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BMG744

Top-down proteomics approaches: (a) to monitor protein purification; (b) to resolve PTM isoforms and protein complexes

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Learning objectives

WEDNESDAY (Jan 9, 2013):

- Rationales for proteomics versus genomics versus Western blots versus immunoprecipitations.
- SMART PROTEOMICS: reduce proteome complexity, increase biological specificity;
- Take advantage of properties of proteins and experimental information to reduce proteome complexity

FRIDAY (Jan 11, 2013):

- How do we know what we have, and what's important
  - SDS-PAGE
  - 2D DIGE but IEF may be enough, depending on question
  - 2D native electrophoresis
- How do we enhance what we have
  - Optimize for PTM/phenotype by titering the stimulus…. TGF-beta?
  - Optimize for signal experimentally: synchronize cells at a particular point in the cell cycle, then release.
  - Optimize immunoprecipitations by minimizing secondary reagents, using sufficient antibody for the initial immune complexes
- Additional quality control issues for proteomic analysis: Randomize; randomize; randomize.

Experimental rationale determines extent and nature of protein purification for proteomic analysis

Different rationales for studying proteins in biological samples:

A. Nothing is known, and all proteins are of interest;

B. Nothing is known, but only proteins that are different between disease and healthy or treated and control or novel versus familiar are of interest;

C. PTM of a single protein or a PARTICULAR PTM category

D. Protein *interactions* involving a specific protein are of interest

Structures important in protein electrophoresis: be able to name them, and know which are NOT used in 2D gels

