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Top-down proteomics using 2D electrophoresis

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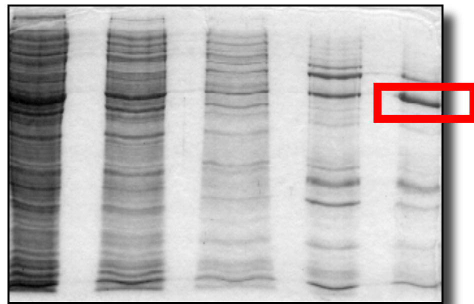
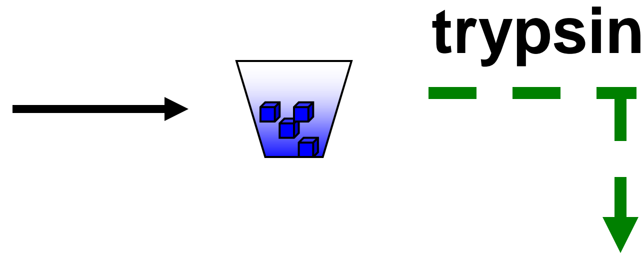
Learning objectives

- **Smart proteomics:**
  - **lowest proteome complexity, highest biological specificity**
- **How do we reduce protein complexity**
  - **Purify proteins according to intrinsic properties**
  - **Purify proteins according to biological properties**
- **Proteomics: global assessment of protein differences in biological samples:**
  - **Electrophoresis:**
  - **Western blot**
  - **Importance of controls, normalization and quality control**
  - **Importance of understanding basic chemistries**

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





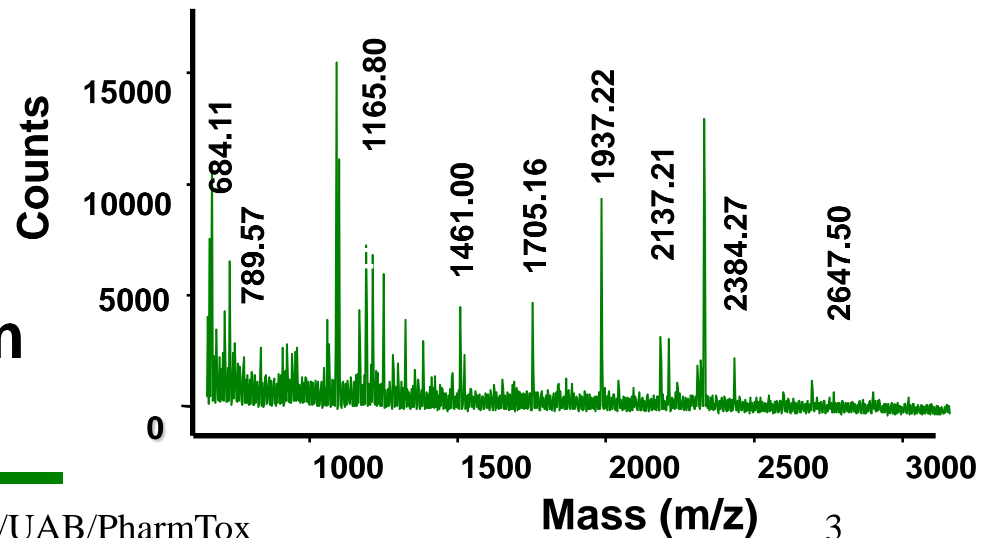
trypsin

MALDI-TOF mass spectrometry

ID of parent polypeptide

MASCOT search engine

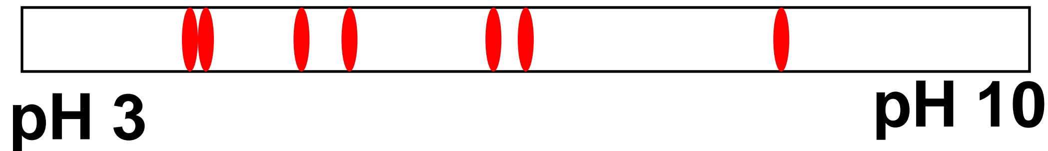
http://www.matrixscience.com



What 2-D electrophoresis involves:

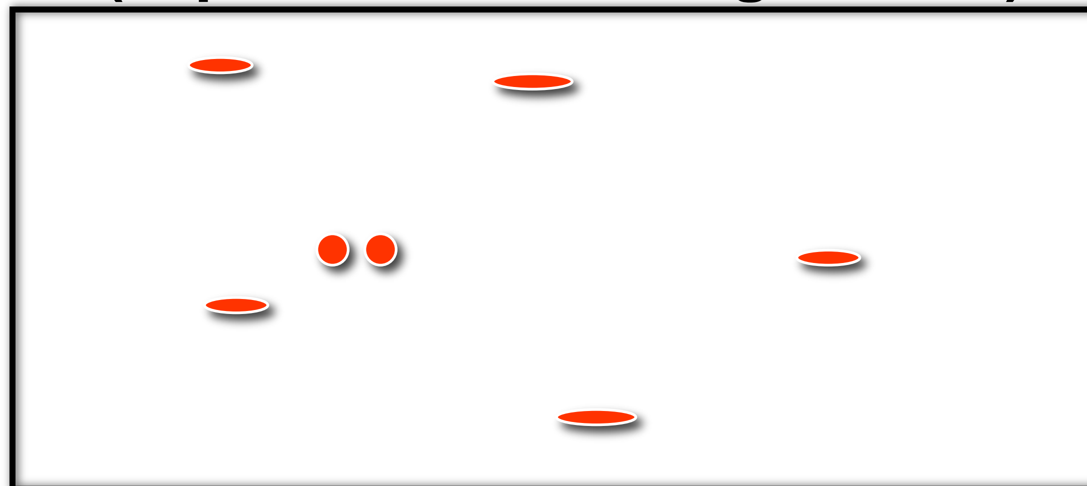
- 1st dimension: **Isoelectric focusing**

  (separation according to charge)



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

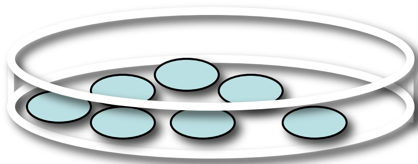
  (separation according to size)



High m.w.

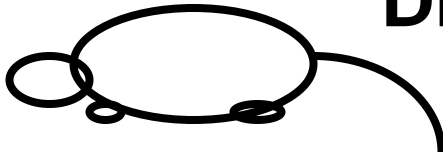
Low m.w.

