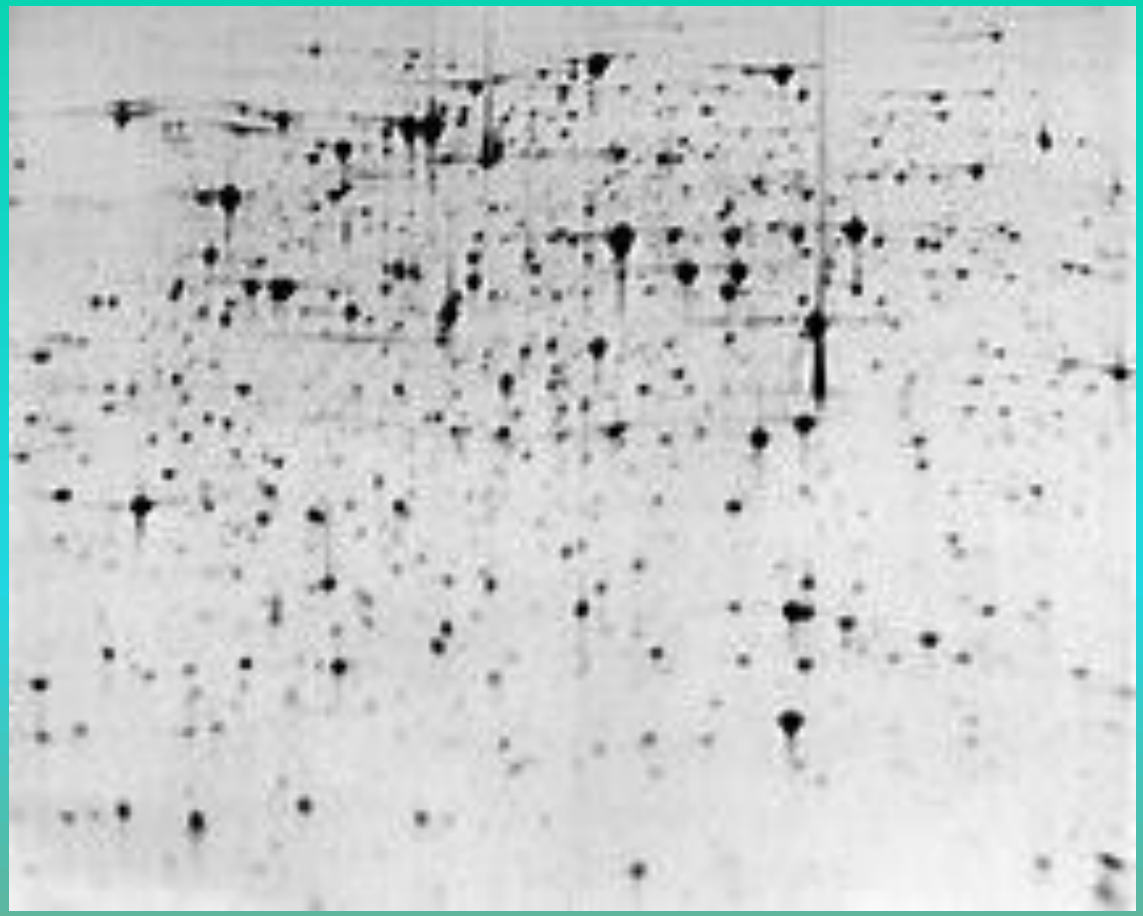
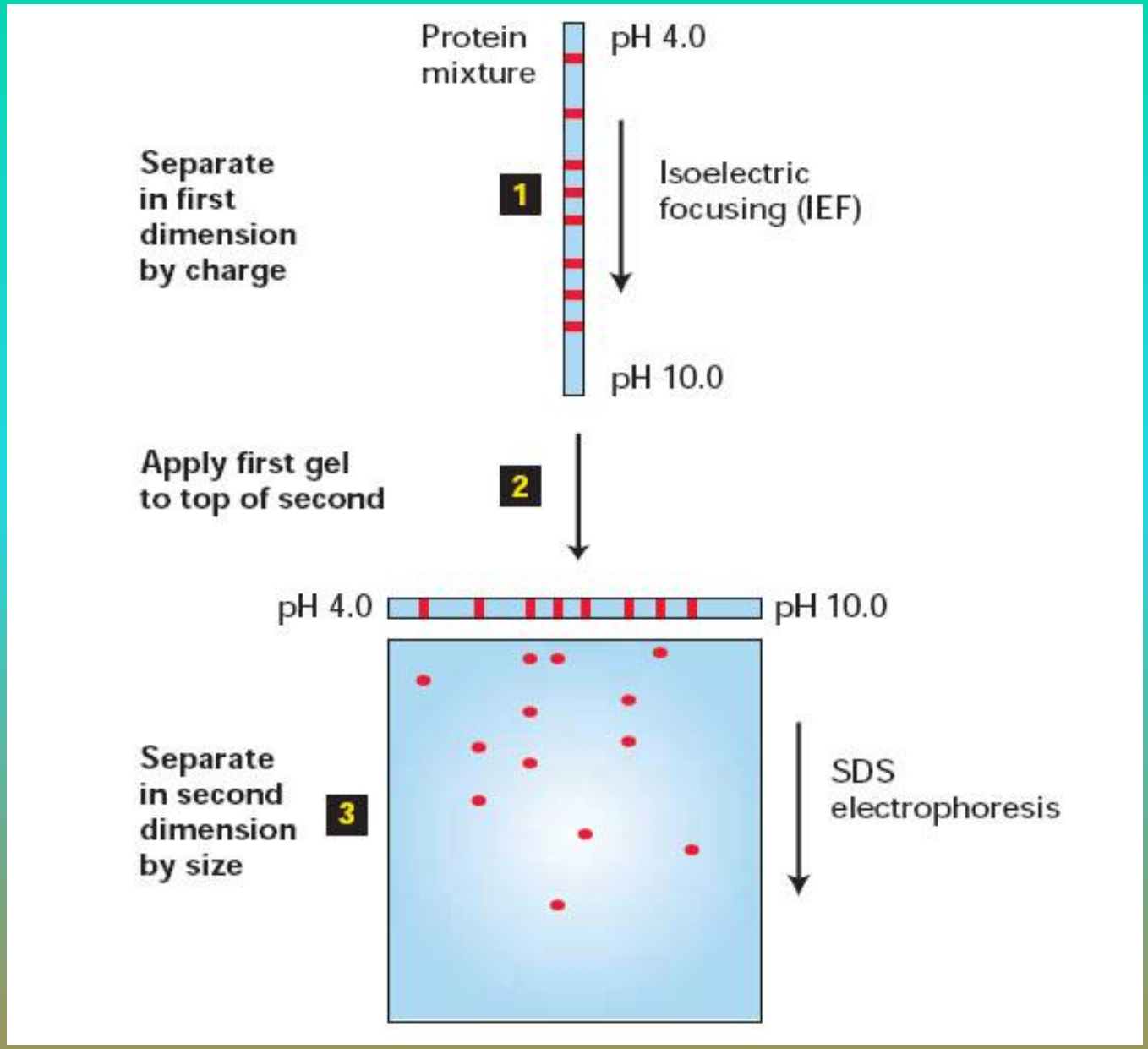
Instrumentation and Biotechniques, DSE-7, Sem VI

