

Jan 10, 2014  
BMG744

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

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1

## Learning objectives

### WEDNESDAY (Jan 8, 2014):

- 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 10, 2014):

- 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.

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2

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

### Different rationales for studying proteins in biological samples:

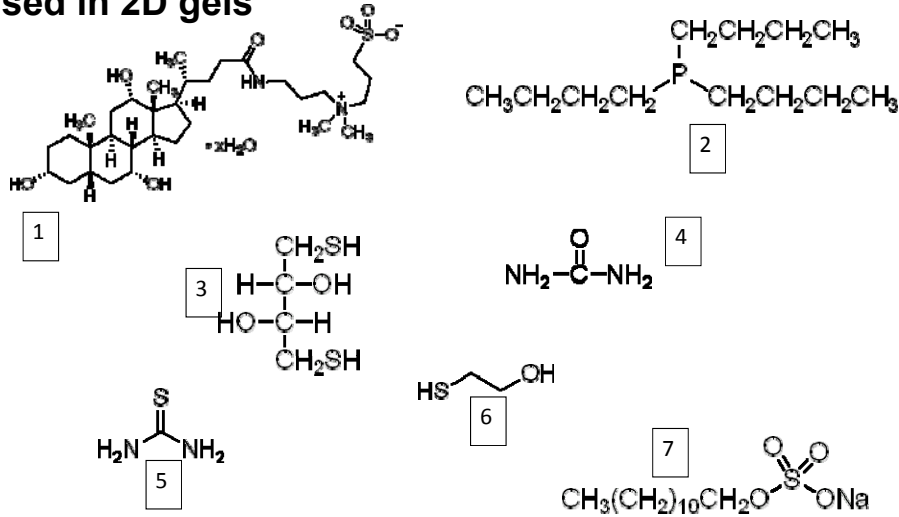
- A. Nothing is known, and all proteins are of interest;
- B. Nothing is known, but only proteins that are different between disease and healthy or treated and control or novel versus familiar are of interest;
- C. PTM of a single protein or a PARTICULAR PTM category
- D. Protein *interactions* involving a specific protein are of interest

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3

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

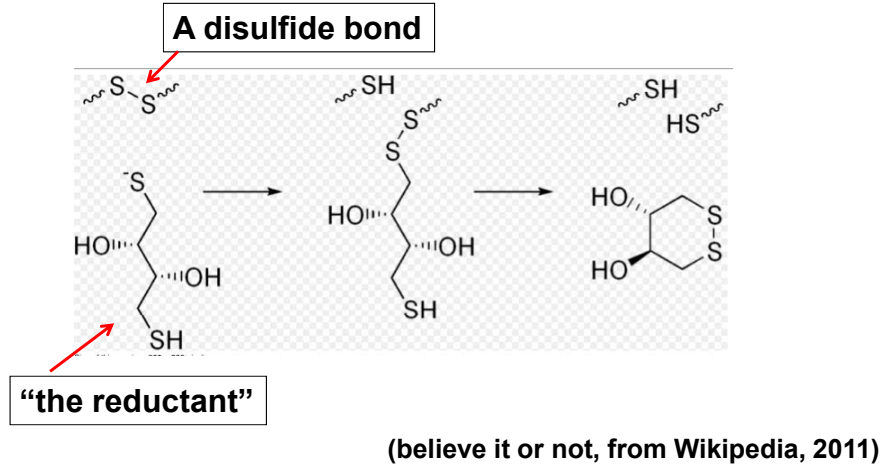


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4

## The action of a “reductant”

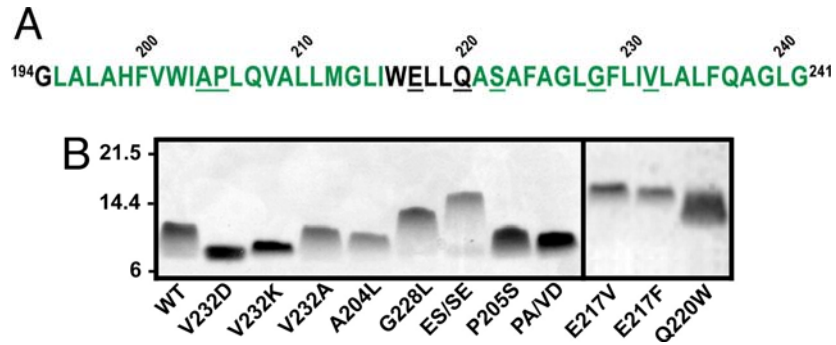


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5

## Different bands on SDS gels of the same protein do not necessarily reflect significant changes in mw;



The observed differences in apparent mw (often several kDa) of the mutants above were due to single amino acid substitutions.

