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

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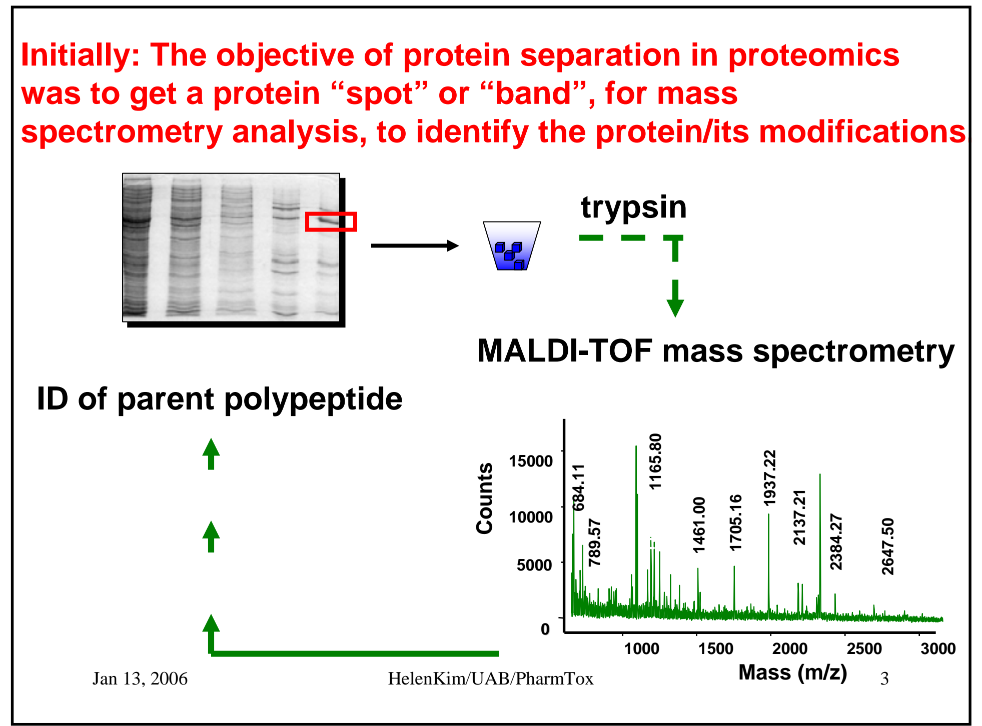
Learning objectives

2-D gel proteomics: What it involves:
- 2D separation
- image, statistical analysis
- MS

Other types of 2D protein separations;
- Free-flow
- 2D Blue native electrophoresis

Other types of protein analysis technologies:
- protein arrays
- antibody arrays



Types of high-throughput separation & analysis technologies

I. 2-dimensional electrophoresis (2-DE)
   - A. "regular" IEF/SDS-PAGE
   - B. 2D-blue-native electrophoresis
   - C. Visualization methods

II. 2-dimensional liquid-based LC/LC

III. Free-flow electrophoresis

IV. "Chip" technology: arrays of ligands for proteins

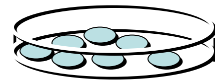
Parameters that govern the choice of protein separation method

- 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

The Elements of any 2-D separation in proteomics

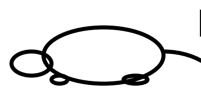
- The experiment!! that generates the “signal”
- Subfractionation to enrich for suspected proteins
- (Trypsin-digestion to generate peptides of the parent proteins)
- Sample work-up
- 2-D separation
- Image or other analysis to identify gel “spot” differences between untreated & treated
- MS of (trypsin-digests) spots/proteins/peptides of interest, to identify and characterize the protein

Sample preparation for 2DE:



Harvest, rinse, and pellet the cells;

or



Dissect out tissue, organ, or fluids;

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

What 2-D electrophoresis involves:

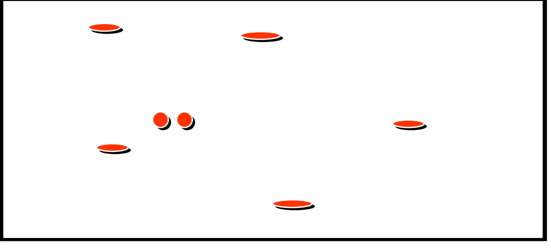
- **1st dimension: Isoelectric focussing**