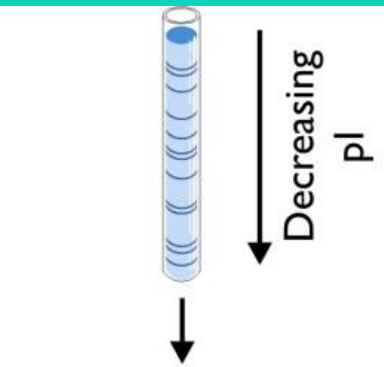


2D GE

SH

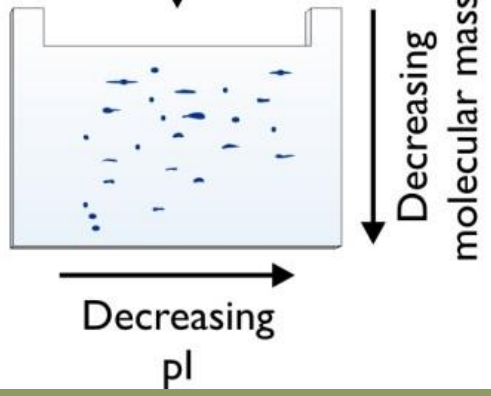

Protein mixture
pH 4.0
Separate in first dimension by charge
1
Isoelectric focusing (IEF)
pH 10.0
Apply first gel to top of second
2
pH 4.0
pH 10.0
Separate in second dimension by size
3
SDS electrophoresis