1

2
$CH_3CH_2CH_2CH_2-P(CH_2CH_2CH_2CH_3)-CH_2CH_2CH_2CH_3$

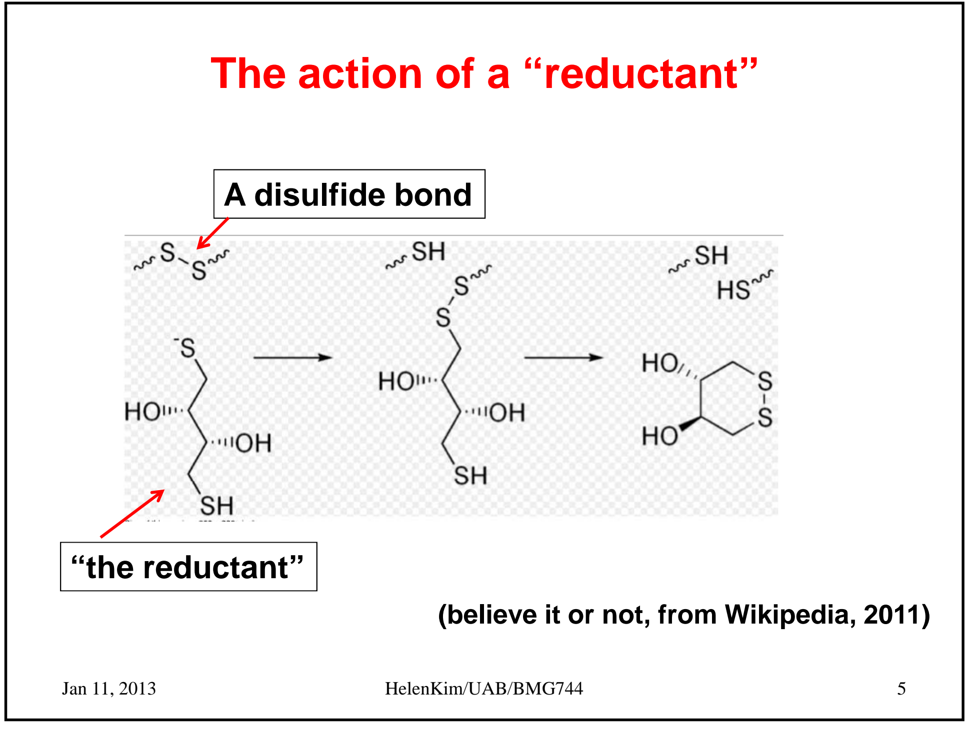
3
CH_2SH / $H-C-OH$ / $HO-C-H$ / CH_2SH

4
$NH_2-C(=O)-NH_2$

5
$H_2N-C(=S)-NH_2$

6
$HS-CH_2CH_2-OH$

7
$CH_3(CH_2)_{10}CH_2O-SO_2-ONa$


The action of a "reductant"
A disulfide bond
"the reductant"
(believe it or not, from Wikipedia, 2011)
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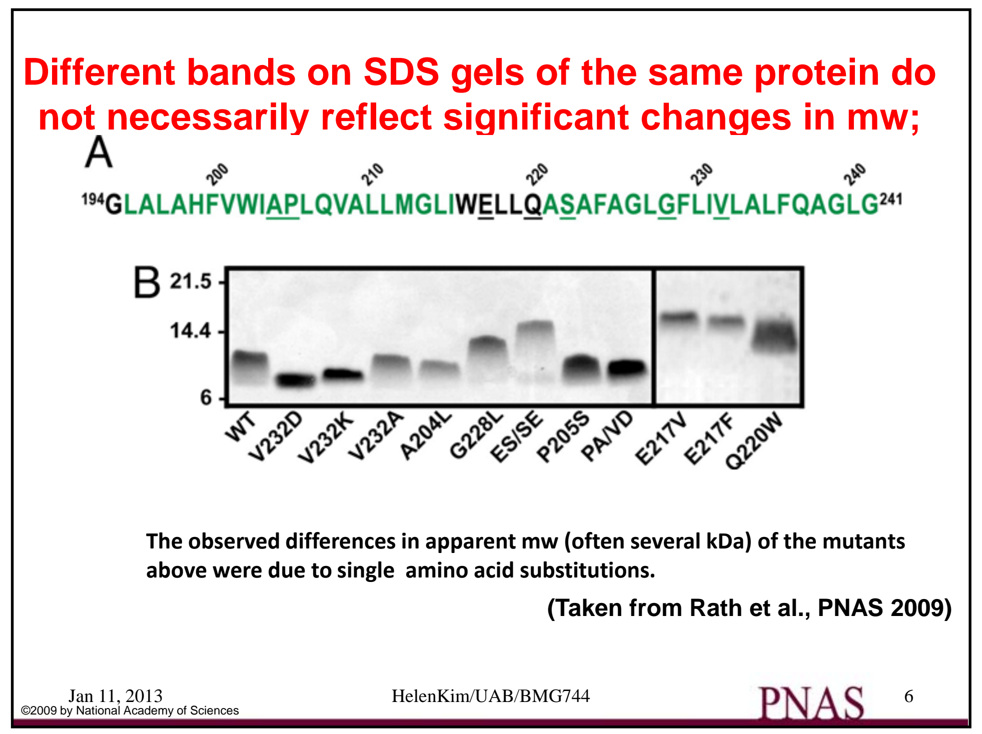
Different bands on SDS gels of the same protein do not necessarily reflect significant changes in mw;
A
194GLALAHFVWIAPLQVALLMGLIWELLQASAFAGLGFLIVLALFQAGLG241
B 21.5
14.4
6
WT V232D V232K V232A A204L G228L ES/SE P205S PA/VD E217V E217F Q220W
The observed differences in apparent mw (often several kDa) of the mutants above were due to single amino acid substitutions.
(Taken from Rath et al., PNAS 2009)
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PNAS
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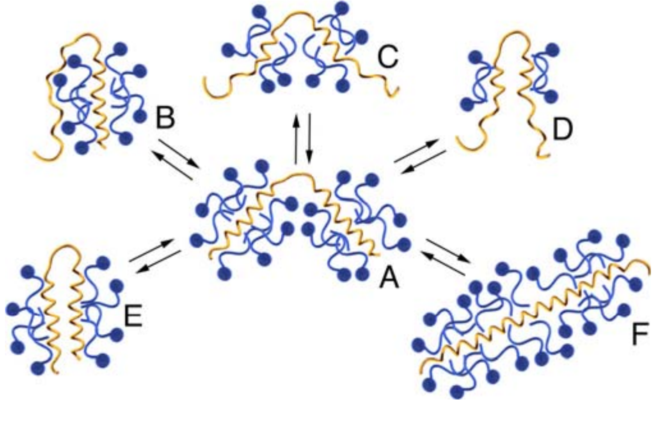

(Taken from Rath et al., PNAS 2009)

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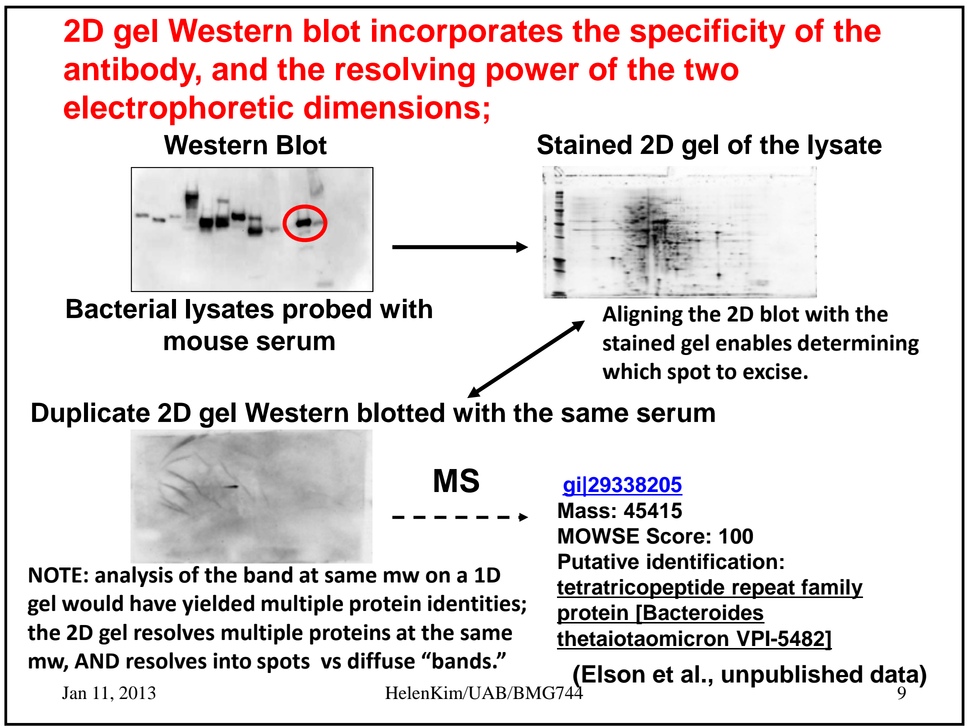
Why do we care about SDS binding and minute differences in protein structure:

