Mass Spectrometry-based Proteomics Workshop

October 20th and 21st 2009

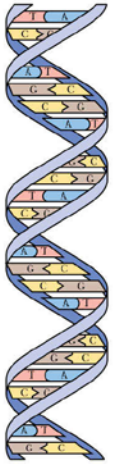
Supported by NSF Plant Genome Program
Instruction: Drs. Joss Rose and Ted Thannhauser and Sheng Zhang
Location: Cornell Proteomics & Mass Spectrometry Core Facility

Proteomics…

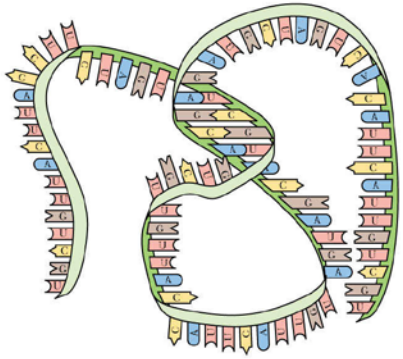
Systematic isolation, visualization and characterization of protein populations
….organisms, tissues, cell types, organelles...

The “-omics” family

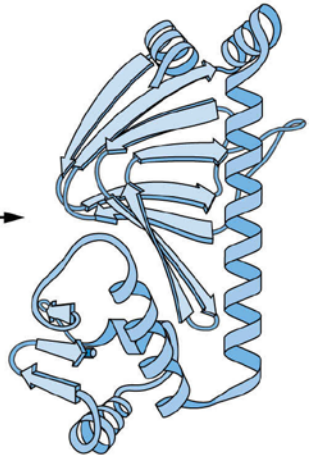
DNA



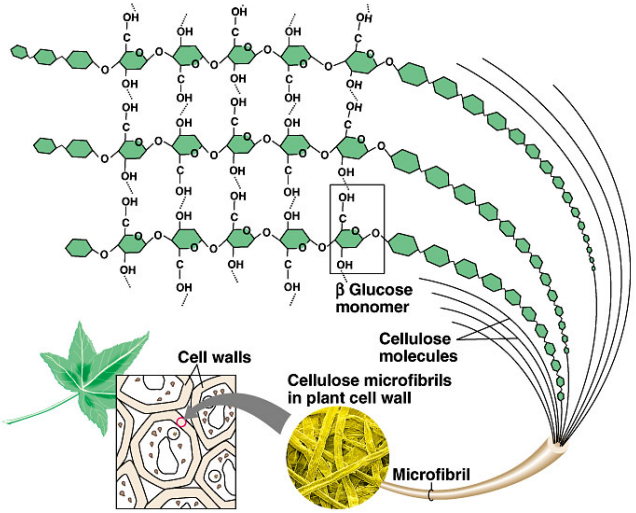
RNA



Protein

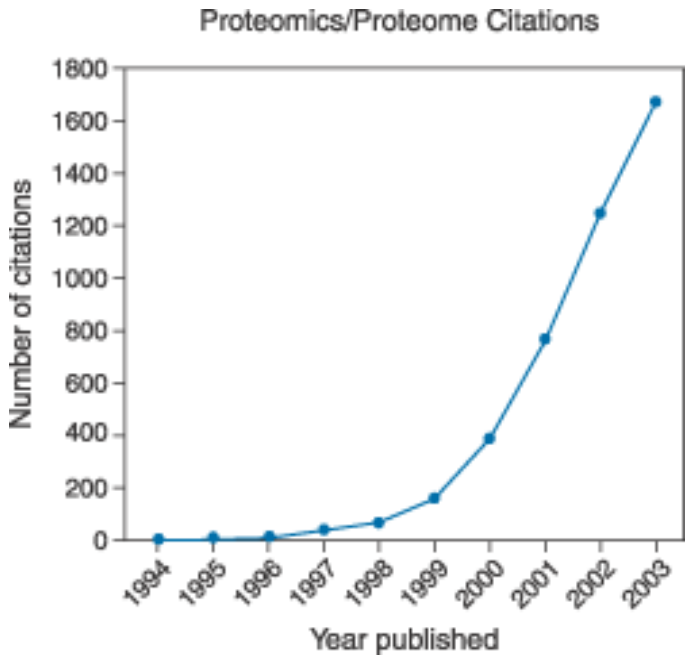


Metabolite



Amino acid	3-Letter code	1-Letter code	Codons
Alanine	Ala	A	GCC, GCU, GCG, GCA
Arginine	Arg	R	CGC, CGG, CGU, CGA, AGA, AGG
Asparagine	Asn	N	AAU, AAC
Aspartic acid	Asp	D	GAU, GAC
Cysteine	Cys	C	UGU, UGC
Glutamic acid	Glu	E	GAA, GAG
Glutamine	Gln	Q	CAA, CAG
Glycine	Gly	G	GGU, GGC, GGA, GGG
Histidine	His	H	CAU, CAC
Isoleucine	Ile	I	AUU, AUC, AUA
Leucine	Leu	L	UUA, UUG, CUA, CUG, CUU, CUC
Lysine	Lys	K	AAA, AAG
Methionine	Met	M	AUG
Phenylalanine	Phe	F	UUC, UUU
Proline	Pro	P	CCU, CCC, CCA, CCG
Serine	Ser	S	UCU, UCC, UCA, UCG, AGU, AGC
Threonine	Thr	T	ACU, ACC, ACA, ACG
Tyrosine	Tyr	Y	UAU, UAC
Tryptophan	Trp	W	UGG
Valine	Val	V	GUU, GUC, GUA, GUG
“Stop”	—	—	UAA, UAG, UGA

Biological Function


Proteomics/Proteome Citations
Number of citations
1800
1600
1400
1200
1000
800
600
400
200
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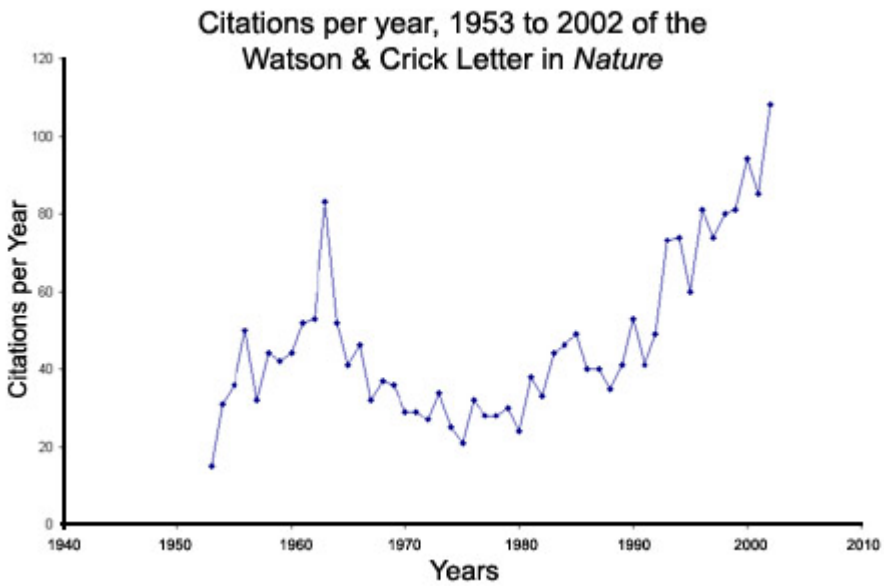
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
Year published


Citations per year, 1953 to 2002 of the
Watson & Crick Letter in *Nature*
Citations per Year
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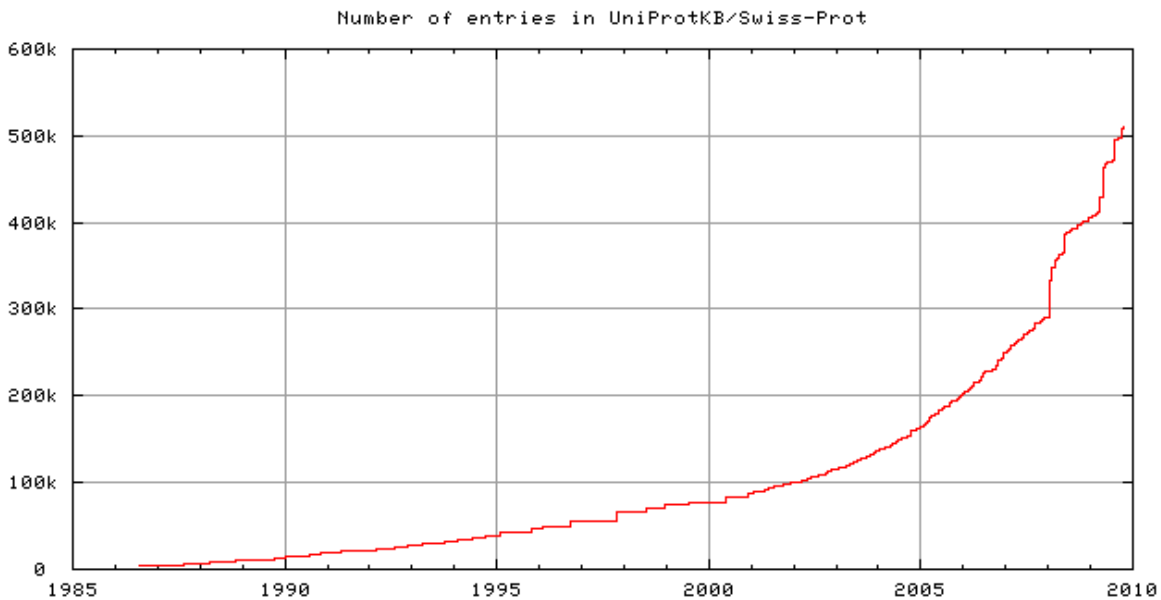
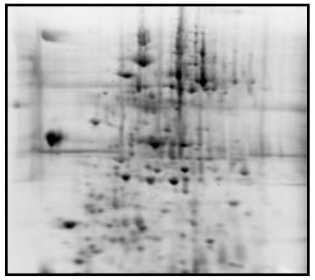
100
80
60
40
20
0
1940
1950
1960
1970
1980
1990
2000
2010
Years


