Applications for 2-D DIGE

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Agenda

1. 2-D DIGE concepts and benefits
2. Biomarkers in colorectal cancer
3. Monitoring effect of drug treatment and diagnosis using PET
4. Changes in tyrosine phosphorylation
5. Selective labeling of cell surface proteins
6. Quantitative fluorescent Western blotting

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1. 2-D DIGE concepts and benefits
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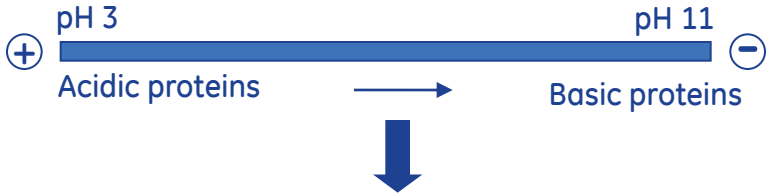
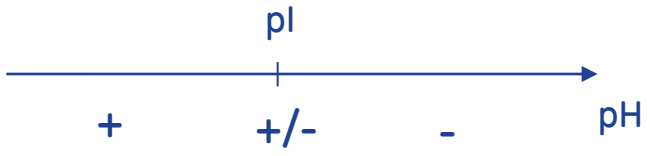
imagination at work

2-D gel electrophoresis

1st dimension isoelectric focusing:

pH gradient in the gel strip – proteins will focus when the pH=pI (no charge)

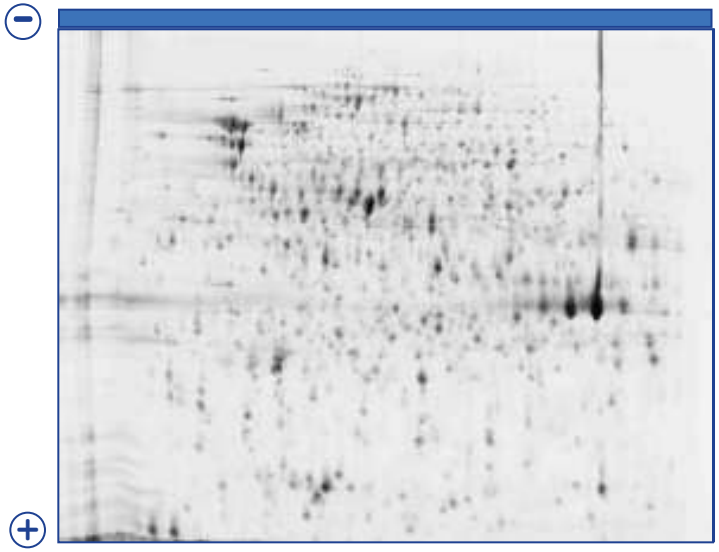




Protein + charged at pH below its pI
Protein - charged at pH above its pI

2nd dimension SDS-PAGE:

proteins will separate according to their size



胶、膜的标记检测技术列表

标记	检测	注释
同位素	胶片曝光 磷屏成像- 储存磷屏	+ 敏感度 + 可进行代谢标记 + 宽动态范围 (~10^5) - 放射性，废料处理问题
银染	光密度	+ 敏感度 + 廉价，易用 - 窄动态范围 (~10^1) 及线性 - 有毒化学试剂
考染	光密度	+ 廉价，易用 - 敏感度 - 窄动态范围 (~10^{1-} 10^2)
化学发光	胶片曝光 CCD相机	+ 敏感度 + 中等动态范围 (~$10^{1.5-}$ $10^{2.7}$) + 廉价，易用
荧光	荧光成像仪	+ 敏感度 + 宽动态范围, CyDyes (~10^4) + 易用 + 可以进行多通路实验 + 环境友好，无毒性

What is Ettan™ DIGE System?

Difference gel electrophoresis (DIGE)

Ettan DIGE System is a leading edge technology for differential analysis of protein abundance using 2-D gel electrophoresis.

- CyDye™ DIGE Fluors for protein labeling
- Imager for image acquisition
- DeCyder™ 2-D Differential Analysis Software for image analysis

What is the key for Ettan™ DIGE?

Traditional 2-D electrophoresis

Time consuming and high experimental variation (single post-stain, biological and technical replicates required)

Ettan DIGE system

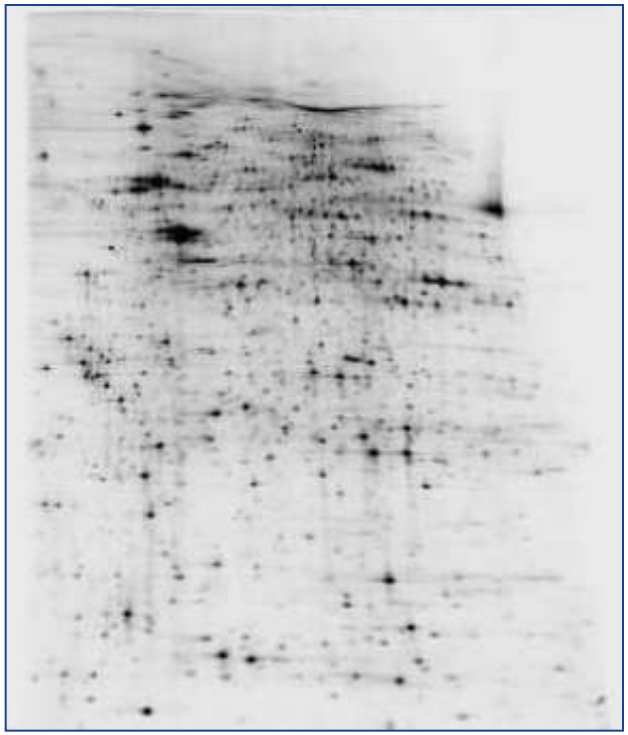
Provide greater accuracy and greatly reduces number of gels needed due to:

- Multiplexing – multiple pre-labeled samples run on same gel
- Internal standard – run on all gels within an experiment
- Experimental design – unique for this technique

Post-staining vs fluorescent pre-labeling – sensitivity

Silver stain of 10.000 cells

Cy5 stain of 5.000 cells



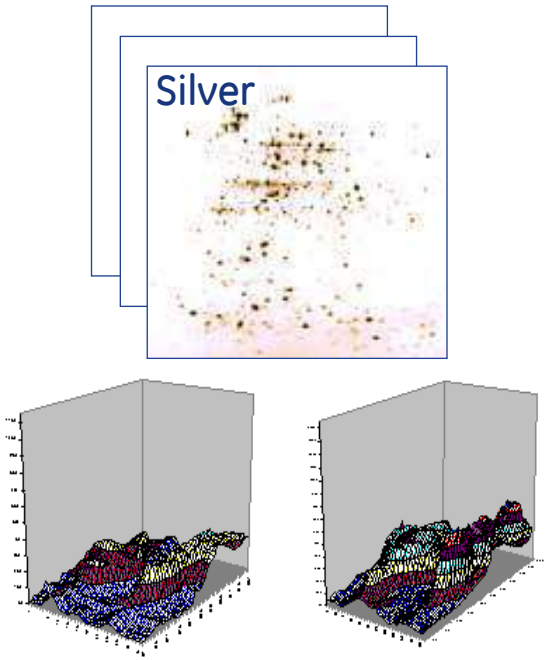
Class leading sensitivity
Only 250 ng protein

Courtesy of Kai Stühler, RU, Bochum, DE

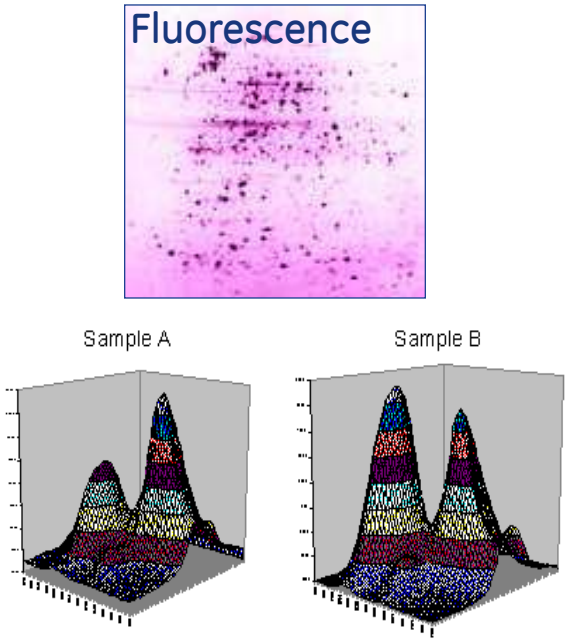
Post-staining vs fluorescent pre-labeling – dynamic range

Fluorescent labelling and staining techniques offer significant increase in **detection levels** combined with **dynamic range** (4-5 orders of magnitude) as compared to for instance classical silver staining techniques

Post staining



Pre-labeling with CyDyes™

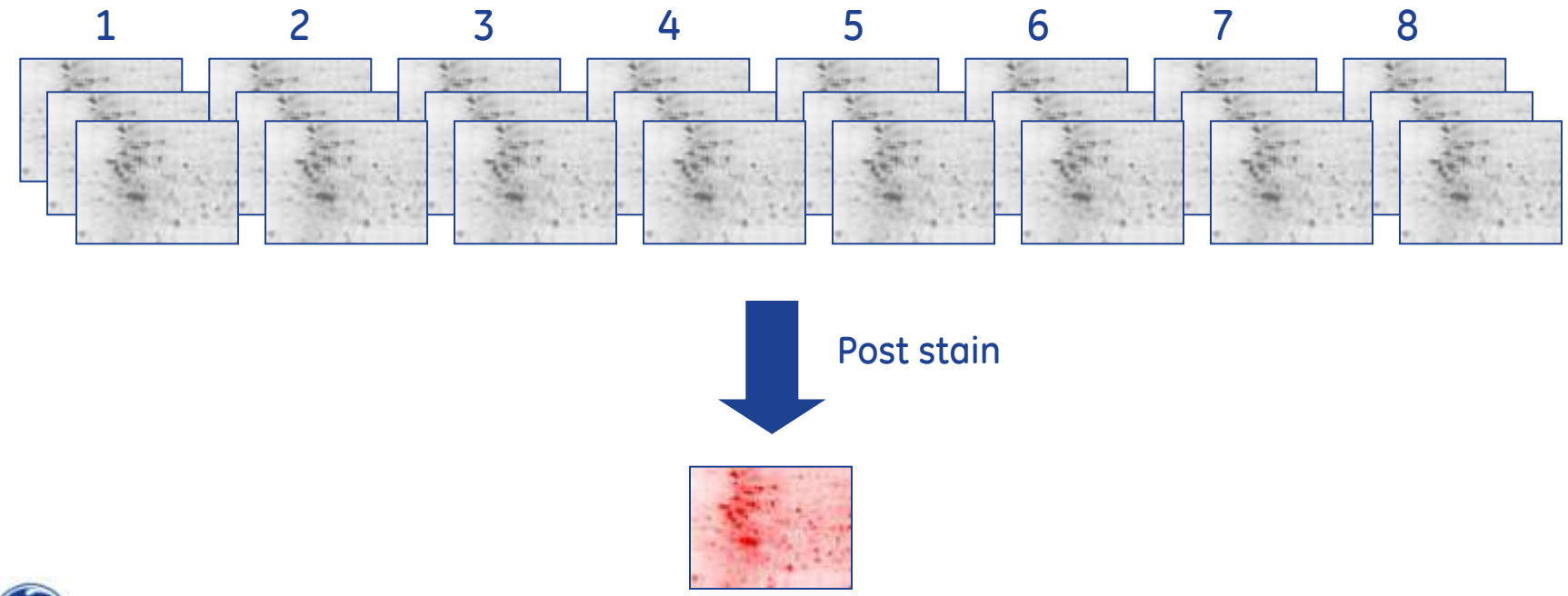


imagination at work

Traditional 2-D vs 2-D DIGE

Traditional 2-D electrophoresis (1-colour)

8 samples:
8 gels x triplicate = 24 gels

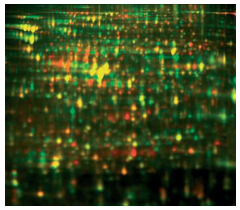
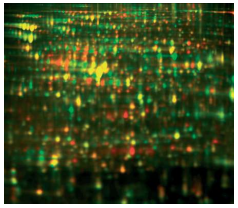
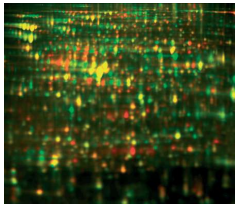
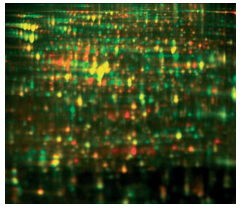


Traditional 2-D vs 2-D DIGE

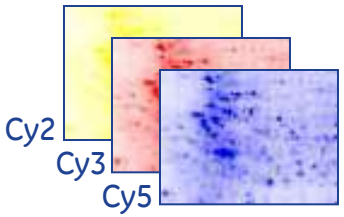
Ettan™ DIGE (3-colour)

8 samples:
4 gels (no gel replicates) = 4 gels

Cy 2™ Internal standard	Cy 2 Internal standard	Cy 2 Internal standard	Cy 2 Internal standard
Cy 3 Sample 1	Cy 3 Sample 3	Cy 3 Sample 5	Cy 3 Sample 7
Cy 5 Sample 2	Cy 5 Sample 4	Cy 5 Sample 6	Cy 5 Sample 8

Imaged using Typhoon™ fluorescent Imager



imagination at work

2-D electrophoresis and 2-D DIGE – what's the difference?

2-D DIGE is the only significant development of 2-DE over the last 20 years!