- **Western blot analysis can detect a polypeptide at an unexpected mw; doesn't mean its mass is different necessarily in the mutant or diseased tissue; it could be due to micro-differences in how the SDS is binding.**
- **Western blot detection of multiple bands may reflect structural or conformational heterogeneity in that antigen in the sample, not cross-reactivity with other proteins, otherwise known as "nonspecific-ness."**
- **Cannot presume a polypeptide will be at or near its predicted or WT mw.**


2D gel Western blot incorporates the specificity of the antibody, and the resolving power of the two electrophoretic dimensions;
Western Blot
Stained 2D gel of the lysate
Bacterial lysates probed with mouse serum
Aligning the 2D blot with the stained gel enables determining which spot to excise.
Duplicate 2D gel Western blotted with the same serum
MS
gi|29338205
Mass: 45415
MOWSE Score: 100
Putative identification:
tetratricopeptide repeat family protein [Bacteroides thetaiotaomicron VPI-5482]
NOTE: analysis of the band at same mw on a 1D gel would have yielded multiple protein identities; the 2D gel resolves multiple proteins at the same mw, AND resolves into spots vs diffuse "bands."
(Elson et al., unpublished data)
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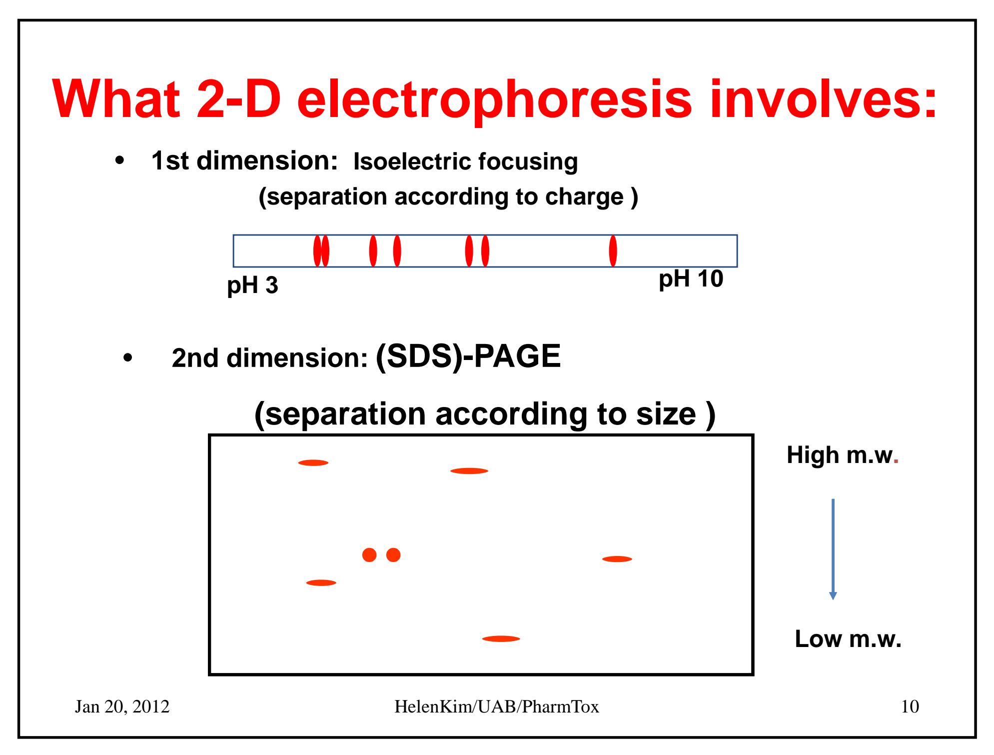
What 2-D electrophoresis involves:
1st dimension: Isoelectric focusing
(separation according to charge)
pH 3
pH 10
2nd dimension: (SDS)-PAGE
(separation according to size)
High m.w.
Low m.w.
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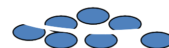
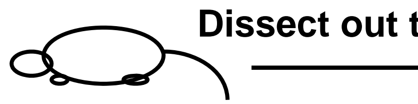
Sample preparation for 2DE:



Harvest, rinse, and pellet the cells;