Number of entries in UniProtKB/Swiss-Prot
600k
500k
400k
300k
200k
100k
0
1985
1990
1995
2000
2005
2010

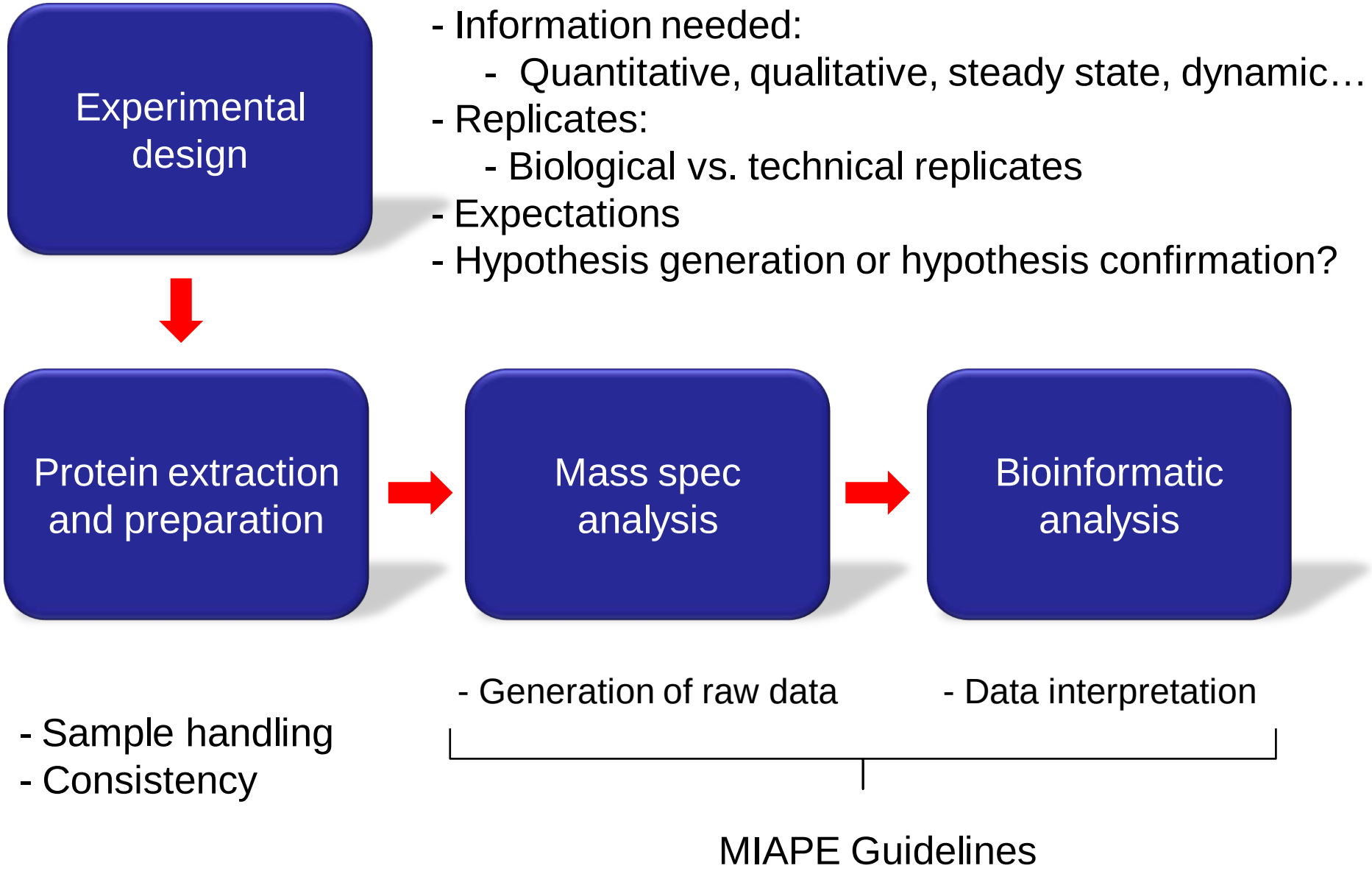
Major Developments in Proteome Analysis

- Protein Extraction and Fractionation



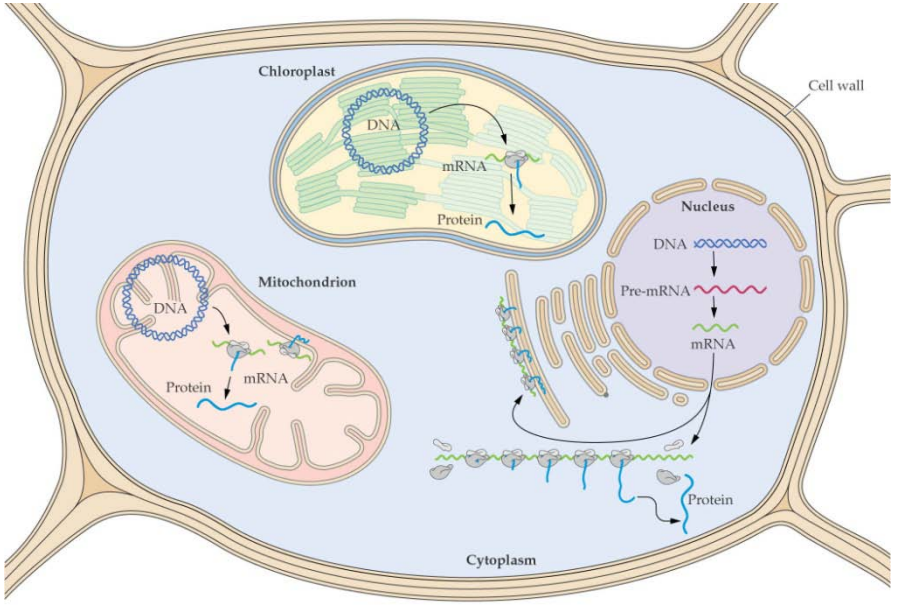
- Mass Spectrometry

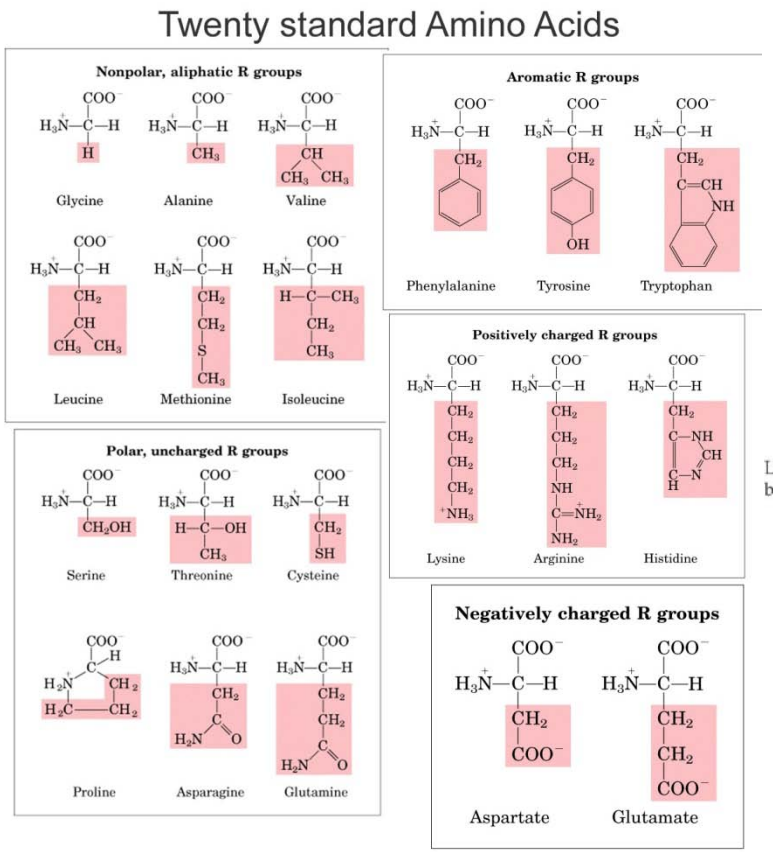
- Bioinformatics

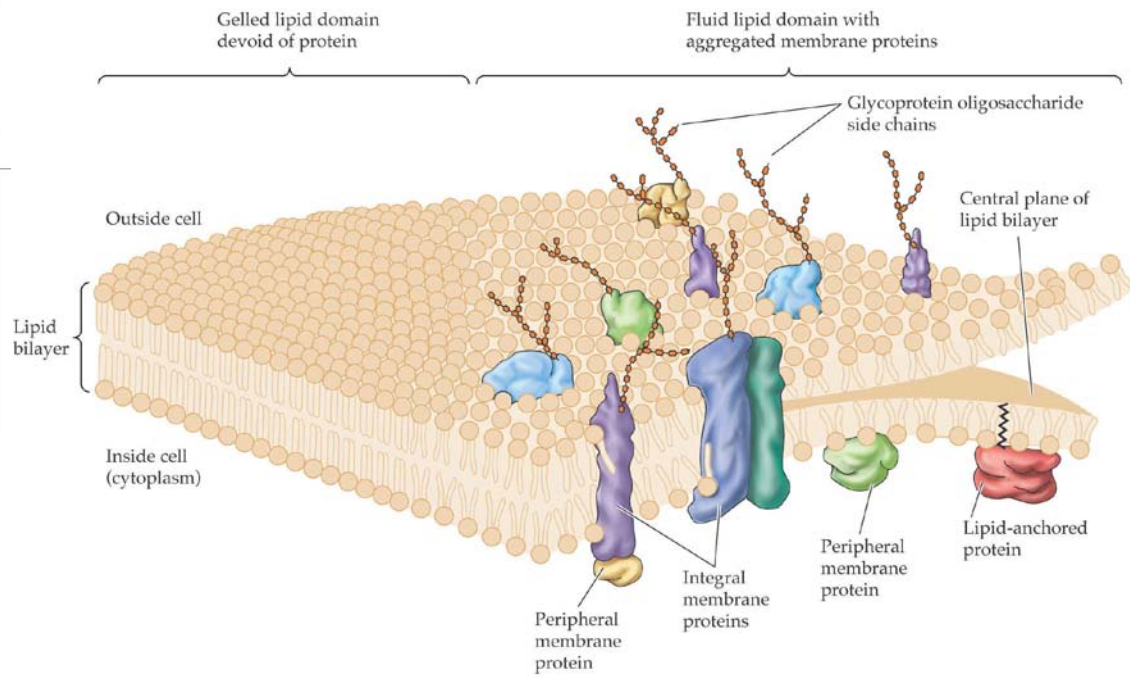

Experimental design
- Information needed:
- Quantitative, qualitative, steady state, dynamic…
- Replicates:
- Biological vs. technical replicates
- Expectations
- Hypothesis generation or hypothesis confirmation?
Protein extraction and preparation
Mass spec analysis
Bioinformatic analysis
- Sample handling