Sample preparation for 2DE:



Harvest, rinse, and pellet the cells;

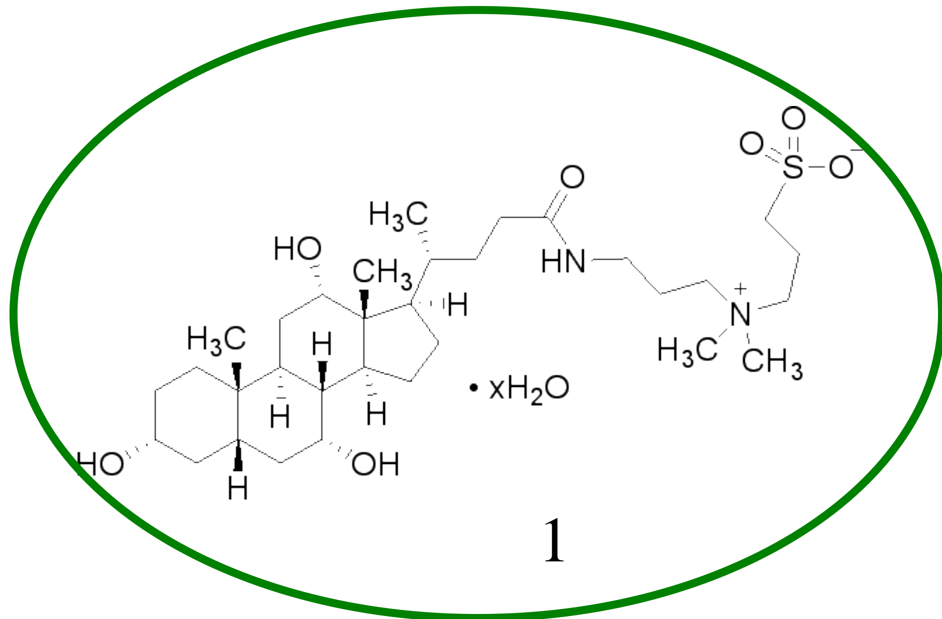
or

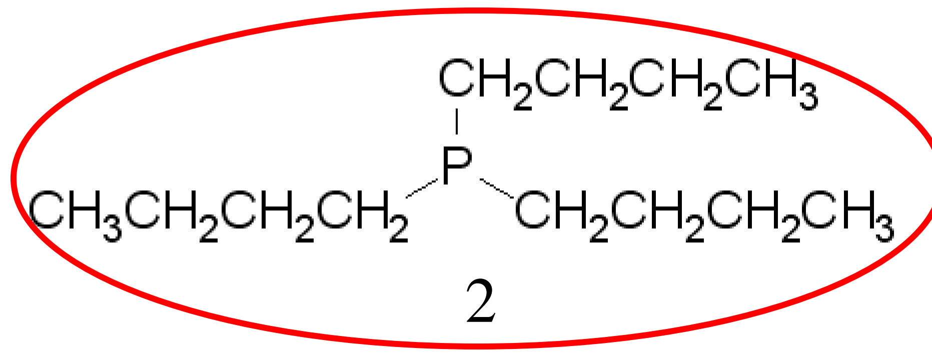


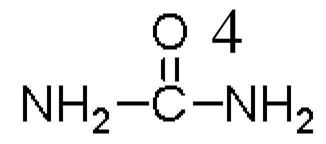
Dissect out tissue, organ, or fluids;

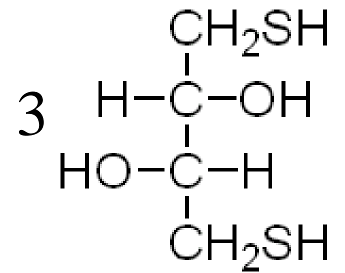
- **Homogenize/lyse in buffer that *dissociates and unfolds the proteins***
  - •High urea usually 5-8 M---unfolds the protein
  - •Sometimes 2 M thiourea--unfolds the protein
  - •1-4% nonionic detergent--solubilizes hydrophobic components
  - •Beta-mercaptoethanol or other reductant
  - •Inhibitors: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

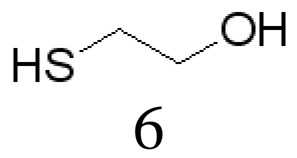
Chemistries relevant in 2D protein electrophoresis