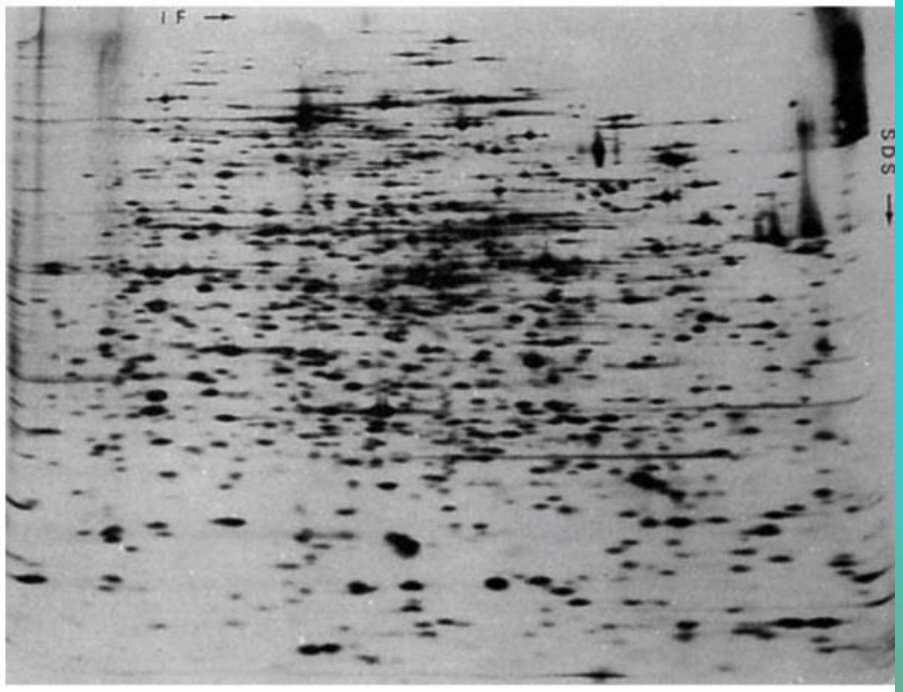
1. Separation of proteins by pI value



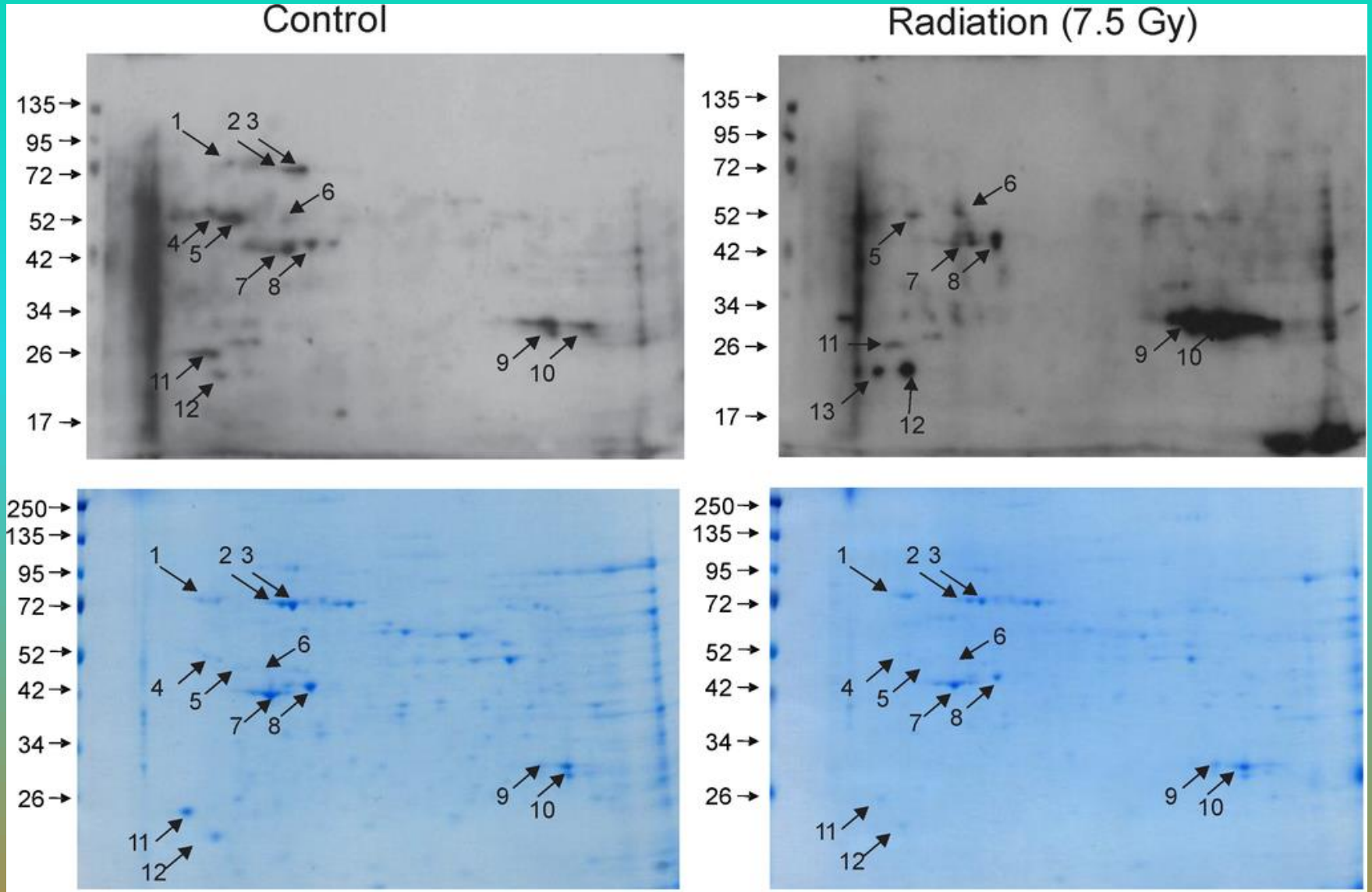
2. Soaking the gel in SDS solution and fitting it on an SDS PA gel