- Consistency
- Generation of raw data
- Data interpretation
MIAPE Guidelines

- Protein Extraction and Analysis

-Biochemical heterogeneity/structural variability: solubility, charge, hydophobicity, size
-Stability and contaminants
-Abundance limitations. Non-amplifiable (no protein PCR!)
-Temporally and spatially complex (different tissues and cell types), native vs. denaturing

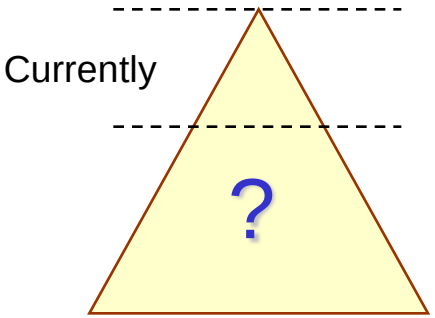




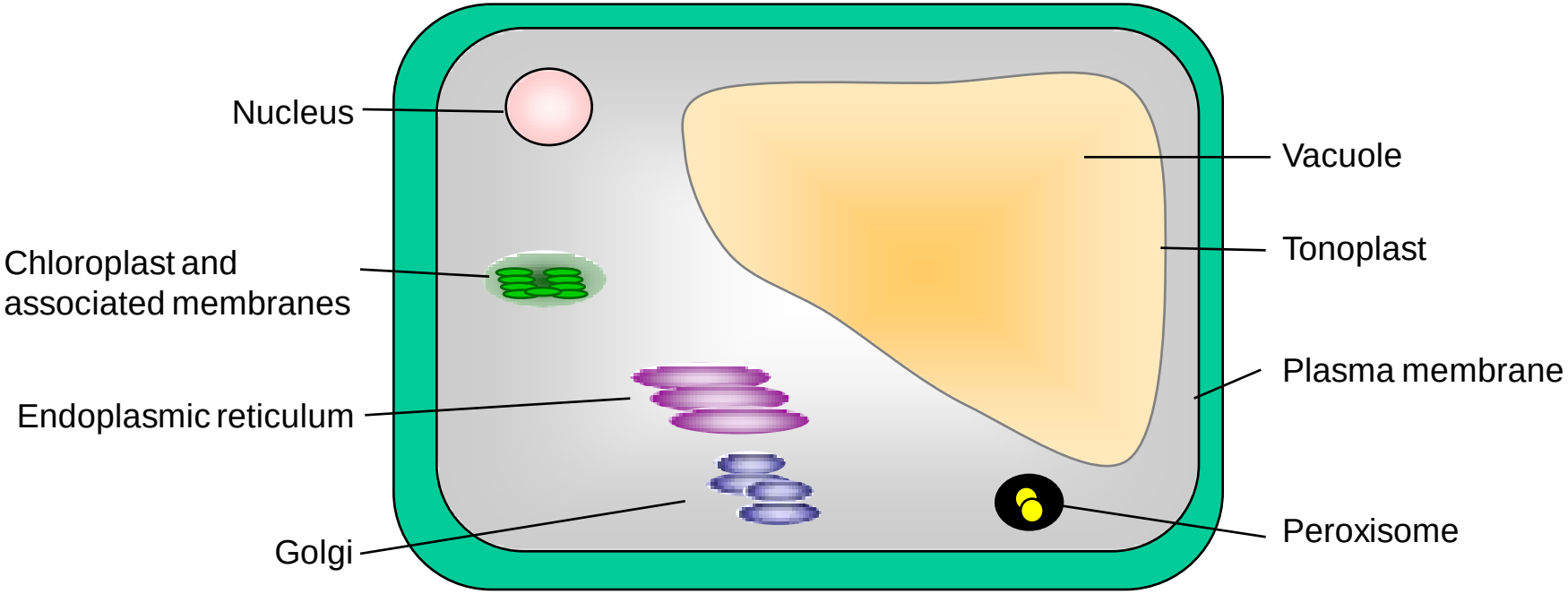


Increasing the proportion of the detectable proteome

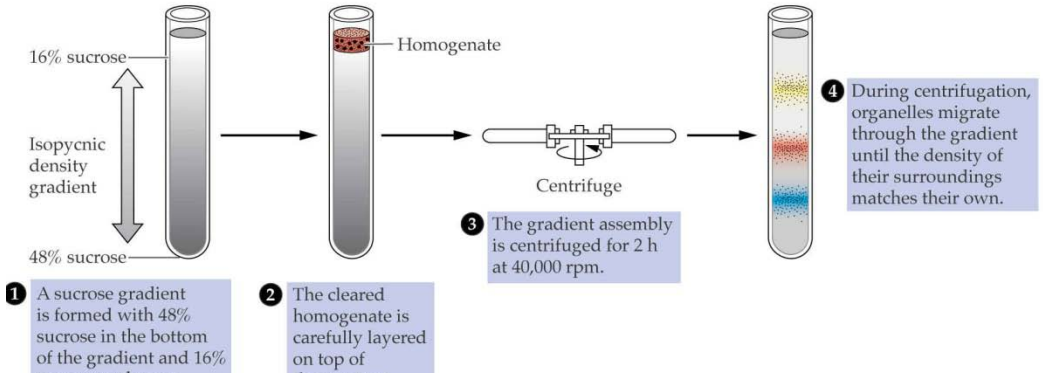
- Biochemical pre-fractionation….liquid chromatography
- Biological pre-fractionation…organs, tissues, cell types, organelles