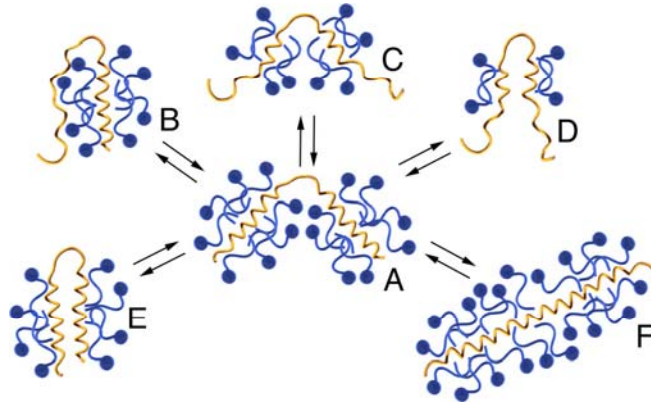
(Taken from Rath et al., PNAS 2009)

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©2009 by National Academy of Sciences

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PNAS 6

**Complexity of SDS binding to proteins, causing different migration on SDS gels, can result from different conformations of hairpin loops.**



(Taken from Rath et al., PNAS 2009)

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PNAS 7

**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.**

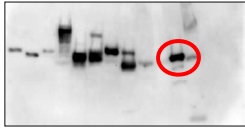
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8

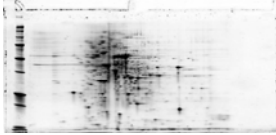
**2D gel Western blot incorporates the specificity of the antibody, and the resolving power of the two electrophoretic dimensions;**

**Western Blot**



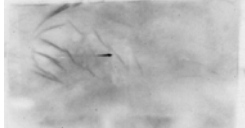
Bacterial lysates probed with mouse serum

**Stained 2D gel of the lysate**



Aligning the 2D blot with the stained gel enables determining which spot to excise.

Duplicate 2D gel Western blotted with the same serum



**MS**

----->

[gil29338205](#)  
 Mass: 45415  
 MOWSE Score: 100  
 Putative identification:  
tetratricopeptide repeat family protein [Bacteroides thetaiotaomicron VPI-5482]

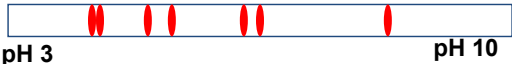
NOTE: analysis of the band at same mw on a 1D gel would have yielded multiple protein identities; the 2D gel resolves multiple proteins at the same mw, AND resolves into spots vs diffuse "bands."

(Elson et al., unpublished data)

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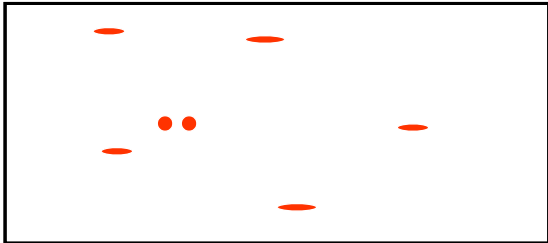
**What 2-D electrophoresis involves:**

- 1st dimension: Isoelectric focusing (separation according to charge)



pH 3 pH 10

- 2nd dimension: (SDS)-PAGE (separation according to size)



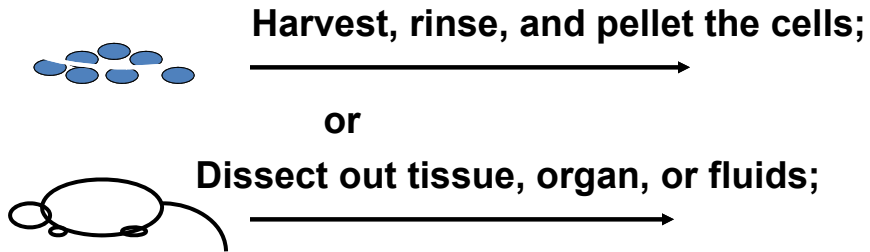
High m.w.

↓

Low m.w.