3. Separating the proteins by molecular mass with SDS PAGE





Approximately 1000 *E. coli* proteins on a single 2D gel


Control
Radiation (7.5 Gy)
135
95
72
52
42
34
26
17
250
1
2 3
4
5
6
7
8
9
10
11
12
13

Introduction

- Conventional electrophoresis techniques can separate up to 100 different proteins on one run.
- Typically, cell or tissue extracts contain thousands of proteins, most of which will not be resolved into single bands using a separation based on any one parameter, such as size or net charge.
- For any one size range, there is a high probability of more than one protein (out of thousands) falling into this range.
- Separation on the basis of two parameters, usually size and isoelectric point, lowers the probability that two proteins will overlap, and allows the resolution of thousands of protein species on one gel.

2D gel electrophoresis

- Introduced by O'Farrell and Klose in 1975.
- p(I)/ pH(I)
- Isoelectric point (IEP)
- 2-D PAGE provides the highest resolution separation method for proteins. Following a first dimension IEF, proteins are subjected to SDS-PAGE in a perpendicular direction.
- The technique is a true orthogonal procedure in that the two separation mechanisms are based on different physical principles and the two separations are done at right angles to one another (they are geometrically orthogonal).

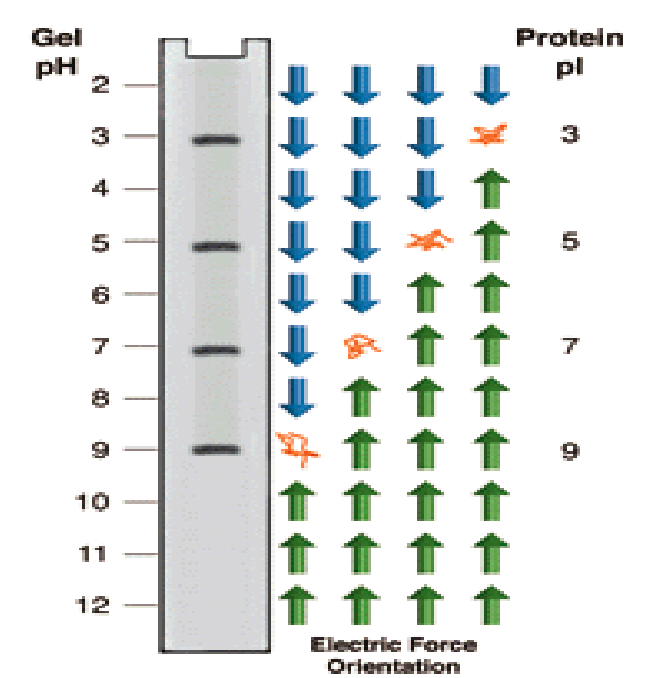
STEPS of 2-D electrophoresis

- Protein sample preparation
- First-dimension electrophoresis
- Second-dimension electrophoresis
- Gel staining and protein visualization
- Gel imaging and protein analysis
- Protein identification

IEF

- IEF, also known simply as electrofocusing, is a technique for separating charged molecules, usually proteins or peptides, on the basis of their isoelectric point (pI), i.e., the pH at which the molecule has no charge.
- IEF works because in an electric field molecules in a pH gradient will migrate towards their pI.

IEF



Isoelectric focusing employs a pH gradient extending the length of an electrophoresis gel.

A protein stops migrating when it enters the zone in which the surrounding pH equals its isoelectric point, pI.