- Massive reduction in costs and time for 2-D electrophoresis
- Massive increase in data quality for protein analysis

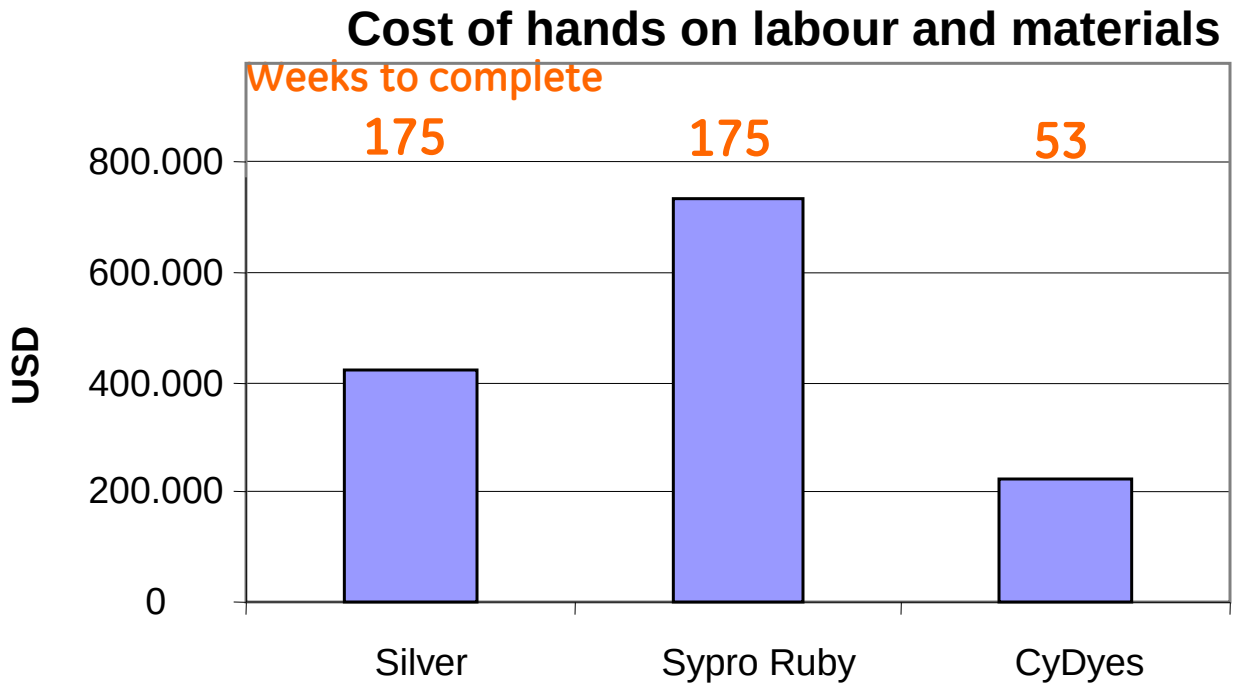
Benefits of 2-D DIGE vs other technologies

- 2-D DIGE vs 2-D electrophoresis – much better data quality and much less time
- 2-D DIGE vs mass spectrophotometry – great complementary technology, studies whole proteins
- 2-D DIGE vs protein arrays – studies whole proteins, antibody-free detection, quantitation, and functional analysis of all proteins

DIGE saves time and money

700 silver-stained and SYPRO™ gels x 3 (necessary replicates)

350 2-D DIGE gels



Save 70% time and 50% costs

Traditional 2-D vs 2-D DIGE – summary

Traditional 2-D (1-colour)

- More gel replicates (24 gels)
- Poor accuracy for quantification
- Slow and labour intensive analysis

Ettan™ DIGE

- Less gels required (4 gels)
- High accuracy for quantitation
- Analysis fast and highly automated

2-D with no internal standard can show inaccurate or incorrect results
→ could lead to *false biological conclusions*

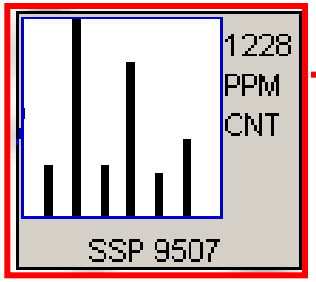
To maximize confidence in results and get the most out of the data
→ *an internal standard MUST be used*

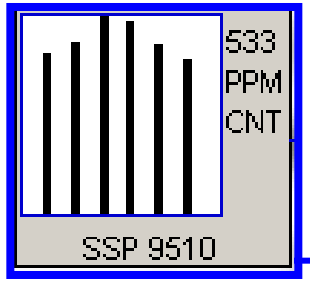
Why do you need an internal standard? Without it, variation is too high

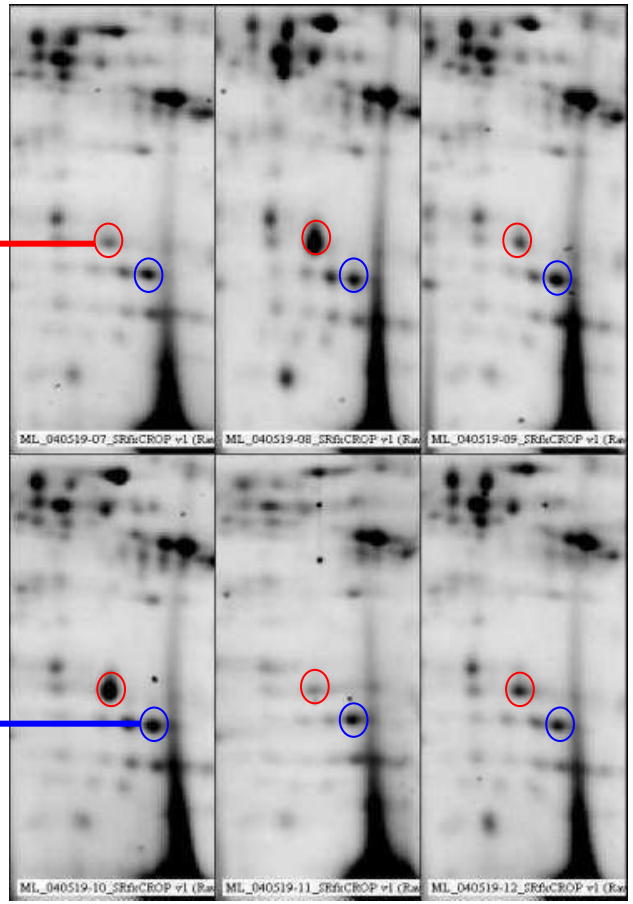
Gel to gel variation

Large gel-to-gel variation

Small gel-to-gel variation





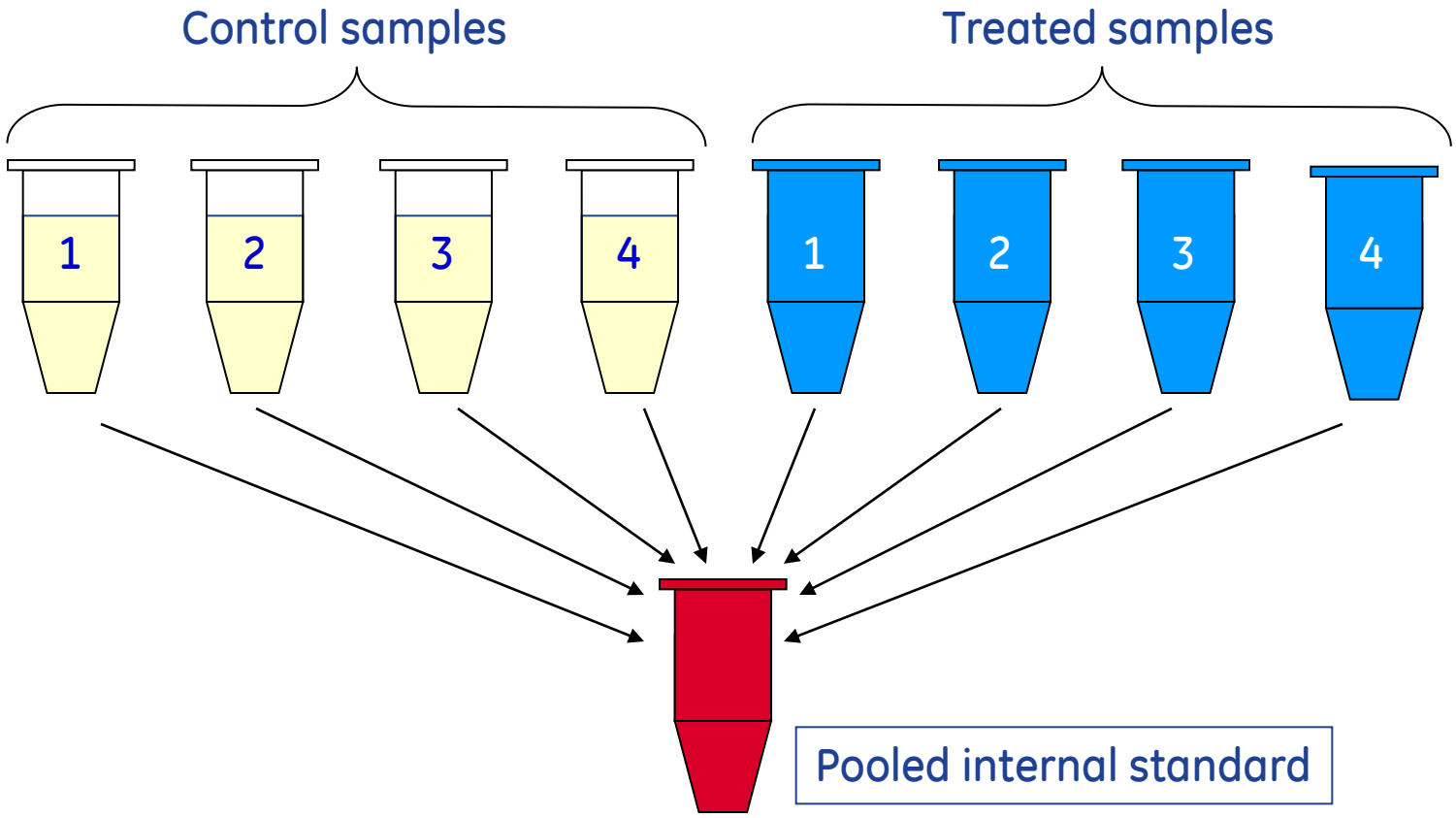


6 replicate gels made from the same sample (SYPRO™ Ruby)

imagination at work

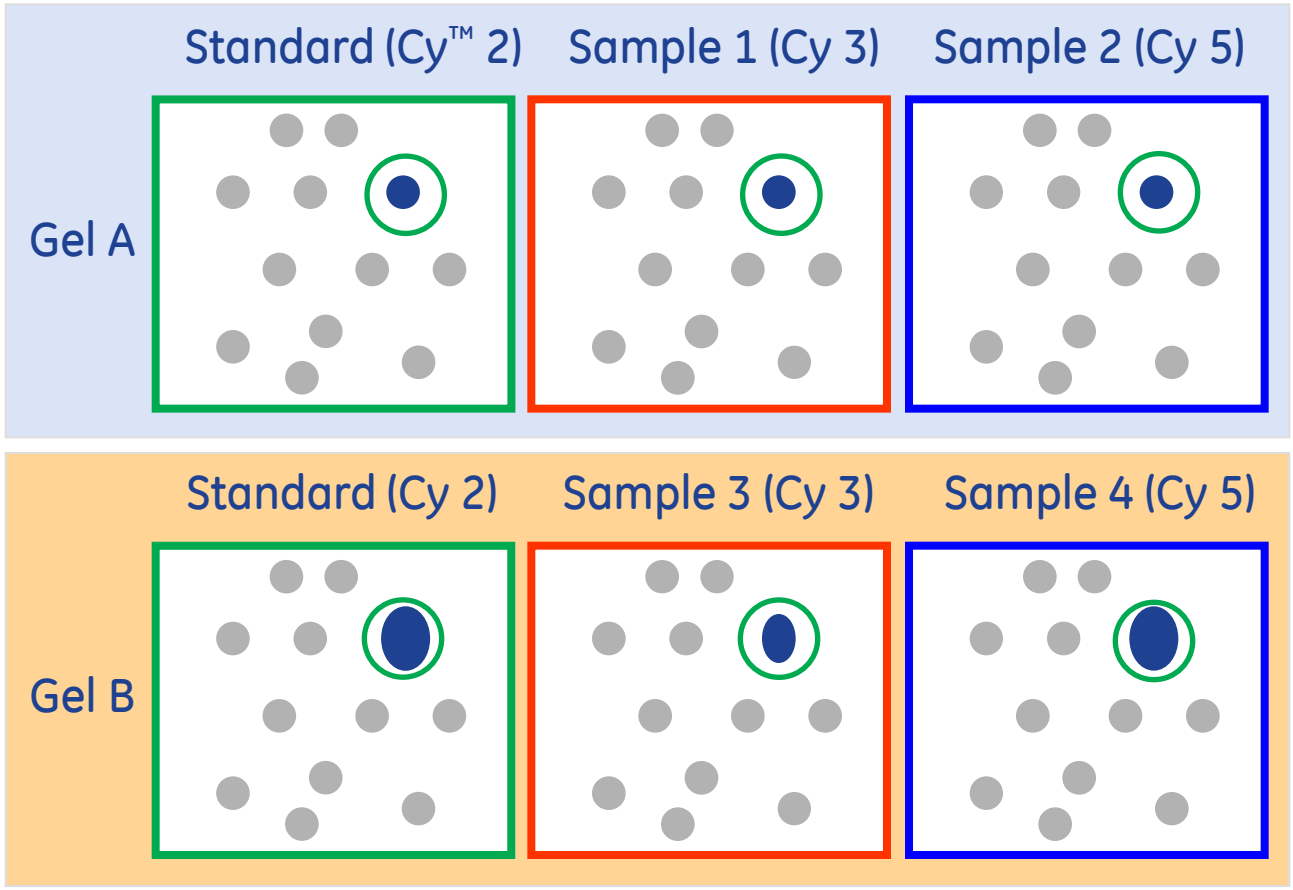
Data courtesy of
Jörgen Östling, AstraZeneca R&D Mölndal, Sweden. June 2004

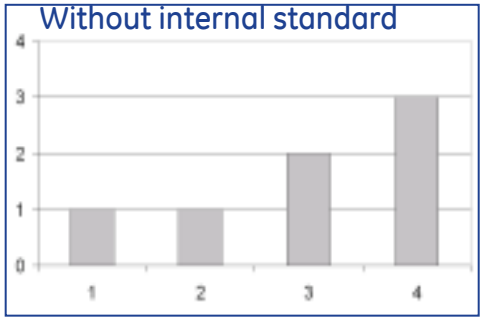
How to prepare an internal standard

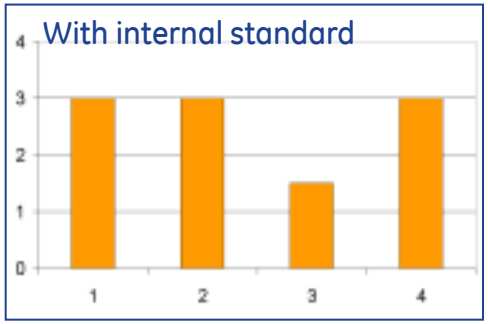


A reference point for ***every*** protein species on ***each*** gel in the experiment

Why do you need an internal standard?
With 2-D DIGE, variation is eliminated







Virtual elimination of gel-to-gel variation reveals induced biological change with statistical accuracy capable of revealing differences in abundance of less than 10% between samples