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## Sample preparation for 2DE:



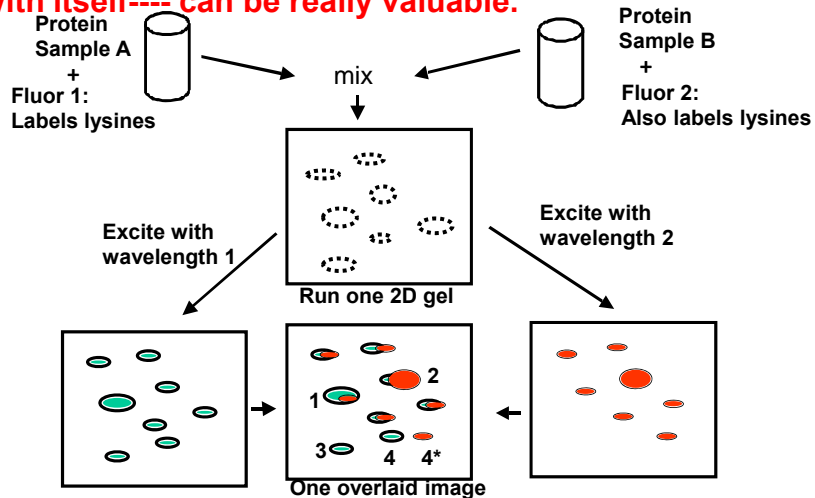
- Homogenize/lyse in buffer that *dissociates and unfolds the proteins*
- High urea usually 5-8 M---unfolds the protein
- Sometimes 2 M thiourea--unfolds the protein
- 1-4% nonionic detergent--solubilizes hydrophobic components
- Beta-mercaptoethanol or other reductant
- Inhibitors: of proteases, kinases,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

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11

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



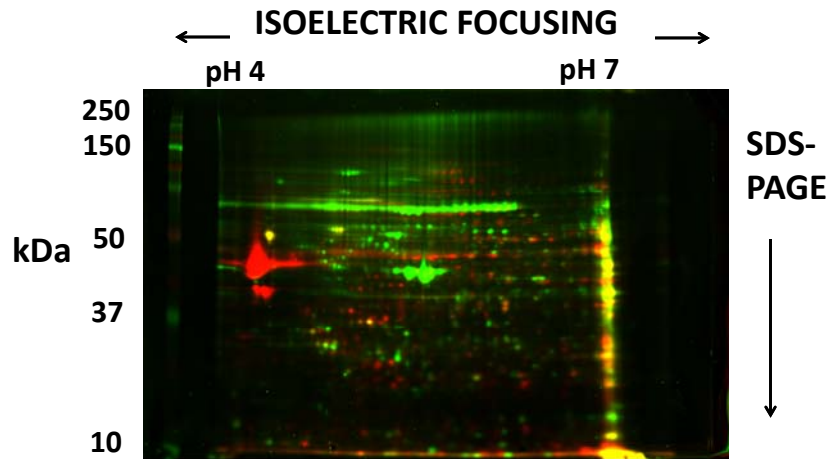
Single biggest bottleneck, even with DIGE: even commercially made gels (1st dimension, 2nd dimension) are not perfect. So, the simpler you can keep the system, the better. If you don't HAVE to use a gradient gel, don't.

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12

## 2D DIGE analysis of proteome differences between biological samples



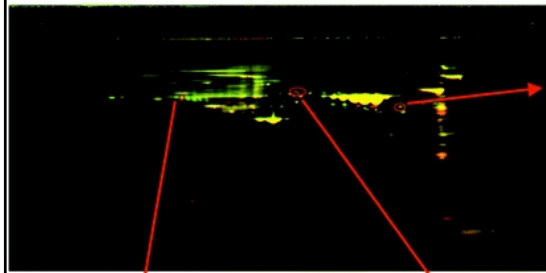
(Diane Bimczok, unpublished data)

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13

## 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 smear 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:

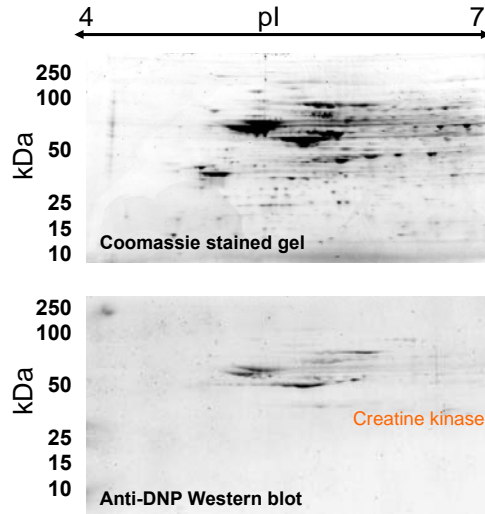
1. each “interesting” protein can be focused in the center of the gel (because its pI can be determined once its identity is known), with isoforms better resolved from each other in the horizontal dimension.
2. These rat tissues were obtained without perfusing the animals, thus the dominant polypeptide “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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14

