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2D gels and other high-resolution separations and analysis of intact proteins in biological samples

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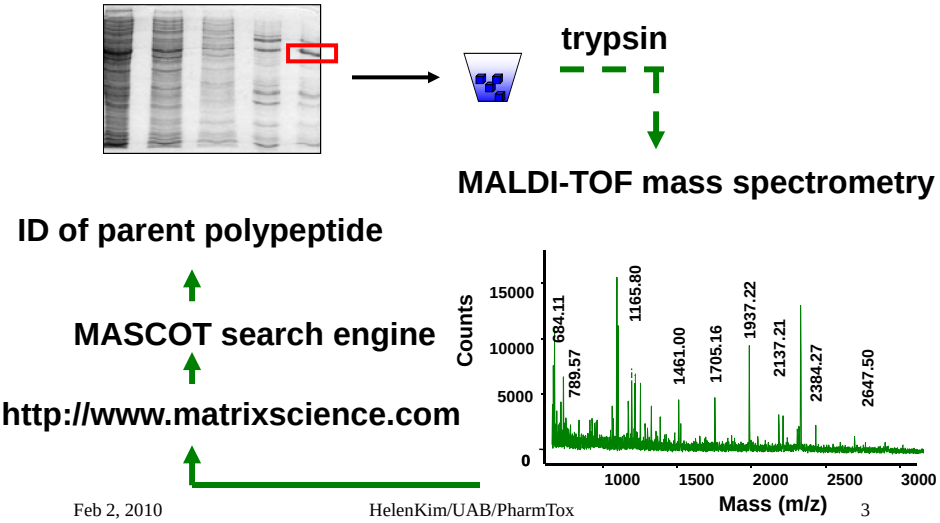
Learning objectives

- **Elements of 2-D gel separation:**
  - **IEF**
  - **SDS-PAGE**
- **Other types of intact protein separations;**
  - **Free-flow**
  - **2D native electrophoresis**
  - **GELFREE with other separations**
- **Basic chemistries that underlie the different types of separations**
- **How do we go from 2D separation patterns to understanding the biology**

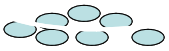
The basic elements of intact protein proteomics: (1) separation, (2) analysis, (3) identification and characterization



Parameters that govern the choice of protein separation method

- Purity of protein
- Speed of purification
- Quantity of protein
- What is the question:most important
  - Discovering a new protein/proteome
  - Identifying protein-protein interactions
  - Identifying potential modifications of known proteins

Sample preparation for 2DE:



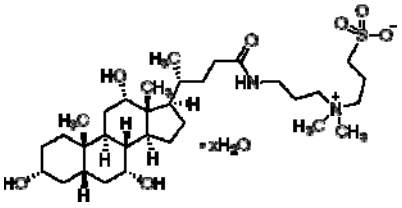
Harvest, rinse, and pellet the cells;

or

Dissect out tissue, organ, or fluids;

- **Homogenize/lyse in buffer that *dissociates and unfolds the proteins***
  - **High urea usually 5-8 M---unfolds the protein**
  - **Sometimes 2 M thiourea--unfolds the protein**
  - **1-4% nonionic detergent--solubilizes hydrophobic components**
  - **Beta-mercaptoethanol or other reductant**
  - **Inhibitors: of proteases, kinases, & phosphatases**
- **Clarify by centrifugation to get rid of insoluble matter;**
- **Protein assay to know how much and how concentrated**

Structures important in 2D electrophoresis to know and understand



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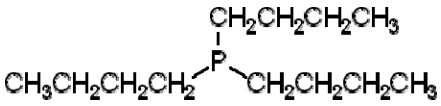

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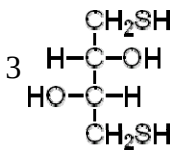

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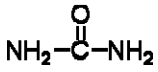

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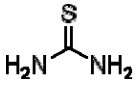

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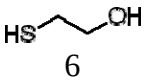