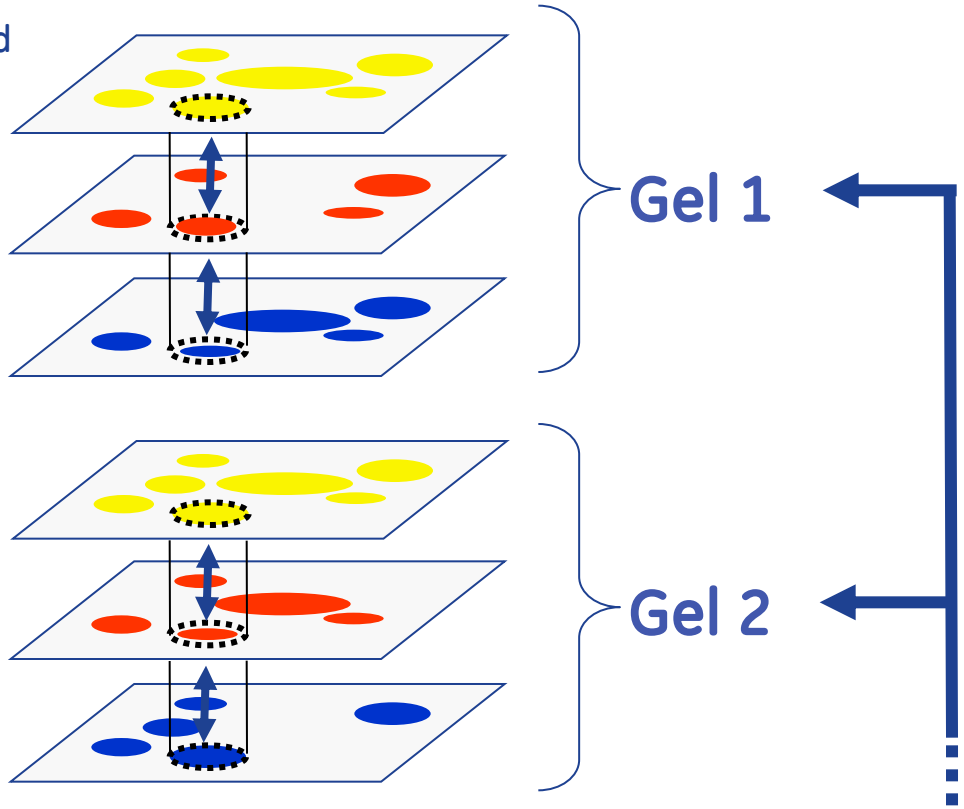
imagination at work

Co-detection within gels, and matching between gels

Image 1: Pooled internal standard
Boundaries transferred to image 2 and 3

Image 2: Sample 1

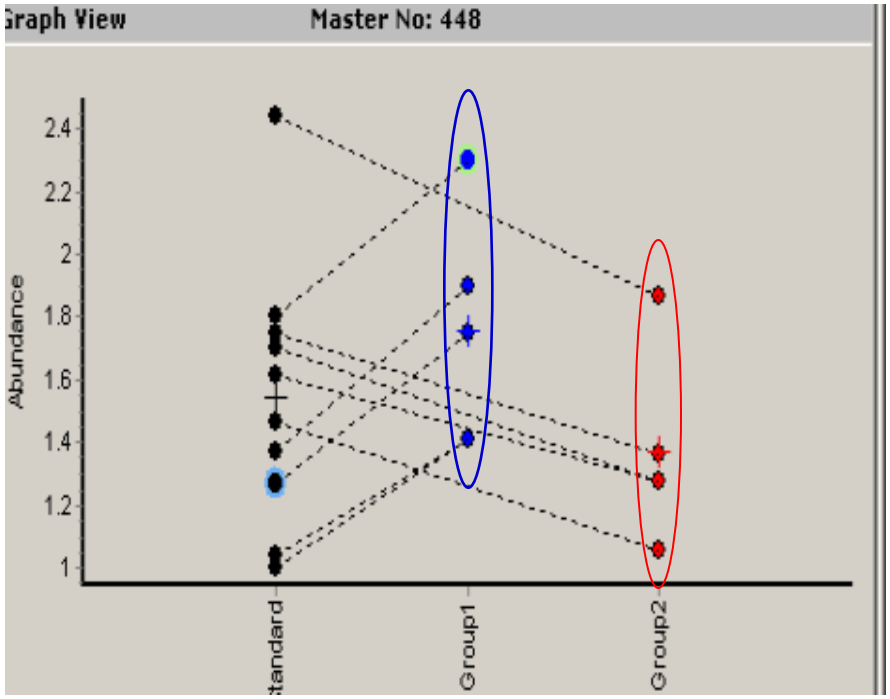
Image 3: Sample 2



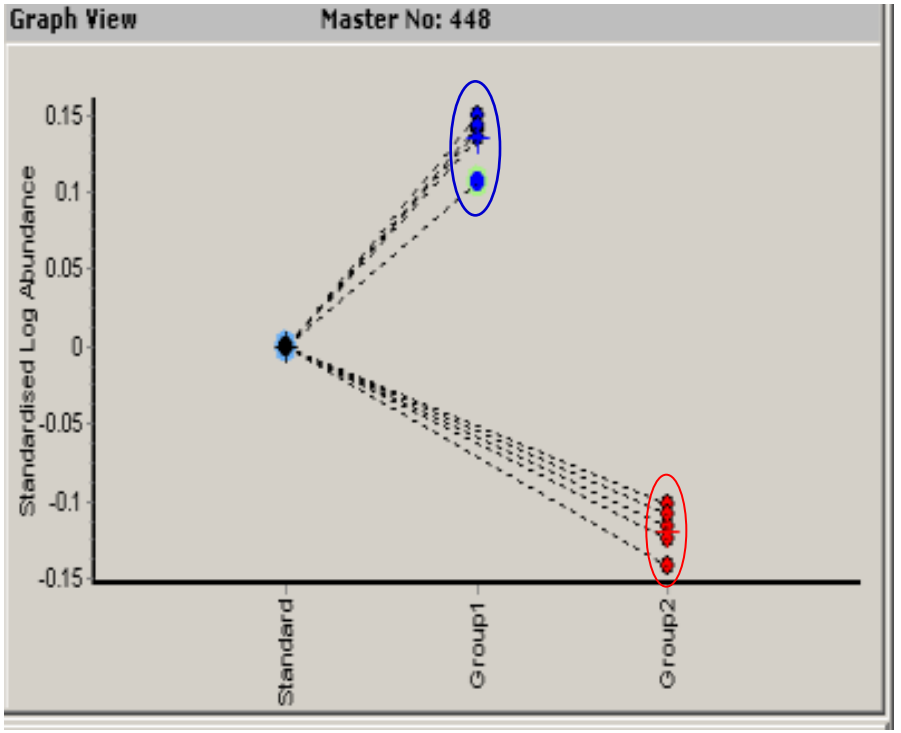
- Boundaries are used for quantitation relative to pooled internal standard
- Matching between gels via pooled internal standard

Internal standard

Normalization of spots between gels



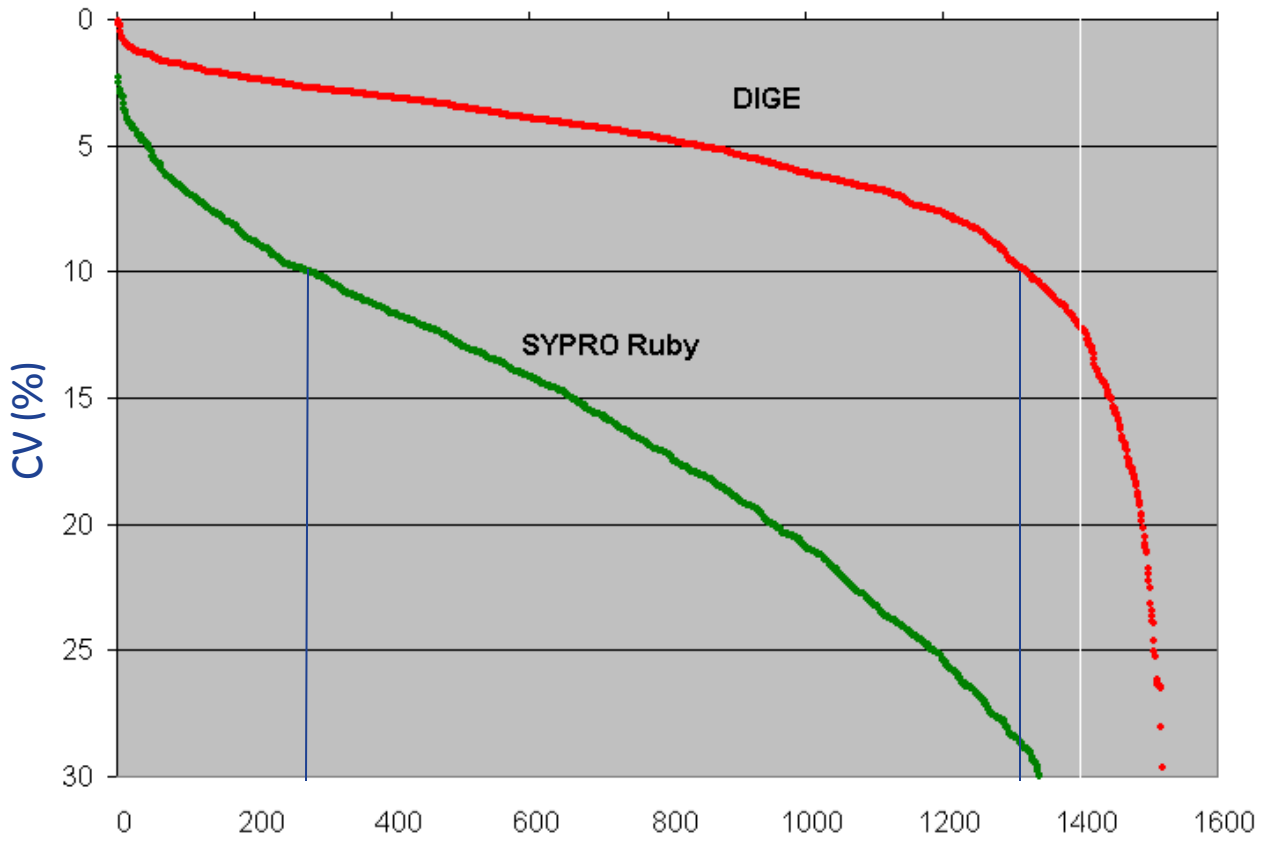
Not normalized to standard



Normalized to standard

2-D DIGE reduces experimental variation in 2-D electrophoresis

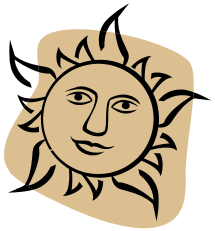
Same sample, 6 gels with good spatial reproducibility, SR vs DIGE

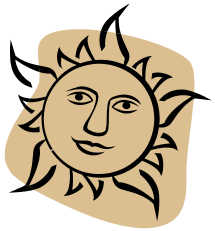


Courtesy of Jörgen Östling, AstraZeneca R&D Mölndal, Sweden

Why switch to 2-D DIGE?

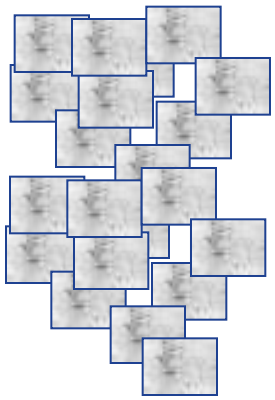
2-D electrophoresis → 2-D DIGE

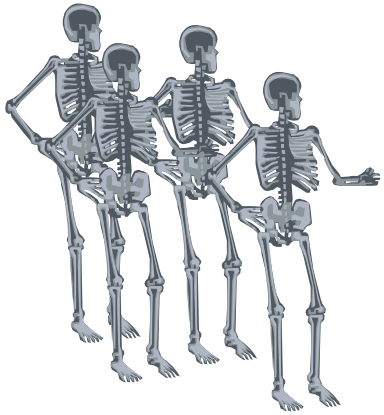


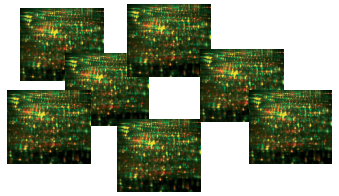


Working overtime

Take a break once in a while







1 year later:
Do we really have a 50% difference???

2 months later:
We have a 10% difference!!!

Ettan™ DIGE components

DIGE approved

Sample labeling

Novel CyDye™ DIGE fluors

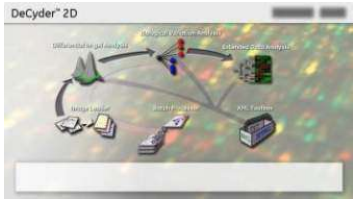
- Highly fluorescent dyes designed specifically for this application
- Sensitive, photostable and spectrally distinct