**2D Western blot analysis detects oxidations of multiple proteins, which can be identified by MS of gel spots from duplicate stained gels**



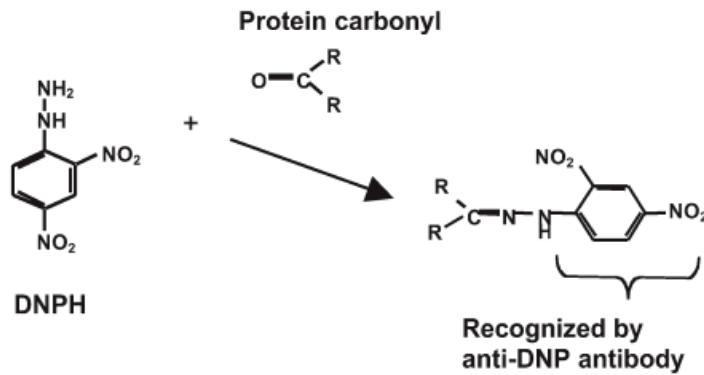
Kim et al., Methods in Aging Research, 2006

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15

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



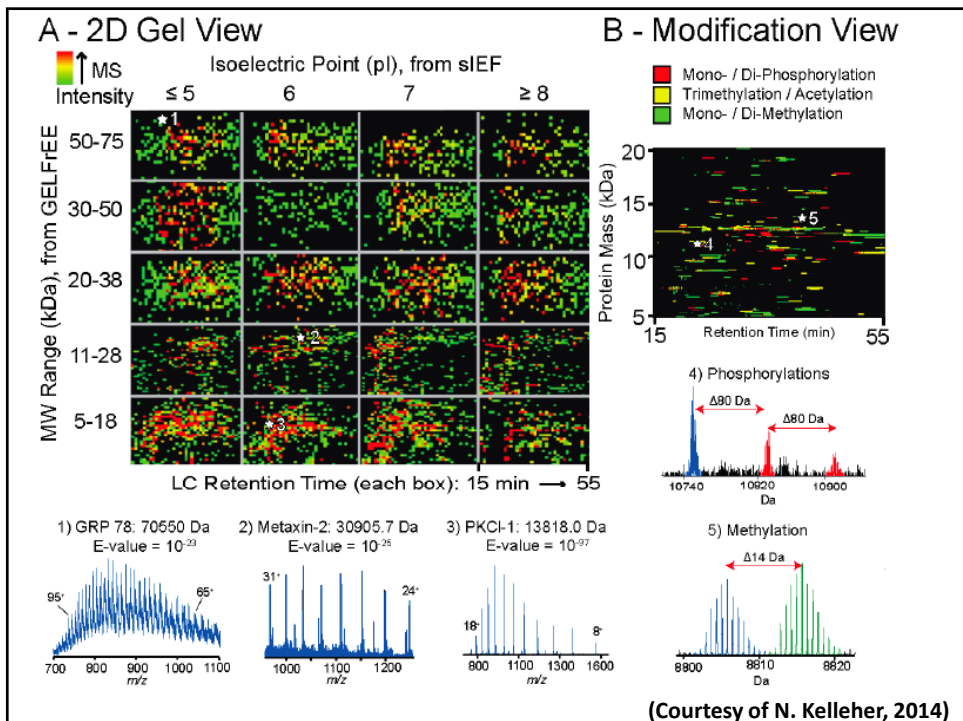
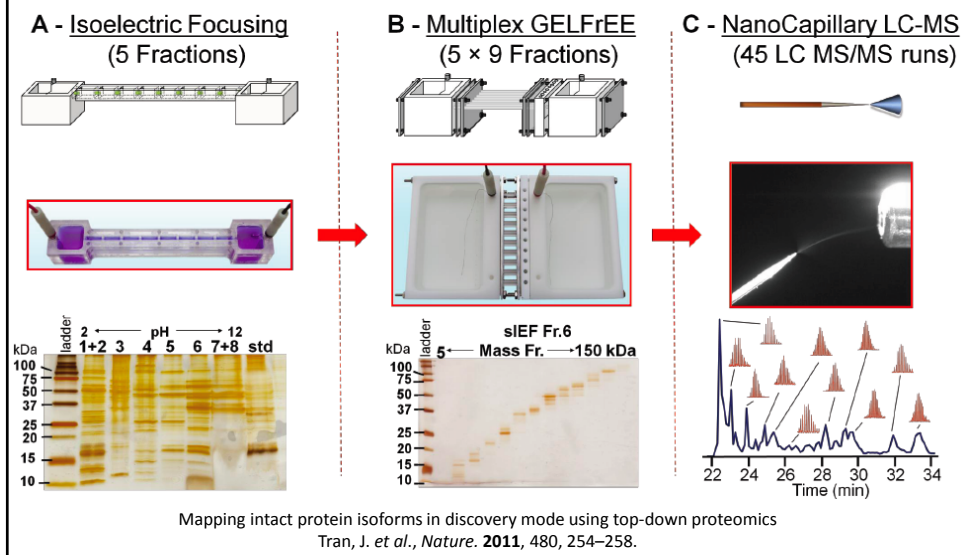
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16