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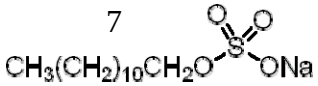

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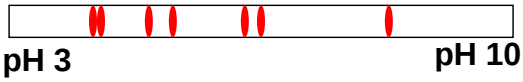
What 2-D electrophoresis involves:

- 1st dimension: **Isoelectric focusing**

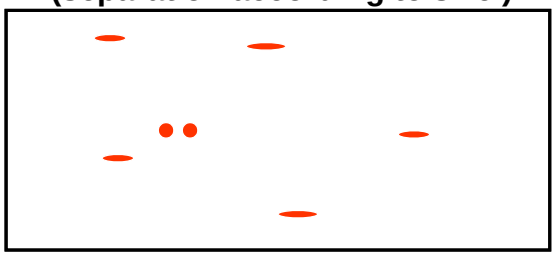
(separation according to charge)



pH 3 **pH 10**

- 2nd dimension: **(SDS)-PAGE**

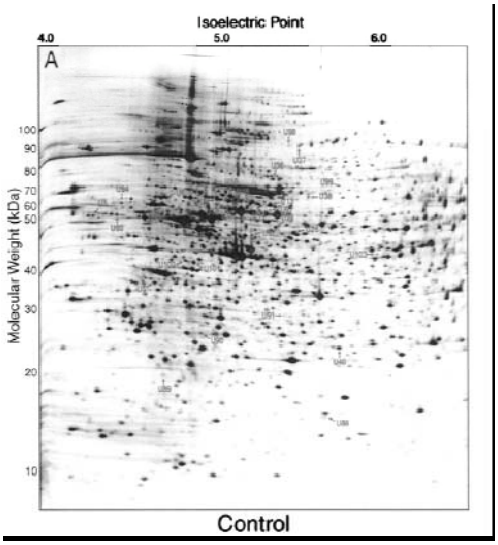
(separation according to size)



High m.w.

Low m.w.

A real 2-D gel



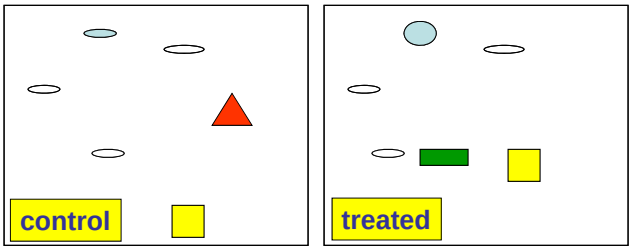
Find this and other 2-D gels at http:www.expasy.org

Lewis et al., [2000] Molec. Cell, 6)

(from Natalie Ahn's lab)

The pattern itself is information; a change in intensity or position of a spot has biological meaning…..similar to astronomy.

Critical part of 2-D gel proteomics: Image analysis



with software: "compare" the images.

Types of information:

- Suggests upregulation of gene
- Suggests new posttranslational modification
- Suggests downregulation of gene
- Suggests "aberrant processing:" the different size and pI indicate part of the protein in control is different from in treated.

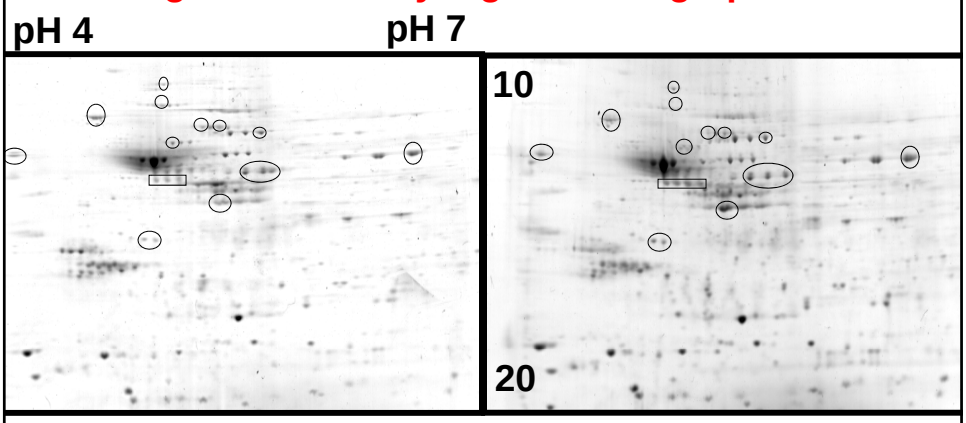
Elements of image analysis of "regular" 2D gels:

1. Compare the 2D displays of spots
2. Determine total spot number for each display
3. Quantify spot intensities, identify differences
4. Identify spots that may have " moved" horizontally; these are candidates for those that are altered in charge, reflecting posttranslational modifications.

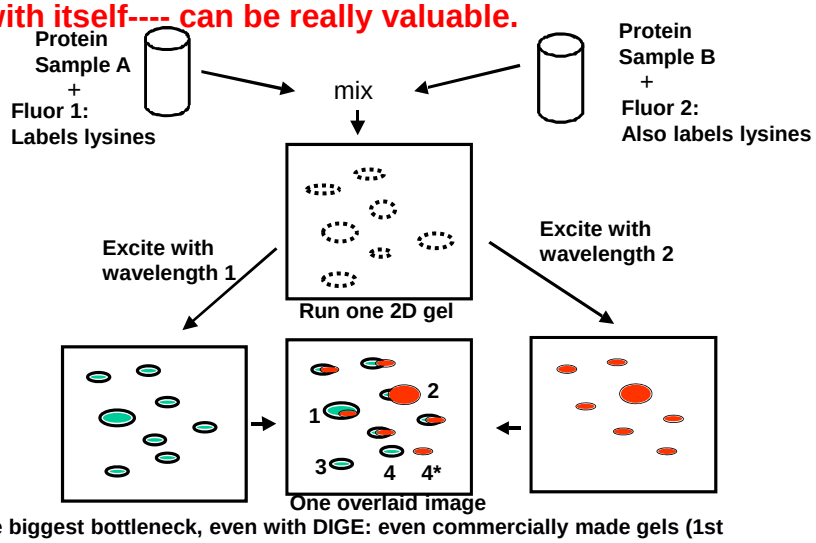
Ultimate and simple goal of image analysis

to answer the question,
"What is changing, and by how much?"

A pair of 2D gels representing rat brain protein changes induced by ingestion of grape seed



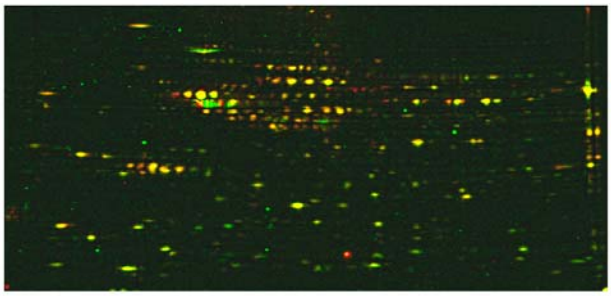
Difference gel electrophoresis (DIGE); a protein migrates with itself---- can be really valuable.



Single biggest bottleneck, even with DIGE: even commercially made gels (1st dimension, 2nd dimension) are not perfect. So, the simpler you can keep the system, the better. If you don't HAVE to use a gradient gel, don't.