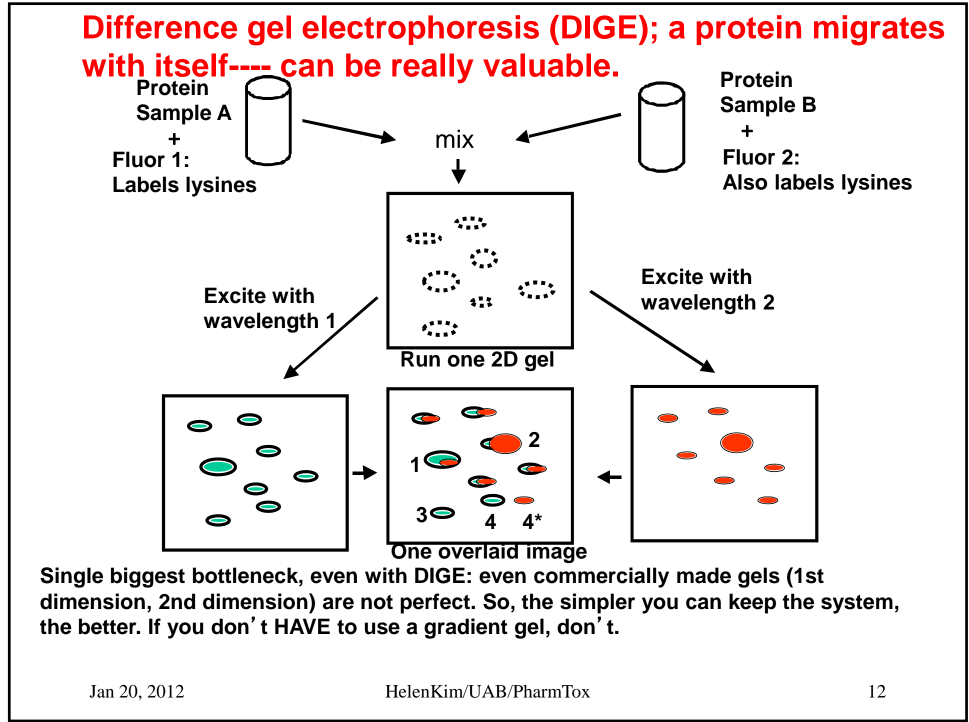
or



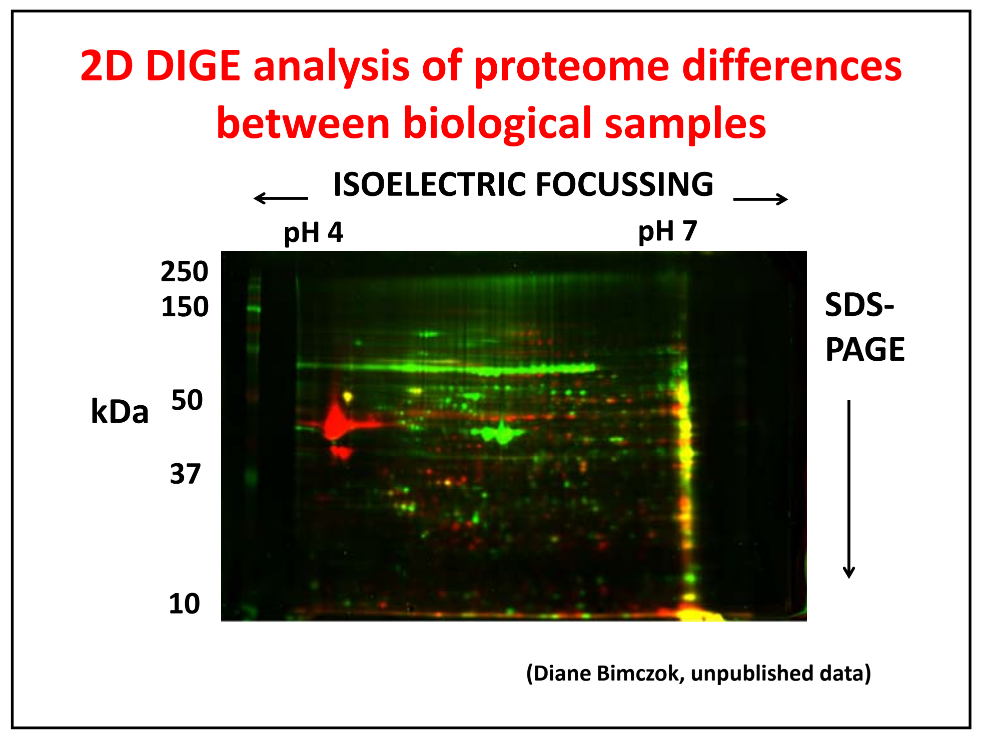
Dissect out tissue, organ, or fluids;