Image acquisition

DIGE enabled Typhoon™ Imager, Ettan™ DIGE Imager

- Designed specifically for this multiplexing technology

Differential analysis

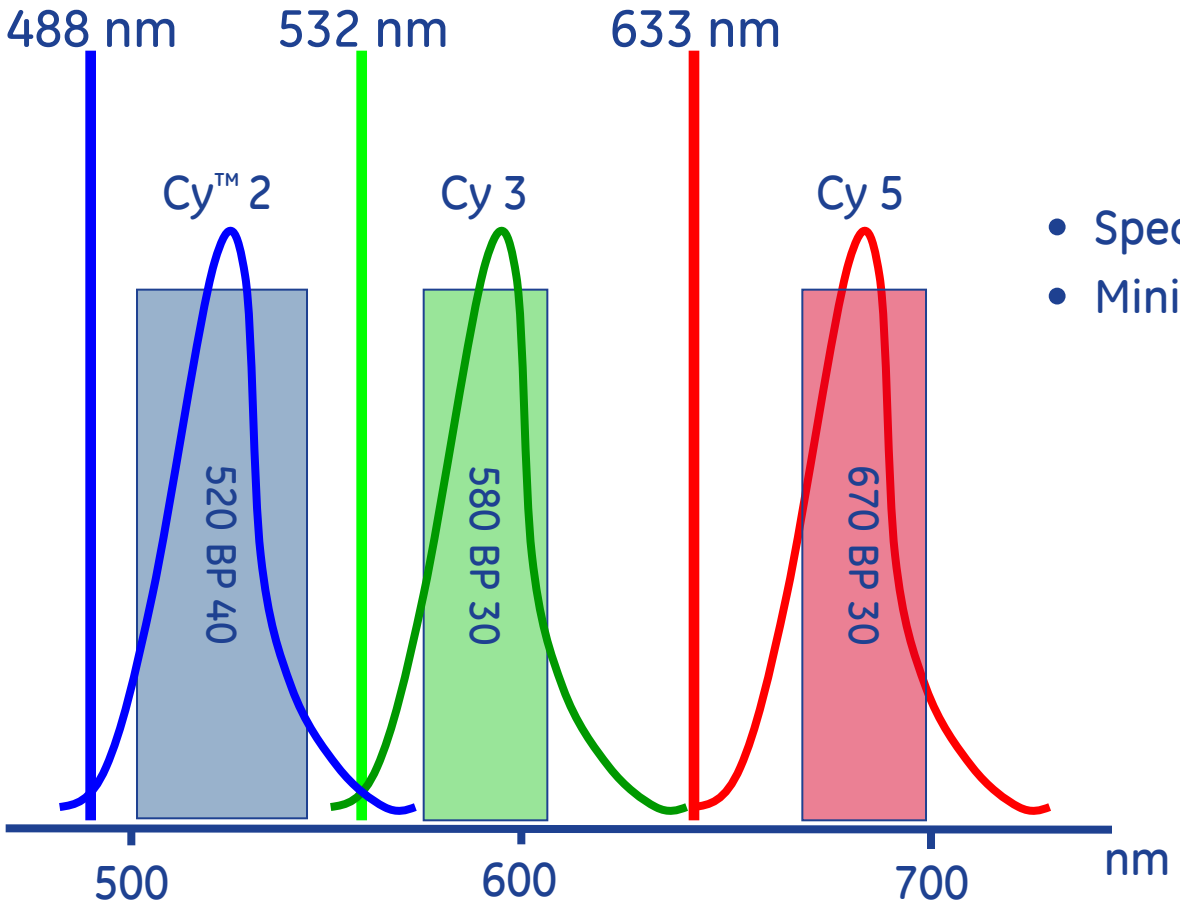


DeCyder™ software

- Designed specifically for this multiplexing technology

imagination at work

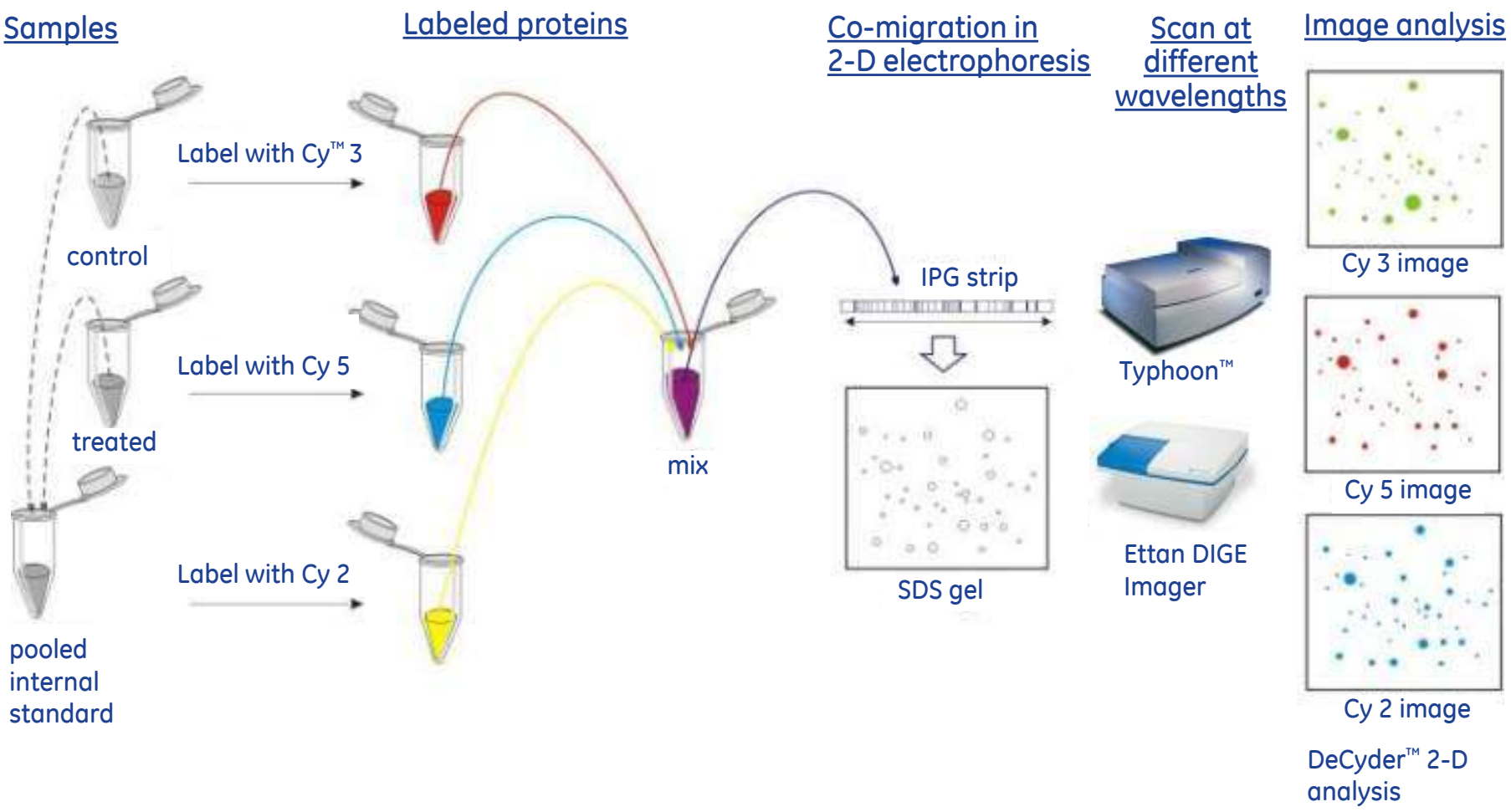
Multiplex detection – fluorescence excitation and emission



- Spectrally well resolved dyes
- Minimal cross-talk

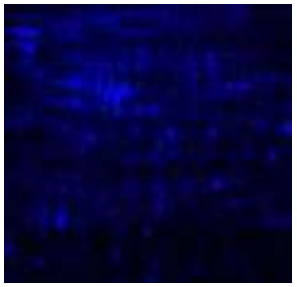
imagination at work

Ettan™ DIGE system – experimental procedure



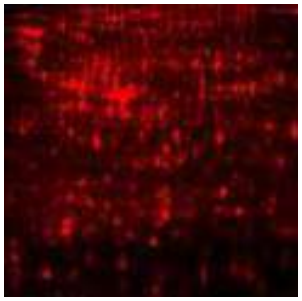
Multiplex detection
three color image from scanner

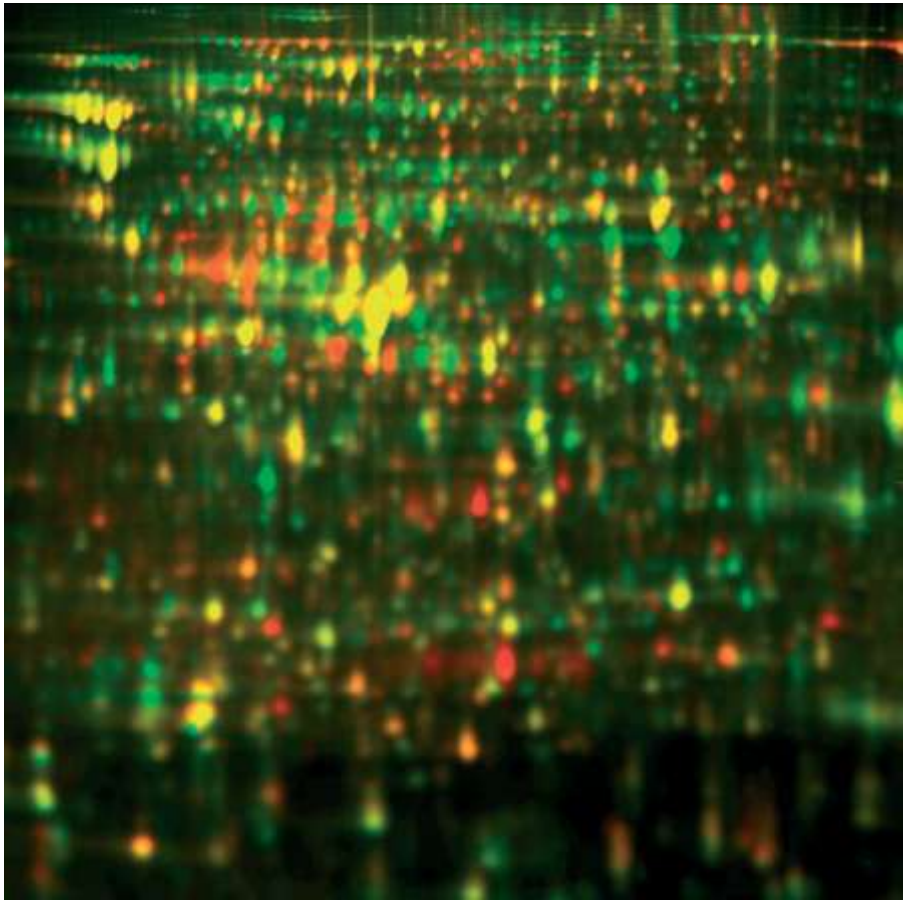
Cy™ 2



Cy 3

Cy 5



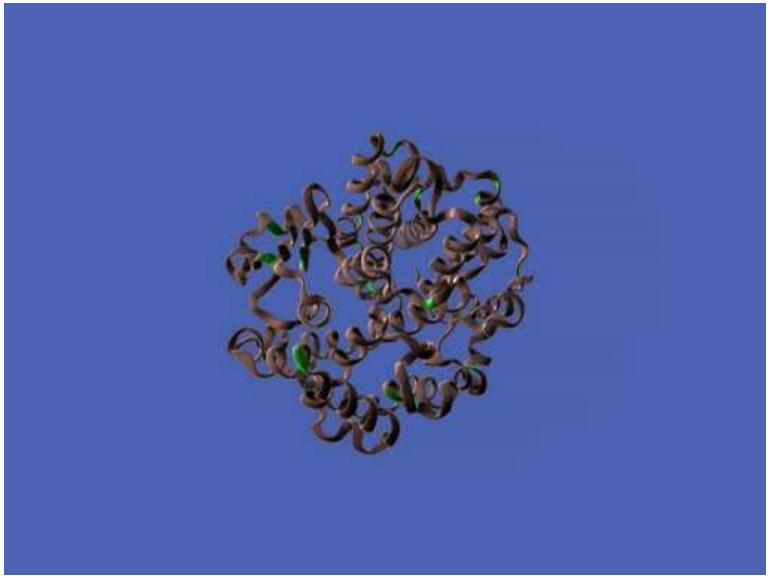


Three color overlay

Minimal CyDye™ DIGE fluors

Minimal labeling

- 50 µg protein
- single label (3 %)
- ε-amino group of lysine



3 dyes: Cy™ 2, Cy 3, Cy 5

- charge matched (+1 charge)
- size matched (~450Da)
- labeled samples co-migrate
- Sensitivity: 0.25 ng
- linear dynamic range: over 4 orders of magnitude

Saturation CyDye™ DIGE fluors

Saturation labeling

- 5 µg protein
- multiple labels (100 % of all cysteines)
- thiol group of cysteine

2 dyes: Cy™ 3, Cy 5

- charge matched (neutral)
- size matched (~680Da)
- Sensitivity: lower than 0.025 ng
- linear dynamic range:
  over 3 orders of magnitude

imagination at work

CyDye™ DIGE Fluors

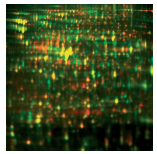
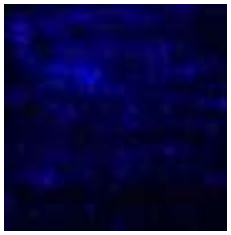
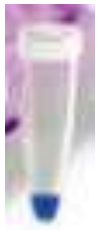
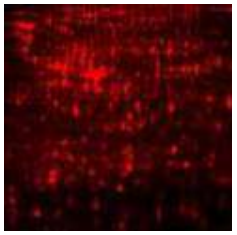
Dye	Visible light		Fluorescence	
Cy™ 2		Yellow		Blue
Cy 3		Red		Green
Cy 5		Blue		Red

Image acquisition

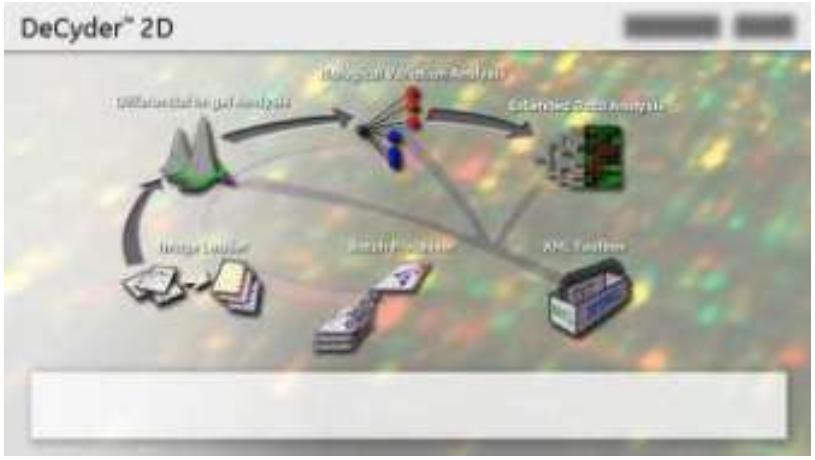
Two imager options for 2-D DIGE

1. Ettan™ DIGE Imager
    - Scanning CCD camera
    - Fluorescence
2. Typhoon™ Imager
    - Laser scanning
    - Fluoresence, phosphorimaging, chemiluminescence

Image analysis

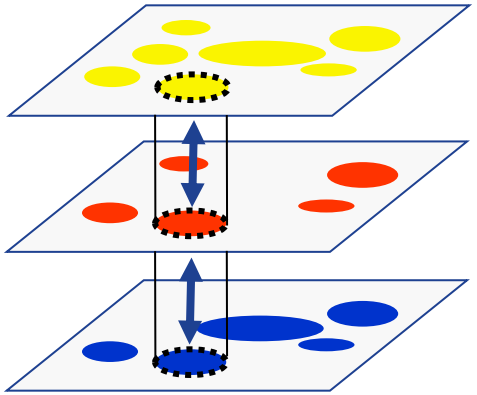
Improved/updated software for image analysis

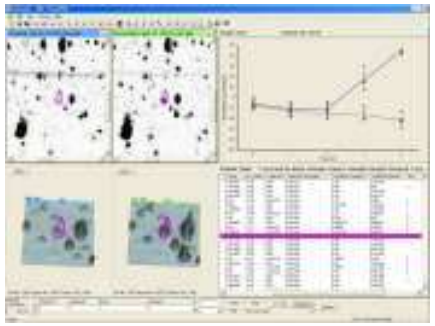
DeCyder™ 2-D Differential Analysis Software v7.0



DeCyder™ 2-D Differential Analysis Software

DeCyder module	Function
DIA (Differential In-gel Analysis)	- Protein spot detection on a single gel - Spot co-detection on all three images - In-gel normalization
BVA (Biological Variation Analysis)	- Matches all gels - Statistics for quantitative comparisons - Internal standard correction
EDA (Extended Data Analysis)	- Multivariate modeling - Expression pattern analysis - Classification