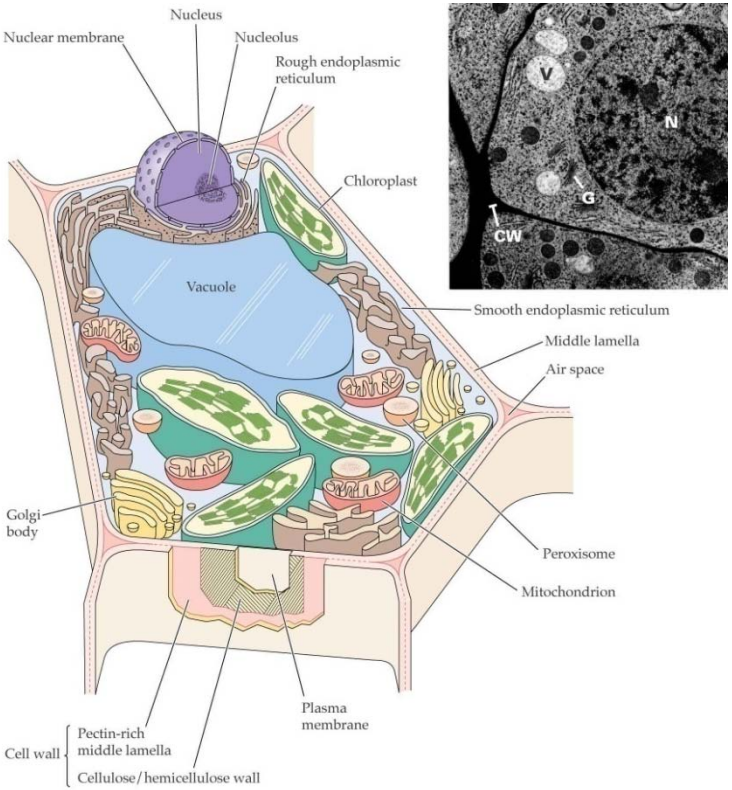


Subcellular Proteomics Projects

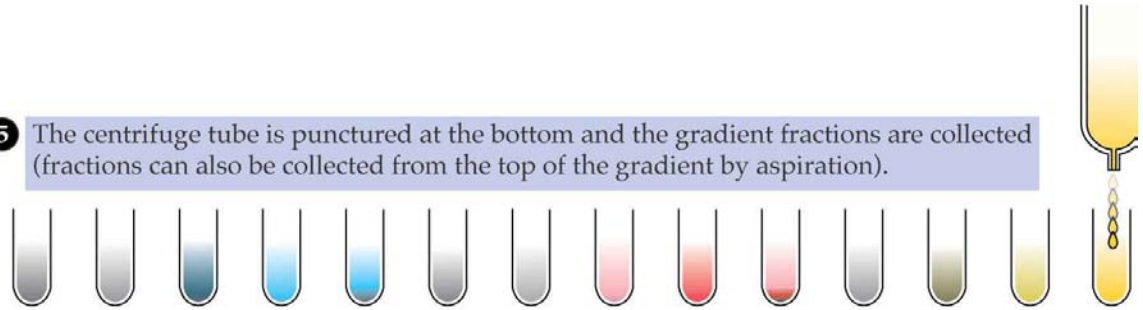


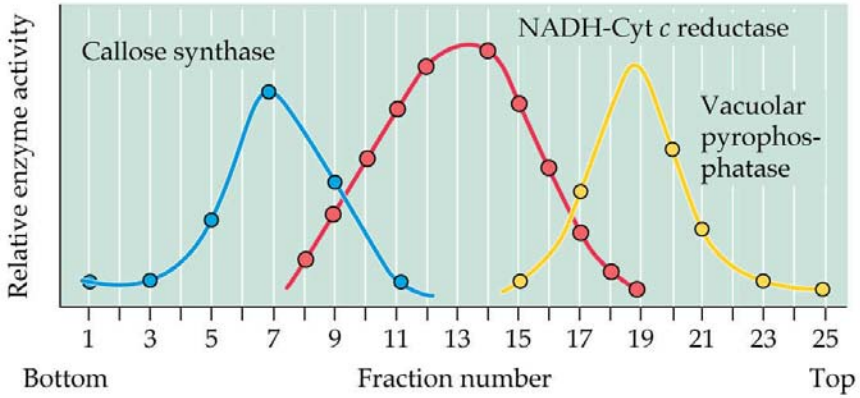
Subcellular Fractionation





5 The centrifuge tube is punctured at the bottom and the gradient fractions are collected (fractions can also be collected from the top of the gradient by aspiration).





6 The fractions are assayed for marker enzyme activity (or used for immunoblots). Here, callose synthase, NADH-cytochrome *c* reductase, and vacuolar pyrophosphatase serve as markers for the plasma membrane, ER, and tonoplast, respectively.

Concentrating Proteins and Buffer Exchange

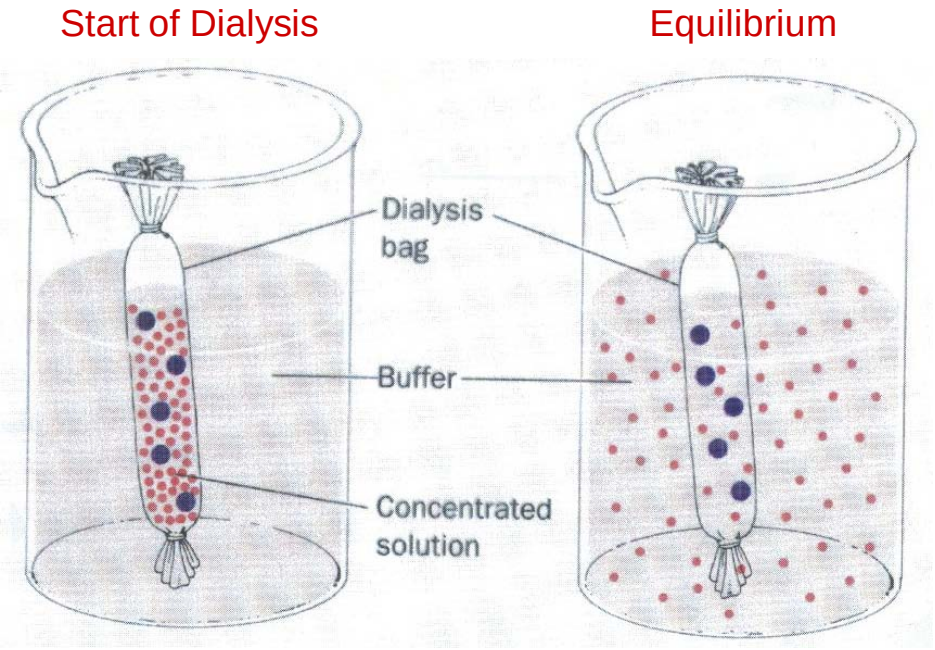
1) Precipitation
   - “salting out”
   - solvent

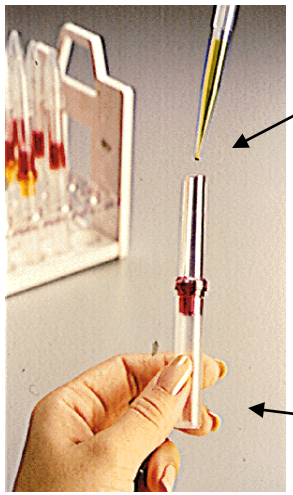
2) Ultrafiltration

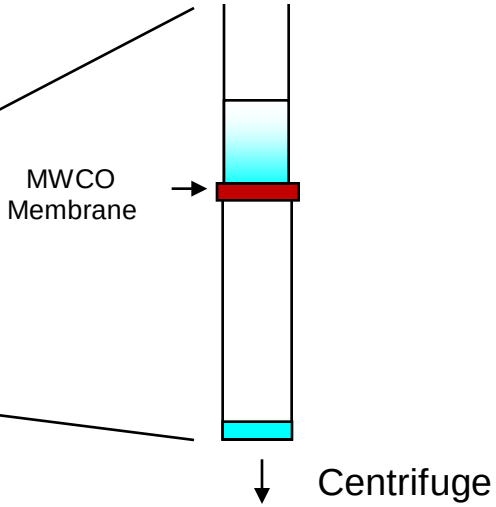
3) Lyophilization

4) Solid PEG dialysis

Dialysis

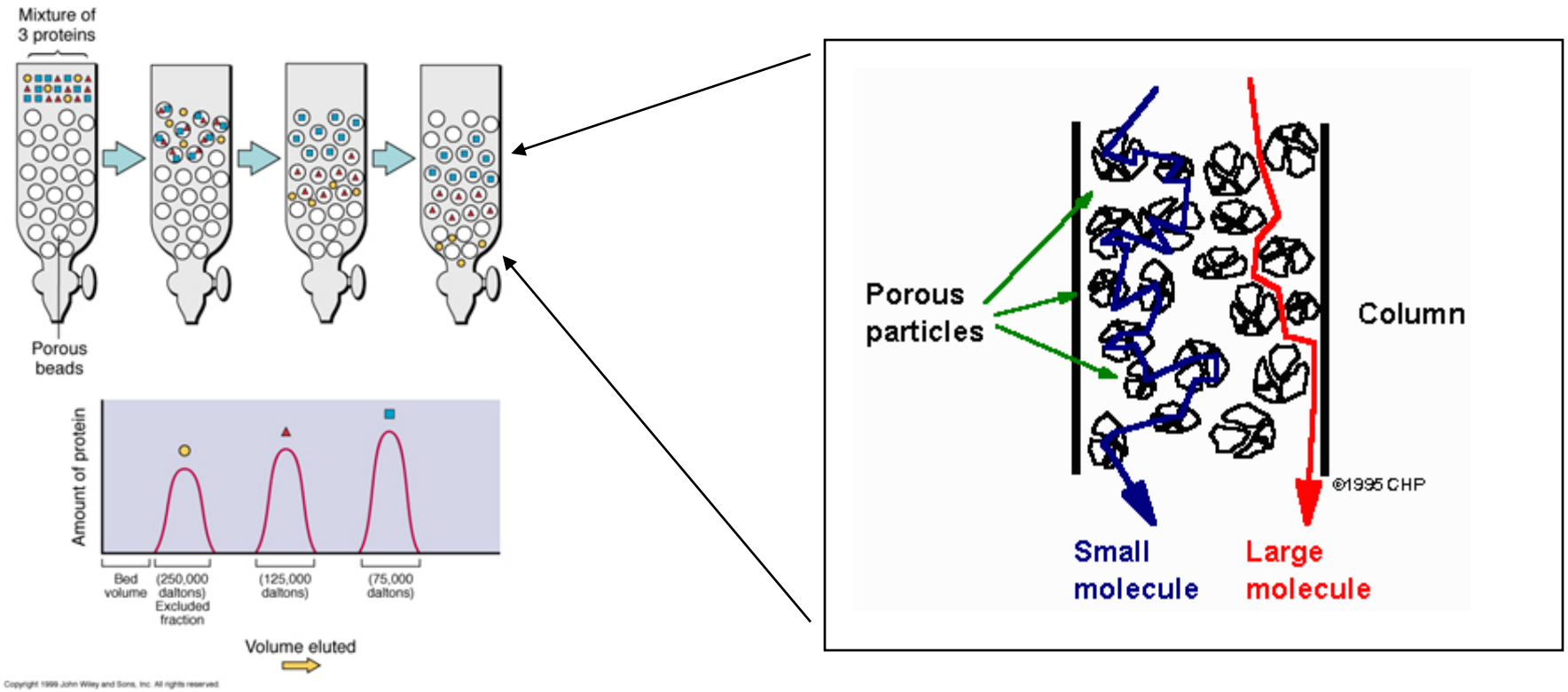






Liquid Chromatography (LC)

- Size Exclusion Chromatography/Gel Permeation Chromatography



Size-exclusion chromatography (SEC), also called gel-permeation chromatography (GPC), uses porous particles to separate molecules of different sizes. It is generally used to separate biological molecules and to determine molecular weights and molecular weight distributions of polymers. Molecules that are smaller than the pore size can enter the particles and therefore have a longer path and longer transit time than larger molecules that cannot enter the particles.

- IEC (Ion exchange chromatography)

Cation and anion exchangers may be broken down further into *weak* and *strong* exchangers (reflecting binding affinity).