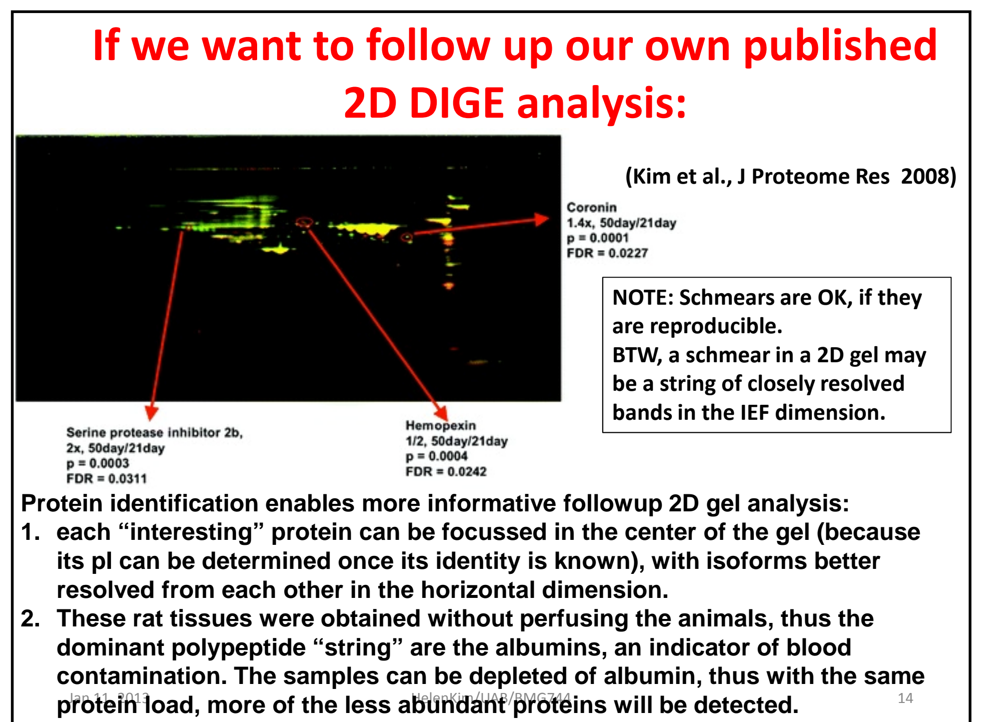
- Homogenize/lyse in buffer that *dissociates and unfolds the proteins*
  - High urea usually 5-8 M---unfolds the protein
  - Sometimes 2 M thiourea--unfolds the protein
  - 1-4% nonionic detergent--solubilizes hydrophobic components
  - Beta-mercaptoethanol or other reductant
  - Inhibitors: of proteases,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

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.


2D DIGE analysis of proteome differences between biological samples
ISOELECTRIC FOCUSSING
pH 4
pH 7
250
150
50
37
10
kDa
SDS-PAGE
(Diane Bimczok, unpublished data)


If we want to follow up our own published 2D DIGE analysis:
(Kim et al., J Proteome Res 2008)
Coronin
1.4x, 50day/21day
p = 0.0001
FDR = 0.0227
NOTE: Schmears are OK, if they are reproducible.
BTW, a schmear in a 2D gel may be a string of closely resolved bands in the IEF dimension.
Serine protease inhibitor 2b,
2x, 50day/21day
p = 0.0003
FDR = 0.0311
Hemopexin
1/2, 50day/21day
p = 0.0004
FDR = 0.0242
Protein identification enables more informative followup 2D gel analysis:
1. each "interesting" protein can be focussed in the center of the gel (because its pI can be determined once its identity is known), with isoforms better resolved from each other in the horizontal dimension.
2. These rat tissues were obtained without perfusing the animals, thus the dominant polypeptide "string" are the albumins, an indicator of blood contamination. The samples can be depleted of albumin, thus with the same protein load, more of the less abundant proteins will be detected.
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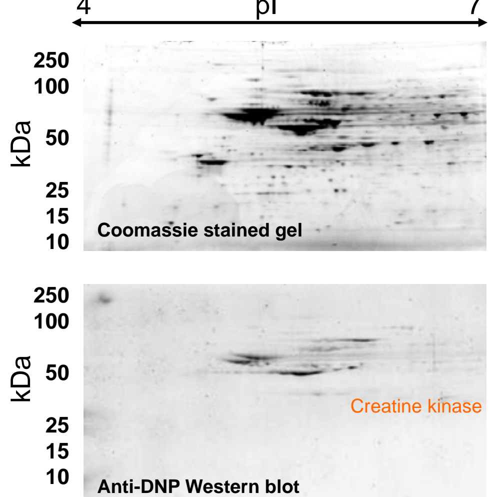
2D Western blot analysis detects oxidations of multiple proteins, which can be identified by MS of gel spots from duplicate stained gels



Kim et al., Methods in Aging Research, 2006

The chemistry behind Western blotting for protein oxidations (oxyblots).

Protein carbonyl

$O{=}CR_2$

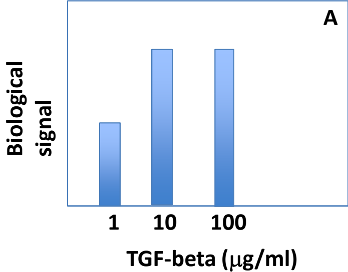
NH_2
NH
NO_2
NO_2
+

DNPH

R
R
C=N–N
H
NO_2
NO_2

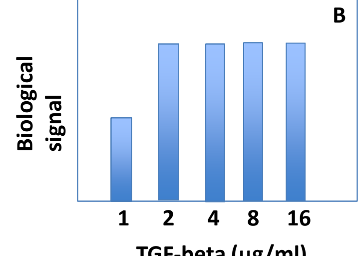
Recognized by anti-DNP antibody

Enhance specificity of your proteomic data by optimizing for your biological signal



(A) tells you that 10 ug/ml TGF-beta gives you a higher signal than 1 ug/ml, but increasing ten fold to 100 ug/ml does not increase the signal.