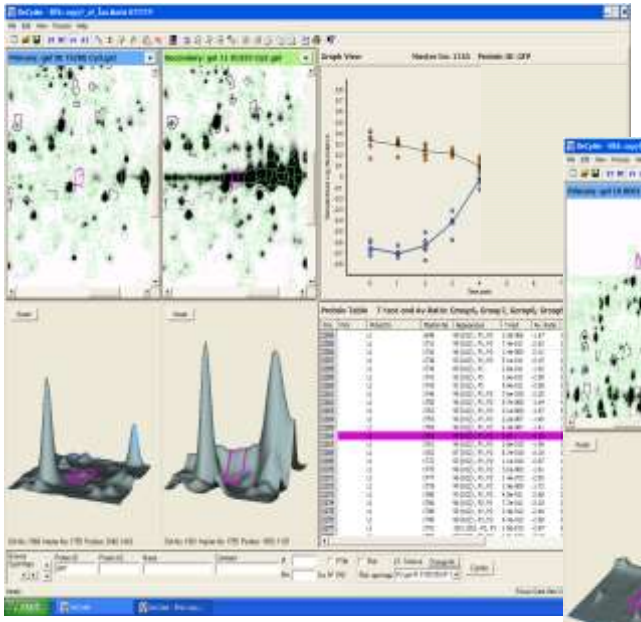




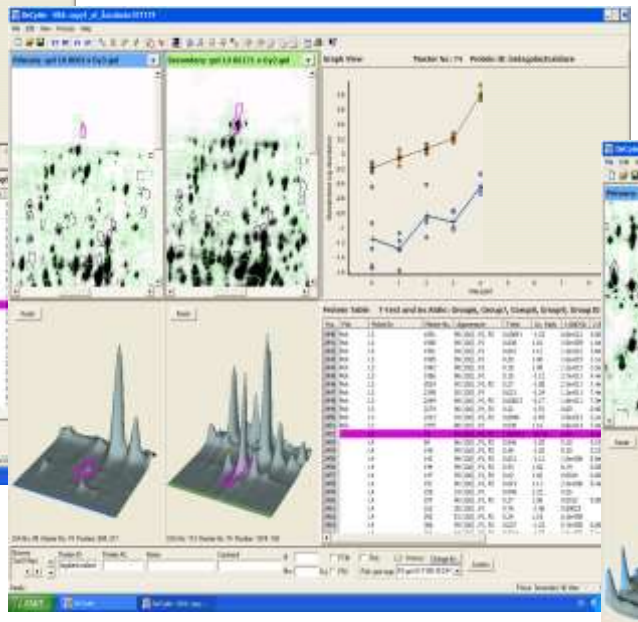
Results - individual protein profile

DeCyder™ 2-D differential analysis software

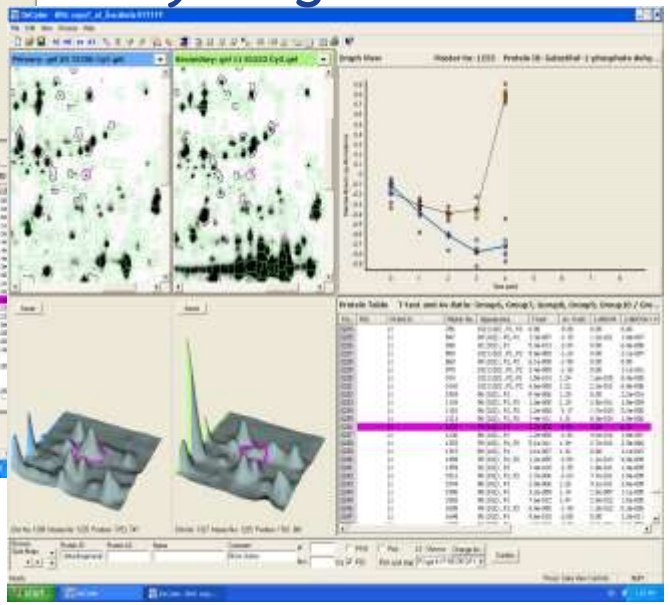
GFP



β galactosidase



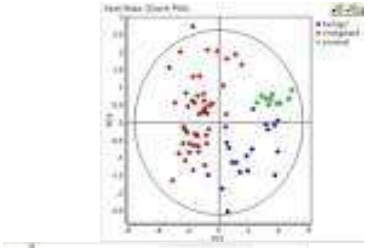
Galactitol-1-phosphate dehydrogenase

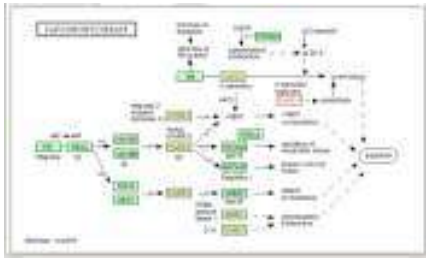


imagination at work

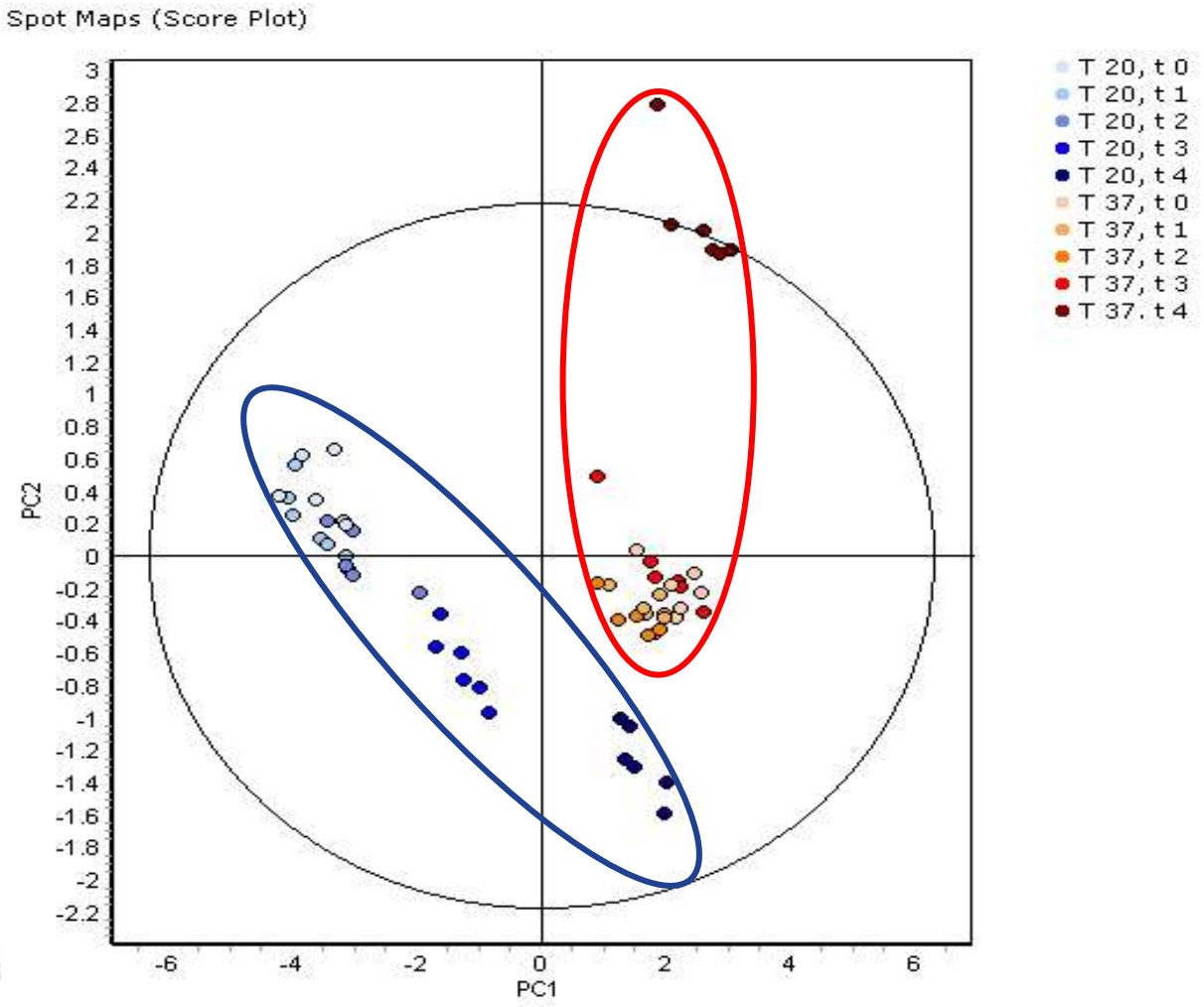
DeCyder™ 2-D Differential Analysis Software

EDA	Function
Principal Component Analysis	- Identify outlayers - Find groupings of the data
Pattern Analysis	- Find proteins with similar expression profiles - Find regulatory pathways - Classification of proteins with respect to their biological function
Discriminant Analysis	- Identify diagnostic markers - Classify unknown samples to known classes



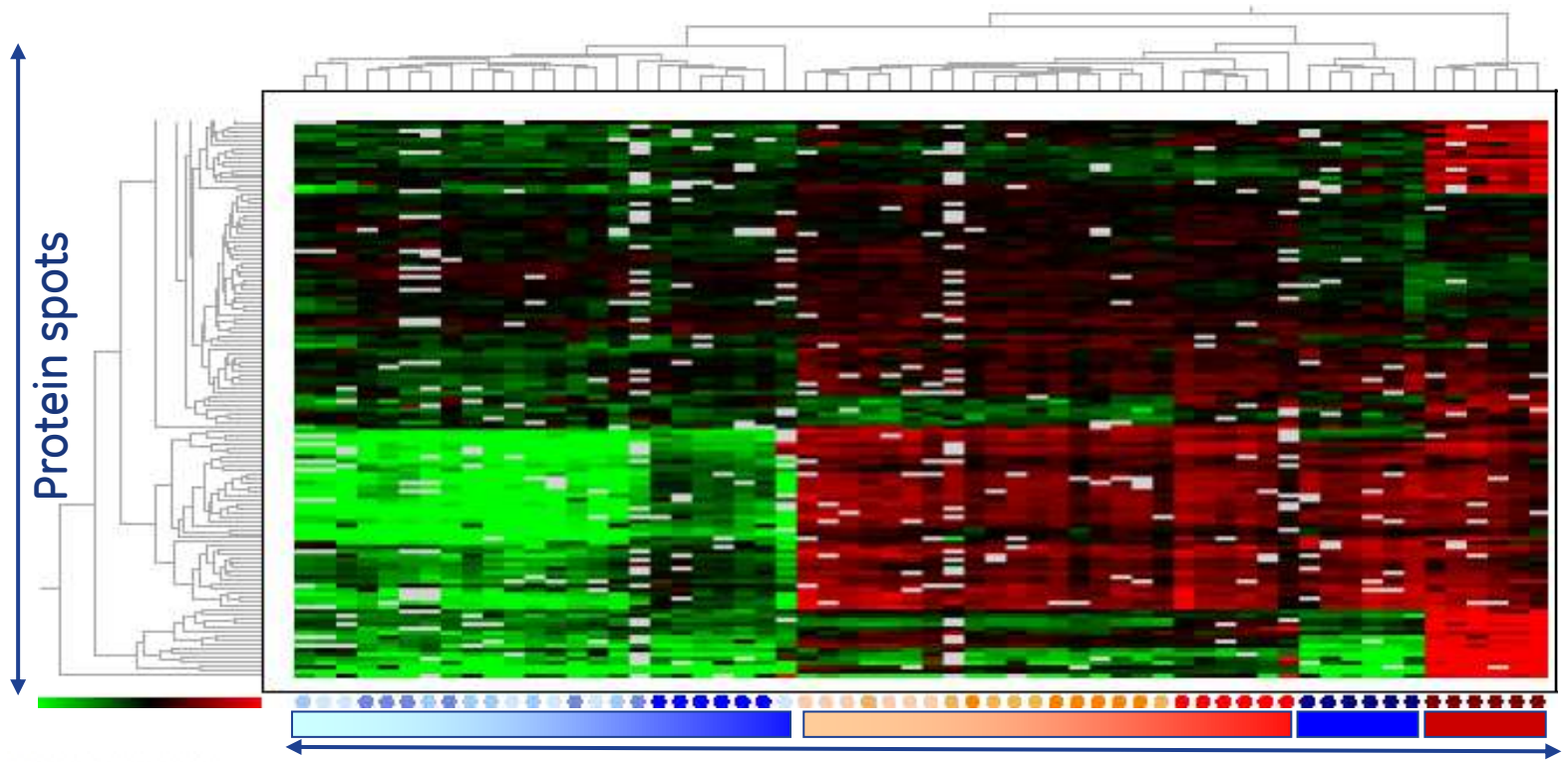


Complexity reduction: PCA analysis using DeCyder™ EDA module



imagination at work

Complexity reduction: hierarchical clustering analysis using DeCyder™ EDA module



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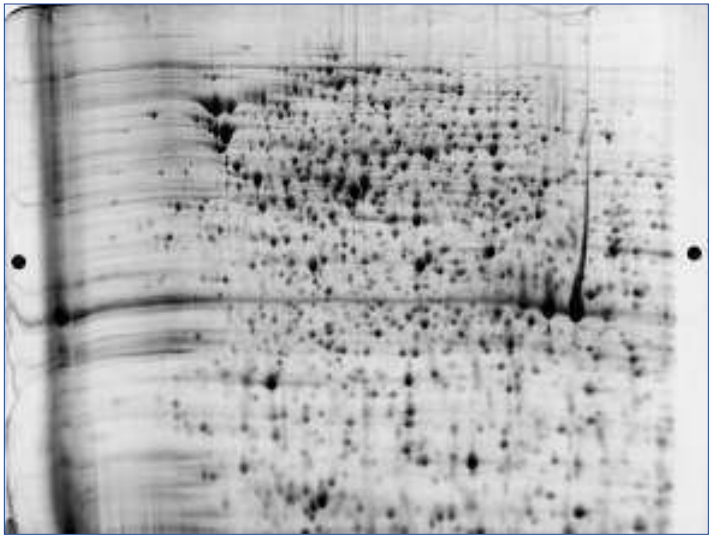
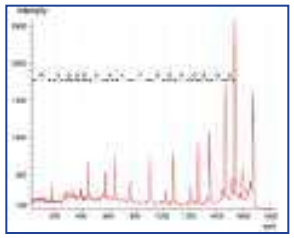
Protein identification

Preparative 2-D gel
(matched against analytical gels)





Automated spot picking

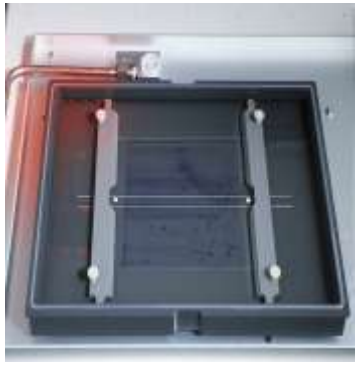
Spot digestion

MALDI target spotting

Identification with MS

Ettan™ Spot Picker

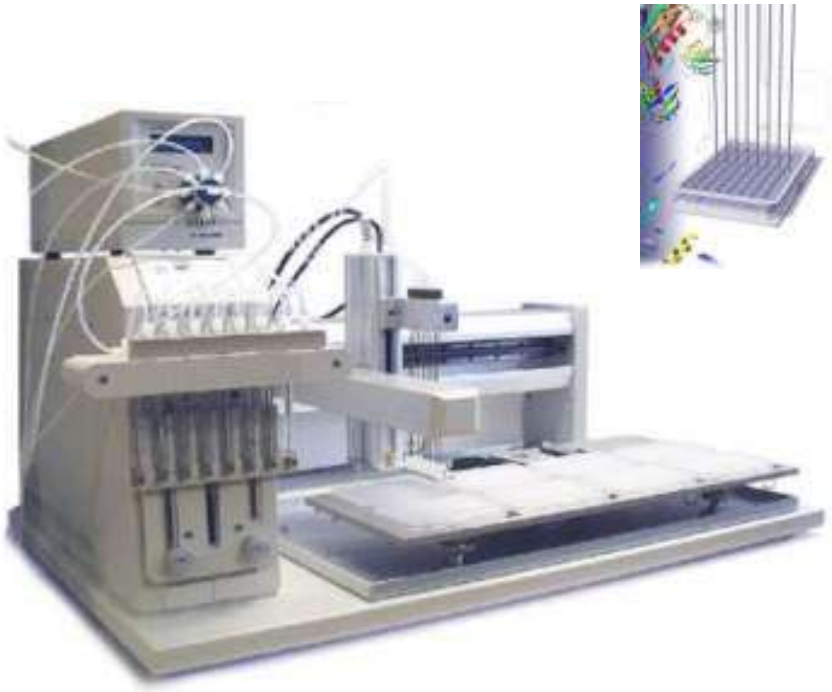
- Automated spot picking and dispensing of protein gel plugs into microplate wells
- Automatically picks selected protein spots from stained or de-stained gels
- Designed for most common 2-D gels
- Transfers from gel to well in less than 10 seconds
- >99% picking efficiency



One gel, 96 spots <30 min

Ettan™ Digester

- Automated spot in-gel digestion of proteins from 2-D gels
- Simple to use
- Standard multi-well plates
- No need for desalting - salt levels controlled by method (low-salt protocol)



384 gel plugs in 8 hrs

User endorsements

*"We do not perform standard 2-D electrophoresis anymore! We only do DIGE because it is **quicker**, **cheaper** and gives us far **higher quality** information."*