  (separation according to charge)

  pH 3 **pH 10**

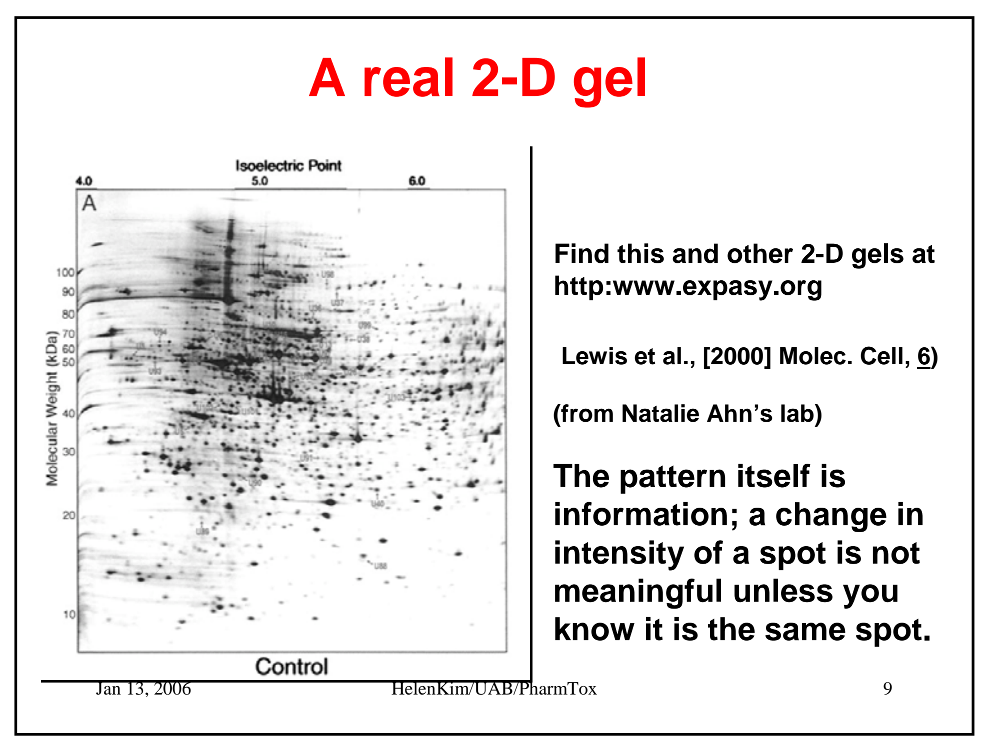
- **2nd dimension: (SDS)-PAGE**

  (separation according to size)



High m.w.

Low m.w.