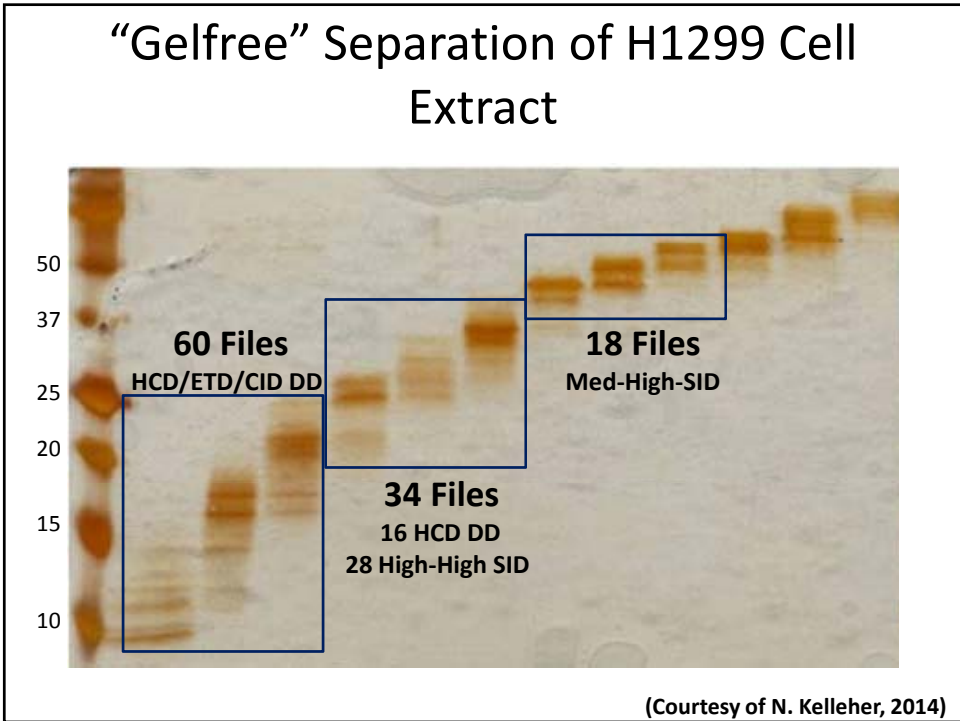
## Top-down proteomics à la Kelleher, where the proteins are kept intact all the way up to the MS; 4-Dimensional Fractionation of Intact Proteins