Type of exchanger	Functional group	Common name
Weak cation exchanger	Carboxymethyl	CM cellulose/sephadex
Strong cation exchanger	Sulfopropyl	SP sephadex
Weak anion exchanger	Diethylaminoethyl	DE cellulose/sephadex
Strong anion exchanger	Quaternary amine	QAE sephadex

Protein A (pI=9.0)

Protein B (pI=7.5)

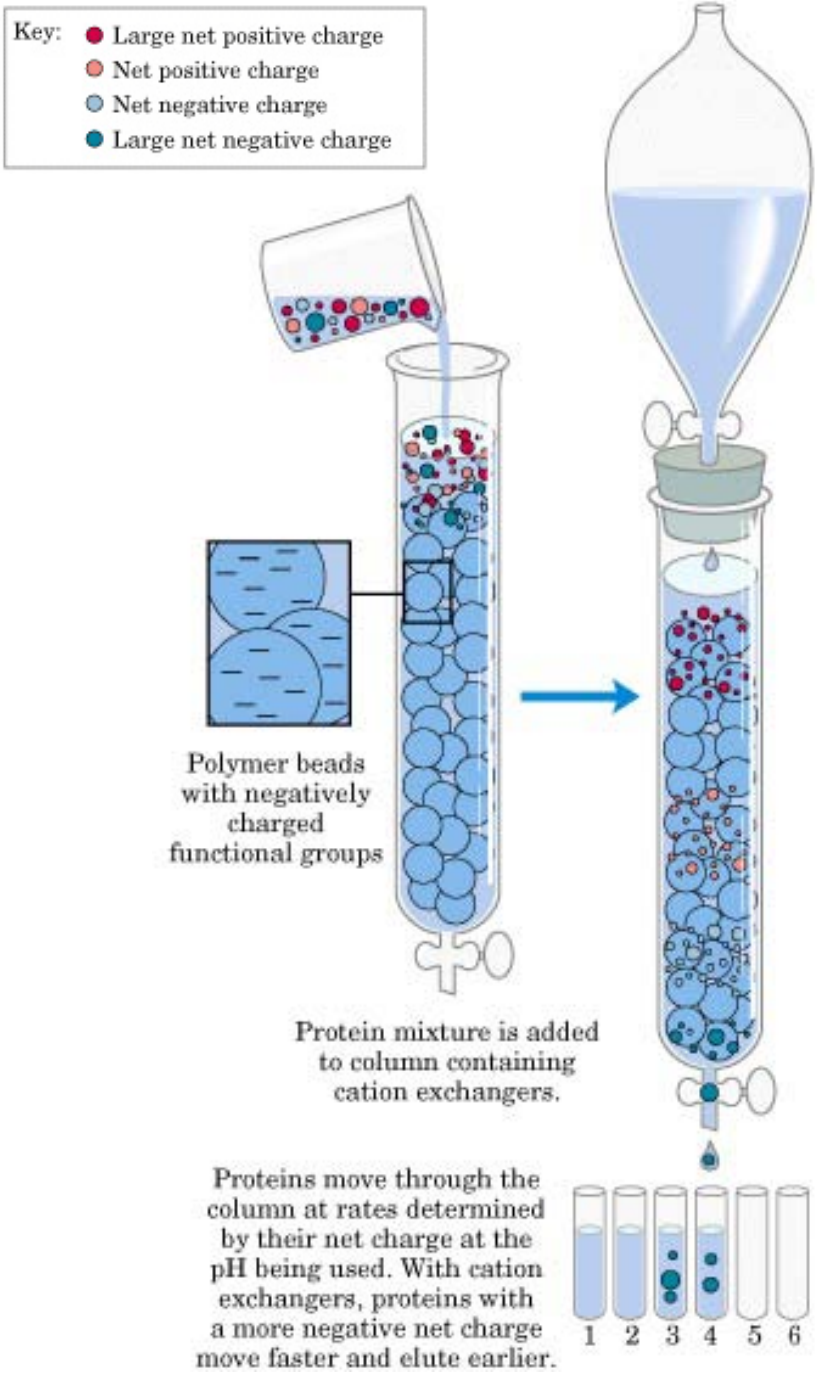
Buffer pH 7.0

Buffer pH is substantially below the pI so protein A will have a *strong* positive charge.

Use a *weak* cation exchange resin

Buffer pH is slightly below the pI so protein B will have a *weak* positive charge.

Use a *strong* cation exchange resin



3) HIC (Hydrophobic Interaction Chromatography)

Theory

1) Water has a high dielectric constant- screens weak intermolecular hydrophobic forces.

2) Addition of salt results in lower protein hydration levels.

3) 'Salting out' salts (eg. $(NH_4)_2SO_4$) structure water around themselves and disrupt the protein hydration shell around the proteins effectively.

4) Exposed hydrophobic sites of protein bind to hydrophobic stationary phase.

The Hofmeister (lyotropic series)

Anions

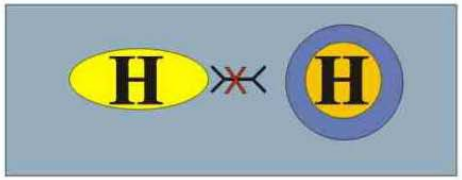
$PO_4^{2-} > SO_4^{2-} > CH_3COO^- > Cl^- > Br^- > NO_3^- > ClO_4^- > I^- > SCN^-$

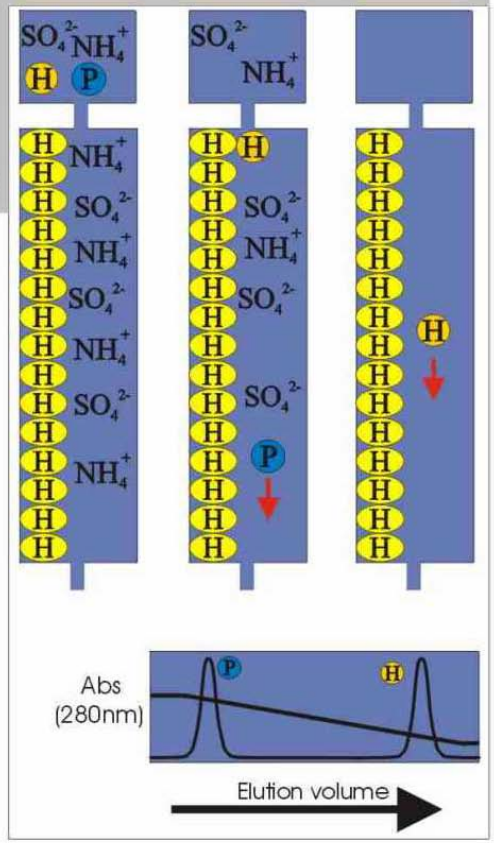
Cations

$NH_4^+ > Rb^+ > K^+ > Na^+ > Cs^+ > Li^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}$

Salting out ions

1) Decrease available H_2O molecules in solution.
2) Increase the surface tension of water.





Elution

1) Decrease salt concentration
2) Decrease polarity of mobile phase
3) Add detergent

Stationary Phase

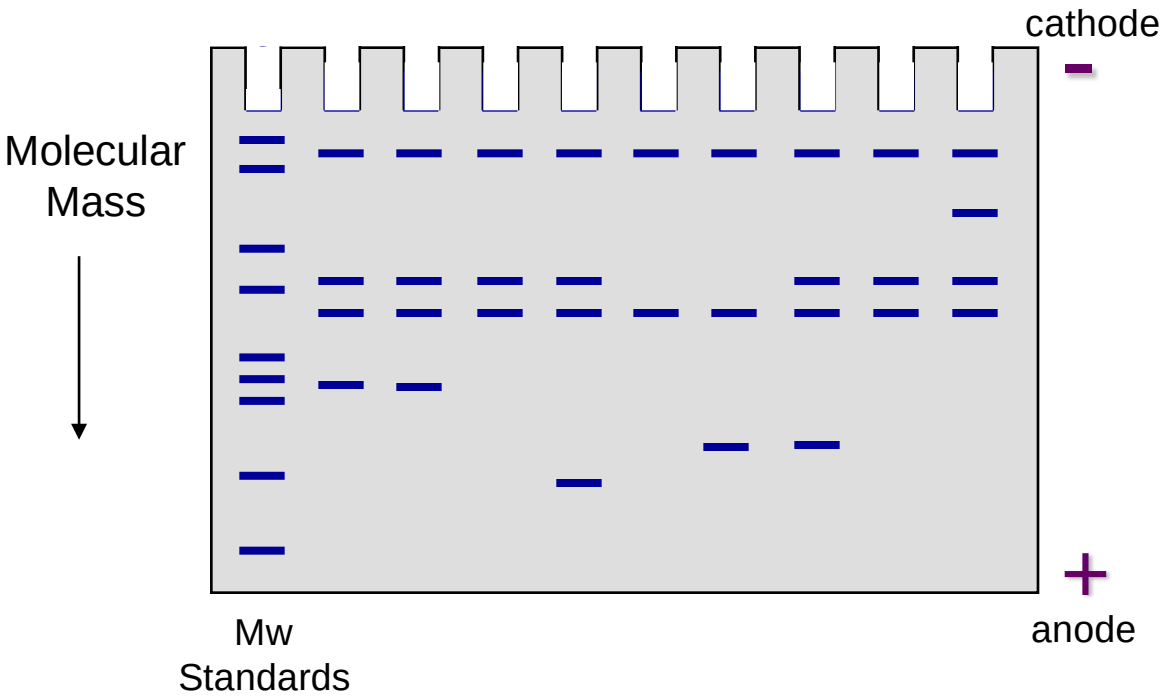
Strength of hydrophobic interaction increases with chain length (alkyl systems). Common chain lengths = C4-10.