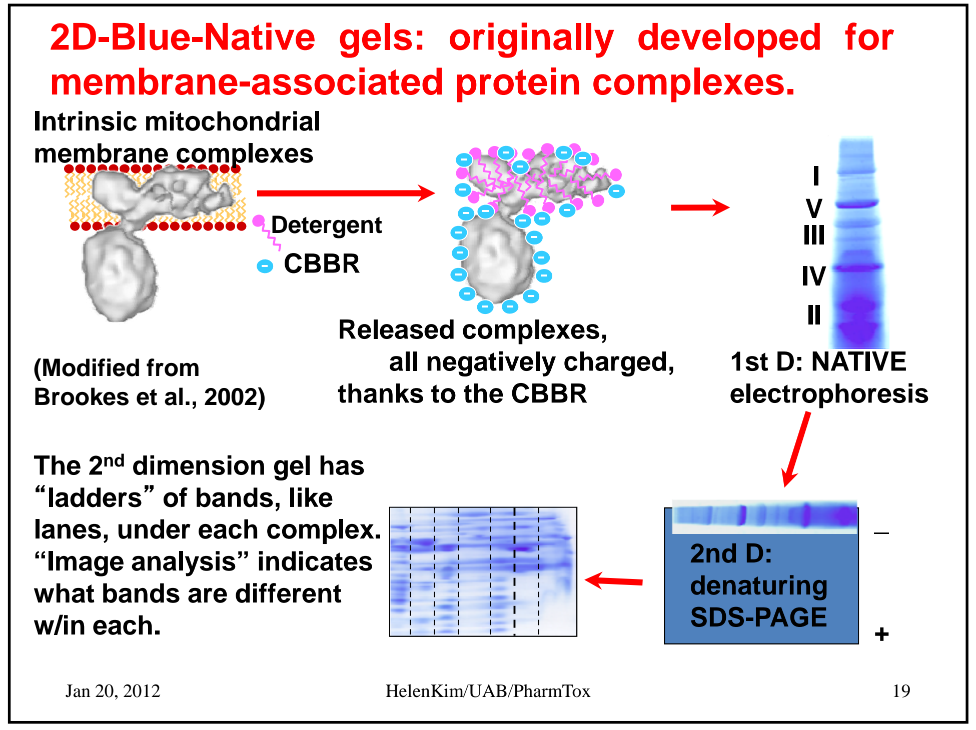
Thus, by having done the titration, you save $$$ by using 10-fold less TGF-beta, but more importantly, may reduce "nonspecific" PTMs and other cellular events unrelated to direct TGF-beta actions by keeping TGF-beta as low as possible.



(B) Repeating the experiment, looking between 1 and 10 ug/ml TGF-beta, shows that only 2 ug/ml is needed. It's a good idea to repeat at least one point beyond the 10, to confirm that the signal has indeed plateaued.

Reduce proteome complexity (and cost) of immunoprecipitation experiments by titrating the immuno-reagents

- Per 1 ug antibody, what volume of Protein A beads do you really need; test 10, 20 and 40 ul of beads with same 1 ug antibody, run on SDS-gel, stain, see where the amount of antibody has saturated; if 10 ul beads is sufficient, why use 40.
- Per 1 mg cell lysate, how much antibody do you need? Test 1,2,4 and 8 ug antibody, with corresponding volumes of Protein A beads to pull the immune complexes down; assess amount of antigen by 1D Western blot of the immune complexes. If 2 ug antibody brings down maximum antigen, why use 8 or 10?