Dr Richard Burchmore,
SHW Functional Genomics Facility,
University of Glasgow

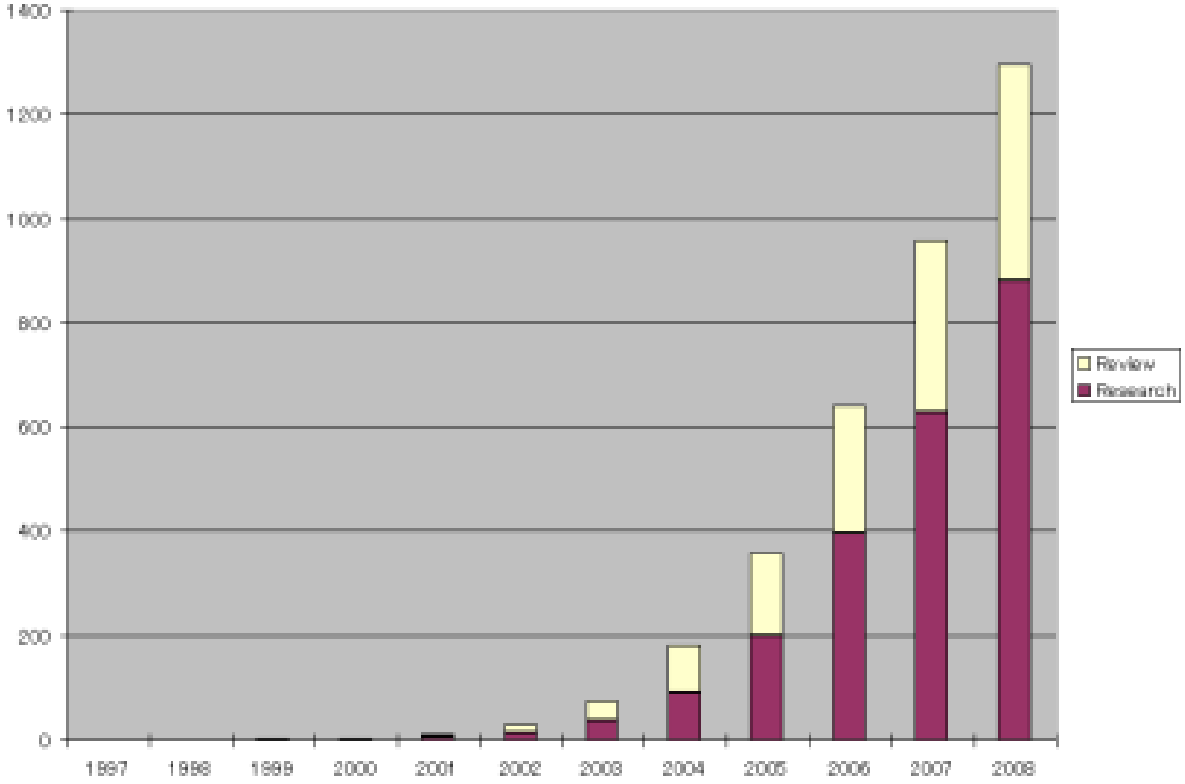
*"...the DIGE technology is **very sensitive** for **quantitative** variation..."*

*"The DIGE analysis showed a much **lower technical variation** (~7%) than the proteomics methods used in other studies (2-D electrophoresis without internal standards). Thus, the internal standard **increases the statistical confidence** of the analysis substantially."*

Prof. Dr. Oehler, Academical Hospital (AKH), Vienna
Winkler W, Zellner M, Diestinger M, Babeluk R, Marchetti M, Goll A, Zehetmayer S, Bauer P, Rappold E, Miller I, Roth E, Allmaier G, Oehler R. Biological variation of the platelet proteome in the elderly population and its implication for biomarker research. Mol Cell Proteomics 7 (2008) 193-203.

2-D DIGE
- an exciting technology that delivers real results

Total number of DIGE publications
~1300 publications



Top 5 categories of DIGE publications:

1. Human medicine
2. Proteomics
3. Molecular biology
4. Plants
5. Environment

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Frost & Sullivan Technology Innovation Award 2007

*"GE's **Ettan™ DIGE System is capable of comparing protein expression patterns from two different samples in a single gel**. This information is **crucial in the search for biomarkers that may change in expression levels during** the initiation or progression of a **disease** from one phenotype to a more malignant phenotype.*

The need to isolate and identify these protein biomarkers that appear or fail to appear is likely to also influence the way patients' treatment protocols are determined."

North American Frost & Sullivan Award for Technology Innovation (2007)

Summary

Advantages with DIGE technology:

- Multiplexing of samples in the same gel (size and charge matched CyDyes™)
- Sensitivity (sub nanogram level)
- Wide dynamic range (4-5 orders of magnitude)
- Detection of small differential changes (down to 10%)
- High statistical accuracy (DeCyder™ 2-D Differential Analysis Software)
- Saves time. Few gels needed. Biological replicates only.

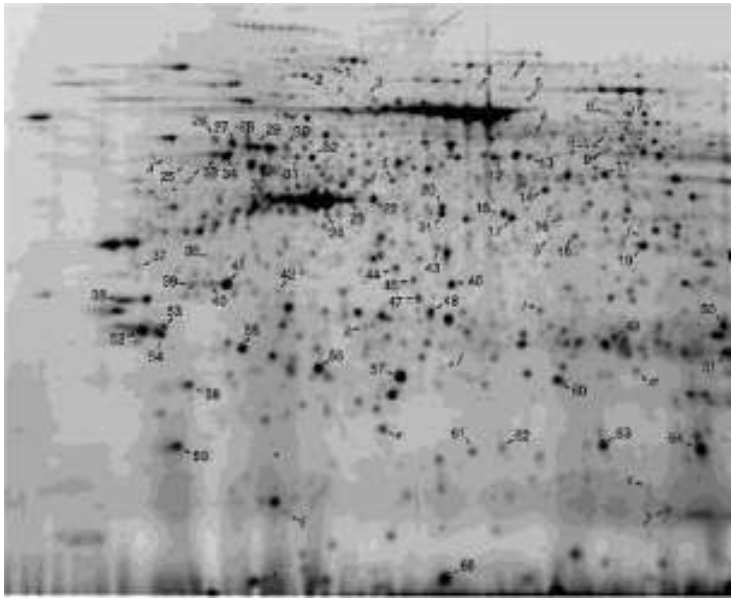
Agenda

1. 2-D DIGE concepts and benefits
2. Biomarkers in colorectal cancer
3. Monitoring effect of drug treatment and diagnosis using PET
4. Changes in tyrosine phosphorylation
5. Selective labeling of cell surface proteins
6. Quantitative fluorescent Western blotting

imagination at work

2. Putative biomarkers in colorectal cancer

- Tumor tissues from 6 patients with different stages of colorectal cancer
- Minimal labeling approach – 50 µg protein, with and without internal std



83 significant changes in expression of proteins were detected ($p<0.015$)

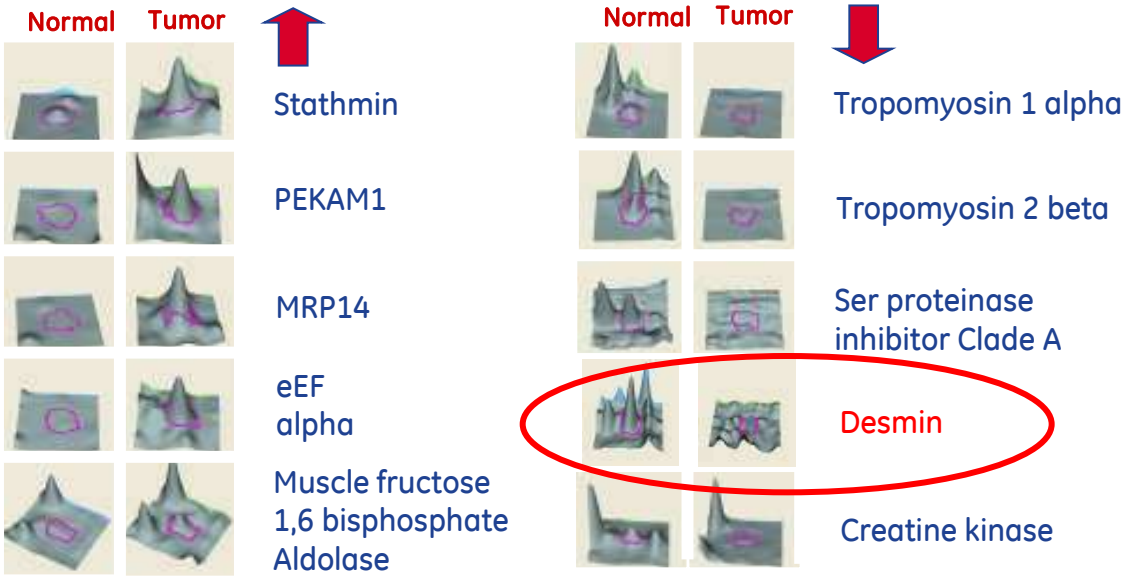
In many cases, identifications were made on low abundant proteins

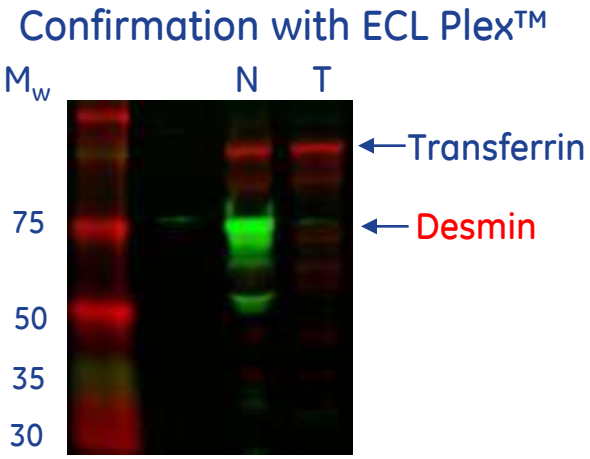
Without the benefit of the internal standard, 42 of 52 identified proteins would have been overlooked => increase the number of real hits

Friedman *et al. Proteomics* (2004) 4:793-811

Putative biomarkers in colorectal cancer

- Minimal labeling approach
- 288 unique proteins identified using MALDI
- 30 unique proteins were differentially regulated
- 15 of the differentially regulated proteins have previously been reported to be involved in cancer





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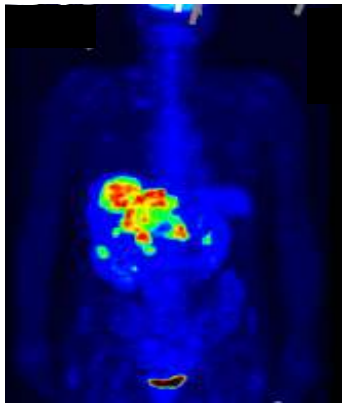
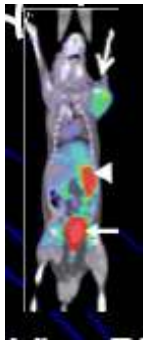
3. Monitoring effect of drug treatment and diagnosis using PET



In Vivo Human PET



In Vivo animal PET

Tumor Biology

Multi cellular tumor spheroid
Patient tissue

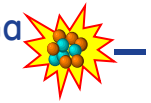
In Vitro PET

2-D DIGE
ECL Plex™

What is Positron Emission Tomography PET?

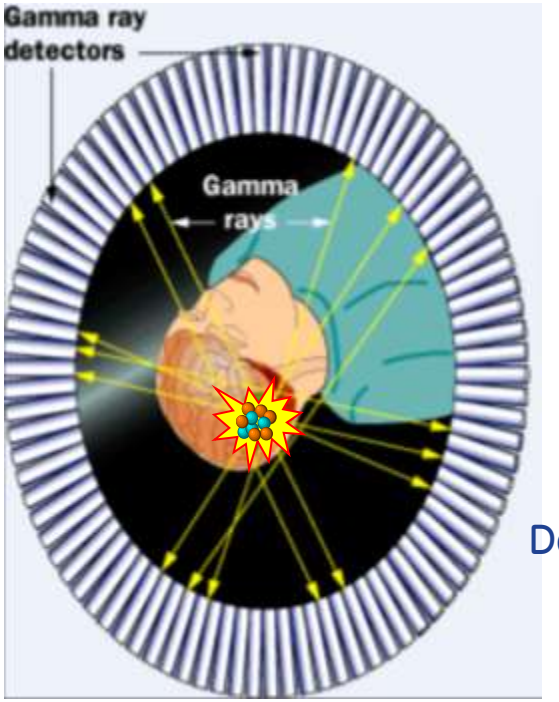
PET-Tracer

^{68}Ga
^{18}F
^{11}C



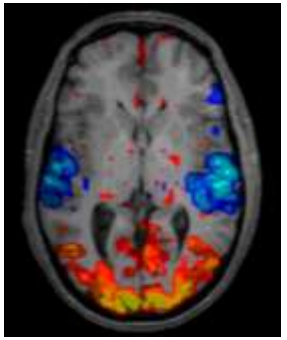
— Biologically active molecule (peptide, protein, drug)

Injection of tracer



Detector rings

Detecting process

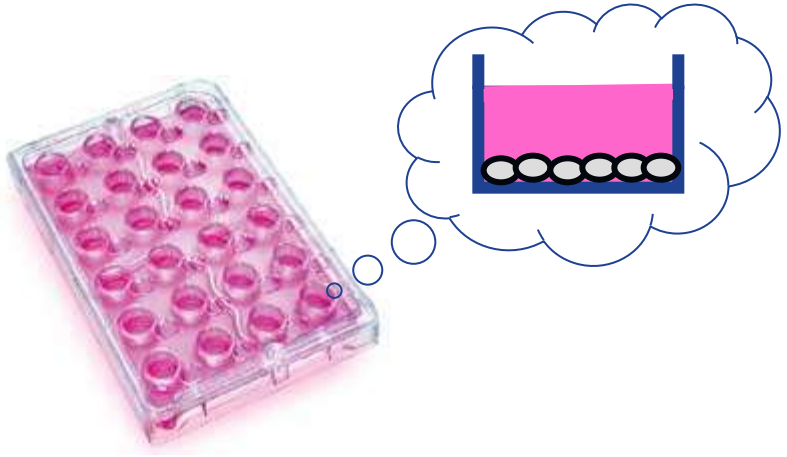


Reconstructed image

In Vitro cell culture techniques

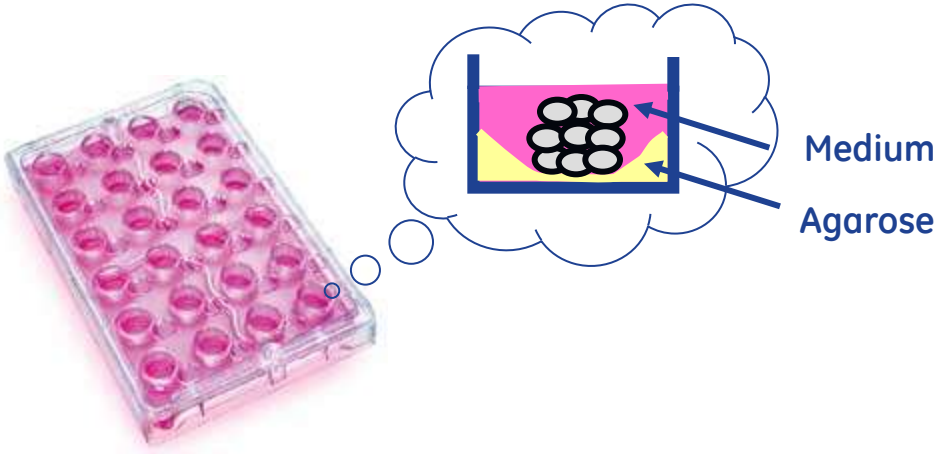
Monolayer

- Single layer
- Two-dimensional



Multicellular Tumour Spheroid (MTS)