Example of DIGE:

Differential protein labeling with Cy3 and Cy5
Superimposed images from the same gel
of normal and cancer cell lines from the breast

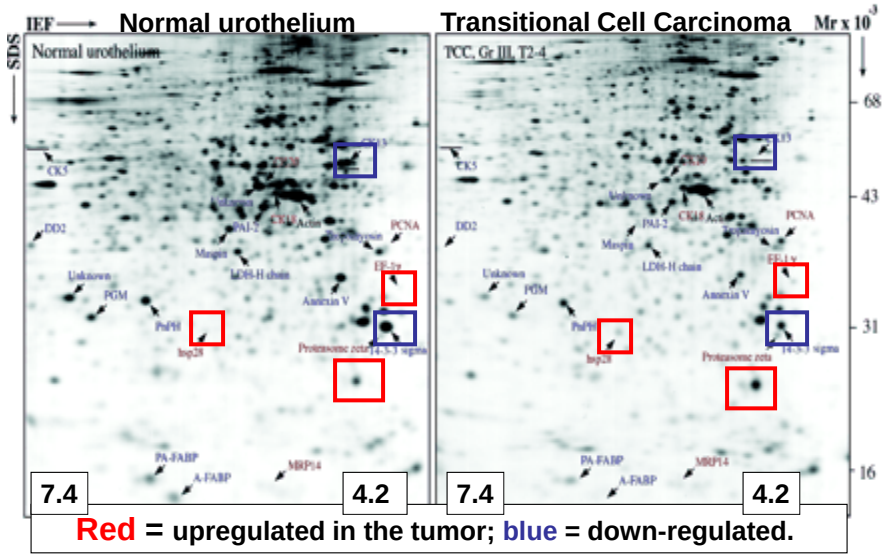


Visually: 100% green spots are specific to normal cells, 100% red spots are specific to cancer cells, degrees of yellow-orange indicate differential expression.

"Mine" your proteomic data

- **Note every difference--eventually it all means something;**
- **But make sure the difference is "real."**
  - **What is the variation in that parameter (mw, pI) for that samespot in that treatment group;**
  - **Quality control issues come into play here;**
    - **Did you design the experiment with a statistician?**
- **Make sure your "basal" mw and pI are consistent with predicted and/or what others have observed;**
- ***Then* you can conclude that a difference in pI, for example, indicates a change in modification**
- **If some/all of a spot is found at a pI different from predicted, it may be constitutively modified in the "unstimulated"/"normal" group**

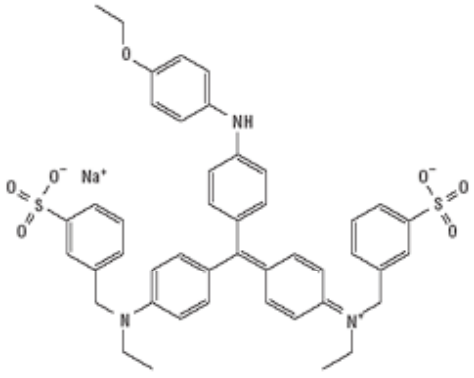
Metabolic labelling can enhance 2D gel analysis: i.e. ^{35}S-methionine-labelling



Red = upregulated in the tumor; blue = down-regulated.

(Celis et al., Mol.Cell Proteomics, 2002, 1:4)

Visualizing 2D gels: Coomassie Brilliant Blue

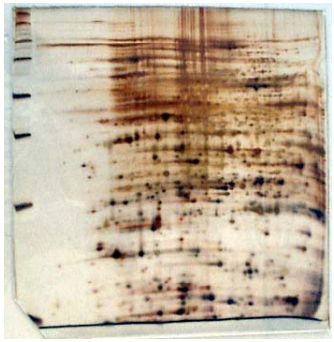


Coomassie Brilliant Blue R-250 Dye
M.W. 825.97

In acidic conditions, the anion of CBBR combines with the protonated amino groups on proteins via electrostatic interactions.

- Inexpensive
- Image readily acquired by scanning at visible wavelengths
- No covalent mass change

Silver stain



Silver ions (from silver nitrate) are chemically reduced to metallic silver on lysine residues.

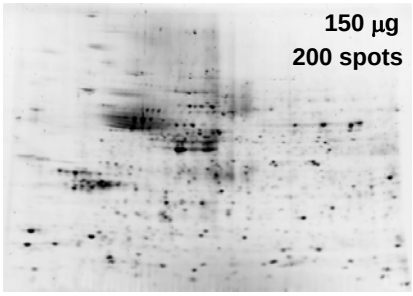
This is the most sensitive protein stain, but also the least useful for quantitation because of its low dynamic range.