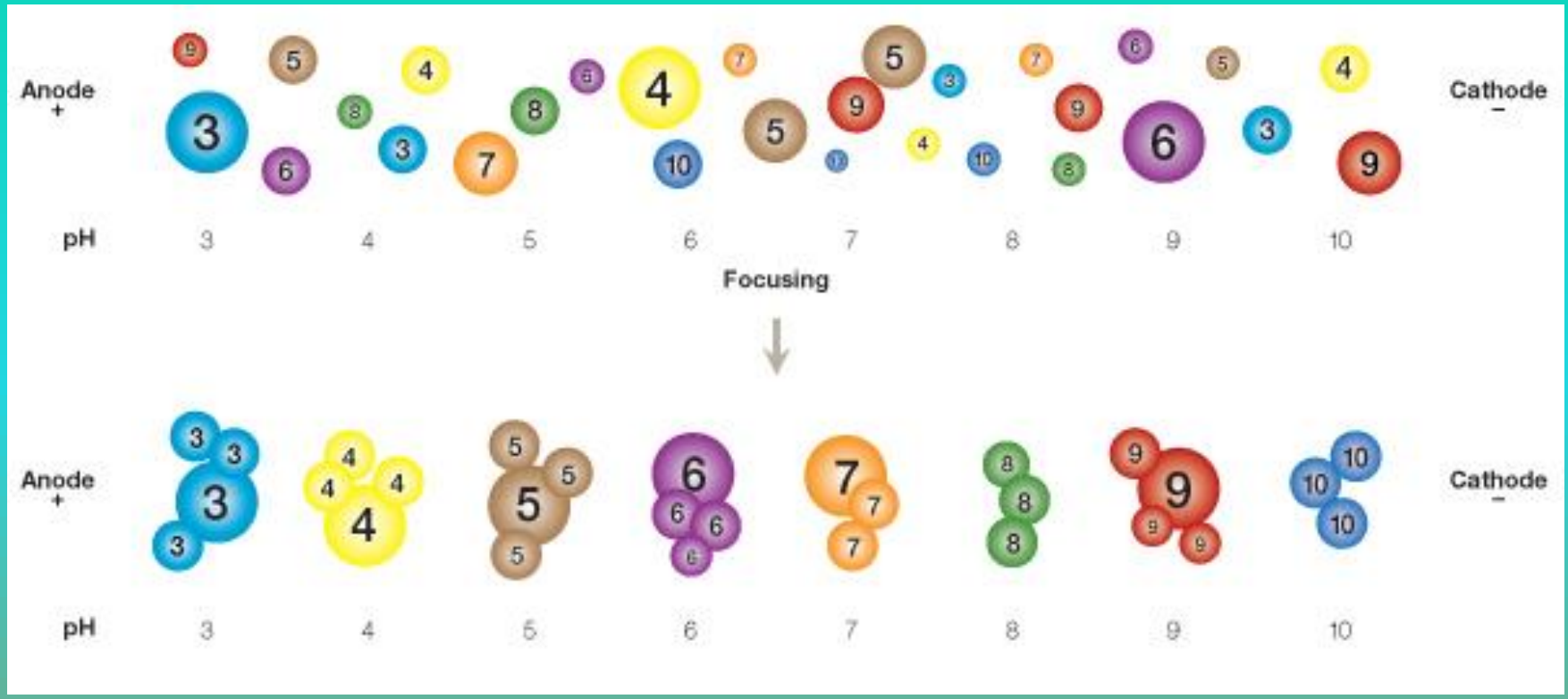
At any other point in the gradient, the protein acquires a charge which causes it to migrate toward its pI (green and blue arrows).

IEF

- A commercially available immobilized pH gradient (IPG) strip is used.
- The IPG strip consists of an acrylamide gel that contains wide pores to prevent a **sieving effect** based on protein mass, with a pH gradient.
- Various gradients are available,
- wider gradients: For eg: pH 3-10 are used for whole proteome analysis,
- Narrower ranges: (like pH 5-8) are used for more specialist applications.

Ampholytes

- The pH gradient in IEF gels is generated by the inclusion of ampholytes, low molecular weight amphoteric molecules.
- A mixture of ampholytes is used, each having a different pI.
- Like protein molecules, the ampholytes migrate through the gel until they reach a region where the pH is equal to their pI.
- Unlike the proteins, the ampholytes are present in high enough concentration to change their local pH.
- The gel is set up with a uniform mixture of ampholytes throughout, and its anodic and cathodic ends are immersed in dilute acid and base respectively.
- Ampholytes near the ends of the gels will be positively charged near the positive electrode, and negatively charged near the negative electrode. They therefore begin to migrate into the gel, with the most charged (ie the ones furthest from their pI) moving the fastest. Over time they separate into zones of defined pH. If the ampholyte system is well designed, a smooth gradient of pH is created, with no abrupt charges, or "steps."


Anode
+
Cathode
−
pH
3
4
5
6
7
8
9
10
Focusing
Anode
+
Cathode
−
pH
3
4
5
6
7
8
9
10