- Three-dimensional
- Similar to tumours



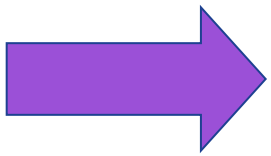
In Vivo/*In Vitro* PET /Spheroid model

Analyze……

- Tumor growth
- Tumor size
- Tumor PET tracer uptake
- Tumor apoptosis level

- 2-D DIGE proteome changes

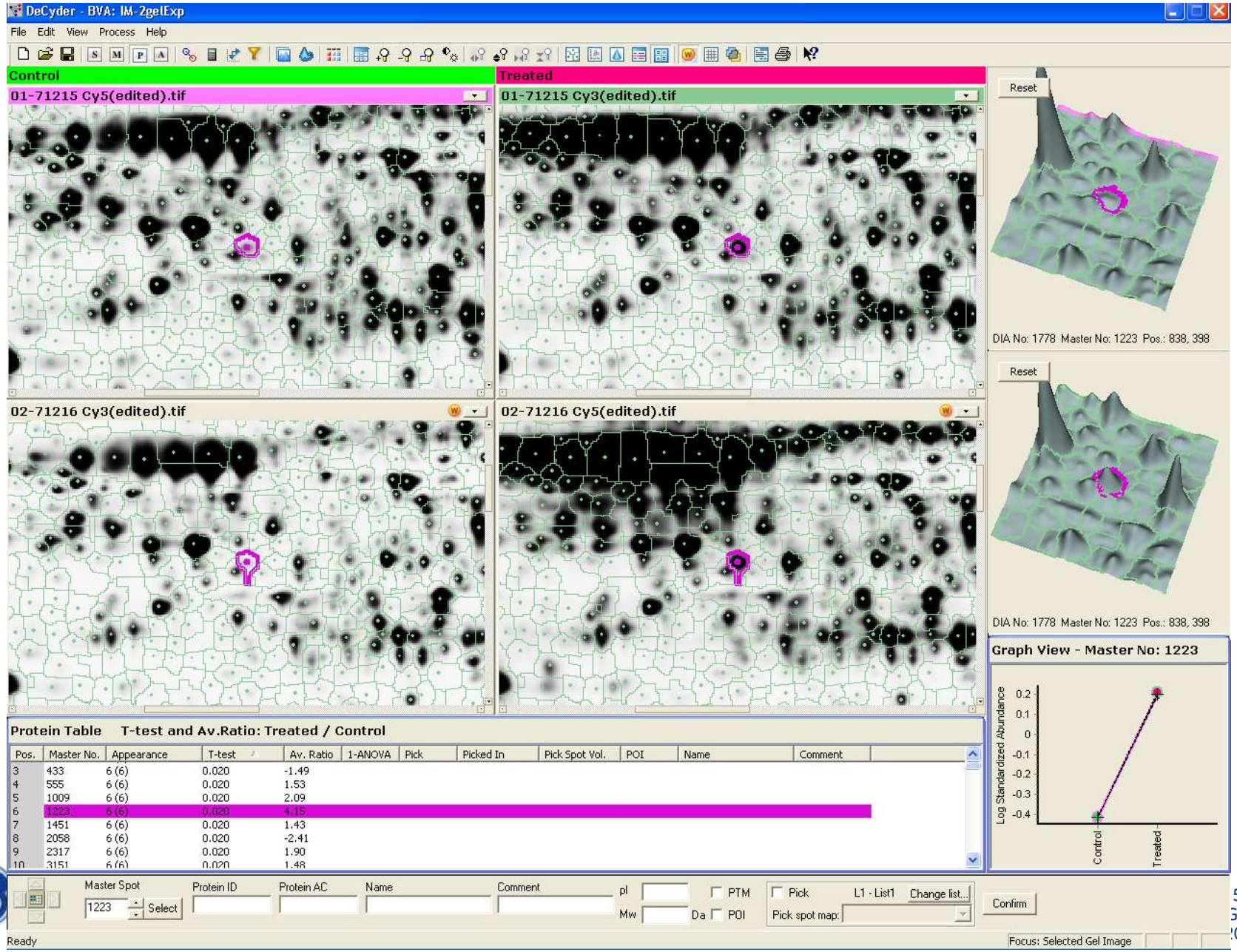
……..and combine information for increased knowledge



Optimize and improve diagnosis and treatment of cancers

Results from 2-D DIGE analysis of control and treated spheroids

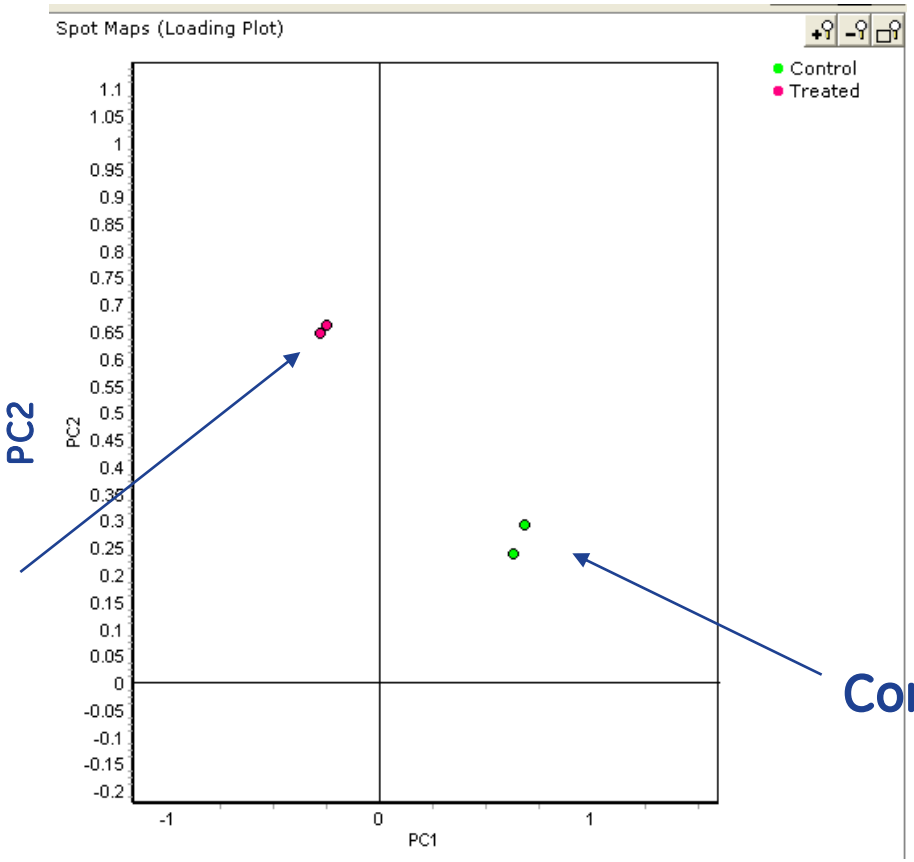
447 differentially expressed proteins



447 differentially expressed proteins

Principal Component Analysis

T-test p <0.05



Treated

Control

PC1

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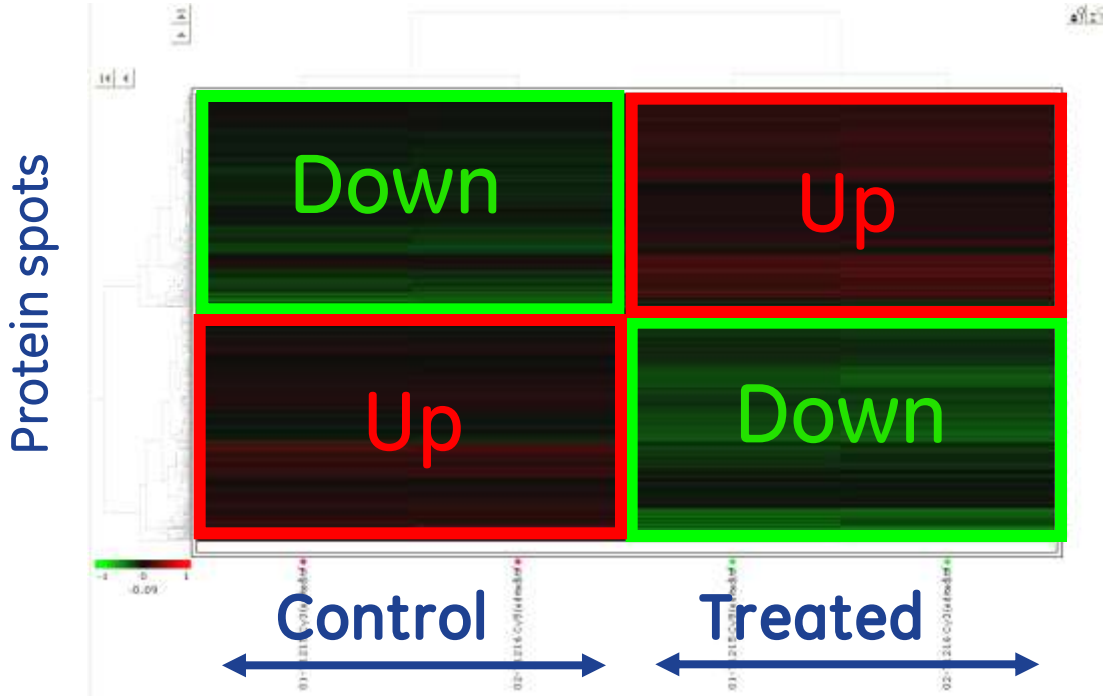
Hierarchical clustering analysis heat map



3. Summary
Monitoring effect of drug treatment and diagnosis using PET

- A combination of 2-D DIGE and PET analysis may increase possibilities to optimize personalized diagnosis and treatment of cancers

  -Give ideas for new PET tracers

  -Study effect of different treatments, disease stages etc.

  -Give ideas for new drug targets

  -Biomarkers

- 2-D DIGE can be used to compare spheroid and patient tissue protein profiles (validate model system)

Agenda

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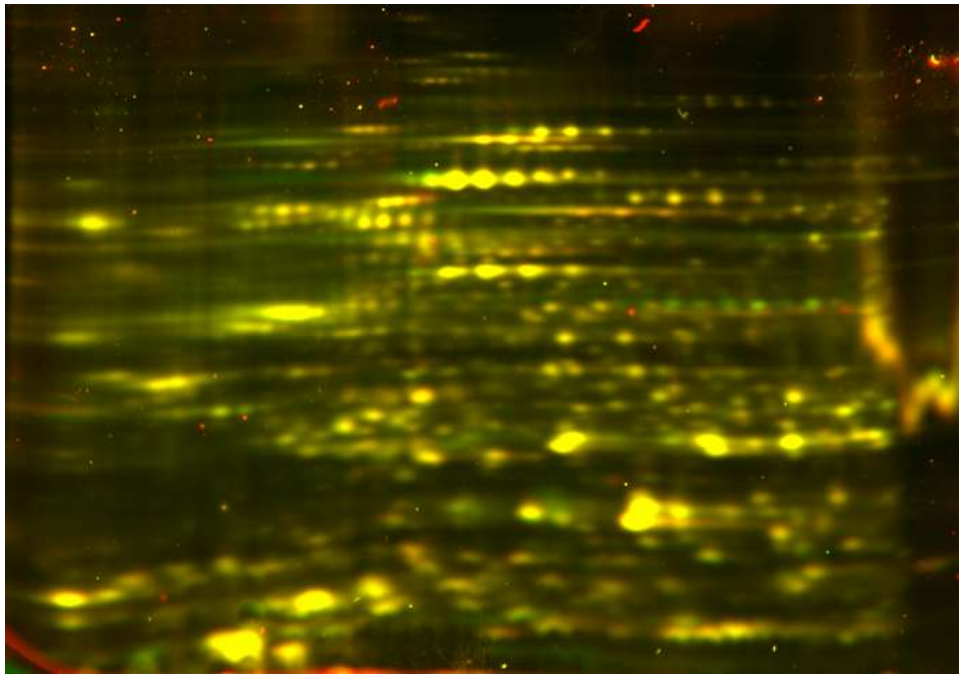
imagination at work

4.Changes in tyrosine phosphorylation in cancer cells upon drug treatment

Phospho tyrosine proteins are VERY low abundant



Control (green), treated (red), overlay (yellow)

No differences detected in total protein samples using DeCyder™ 2-D software

imagination at work

Data courtesy: Dr. Sara Lind, Rudbeck laboratory, Uppsala , Sweden

Products for enrichment of phosphoproteins and -peptides

Magnetic beads

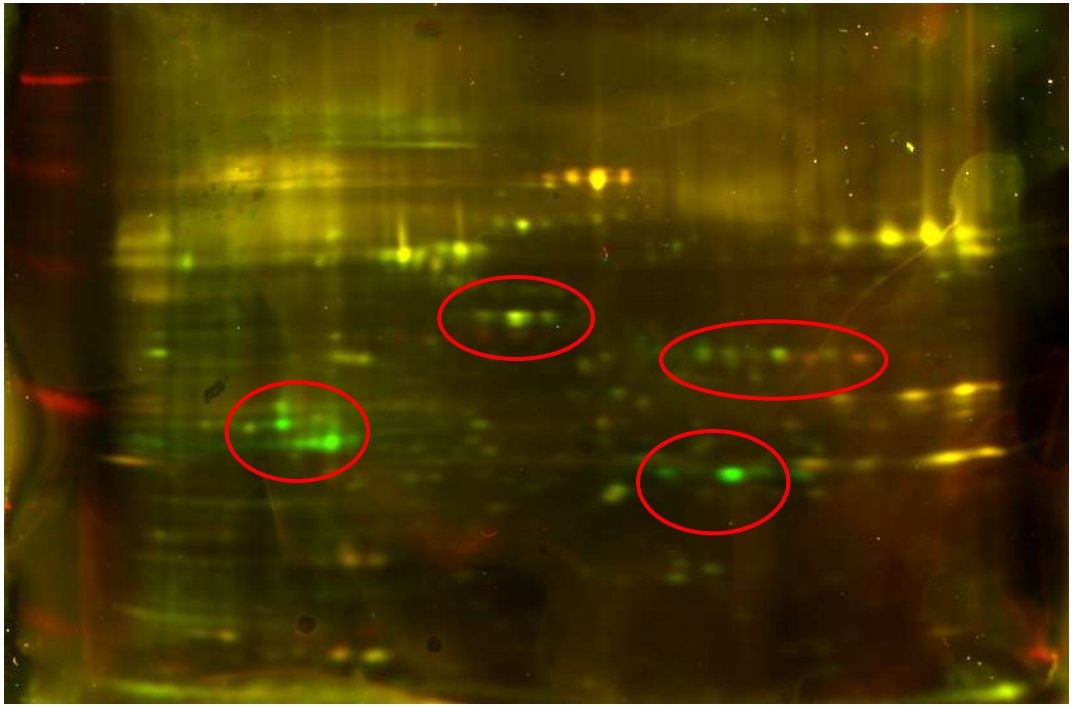
TiO_2 Mag Sepharose™
NHS Mag Sepharose
Protein A Mag Sepharose
Protein G Mag Sepharose