A real 2-D gel
Isoelectric Point
4.0
5.0
6.0
A
Molecular Weight (kDa)
100
90
80
70
60
50
40
30
20
10
Control
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 of a spot is not
meaningful unless you
know it is the same spot.
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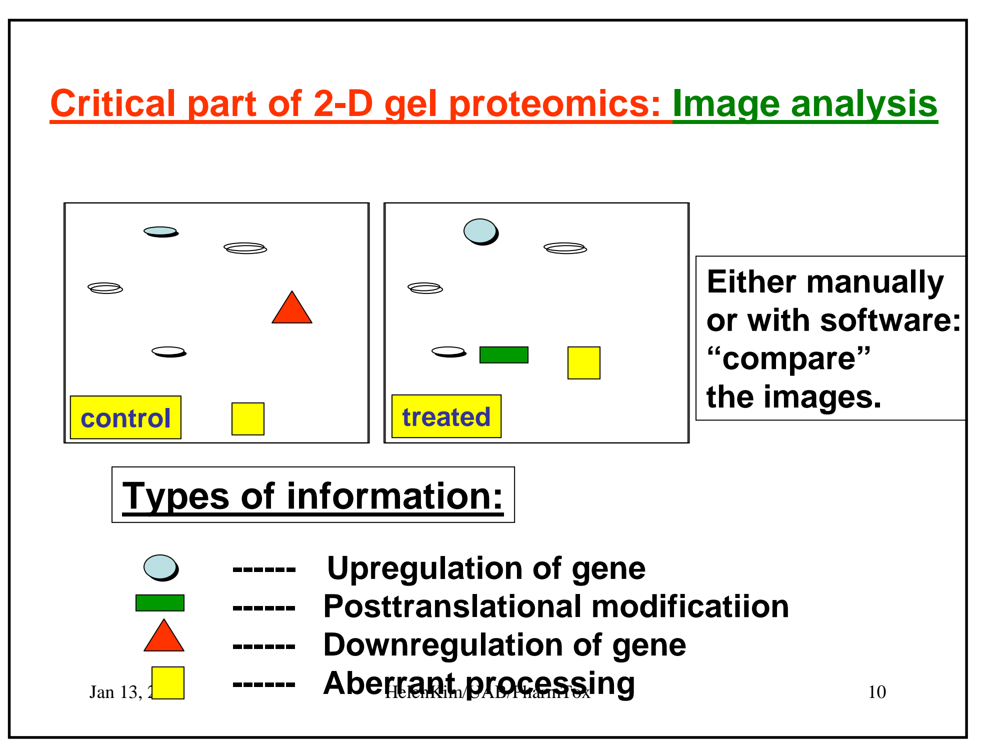
Critical part of 2-D gel proteomics: Image analysis
control
treated
Either manually
or with software:
"compare"
the images.
Types of information:
------ Upregulation of gene
------ Posttranslational modificatiion
------ Downregulation of gene
------ Aberrant processing
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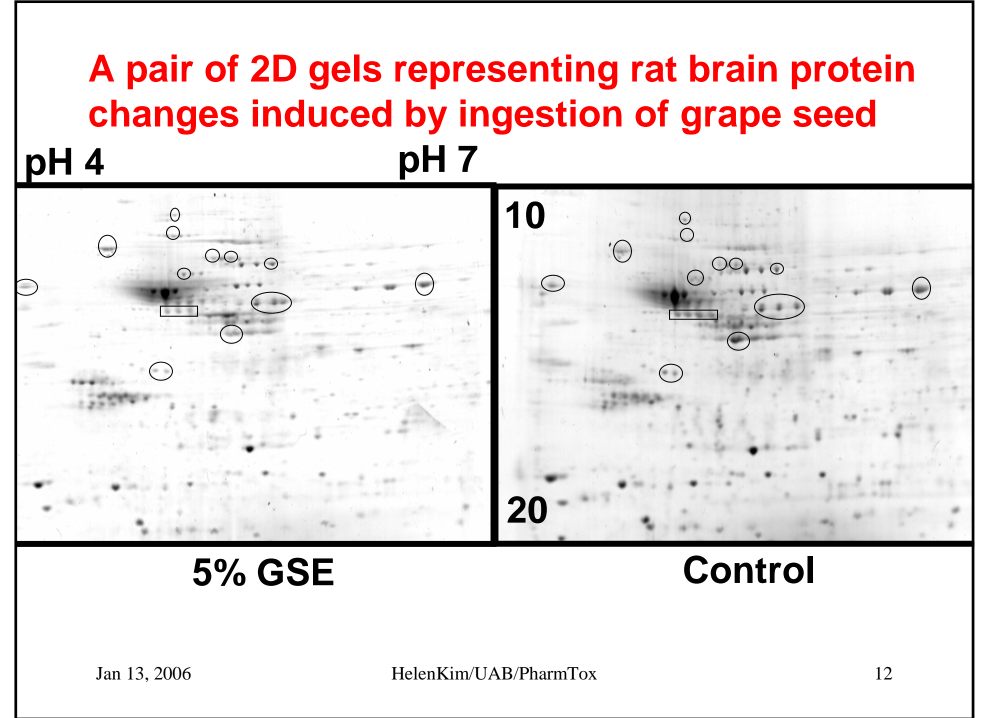
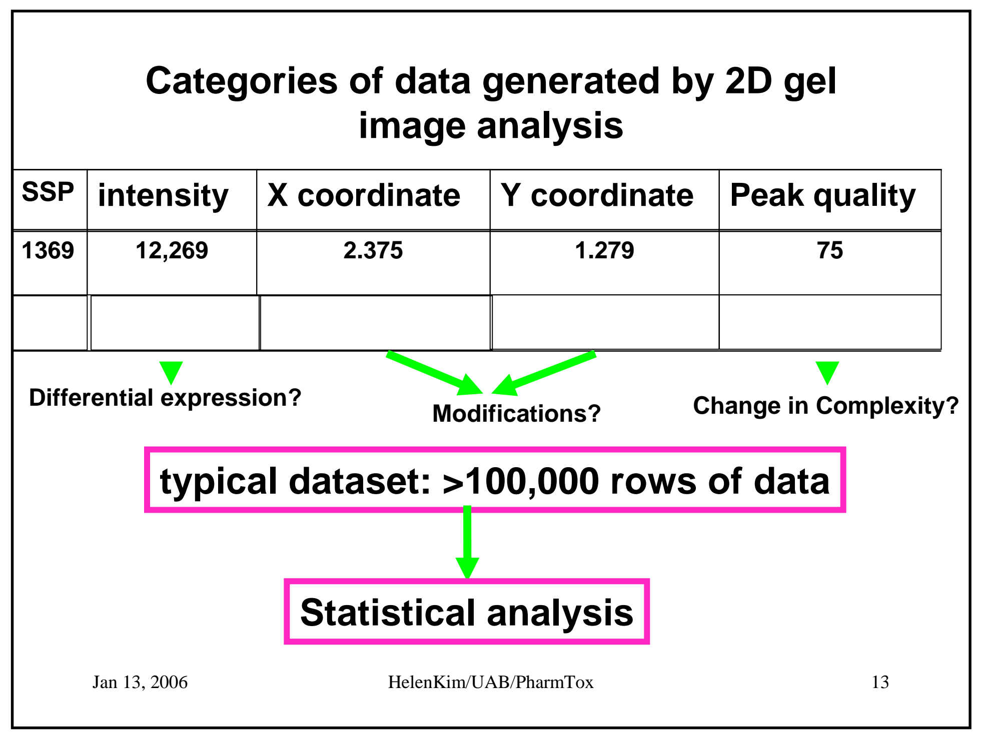
Elements of image analysis of "regular" 2D gels:

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 may have alterations in charge.