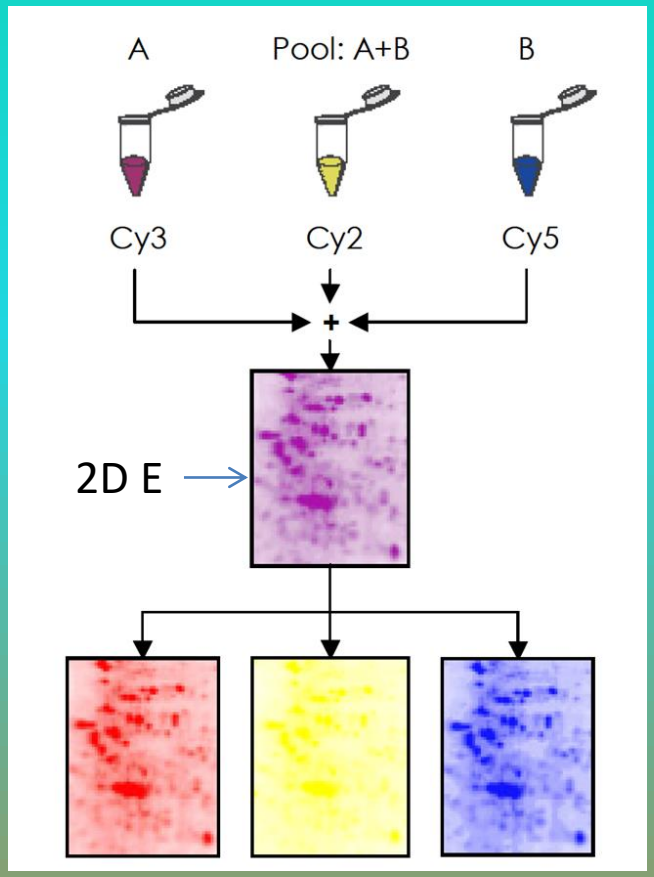
- Various mixtures of amphoteric substances have been used as ampholytes: amino acids, proteins, and poly acidic poly basic synthetic molecules.
- Natural amino acids have poor conductivity and poor buffering capacity in their zwitterionic state, making them poor candidates.
- Proteins can be good ampholytes, but they interfere with analysis of the sample, by introducing new proteins into the mixture.
- Polycarboxylic acid polyamines are the most commonly used ampholytes.
  - have excellent buffering capacity & conductivity across a broad pH range,
  - Usually provided in a molecular weight range of 300 - 500, which is small enough to avoid interference with most subsequent processing.
- Their sole disadvantage is that they may bind tightly to the proteins, due to ionic interactions, and can be very difficult to remove.