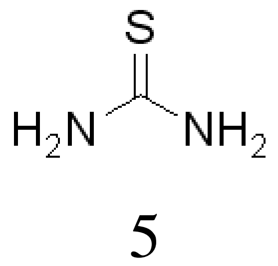


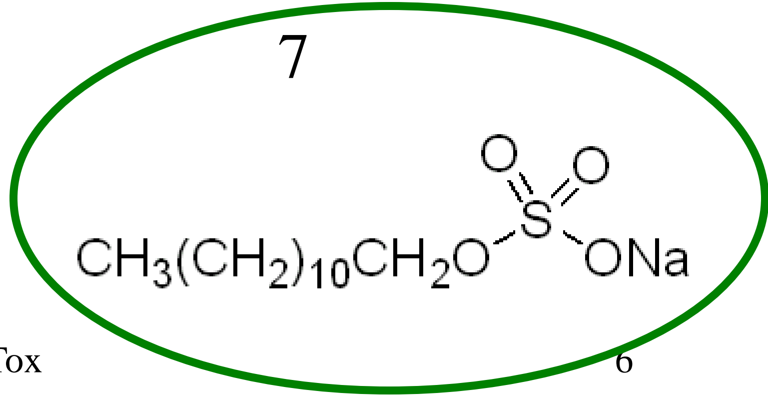




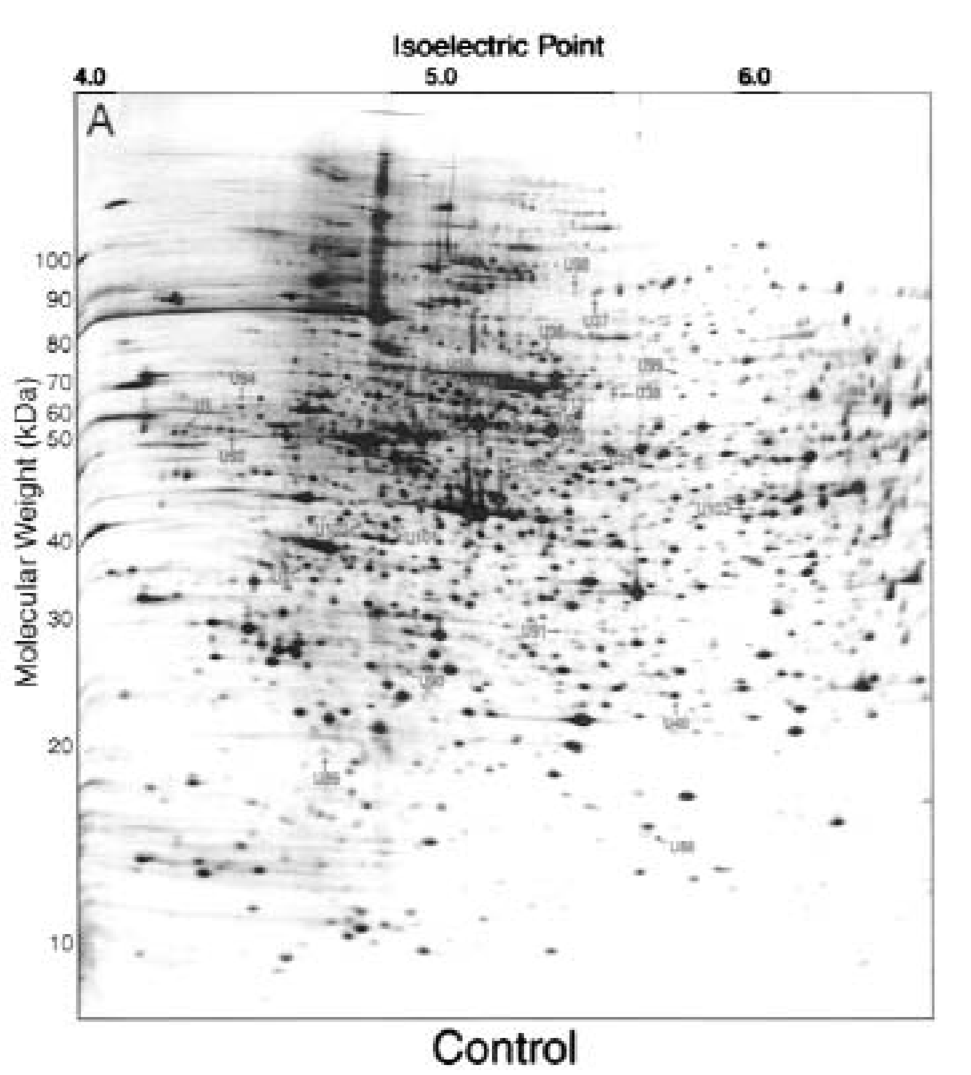








A real 2-D gel



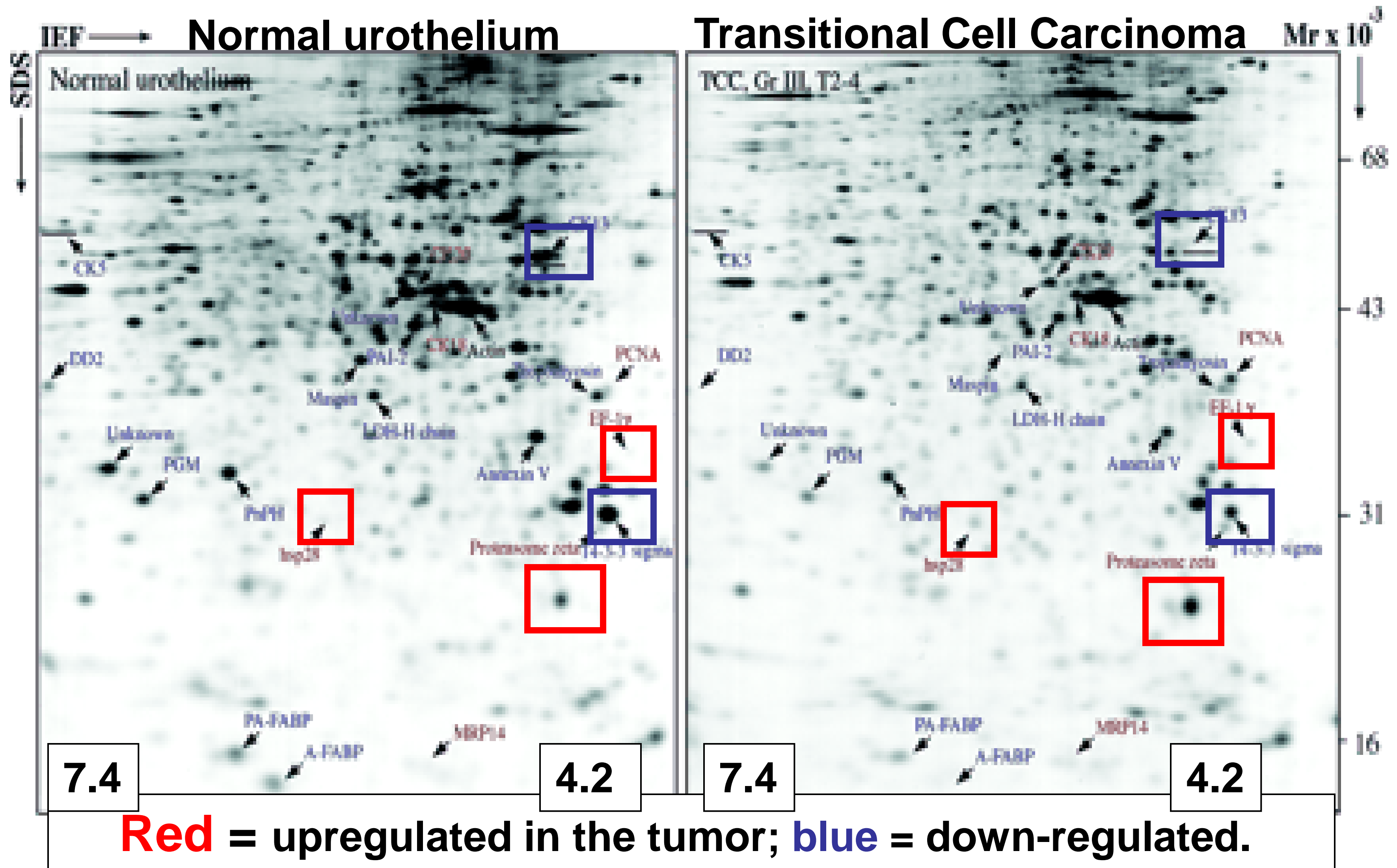
Find this and other 2-D gels at http:www.expasy.org

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.