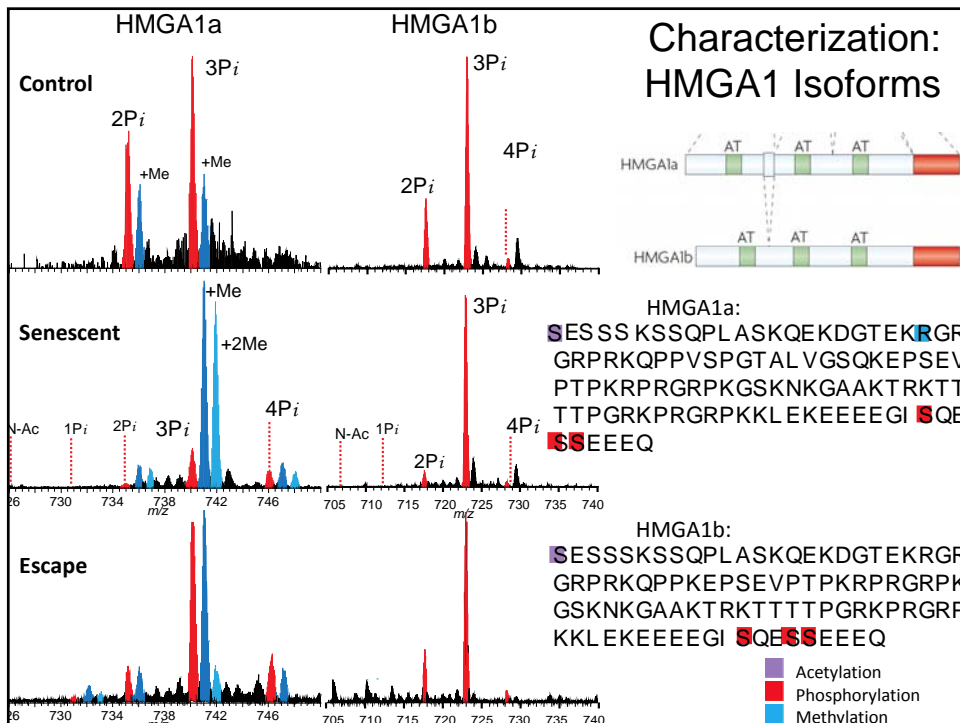
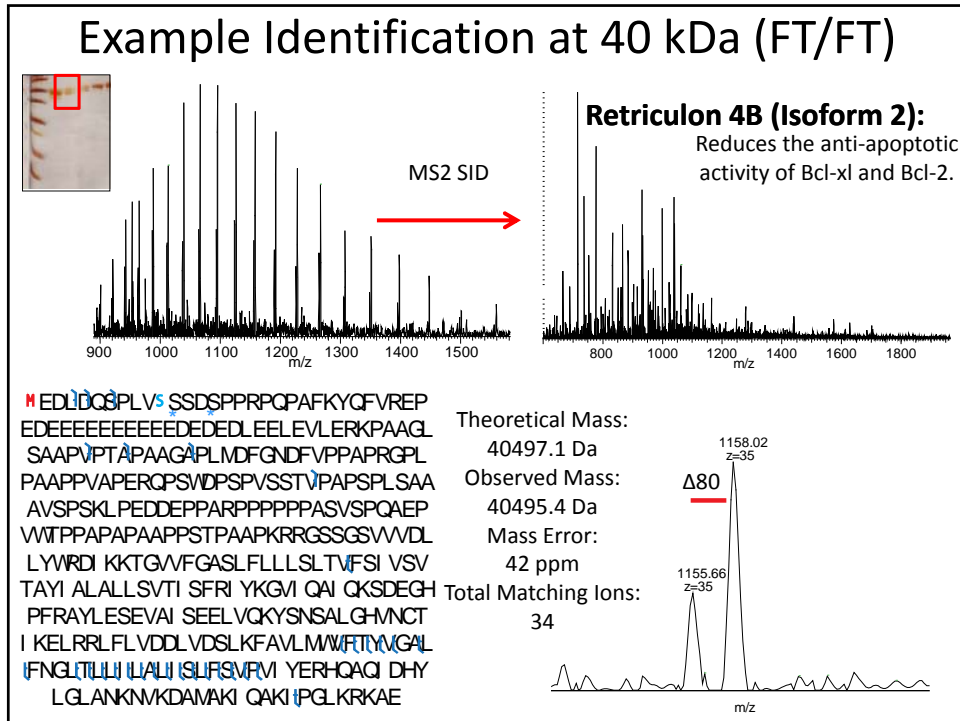


### An Example Results Set...

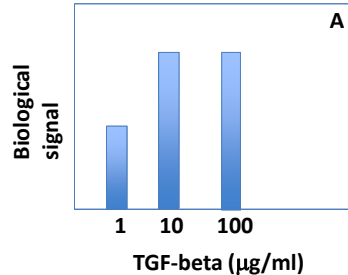
	# unique base sequences	# of isoform s	# of genes	# of transcripts
H2A.O	1	(2)	[1]	
	<1>			
H4	1	(14)	[1]	
	<1>			
Ubiquitin	1	(2)	[4]	
	<4>			
GAL-P	1	(3)	[1]	
	<2>			
HMGA1	1	(10)	[1]	
	<2>			
	•	•	•	•
	•	•	•	•
	•	•	•	•
<b>Σ</b>	1500	(4000)	[1550]	<1700>