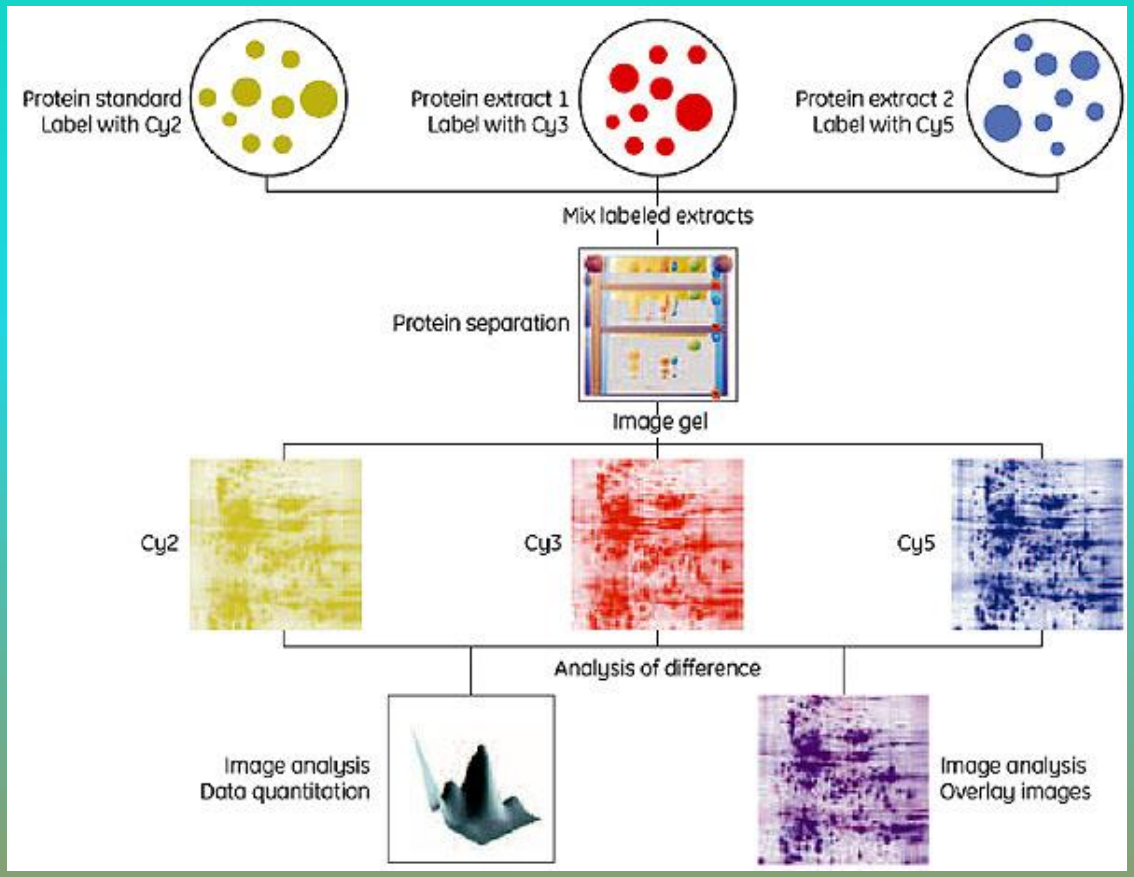
Ampholytes

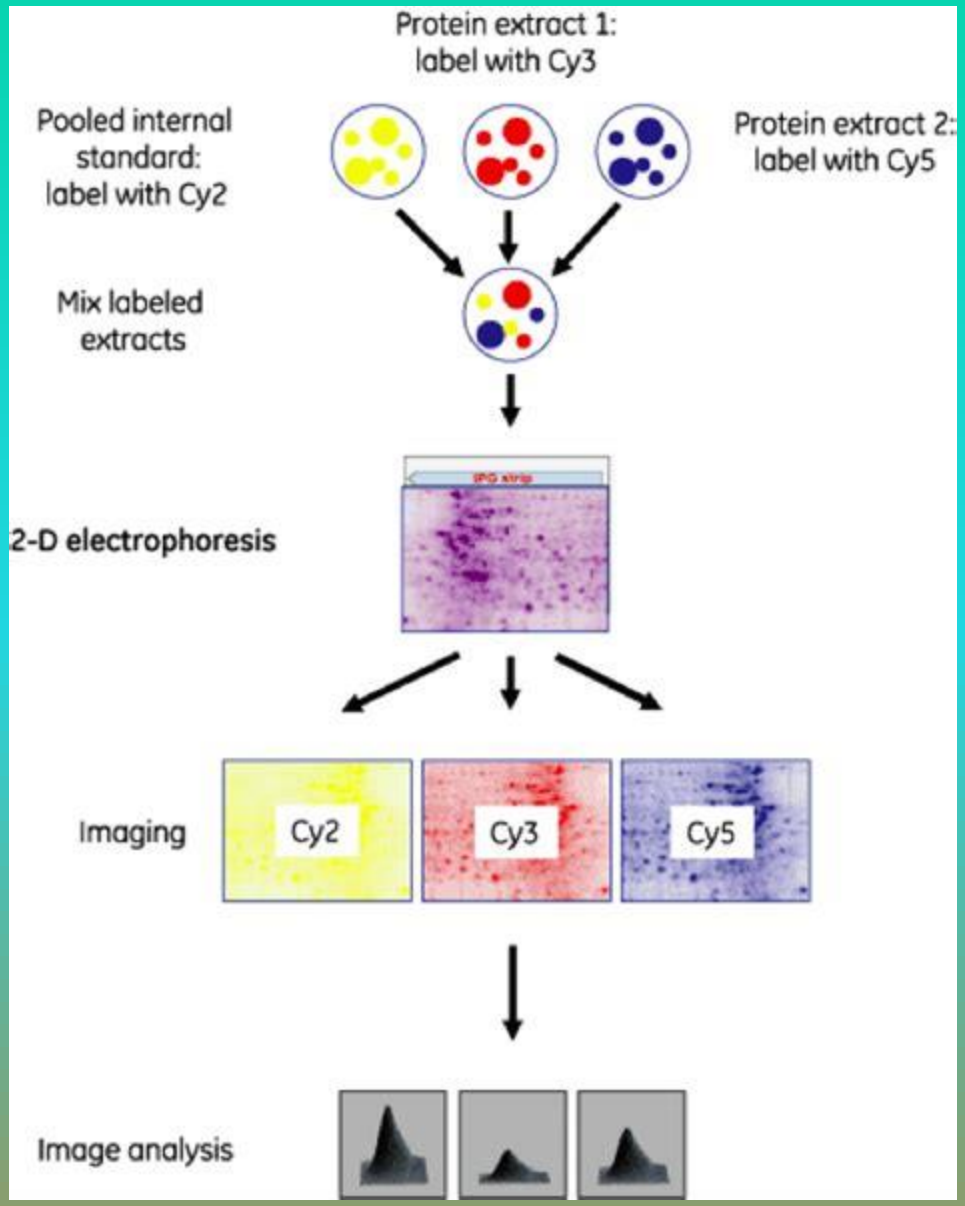
- The sample is usually combined with carrier ampholytes to assist in migration.
- Ampholytes are a mixture of charged molecules with a range of pIs that matches the pI range of the IPG strip.
- The migration of the ampholytes encourages the sample molecules to move along the pH gradient.
- Commercial ampholyte mixtures of a variety of pI ranges are available.
- After separation across the pH gradient, the sample is further separated (in 2D-PAGE) or analyzed (in the case of fractionation for mass spec).