2D gel image courtesy of the U. Va. Mass Spectrometry Shared Facility, 2006

Advantages and limitations of the types of stains

	Sensitivity	Dynamic range	MS- compatible
CBBR	8 ng	10-30 x	yes
Silver	1 ng	< 10 x	Not without special precautions
Fluorescent	2 ng	3 orders of magnitude	yes

Proteins, proteins everywhere, but where's my receptor?



The genome predicts: 20,000-50,000 polypeptides.

So, 200 spots is <1% of the total proteome.

2D gel of rat brain, stained with Sypro Ruby

Conclusion: A fluorescently stained 2D gel of an unfractionated sample, only allows detection of the "low hanging fruit."

Central issue in proteome analysis: dynamic range

In cells: protein amounts vary over a wide *dynamic range*:

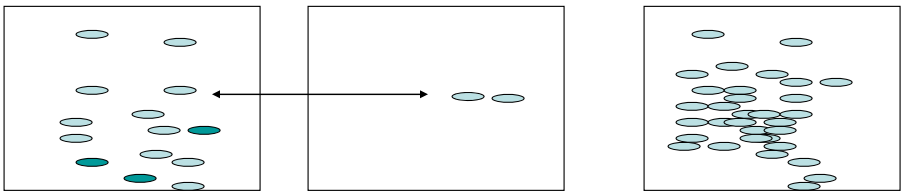
In blood, albumin is 3.5 g/100 ml (35 g/L = 0.5 mM) (10^{-3} M), whereas cytokines are pM (10^{-12} M)

- **This is a difference of nine (9) orders of magnitude.**
- **A 2D gel that is overloaded with respect to an abundant protein, may have *barely detectable* amounts of a low abundance protein……**
  - **If you can't see it, you don't know a protein is there;**
  - **Even if you know it's there, you can't do MS, because there isn't enough protein.**

- **No one stain will detect 9 orders of magnitude differences in abundance of proteins.**

For greatest sensitivity, and the most biological information:

Combine 2D proteomics with "conventional" approaches;
Nothing is quite as conclusive as a good Western blot



Western blot for phosphoproteins

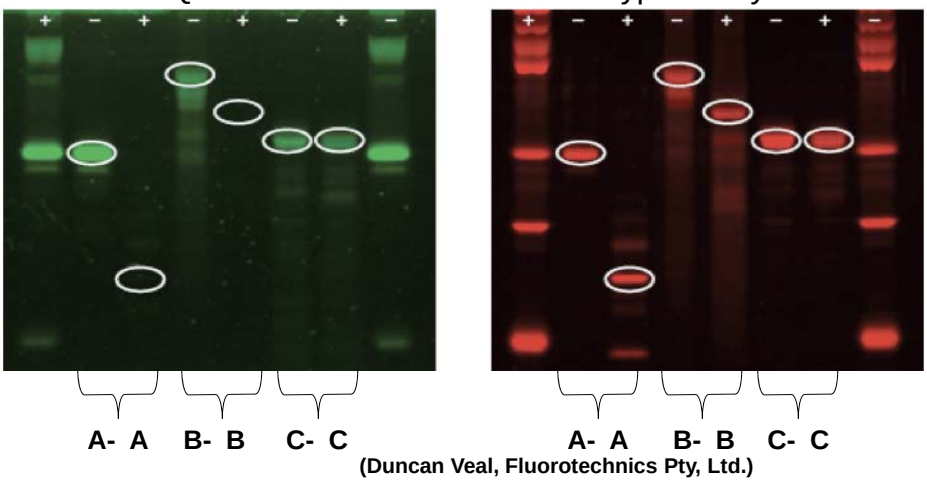
Western blot for a particular protein

Stained gel

Multiplex Proteomics: ProQ Emerald followed by Sypro Ruby can identify multiple glycosylated proteins at once