Membrane proteins are not v. soluble at high salt concentrations. - Use long chain lengths to increase HI.

Aromatics and phenyl (~ equivalent to C5) groups also used.

Ben Hankamer 1999

1-D SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)



- Extraction
  (stability, subcellular fractionation, purification of protein complexes)
- Solubilization in Laemmli buffer
  (SDS, reducing agents) or non-denaturing buffer
- Electrophoresis
  (basic buffer plus/minus SDS)
- Zymograms
  (activity assays)
- Staining
  (coomassie, silver and fluorescent dyes)

Transfer to membrane

Band excision

Western analysis

Polyclonal Ab production

Protein sequencing

Denaturing electrophoresis of proteins

SDS:

- anionic detergent
- effective solubilizing agent for a wide range of proteins
- denaturing agent
- most proteins bind SDS in a fixed ratio of 1.4g SDS:1g protein so a negative charge is conferred in direct proportion to the polypeptide mass. Therefore, proteins migrate in SDS-PAGE gels according to their molecular weight and not their intrinsic charge

Reducing agents (dithiothreitol [DTT] or β-mercaptoethanol):

- disrupt disulphide bonds

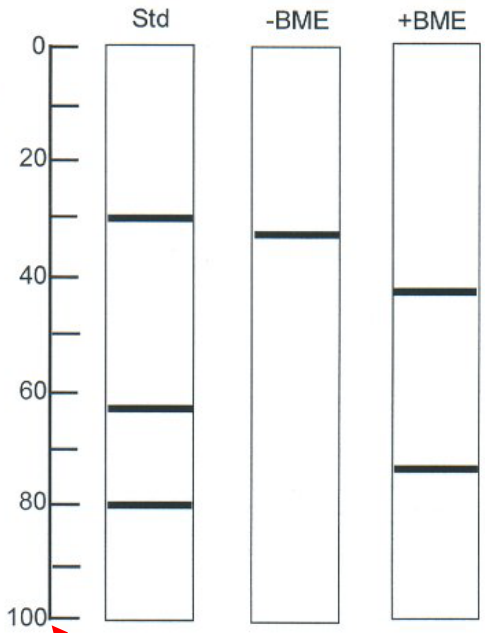
Native gel electrophoresis of proteins

- proteins separated on basis of molecular weight and intrinsic charge
- may allow proteins to maintain biological activity
- only applicable to proteins that are soluble and will not precipitate or aggregate during electrophoresis

DETERMINE MOLECULAR WEIGHT BY SDS PAGE

In lane 1 we run three standards: BSA, chymotrypsinogen, and lysozyme
In lane 2 we run an unknown in the absence of BME
In lane 3 we run the unknown with BME

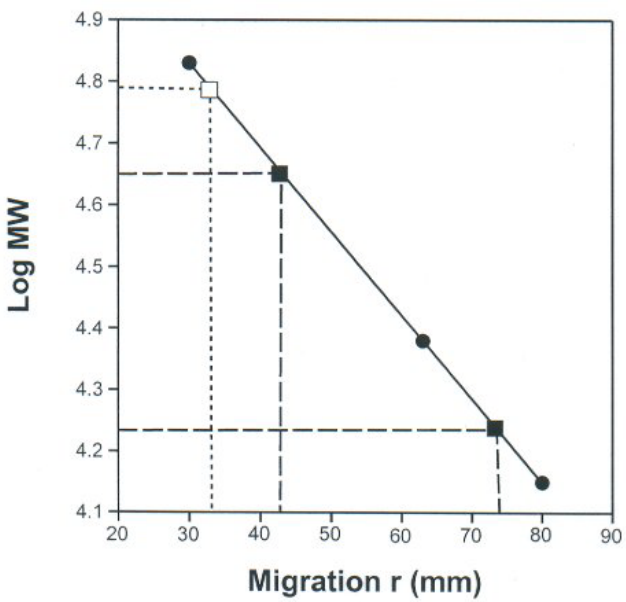
We observe the data shown at the right and tabulated below.



Protein	MW	log MW	r (MM)
BSA	67,000	4.83	30
chymotrypsinogen	24,000	4.38	63
lysozyme	14,000	4.15	80
unknown -BME	?	?	33
unknown#1 +BME	?	?	43
inknown#2 +BME	?	?	74

r=Migration distance (mm)

Graphing the data we see:



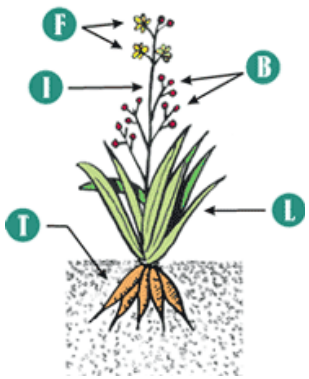
The log MW of unknown -BME = 4.79 ∴ MW = 62,000

With BME log MW #1 = 4.65; MW = 45,000

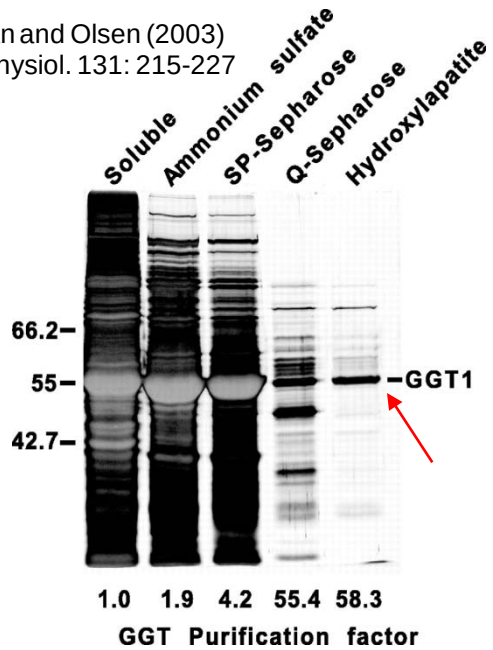
log MW #2 = 4.23; MW = 17,000

Conclusion: unknown is heterodimer with one chain of 45 kDa and one of 17 kDa, linked by a disulfide bond.