2D-Blue-Native gels: originally developed for membrane-associated protein complexes.
Intrinsic mitochondrial membrane complexes
Detergent
CBBR
Released complexes, all negatively charged, thanks to the CBBR
I
V
III
IV
II
(Modified from Brookes et al., 2002)
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.
2nd D: denaturing SDS-PAGE
–
+
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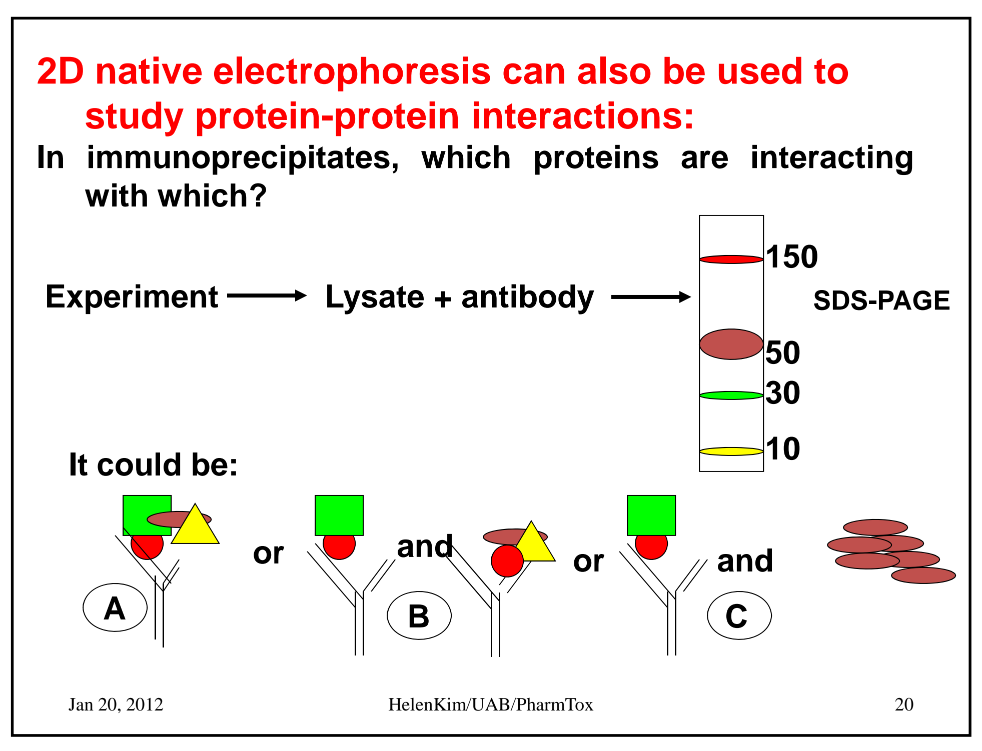
2D native electrophoresis can also be used to study protein-protein interactions:
In immunoprecipitates, which proteins are interacting with which?
Experiment
Lysate + antibody
150
SDS-PAGE
50
30
10
It could be:
or
and
or
and
A
B
C
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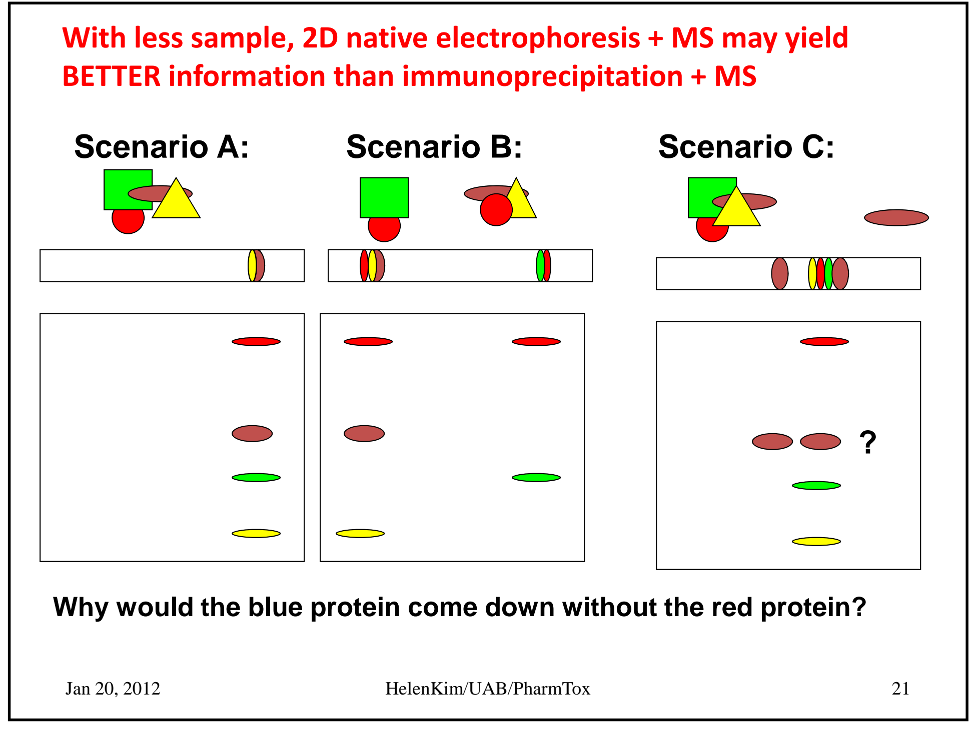
With less sample, 2D native electrophoresis + MS may yield BETTER information than immunoprecipitation + MS
Scenario A:
Scenario B:
Scenario C:
?
Why would the blue protein come down without the red protein?
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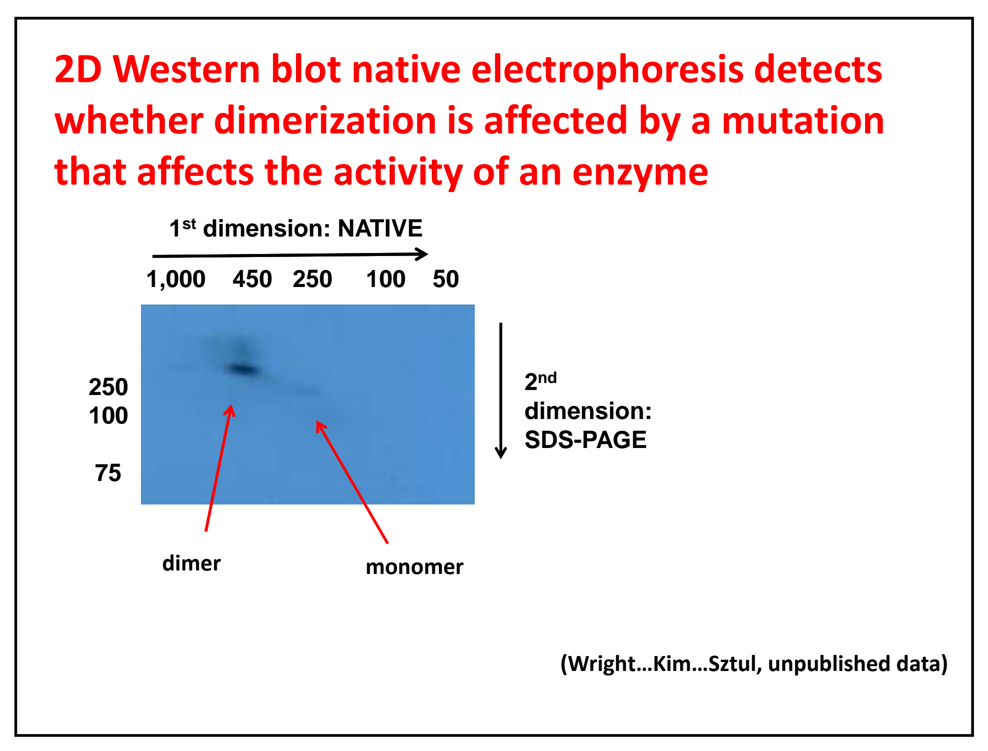
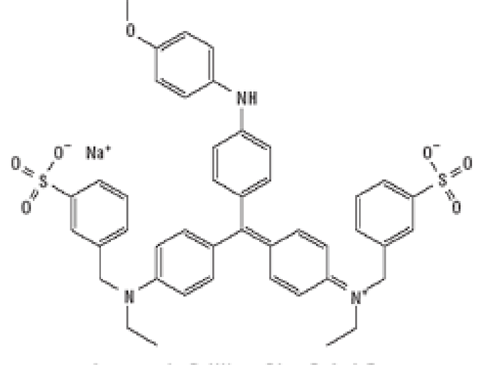