Ultimate and simple goal of image analysis

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




Categories of data generated by 2D gel image analysis
SSP
intensity
X coordinate
Y coordinate
Peak quality
1369
12,269
2.375
1.279
75
Differential expression?
Modifications?
Change in Complexity?
typical dataset: >100,000 rows of data
Statistical analysis
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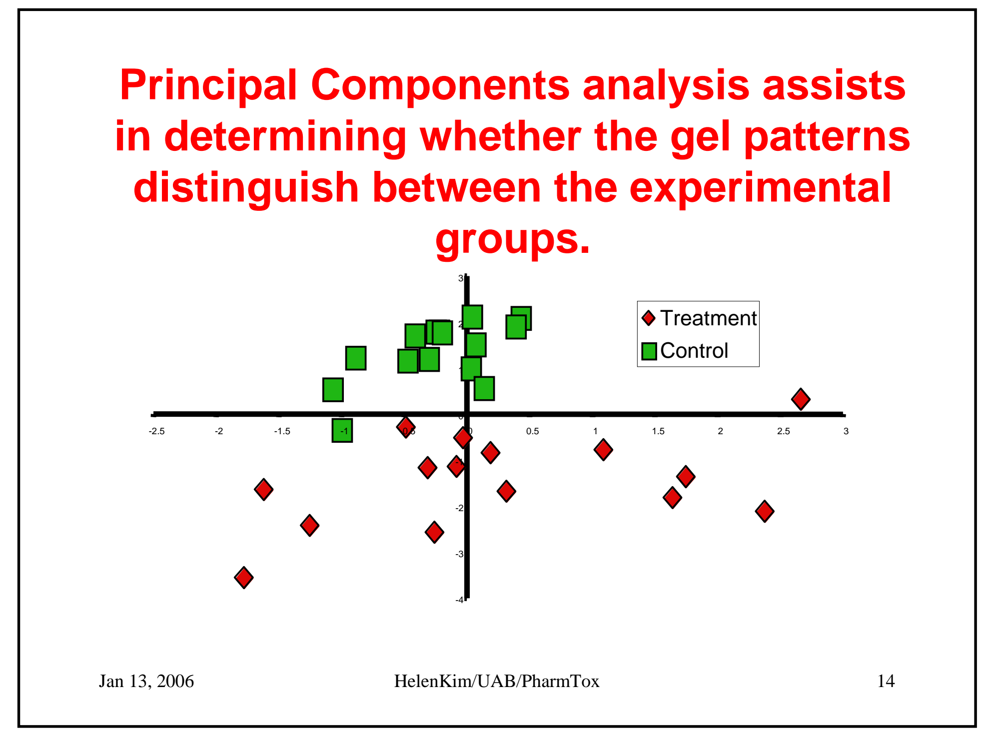
Principal Components analysis assists in determining whether the gel patterns distinguish between the experimental groups.
Treatment
Control
-2.5
-2
-1.5
0.5
1
1.5
2
2.5
3
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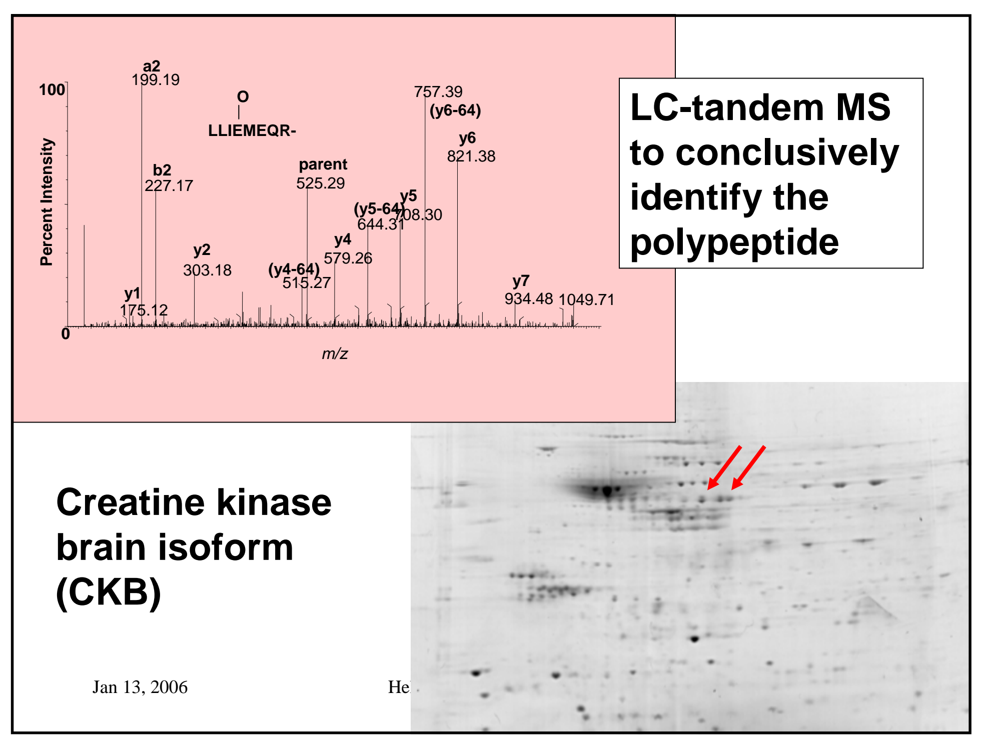
LC-tandem MS to conclusively identify the polypeptide
Creatine kinase brain isoform (CKB)
100
Percent Intensity
0
m/z
a2
199.19
b2
227.17
y1
175.12
y2
303.18
LLIEMEQR-
parent
525.29
(y4-64)
515.27
y4
579.26
(y5-64)
644.31
y5
708.30
757.39
(y6-64)
y6
821.38
y7
934.48
1049.71
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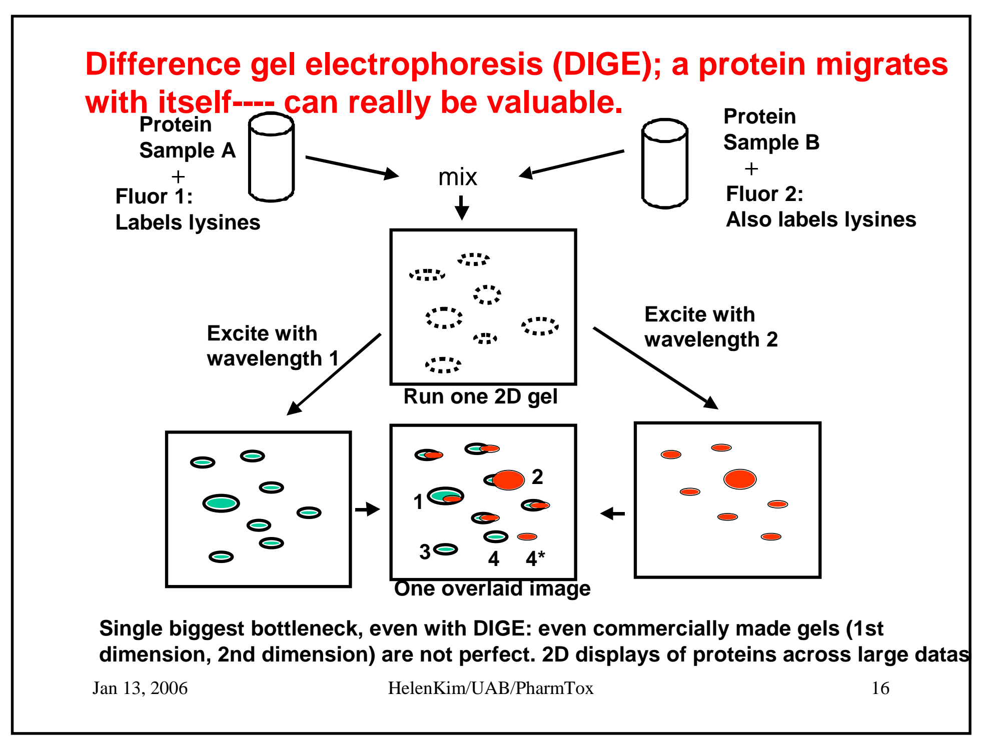
He