Protein Sequencing and Identification



Liepman and Olsen (2003)
Plant Physiol. 131: 215-227

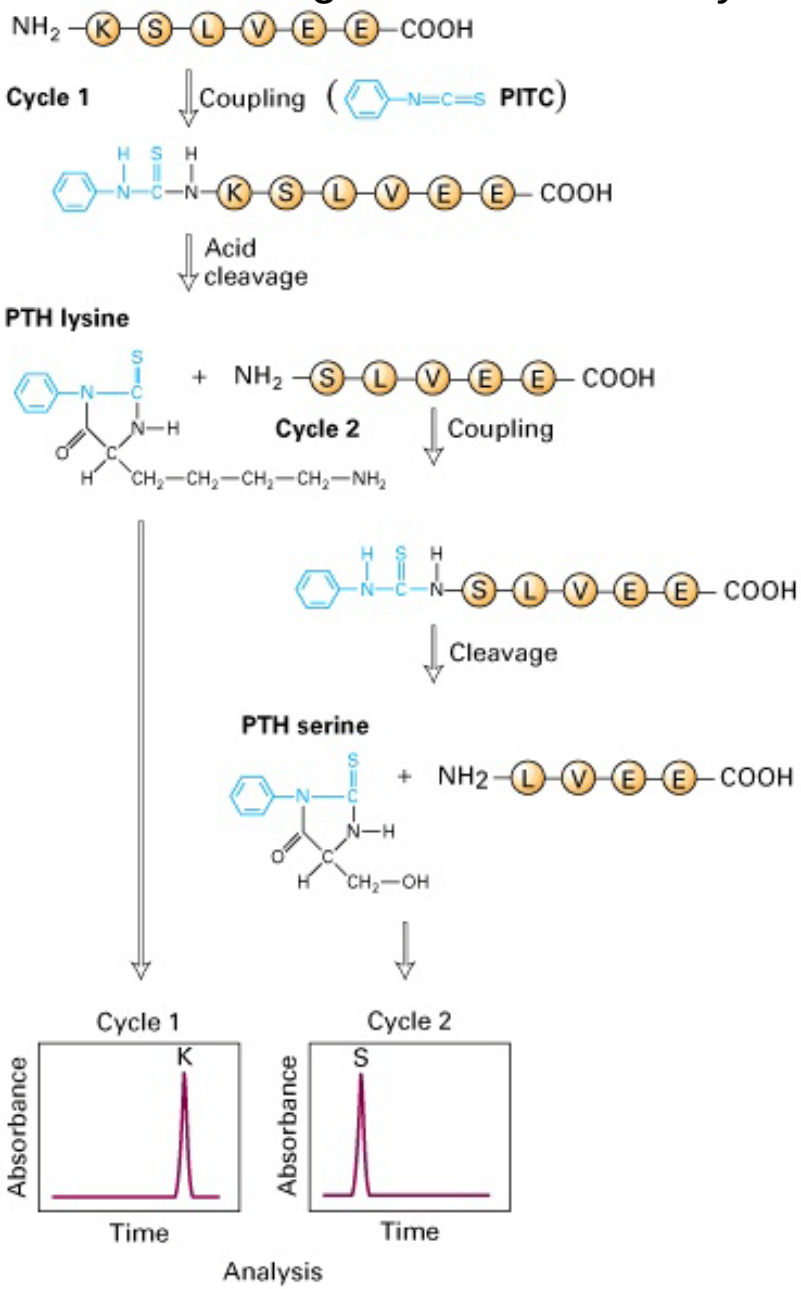


Biological or Biochemical Activity

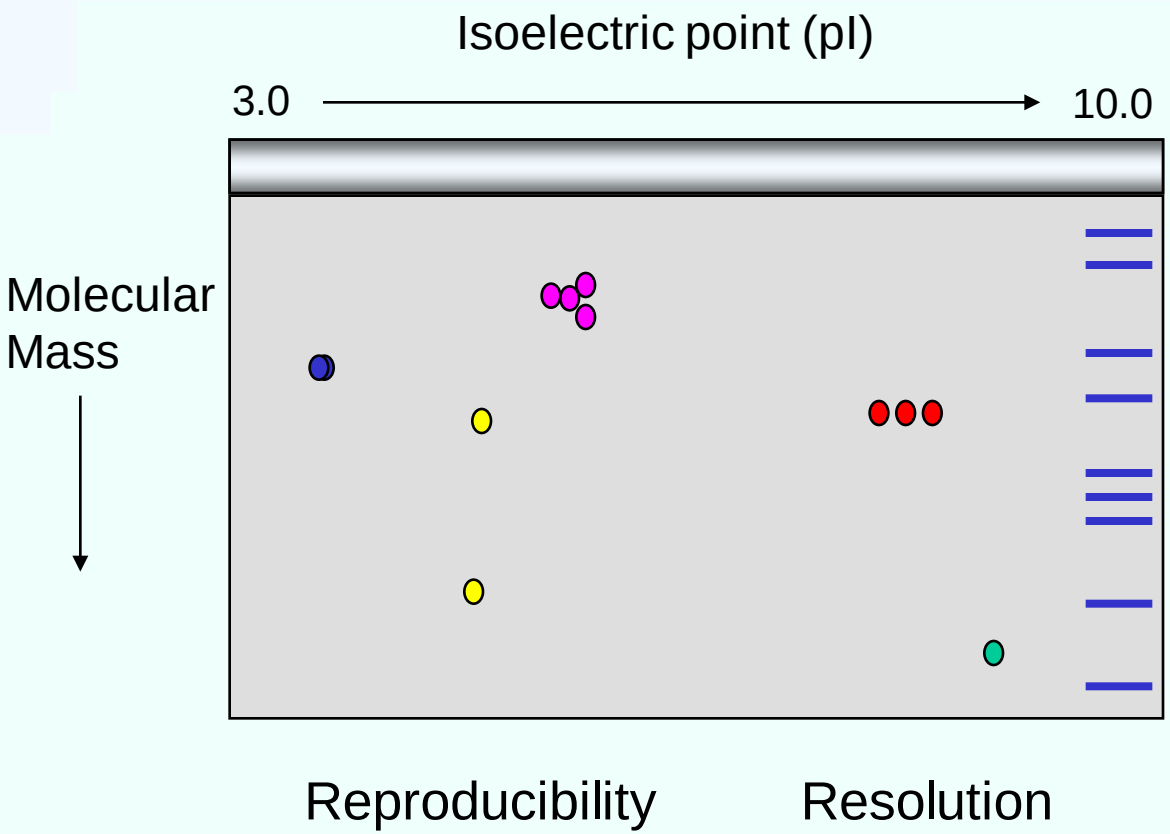
Several Approaches…..

Protein Sequence

N-terminal Sequencing
Edman Degradation Chemistry

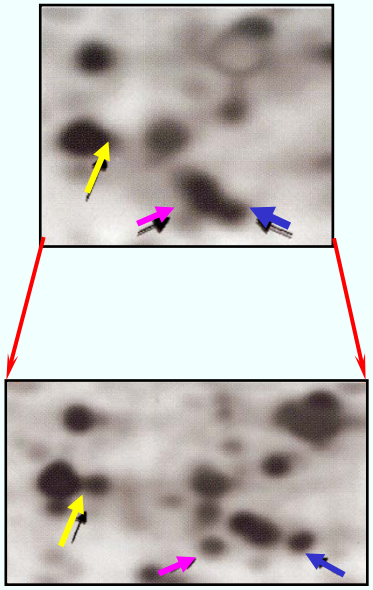


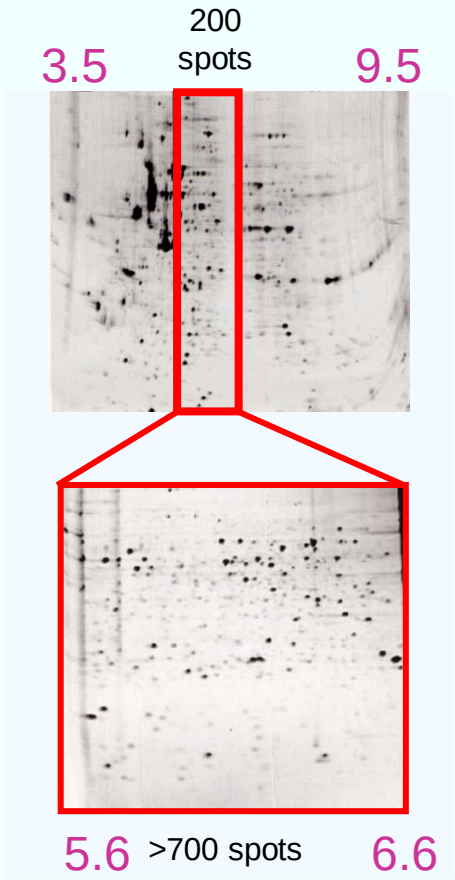
2-D Gel Electrophoresis

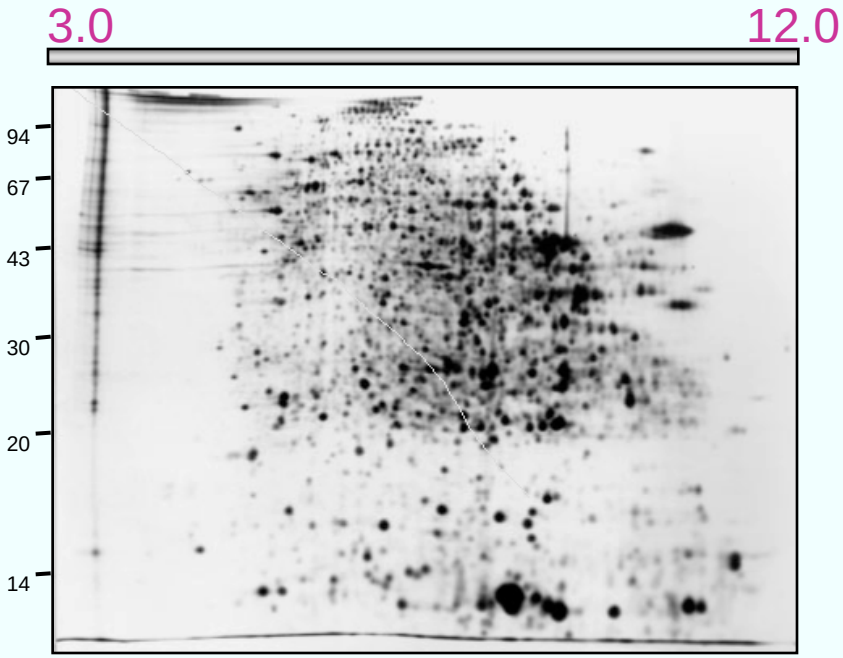


1980s: Immobilized pH gradient (IPG) technology

- 1000s of spots on a single gel
- highly reproducible
- load mgs of protein
- narrow range pI analysis





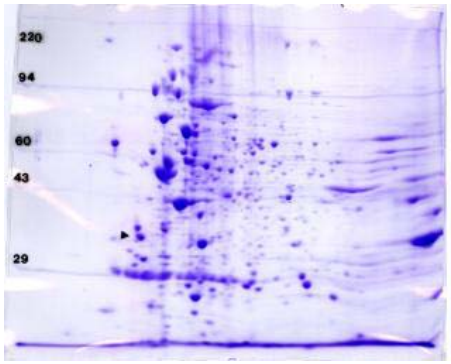


Gorg et al. (1999) Electrophoresis 20: 712-717

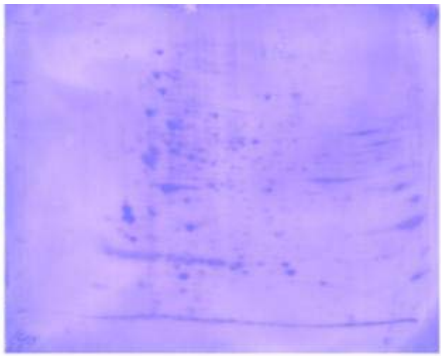
Limitations include hydrophobic
low abundance and insoluble proteins

Detection stains: Sensitivity vs. quantitation

Coomassie-stained 2D gel

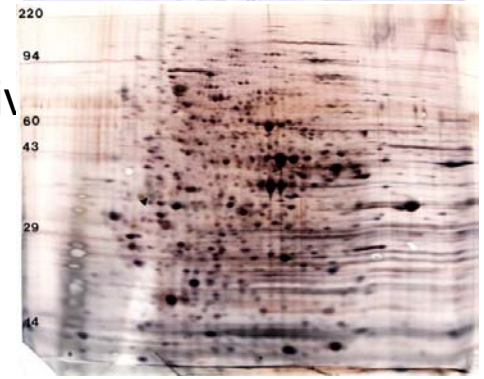


PVDF blot: sequencing/MS



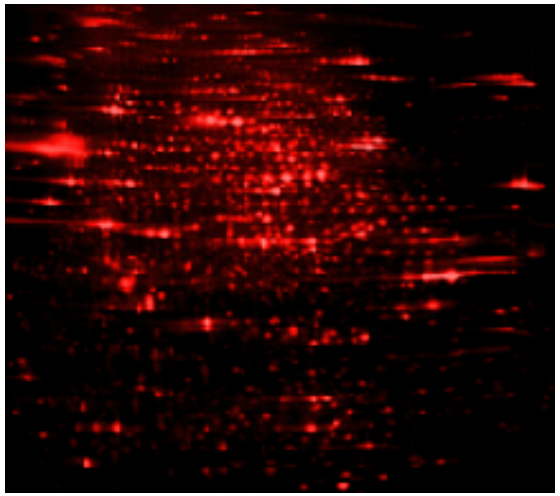
Coomassie Blue (various formulations, cheap, easy, less sensitive)