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)

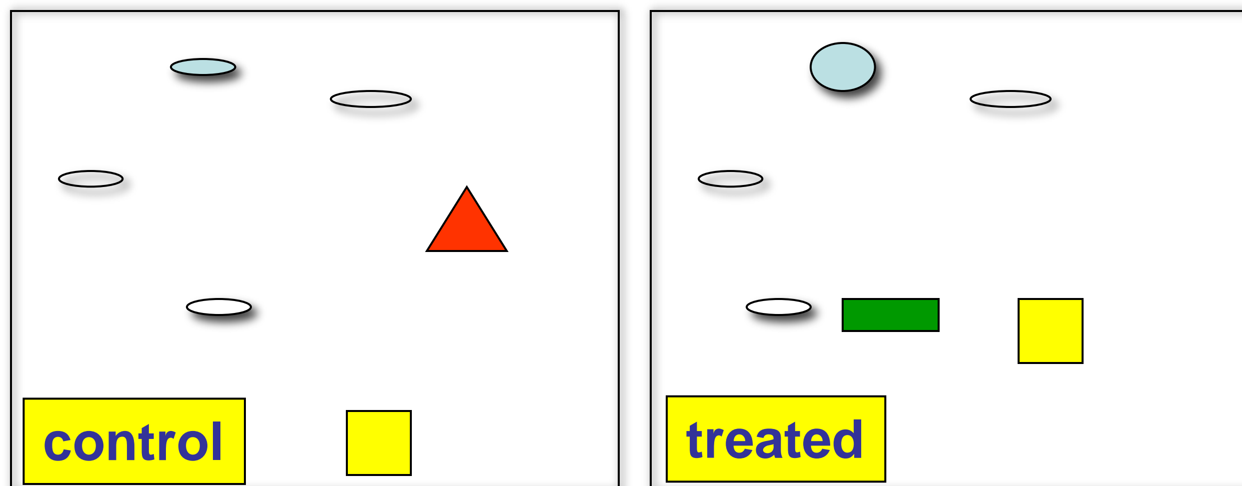
Elements of image analysis of “regular” 2D gels:

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?”

Extracting data out of 2D gels: Image analysis

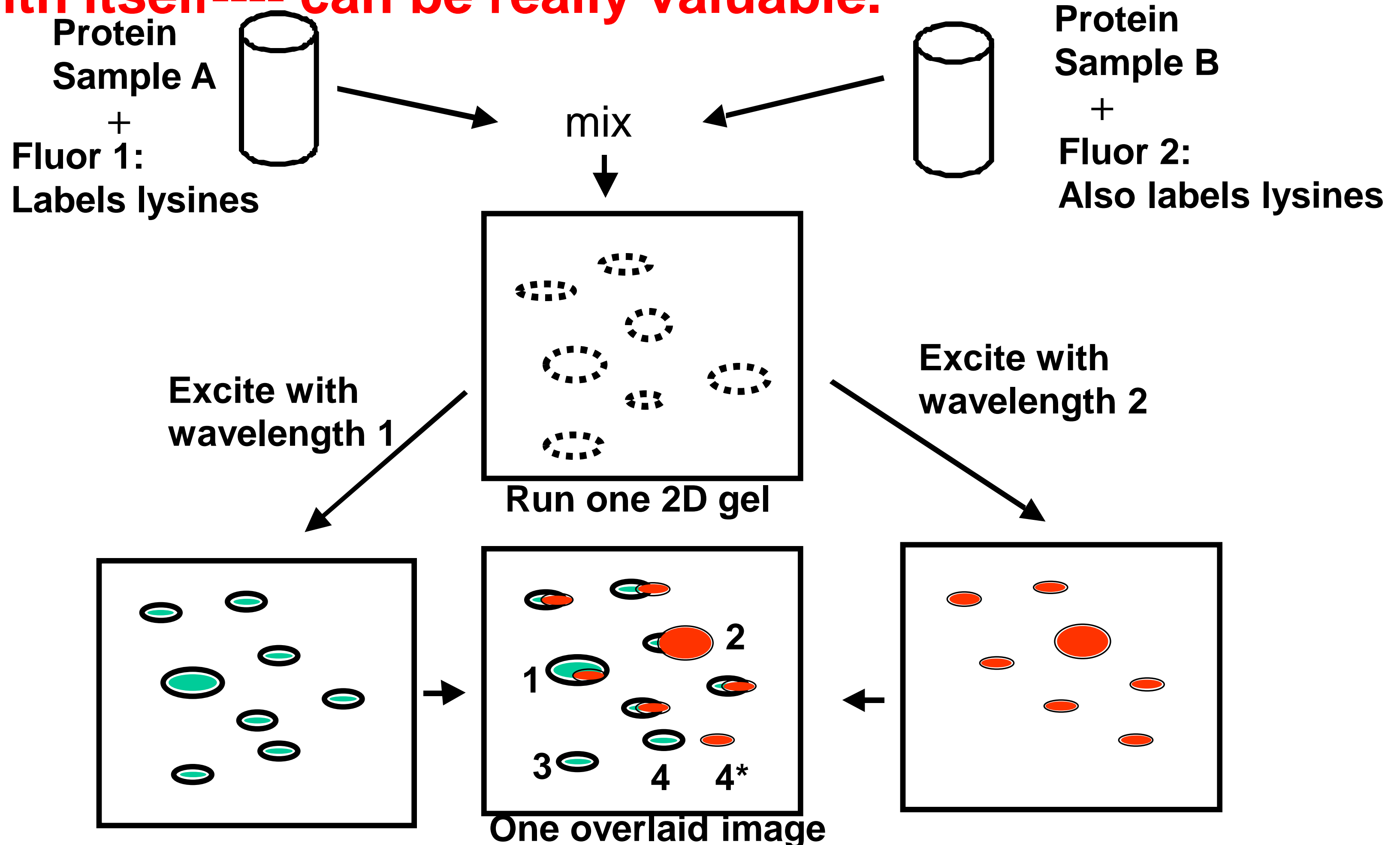


with software: "compare" the images.

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.

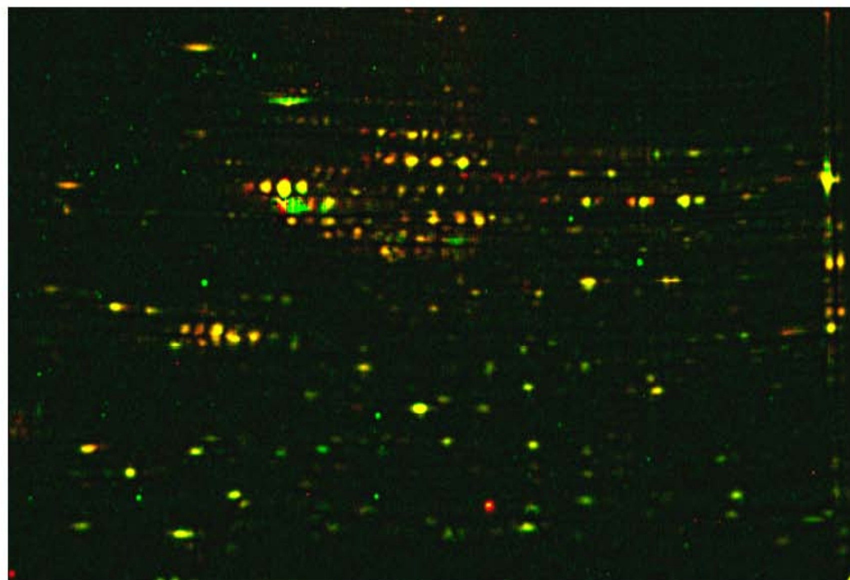
Difference gel electrophoresis (DIGE); a protein migrates with itself---- can be really valuable.