Difference gel electrophoresis (DIGE); a protein migrates with itself---- can really be valuable.
Protein Sample A + Fluor 1: Labels lysines
mix
Protein Sample B + Fluor 2: Also labels lysines
Excite with wavelength 1
Run one 2D gel
Excite with wavelength 2
1
2
3
4
4*
One overlaid image
Single biggest bottleneck, even with DIGE: even commercially made gels (1st dimension, 2nd dimension) are not perfect. 2D displays of proteins across large datas
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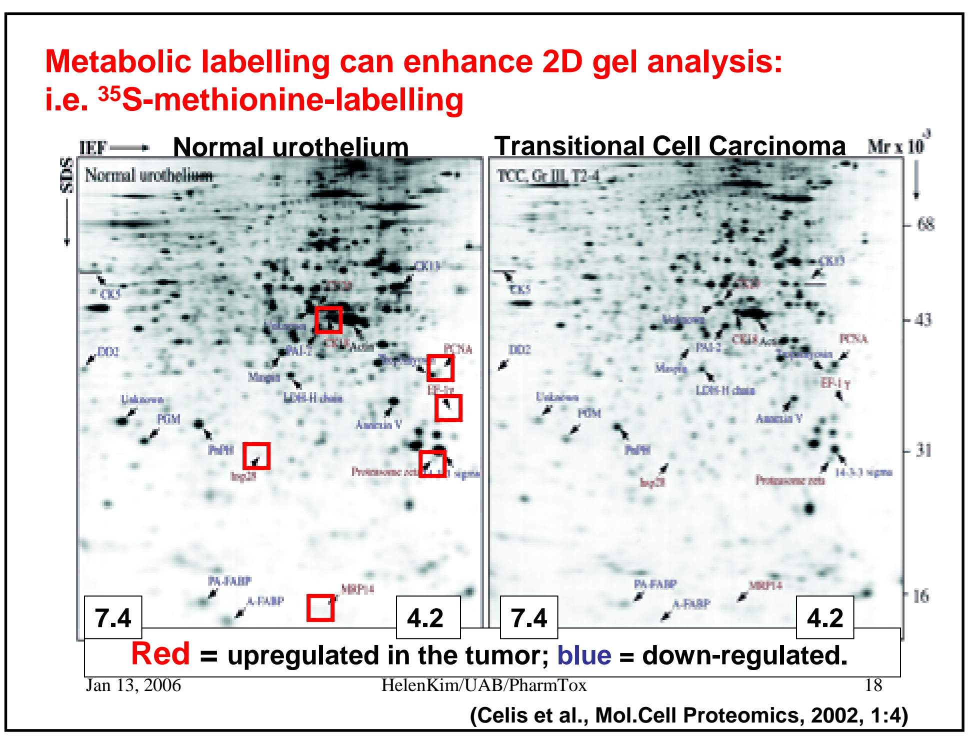
Database obtained from proteomics analysis of breast cancer cell lines