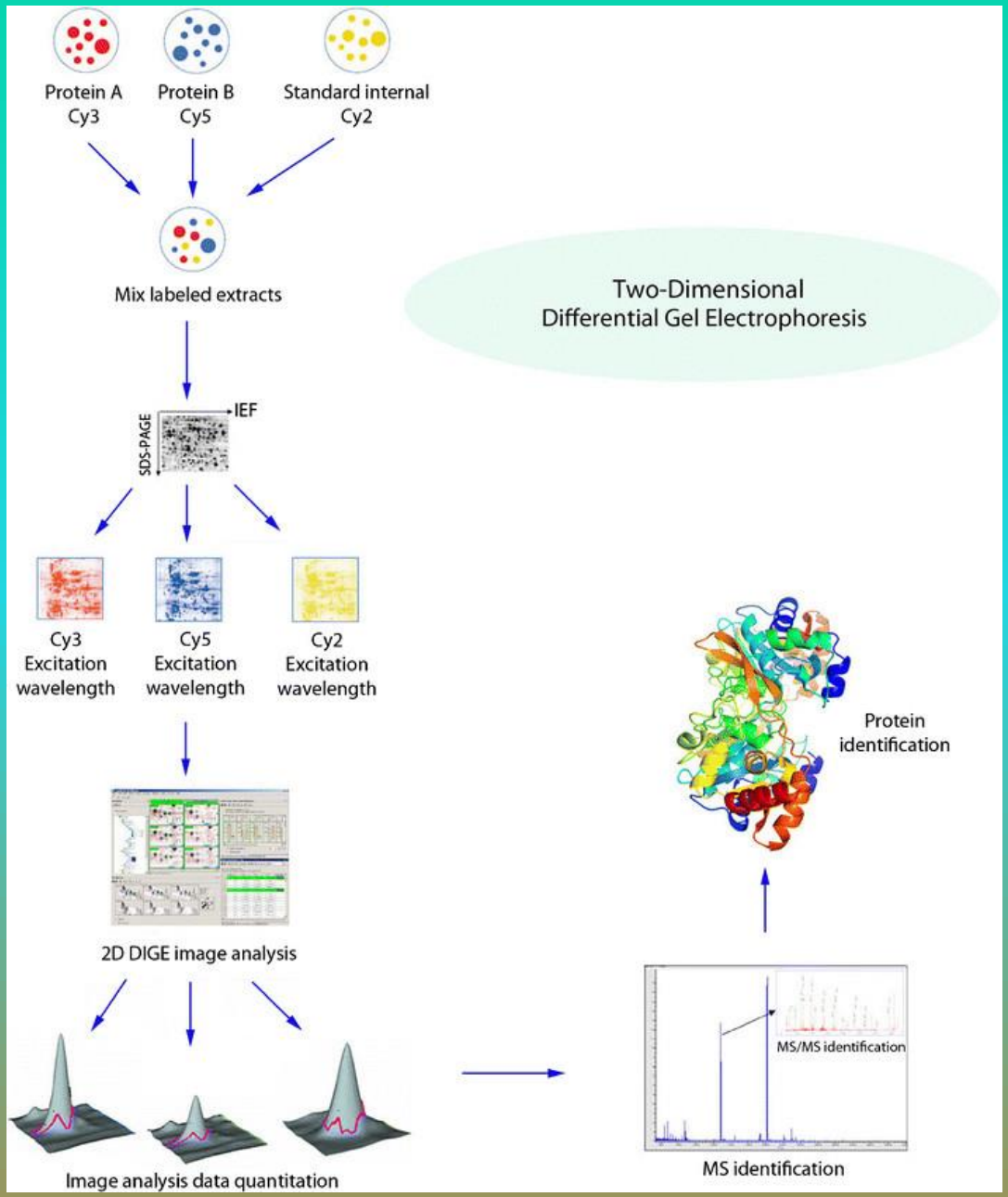
Application of 2D-GE

- To study the entire proteome of an organelle or bacterium.
- To identify and determine differential abundance of proteins (for various downstream applications like Biomarker discovery, Product characterization (Antibodies and drugs in pharma production/batch to batch variations etc.)
- To examine post-translation modifications (PTMs) of proteins (phosphorylation, methylation, glycosylation and acetylation etc).
- Fractionation of proteins or peptides prior to mass spectophotometry


A
Pool: A+B
B
Cy3
Cy2
Cy5
2D E


Protein standard
Label with Cy2
Protein extract 1
Label with Cy3
Protein extract 2
Label with Cy5
Mix labeled extracts
Protein separation
Image gel
Cy2
Cy3
Cy5
Analysis of difference
Image analysis
Data quantitation
Image analysis
Overlay images


Protein extract 1:
label with Cy3
Pooled internal
standard:
label with Cy2
Protein extract 2:
label with Cy5
Mix labeled
extracts
2-D electrophoresis
Imaging
Cy2
Cy3
Cy5
Image analysis


Protein A
Cy3
Protein B
Cy5
Standard internal
Cy2
Mix labeled extracts
Two-Dimensional
Differential Gel Electrophoresis
IEF
SDS-PAGE
Cy3
Excitation
wavelength
Cy5
Excitation
wavelength
Cy2
Excitation
wavelength
Protein
identification
2D DIGE image analysis
MS/MS identification
Image analysis data quantitation
MS identification

Applications DIGE

- Introduced by Minden et al 1997
- widely adopted in most areas of biology and medicine.
- DIGE has become an important tool among proteomics technologies for studying the mechanisms of disease, pinpointing new therapeutic targets or finding potential biomarkers.
- DIGE has been applied to cancer research, renal physiology, plant biology and the elucidation of signal transduction pathways.