ProQ Emerald

Sypro Ruby

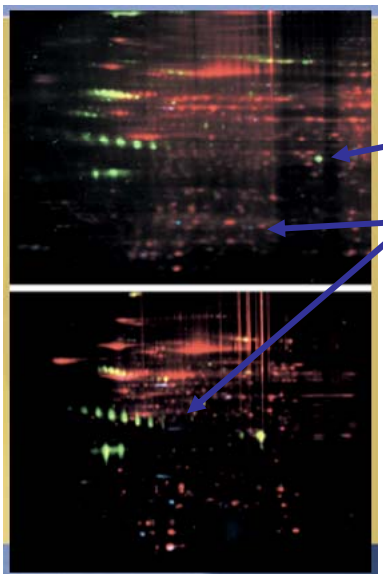


(Duncan Veal, Fluorotechnics Pty, Ltd.)

Multiplexing: valuable when sample is scarce or difficult to obtain

Normal liver

Liver tumor



Sypro Ruby: total protein

Pro Q Emerald: glycosylated

Pro Q Diamond

The value of fluorescent dyes: each can be detected separately from others on the gel, due to different excitation and emission spectra.

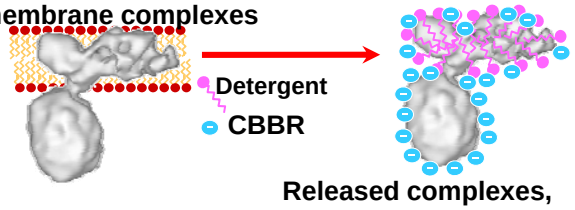
Modified from Duncan Veal, Fluorotechnics Pty, Ltd.

SUMMARY of 2D gel stains

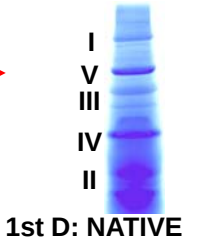
- **Protein stains differ according to**
  - **Sensitivity/Dynamic range/MS-compatibility/Ease of capture of information**
- **The fluorescent dyes offer unparalleled protein analytical capabilities due to the wide dynamic range, and their MS compatibility;**
- **"Multiplexing" allows analysis of subproteomes in the same gel, maximizing use of scarce samples;**
- **Yet, each stain has utility depending on experimental goals.**

2D-Blue-Native gels: for membrane-associated protein complexes.

Intrinsic mitochondrial membrane complexes



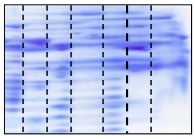
Released complexes, all negatively charged, thanks to the CBBR



1st D: NATIVE electrophoresis

(Modified from Brookes et al., 2002)

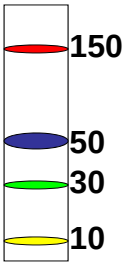
This type of 2D gel has "ladders" of bands.



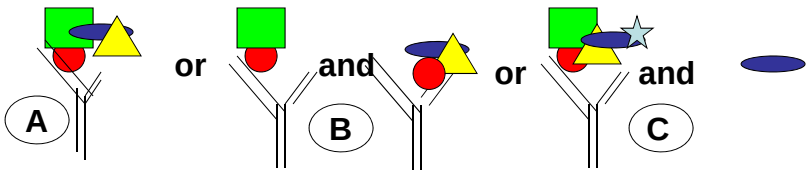
2D native electrophoresis has value beyond identifying components of membrane complexes:

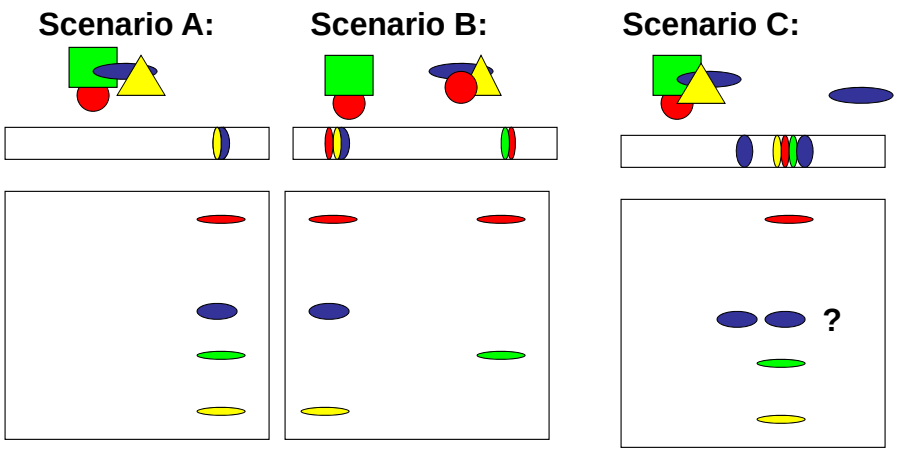
In immunoprecipitates: Which proteins are interacting with which?

Experiment → Lysate + antibody →



It could be:

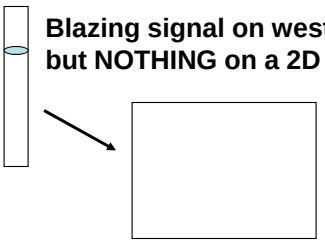




When would you see the same protein in two lanes in the 2nd D?

Issues in 2D gel analysis:

I. Now you see it, now you don't:



Blazing signal on western blot of 1D SDS gel;
but NOTHING on a 2D gel of the same immunoprecipitate:

Explanations?

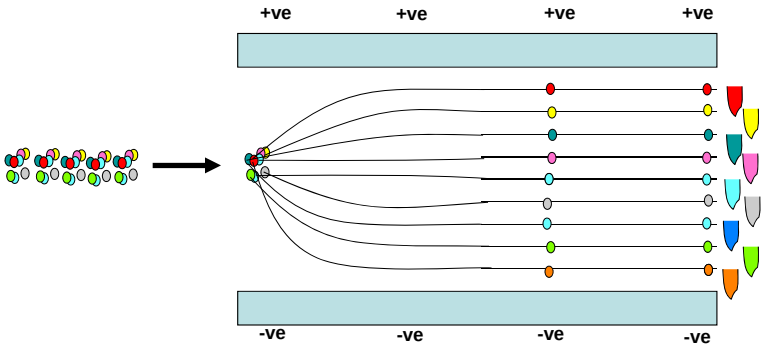
1. Solubilization by SDS but maybe not by urea;
2. Quantity---Western blots more sensitive than people realize; need to scale up at least 10-50 fold for detection by stain

Why do I need to run a 2D gel anyway?