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.

When DIGE works really well:

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



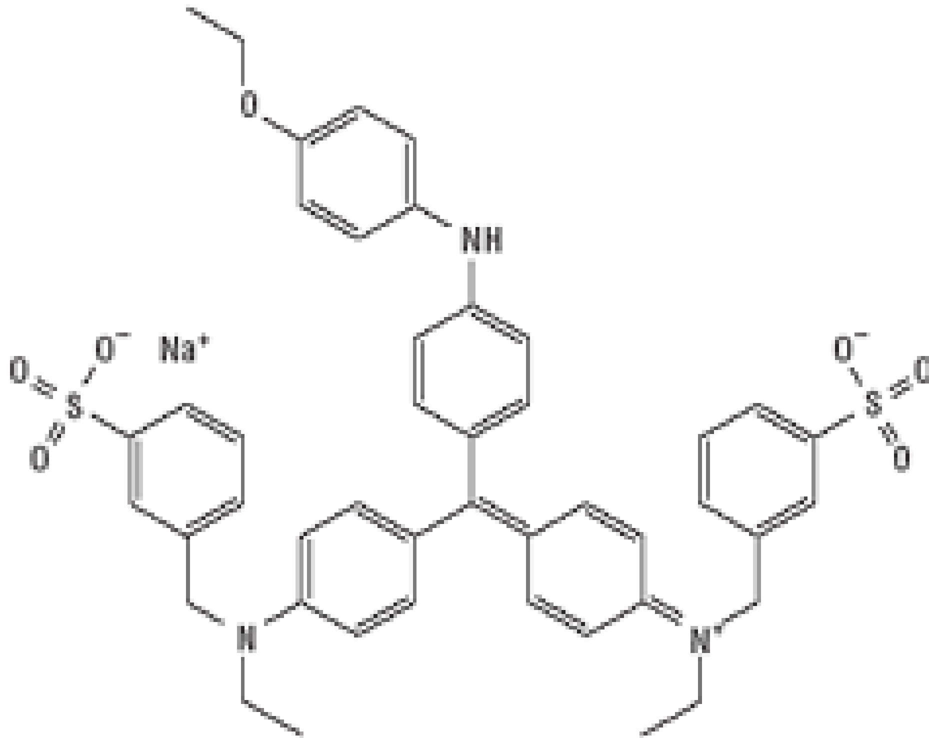
(2D DIGE of rat mammary homogenates [Deshane and Kim] courtesy of BioRad, 2006)

100% green spots are specific to one experimental group,
100% red spots are specific to the other,
degrees of yellow-orange indicate differential expression in both.

"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 same spot 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 always found at a pI different from predicted, it may be constitutively modified in the "unstimulated"/"normal" group**

Visualizing 2D gels: Coomassie Brilliant Blue



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

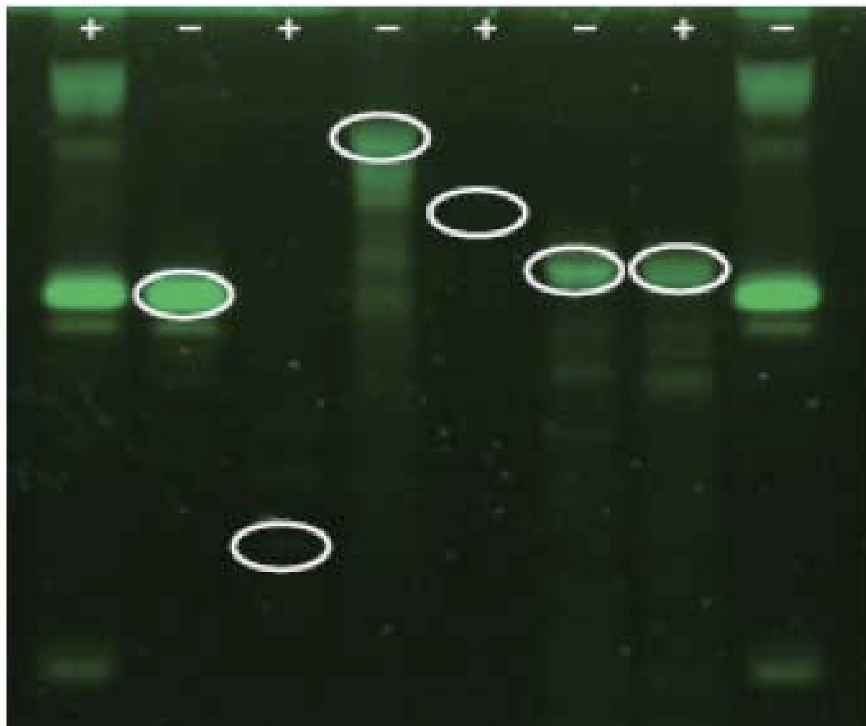
- Inexpensive
- Image readily acquired by scanning at visible wavelengths
- No covalent mass change