(Courtesy of N. Kelleher, 2014)





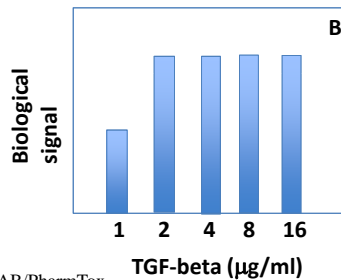
## Enhance specificity of your proteomic data by optimizing for your biological signal



(A) tells you that 10 µg/ml TGF-beta gives you a higher signal than 1 µg/ml, but increasing ten fold to 100 µg/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 µg/ml TGF-beta, shows that only 2 µg/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.



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23

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

- Per 1 µg antibody, what volume of Protein A beads do you really need; test 10, 20 and 40 µl of beads with same 1 µg antibody, run on SDS-gel, stain, see where the amount of antibody has saturated; if 10 µl beads is sufficient, why use 40.
- Per 1 mg cell lysate, how much antibody do you need? Test 1, 2, 4 and 8 µg 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 µg antibody brings down maximum antigen, why use 8 or 10?

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24

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

**Intrinsic mitochondrial membrane complexes**

(Modified from Brookes et al., 2002)

Released complexes, all negatively charged, thanks to the CBBR

1st D: NATIVE electrophoresis

The 2<sup>nd</sup> 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 → SDS-PAGE

It could be:

A or B or 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**

1<sup>st</sup> dimension: NATIVE

1,000 450 250 100 50

250  
100  
75

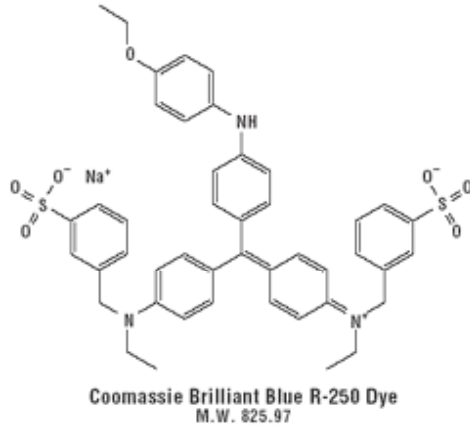
dimer monomer

2<sup>nd</sup> dimension: SDS-PAGE

(Wright...Kim...Sztul, unpublished data)

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## 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

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29

## Advantages and limitations of the types of stains

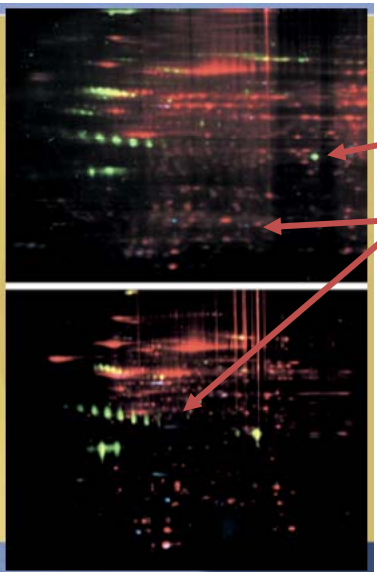
	Sensitivity	Dynamic range	MS- compatible
CBBR	8 ng	10-30 x	yes
Silver	1 ng	< 10 x	Not without special precautions
Fluorescent	2 ng	3 orders of magnitude	yes

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30

**Multiplexing: valuable when sample is scarce or difficult to obtain**



Normal liver

Disease liver

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.

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## 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 enrichment and separations for proteomics analysis:**

- **Effective generation of samples for proteomic analysis results when experimental parameters are optimized for maximum signal and the least “background.”**
- **Everything costs; mass spectrometry, gels, immuno-reagents; optimizing and titrating parameters usually means lower costs in the long run.**
- **Topdown proteomics (intact proteins separated by multiple orthogonal dimensions (sIEF, GELFREE, SDS-PAGE, then LC-MS/MS (á la Kelleher), enables high throughput discovery of proteins and “proteoforms”;**
- **However, physical gels may enable faster visualization of proteins with PTM differences to focus efforts on;**

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33