Sample	Spot#	Protein Name	MOWSE	Accession#	Obs: kDa	Pred: kDa	Obs: pI	Pred: pI
MCF-7	BR-8	Cytoskeletal keratin-7	132	gi12803727	20.7	51.3	8.6	5.3
MCF-10AT	BR-9	Alpha-1 anti-trypsin	90	gi1942953	19.7	44.3	9.6	
	BR-1	Human Cytoskeletal keratin-8	94	gi87303	56.2	53.5	5.9	5.6
	BR-6	Human hypo:XP109048	74	gi18573275	23.4	24.1	6.3	
	BR-5	IgG heavy chain variable region	60	gi5106591	18.1	6.4	5.7	
	BR-7	Intact recomb: alpha-1 antitrypsin mutant F-L	105	gi1942953	37.0	44.3	6.9	
	BR-4	IgG heavy chain var:reg	65	gi5106591	10.0	6.4	4.6	
MCF-10A	BR-3	Human cytokeratin-8	65	gi87303	56.0	53.5	5.8	5.6
	BR-2	DNA replication silencing factor MCM-4	58	gi1705520	22.8	96.5	6	6.6

(Deshane, Johanning, and Kim, unpublished data)



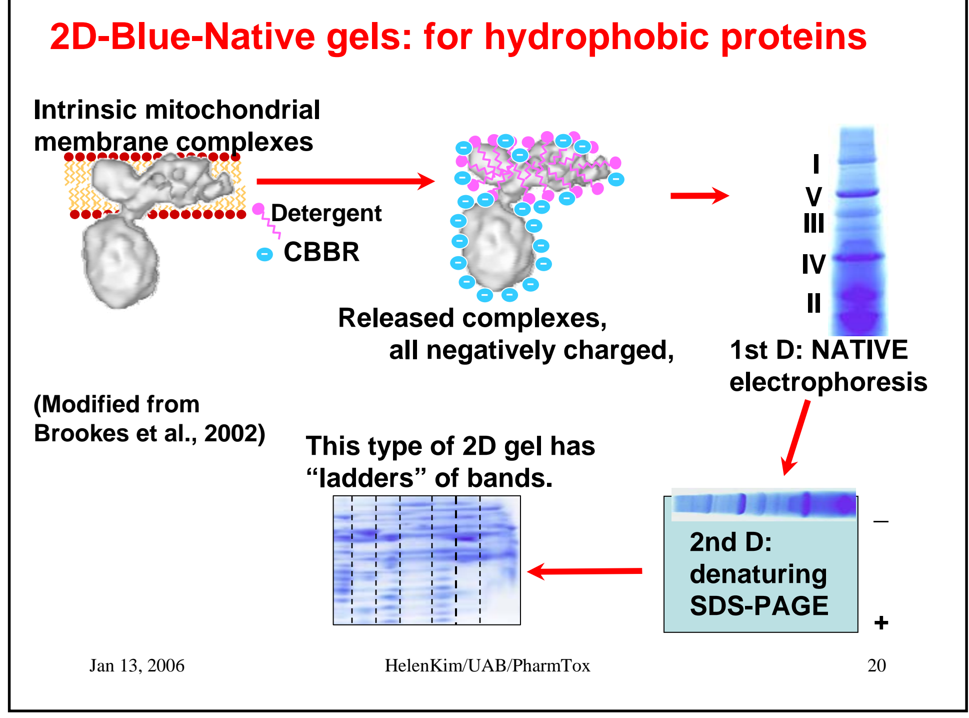
Make use of databases and the internet:

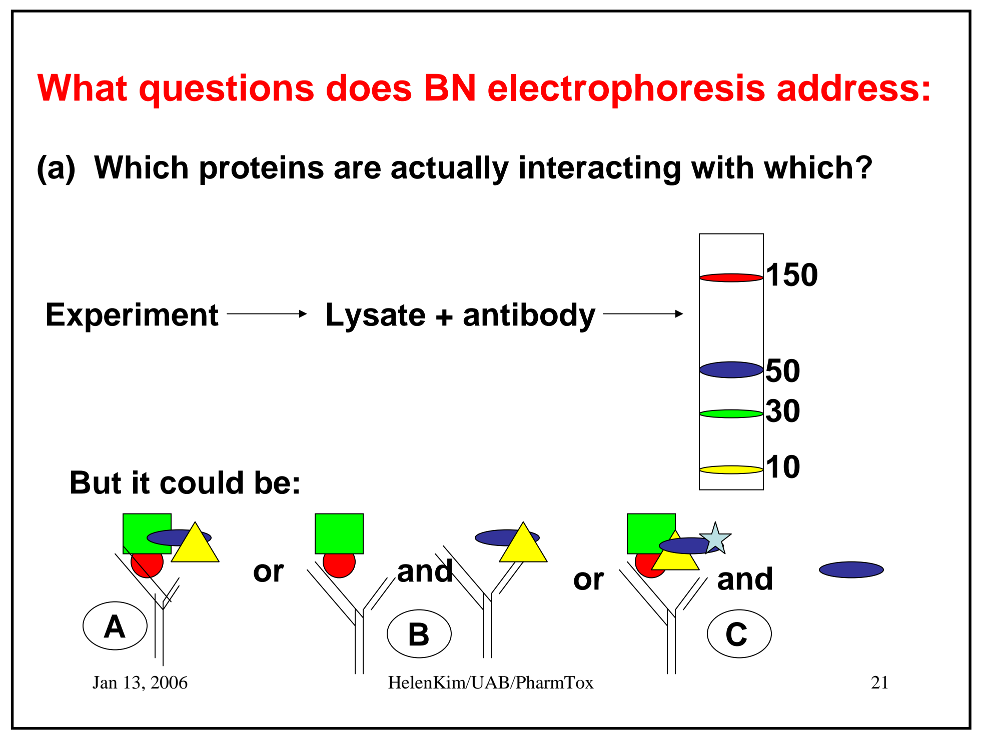
I. Check existing databases and web-links:
  www.expasy.org
    many are annotated
    helpful links:
      proteomics tools

II. Keep up with the literature/ competition:
  Electrophoresis
  Proteomics
  Molecular & Cellular Proteomics
  J. Proteome Research

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

2D-Blue-Native gels: for hydrophobic proteins




What questions does BN electrophoresis address:
(a) Which proteins are actually interacting with which?
Experiment
Lysate + antibody
150
50
30
10
But it could be:
or
and
or
and
A
B
C
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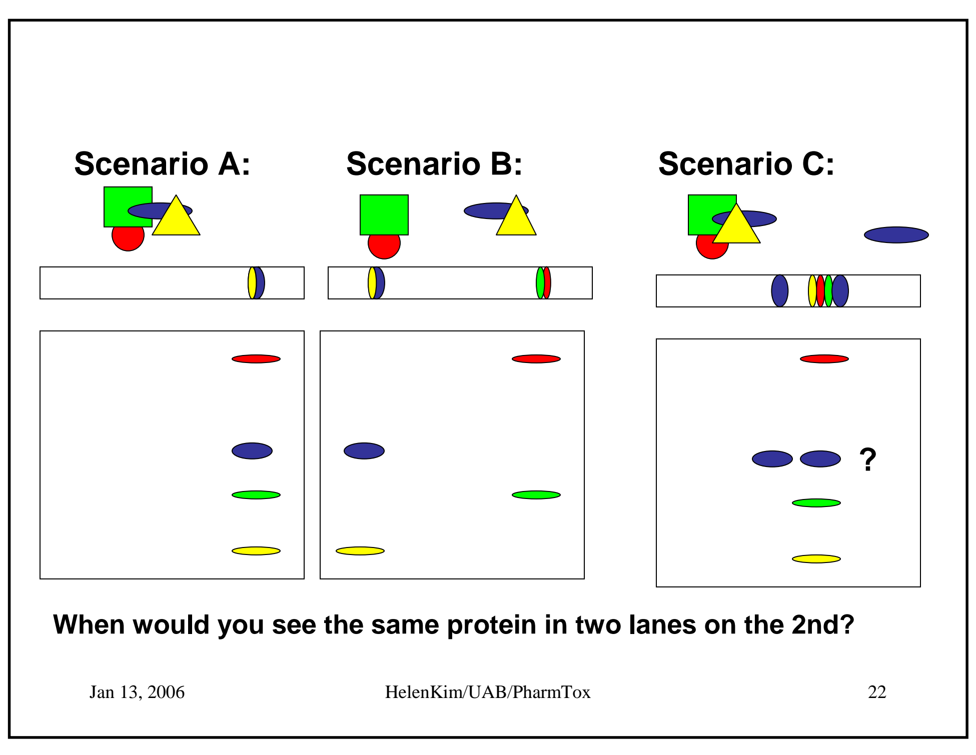
Scenario A:
Scenario B:
Scenario C:
?
When would you see the same protein in two lanes on the 2nd?
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Take home message