Advantages and limitations of the types of stains

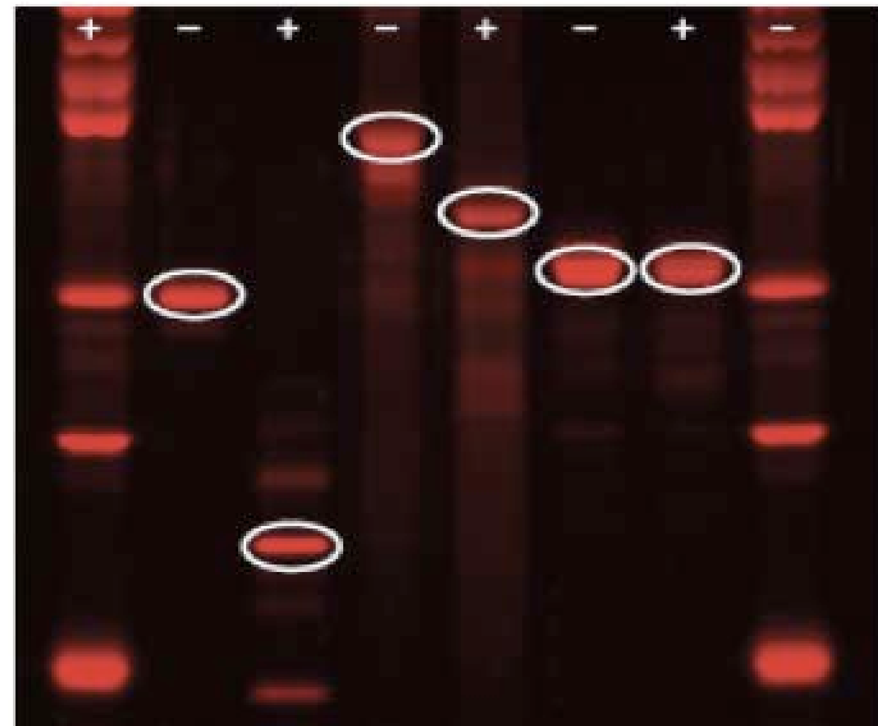
	Sensitivity	Dynamic range	MS- compatible
CBBR	8 ng	10-30 x	yes
Silver	1 ng	< 10 x	Not without special precautions
Fluorescent	2 ng	3 orders of magnitude	yes

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

ProQ Emerald



Sypro Ruby



A- A B- B C- C

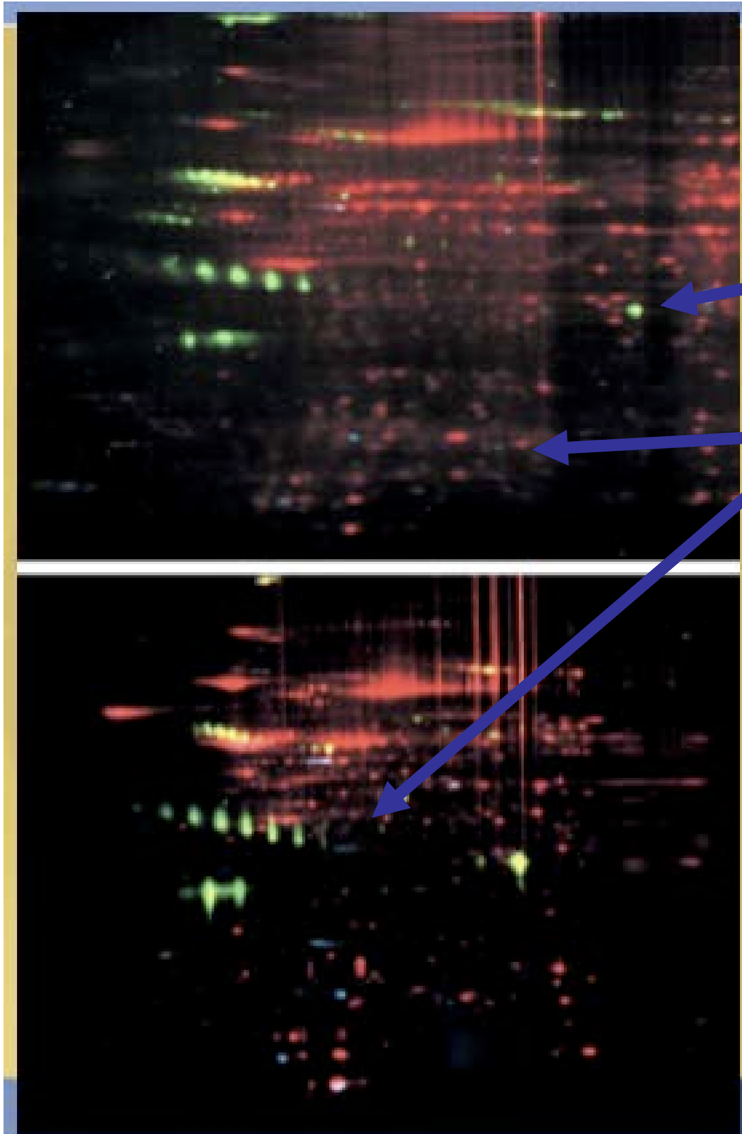
A- A B- B C- C

(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

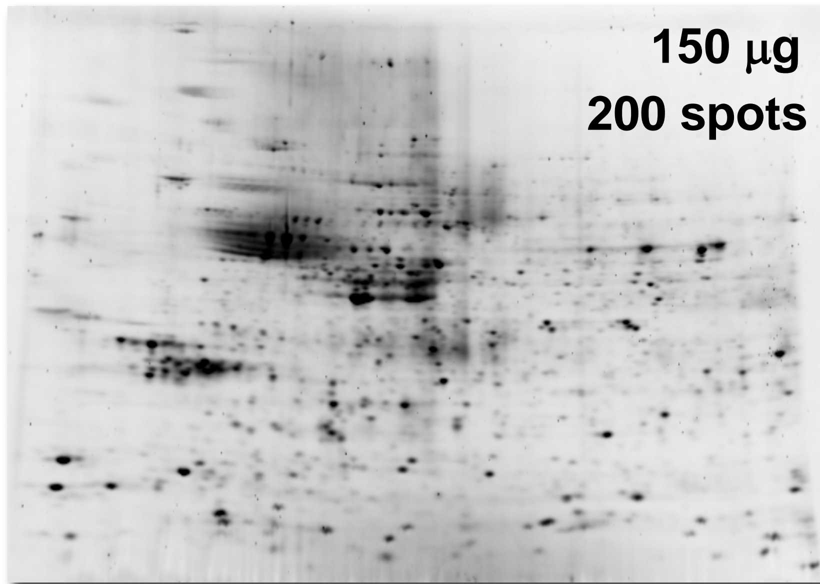
The value of fluorescent dyes: each can be detected separately from others on the gel, due to different excitation and emission spectra.

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.
- Yet, each stain has utility depending on experimental goals.
- "Multiplexing" allows analysis of subproteomes in the same gel, maximizing use of scarce samples.

Rationale for subfractionation, even if you see lots of spots



2D gel of rat brain, stained with Sypro Ruby

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

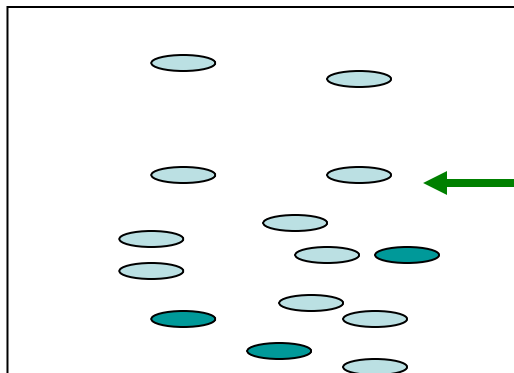
200 spots is <1% of the total proteome.