1. Information about what proteins that associate with the antigen;

2. Separate multiple proteins in that one band

Free flow electrophoresis

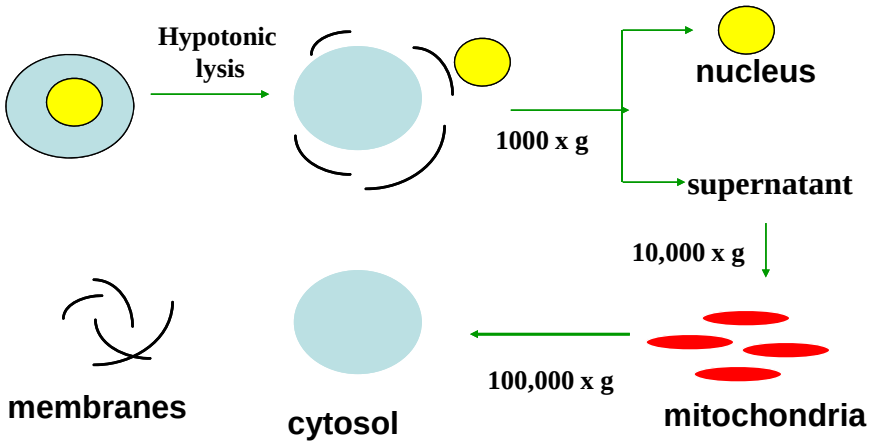


There are subpopulations of mitochondria in most cells, each with a different net charge. These can be passed through a "chamber" which has a potential difference across the sides of the chamber. Each particle will find a position where its surface charge corresponds to that of the gradient, and move along that position parallel with others moving at their own positions. This allows separation of multiple subgroups of mitochondria and physical collection into 96-well plates at the end of the free-flow chamber.

Bottom line for effective 2D gel separation and analysis

- **Reduce proteome complexity by incorporating biological information or properties :**
  - **Intracellular location--subcellular fractions**
  - **Protein-protein interactions--immunoprecipitations, BN gels**
  - **Different states of oligomerization in vivo: microtubules**

Various ways to reduce proteome complexity:
Subcellular fractionation by differential centrifugation

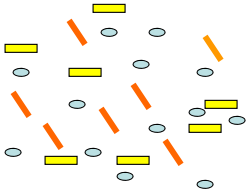


The good news: subcellular proteomes are readily "catalogued."

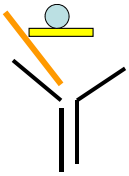
Compartment	# polypeptides in SWISSPROT as of 2000
Mitochondria (1000/cell)	269------5% of total
Lysosome (400/cell)	50------1% of total
Peroxisome	35------0.6%
ER and Golgi apparatus	157------3%
Nuclei (5% cell volume)	964-----17%
Others (cytosol, membrane)	4228----75%
	total:5703

(Jung et al. [2000] Electrophoresis)
Note date: this is old!!! But the principle is the same

Biological specificity of antibodies is invaluable in reducing the complexity of the proteome to be analyzed



A cell lysate: 6,739 polypeptides



An immune complex of 1-10 polypeptides (why might there be more than one polypeptide?)

Which sample would you rather deal with on a 2D gel?

Make use of databases and the internet:

I. Check existing databases and web-links:

www.expasy.org

many are annotated

helpful links:proteomics tools

II. Keep up with the literature/ competition:

J. Biol. Chem.

Proteomics

Molecular & Cellular Proteomics

J. Proteome Research

J. Agric. Food Chem.

III. Use genomics information when available:

The polypeptide sequence (from the cDNA) can predict electrophoretic parameters-- m.w. & pI;

helpful in setting up 2D gel conditions

Take home message

- 2D electrophoretic patterns yield mass and charge information, thus expression or posttranslational modification differences;
- This biological information is not easily obtained in analysis of digests.
- Other newer protein separation approaches can be valuable in reducing complexity, or in biological information;
- Choice of separation governed by
  - Abundance of sample
  - Question being asked
  - What technologies you can access readily
  - What you can afford

Future directions in intact protein analysis

I. Subcellular fractionation will regain importance in proteome analysis;
II. While automated 2D LC/LC-MS/MS may appear more highthroughput for "discovery," every resolved spot on a 2D gel is a purification, *and* a discovery;
III. 2D gel positional information, *without protein identities*, is information itself.
IV. Where 2D gels may play larger roles is in validation of results generated by other approaches;

Final thoughts

- What proteomics technology gives back is like any other analytical approach: the quality is as good as what you put in;
- Be mindful of distinguishing between low abundance proteins vs low level contaminants;
- Keep in mind “conventional” approaches like Western blotting to validate proteomic results;
- Purify, purify, purify before running any proteomic experiment.
- Formulating a hypothesis forces you to incorporate biological information that can enhance proteomic analysis.
- Identifying differentially expressed proteins is a beginning:
  I. What changes are causal to the disease/phenotype
  II. Some are “real” but not causal;
  III. Some could be response of the cells/tissues TO the disease, not causing the disease.