- **Use of proteomics technologies enables *global* analysis of protein changes ;**
- **2D electrophoresis can indicate both differential expression or posttranslational modifications;**
- **Choice of separation governed by**
  - **Abundance of sample**
  - **Question being asked**
  - **Technology available to you**
  - **Cost is a factor;**

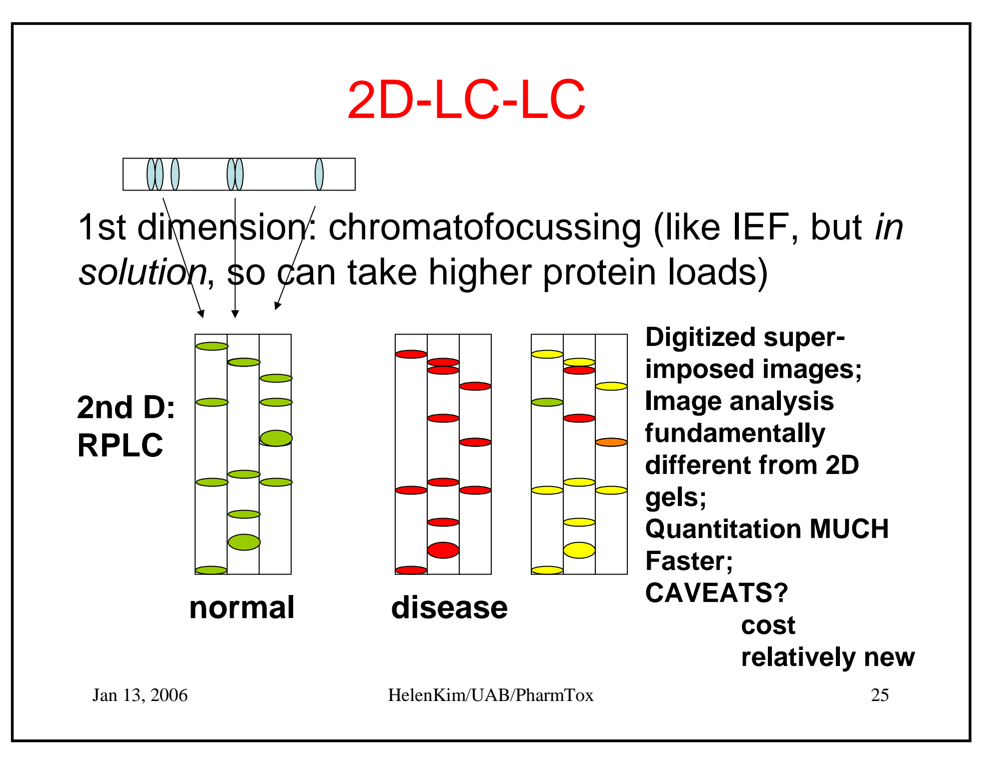
Issues in 2D gel analysis:

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

Blazing signal on western blot but NOTHING on a 2D gel of the same Ip:

Probable answers/what are the solutions:
1. Protein not solubilized by IEF buffer
2. Not enough protein!!!
3. Modification lost during preparation?

II. I see the band on my 1D gel that runs with my western blot band; Why do I need to run a 2D gel anyway?


2D-LC-LC
1st dimension: chromatofocussing (like IEF, but *in solution*, so can take higher protein loads)
2nd D: RPLC
normal
disease
Digitized super-imposed images; Image analysis fundamentally different from 2D gels;
Quantitation MUCH Faster;
CAVEATS?
cost
relatively new
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