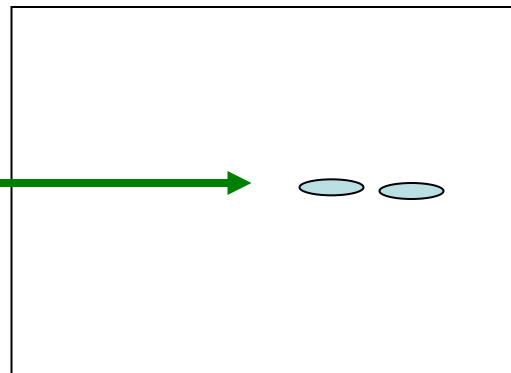
Conclusion: A fluorescently stained 2D gel of an unfractionated sample, only allows detection of the "low hanging fruit."

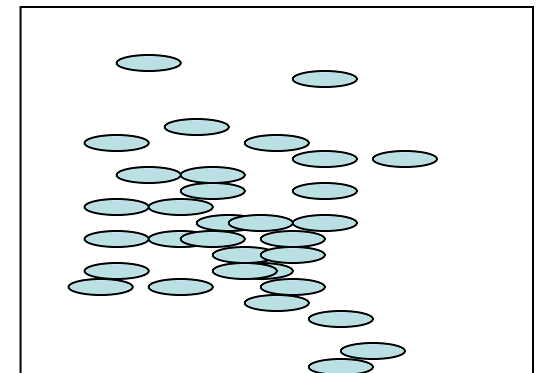
For greatest sensitivity, and the most biological information:

Combine 2D separations with "conventional" approaches;

Nothing is quite as conclusive or helpful as a good Western blot







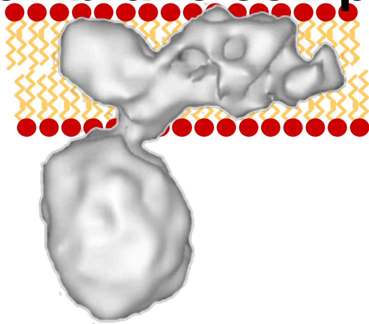
Western blot for phosphoproteins

Western blot for protein X; shows 2 spots; the more acidic is probably phosphorylated; do MS to prove it.

Stained gel

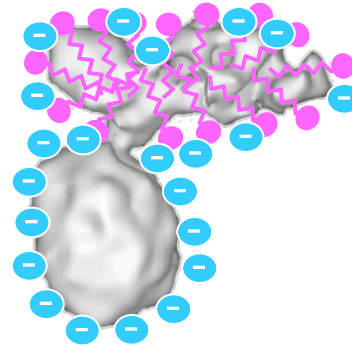
2D-Blue-Native gels: originally developed for membrane-associated protein complexes.

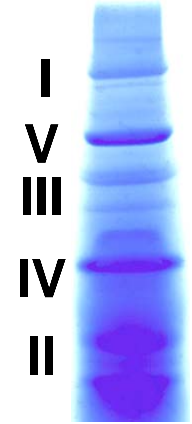
Intrinsic mitochondrial membrane complexes



Detergent

CBBR



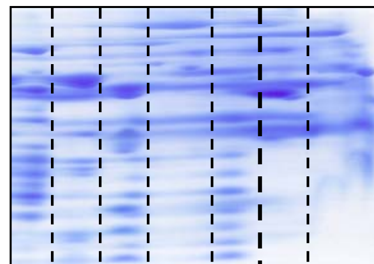


(Modified from Brookes et al., 2002)

Released complexes, all negatively charged, thanks to the CBBR

1st D: NATIVE electrophoresis

The 2nd dimension gel has “ladders” of bands, like lanes, under each complex.
“Image analysis” indicates what bands are different w/in each.