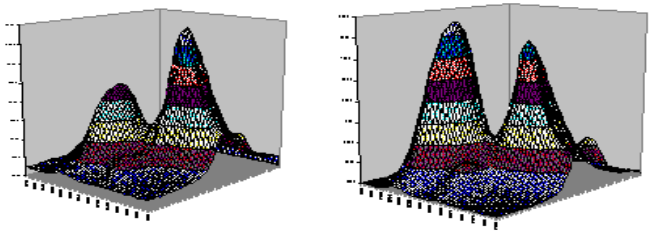
Silver stain (highly sensitive, less quantitativ



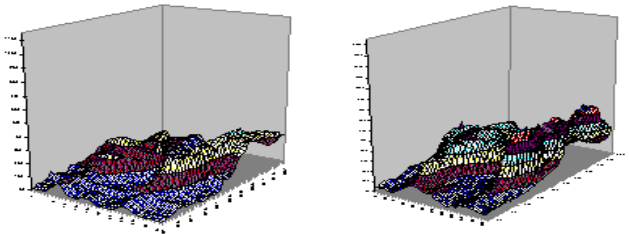
SYPRO Ruby

- low level of detection (1-5 ng)
- compatible with mass spectrometry
- linear over 4 orders of magnitude





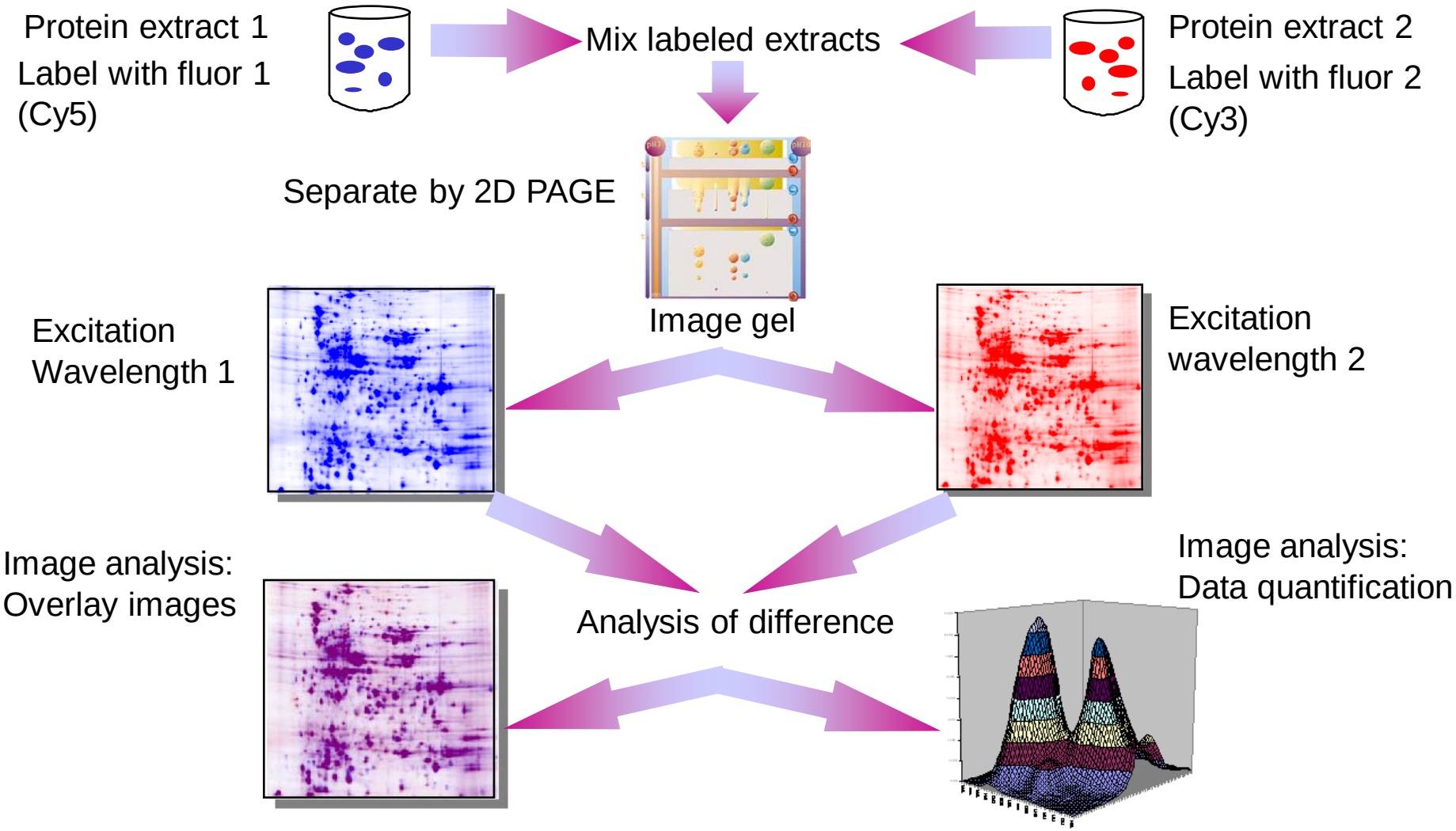
Fluorescent staining

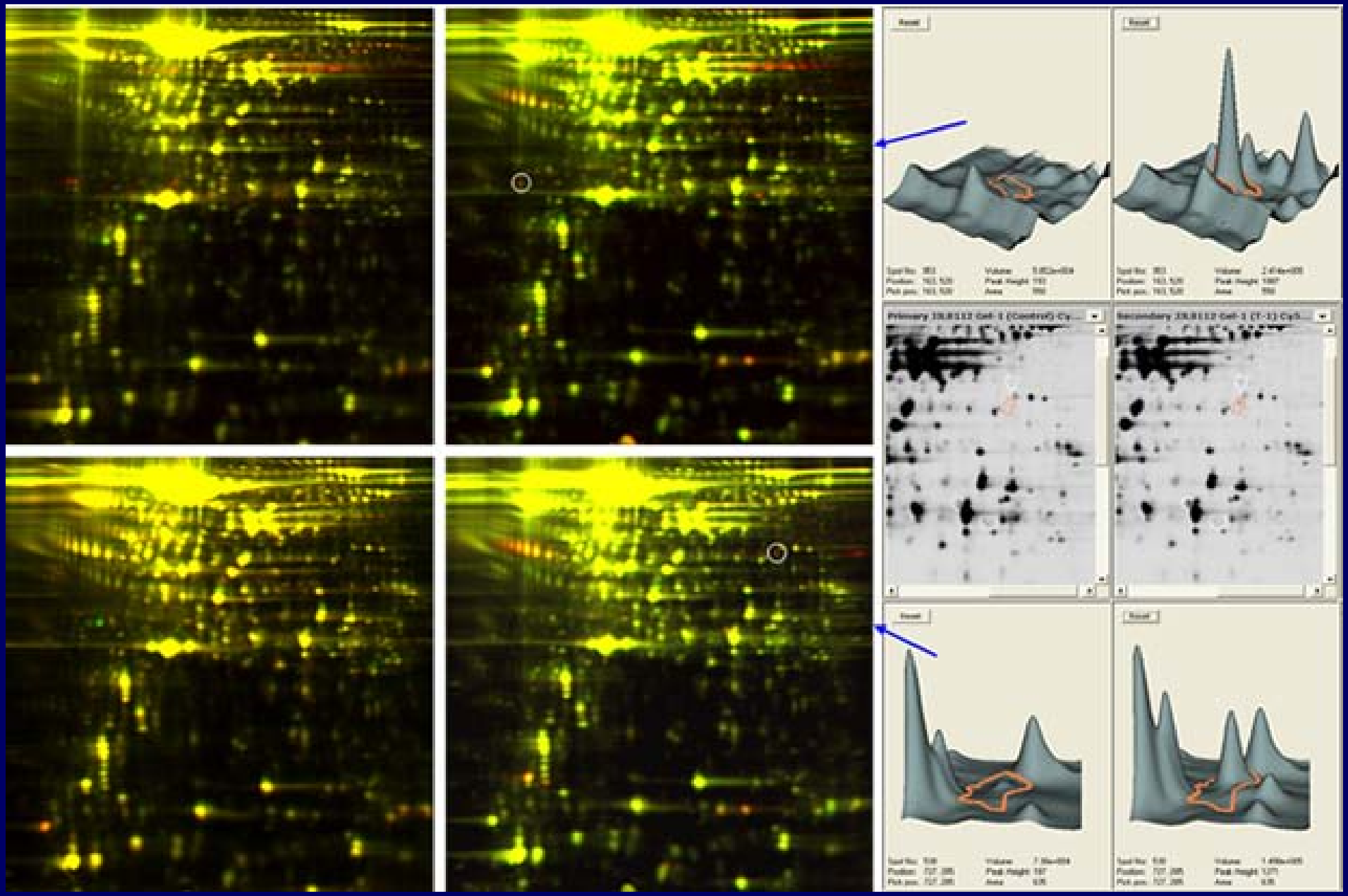


Silver Staining

- 3D plots of same spot areas on gel
- Difference only obvious with fluorescence

Addressing the bottleneck: 2-D Differential In-Gel Electrophoresis (DIGE)





http://www.appliedbiomics.com/img/Serum_Example2.jpg