2D Western blot native electrophoresis detects whether dimerization is affected by a mutation that affects the activity of an enzyme
1st dimension: NATIVE
1,000 450 250 100 50
250
100
75
2nd dimension: SDS-PAGE
dimer
monomer
(Wright…Kim…Sztul, unpublished data)

Visualizing 2D gels: Coomassie Brilliant Blue

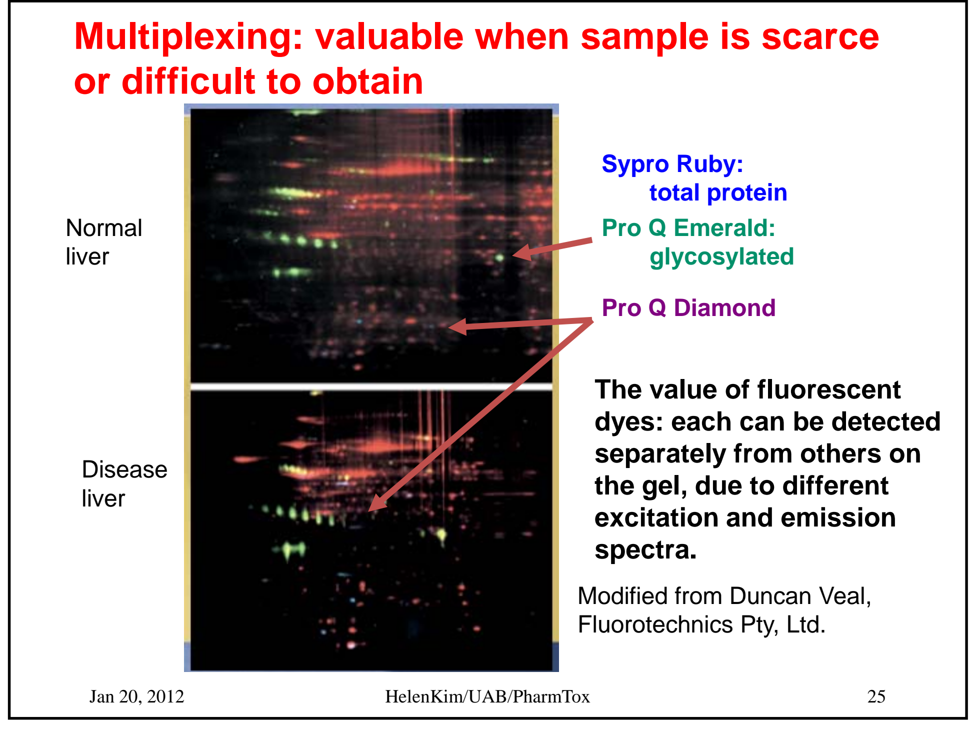


In acidic conditions, the anion of CBBR combines with the protonated amino groups on proteins via electrostatic interactions.

- 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



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.

Take home lessons w/regard to protein preparation for proteomics analysis:

- **Effective generation of protein samples for proteomic analysis results when experimental parameters are optimized for maximum signal and the least "background."**
- **Everything costs; mass spectrometry costs, 2D gels cost, but so do immuno-reagents; optimizing and titrating parameters usually results in lower lab costs in the long run.**
- **High-tech shot-gun mass spectrometry may give you more protein identities in your samples, but less biological information than a set of 2D gels.**