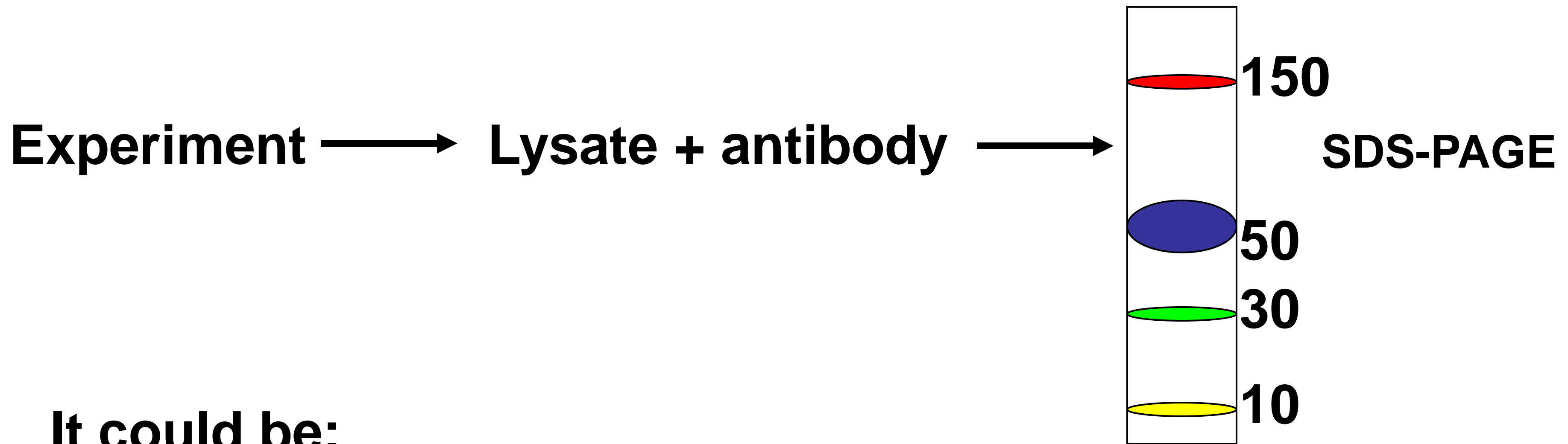


–

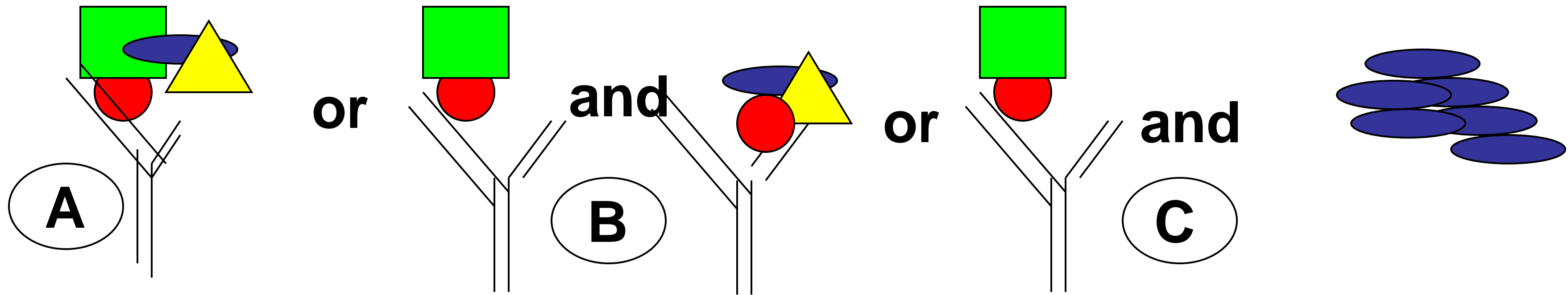
+

2D native electrophoresis to dissect protein-protein interactions:

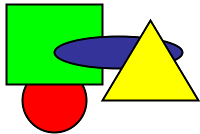
In immunoprecipitates, which proteins are interacting with which?

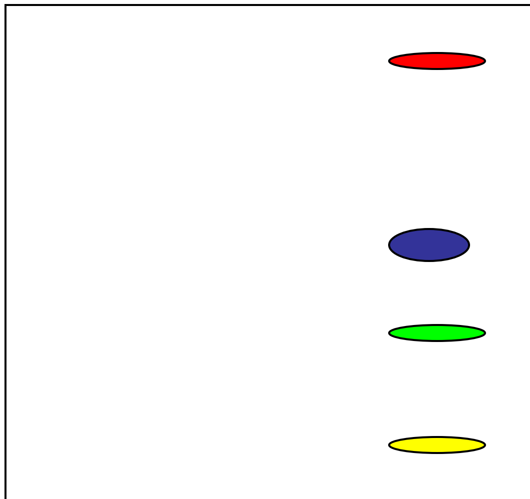


It could be:

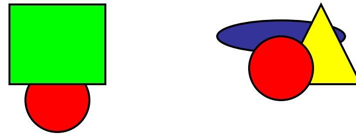


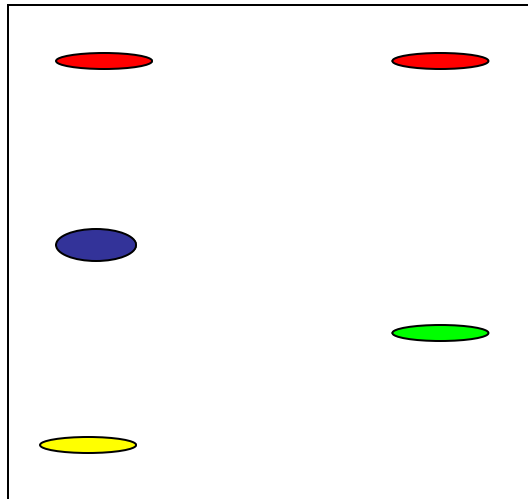
Scenario A:



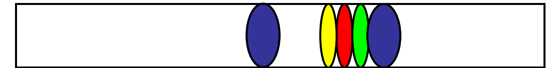


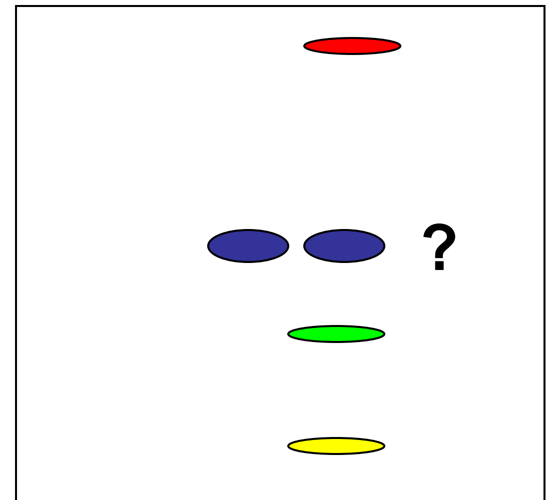
Scenario B:





Scenario C:

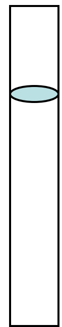




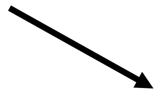
Why would the blue protein come down without the red protein?

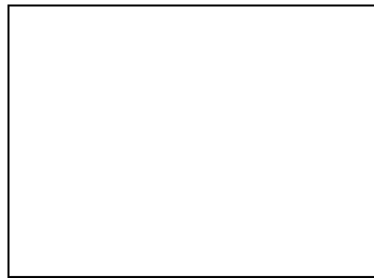
Issues in 2D gel analysis:

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

GELfREE: SDS-PAGE that generates multiple aliquots of “bands” for downstream analysis

- Short resolving tube gel below the stacking gel; cassette runs horizontally, which allows for:
- Large (200 ul vs 15-50 ul) loading chamber;
- Can collect multiple fractions in the outlet chamber according to set times;
- Can run up to 8 channels, either all loaded with the same sample, or with different samples

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 intensity and charge information, thus expression or posttranslational modification information;
- This biological information is not easily obtained by MS analysis of pre-digested fragments.
- Other newer protein separation approaches can be valuable in reducing complexity, or in generating sufficient amounts of materials for downstream analysis: 2D BN, GELfREE.
- Choice of separation governed by
  - Abundance of sample
  - Question being asked
  - What technologies you can access readily
  - What you can afford

Future role of top-down proteomic approaches in protein analysis

I. **Subcellular fractionation will regain importance in proteome analysis;**
II. **While automated hands-off LC/LC-MS/MS may appear more highthroughput for "discovery," every resolved spot on a 2D gel is discovery, but also *purification*;**
III. **2D gel positional information, *without protein identities*, is information itself.**

Notes on quality control

- **Quality control in proteomics has to be kept in mind throughout an experiment, not just when you get to "the analysis."**
- **Reduce variance where you can---**
  - **Maybe share a protocol, but each person does all of one step, don't share the same step;**
  - **Use the same pipettor and small instruments like vortexor for a dataset;**
  - **Make up enough stock solutions for the experiment;**

More notes on quality control

- **Eliminate variance where you can:**
  - **Use the same vendor for disposables;**
  - **Don’t store some samples overnight at -80, but others process right away;**

- **Enhance for the likelihood of detecting difference(s):**
  - **Optimize the cell culture experiment with respect to concentration of bioactive agent, time of exposure, and point in cell cycle**