Spin columns and multiwell filter plates

Phos SpinTrap™ Fe
NHS HP SpinTrap
Protein A SpinTrap™
Protein A MultiTrap
Protein G SpinTrap
Protein G MultiTrap

imagination at work

Phospho tyrosine enriched samples

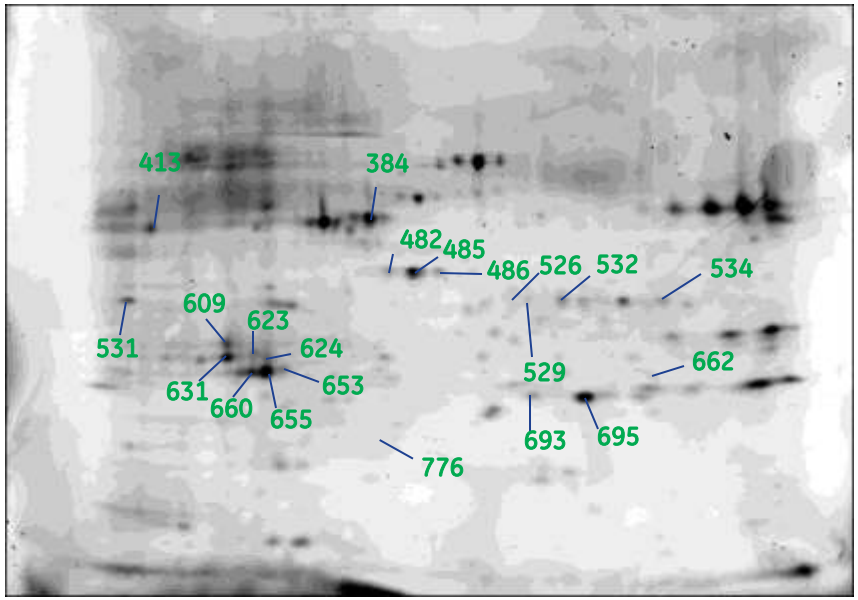


Control (green), treated (red), overlay (yellow)
Protein G Mag Sepharose™

Large differences in tyrosine phosphorylation detected in enriched samples

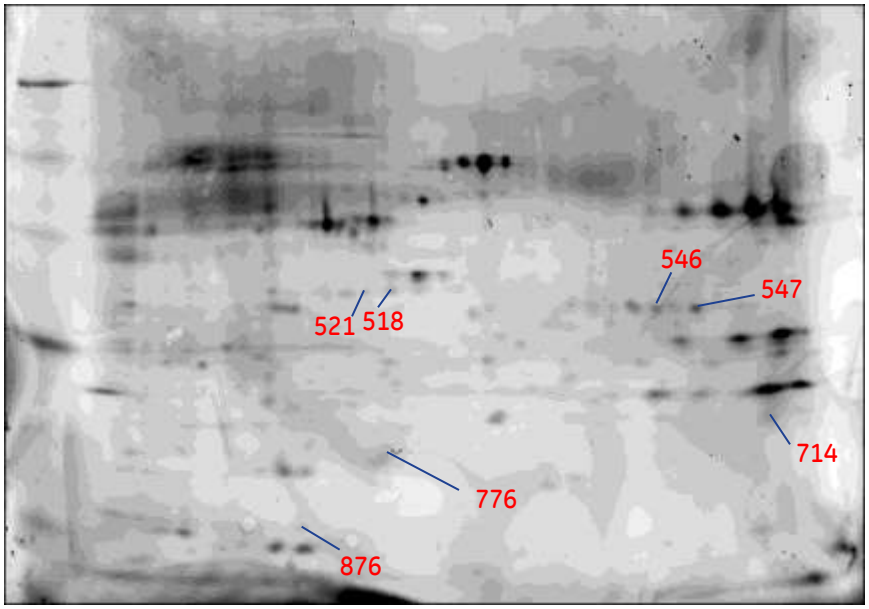
Differential protein expression by DeCyder™ 2-D analysis

Control



Down regulated upon drug treatment

Treated with drug

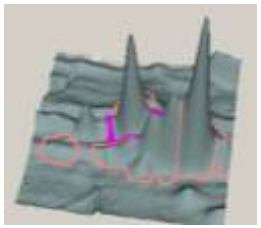
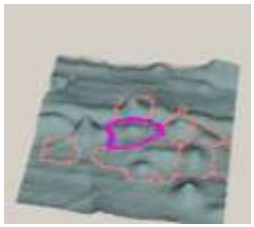
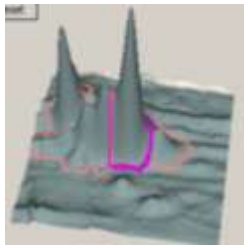
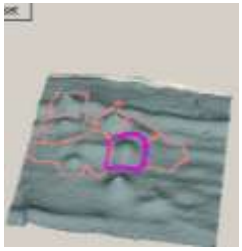
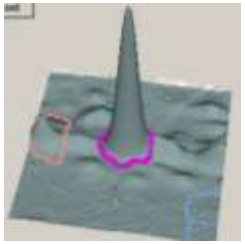
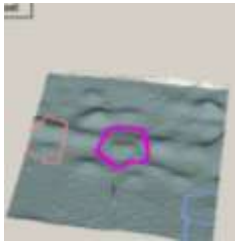


Up regulated upon drug treatment

Many phospho tyrosine proteins are down regulated upon drug treatment

imagination at work

Down regulated phospho tyrosine proteins

Spot no.	Control	Treated	Fold change
631			-5.14
655			-11.52
695			-11.13

imagination at work

2-D Western blotting

Total protein pre-labelled with Cy™3
Unlabeled total protein

↓

2-D electrophoresis

↓

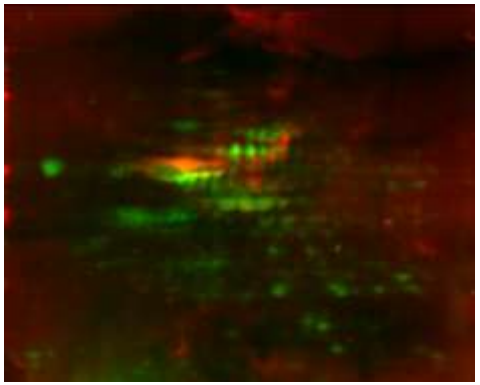
Transfer to membrane

↓

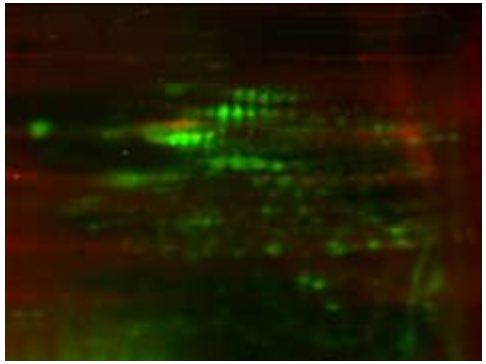
Antibody probing

Anti phospho tyrosine primary
ECL Plex Cy 5 secondary

Control



Drug treated



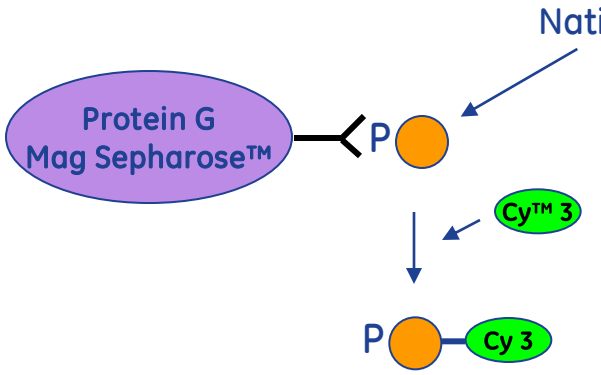
Membrane image
Cy 3/Cy5 overlay

Decrease in antibody detected tyrosine phosphorylation upon drug treatment

imagination at work

Comparison of CyDye labeling and Western signal

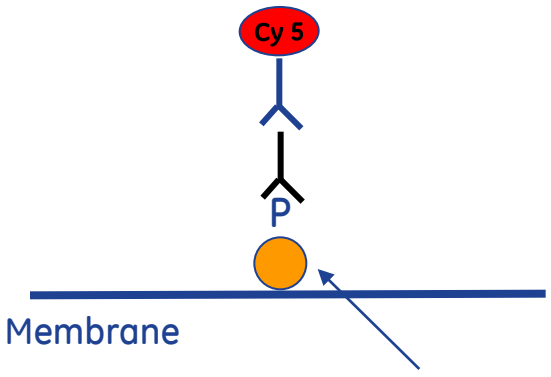
Antibody bound to Mag Sepharose



Phospho tyrosine protein enriched samples labeled with Cy™ 3 (green)

Same antibody used in both methods

Antibody used for probing Western membrane



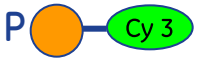
Phospho tyrosine proteins by Western blotting using ECL Plex™ Cy 5 (red)

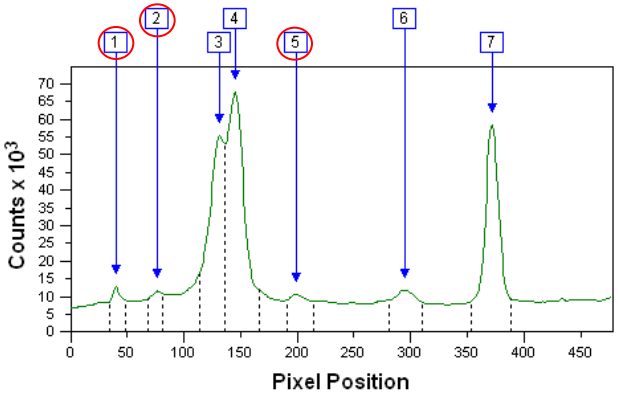
imagination at work

Results
Membrane image

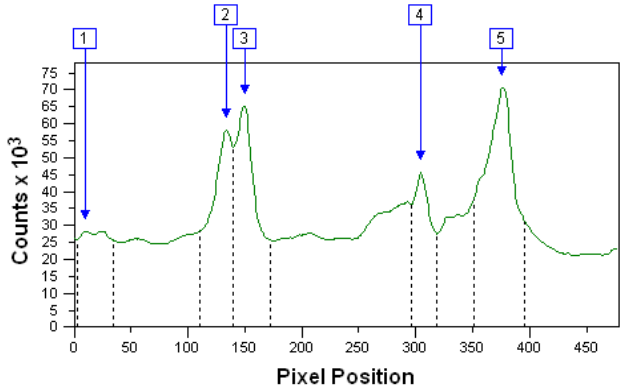
Good agreement between antibody enriched proteins and Western signals

-Detection of same proteins
-Better signal to noise





Cy™ 3



Cy 5

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4. Summary
Changes in tyrosine phosphorylation

- Enrichment necessary to detect differences in very low abundant proteins
- Phospho tyrosine enriched proteins were decreased in response to drug treatment
- CyDye™ labeled enriched proteins and Western signals in good agreement

Agenda

1. 2-D DIGE concepts and benefits
2. Biomarkers in colorectal cancer
3. Monitoring effect of drug treatment and diagnosis using PET
4. Changes in tyrosine phosphorylation
5. Selective labeling of cell surface proteins
6. Quantitative fluorescent Western blotting

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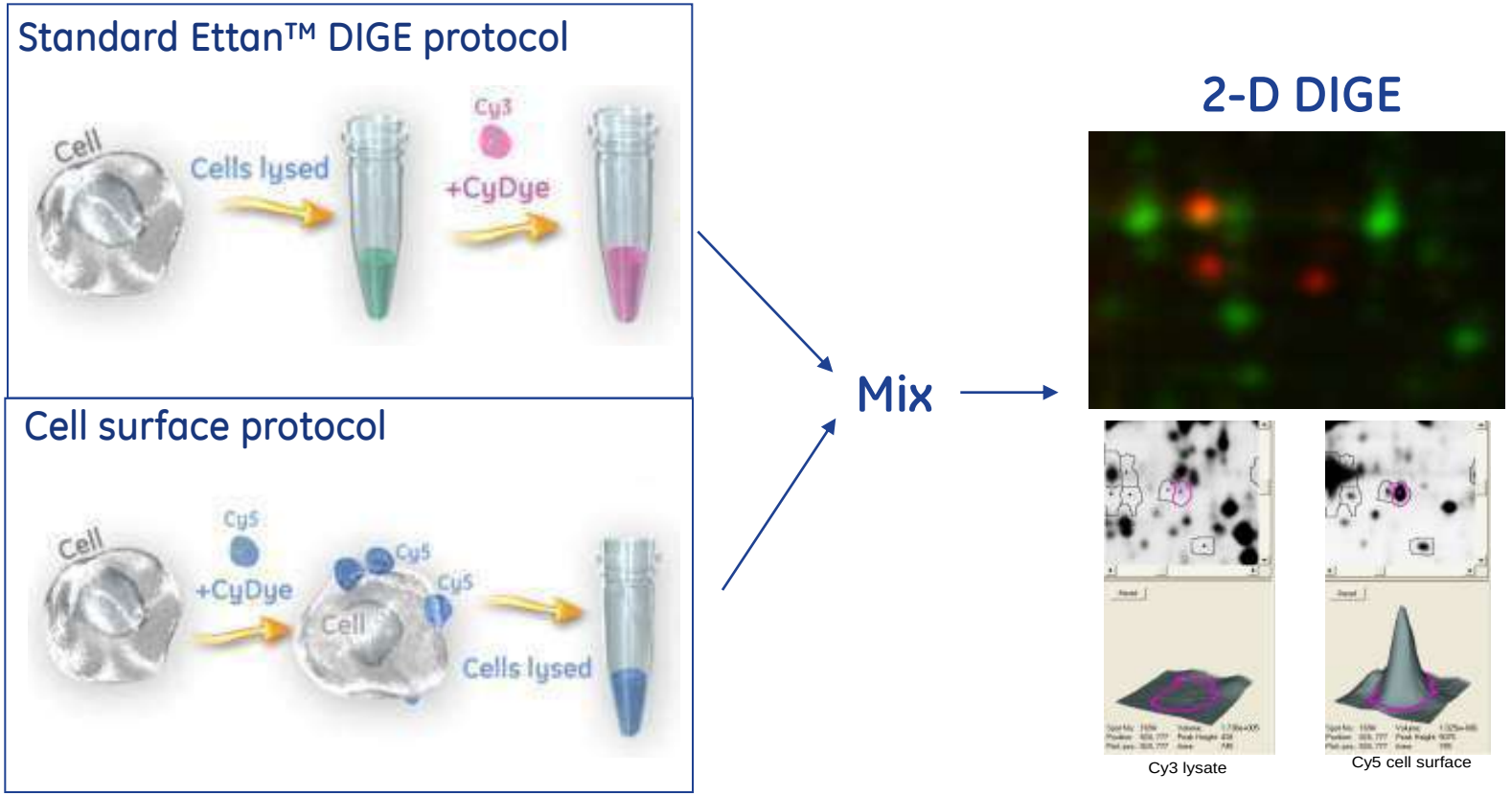
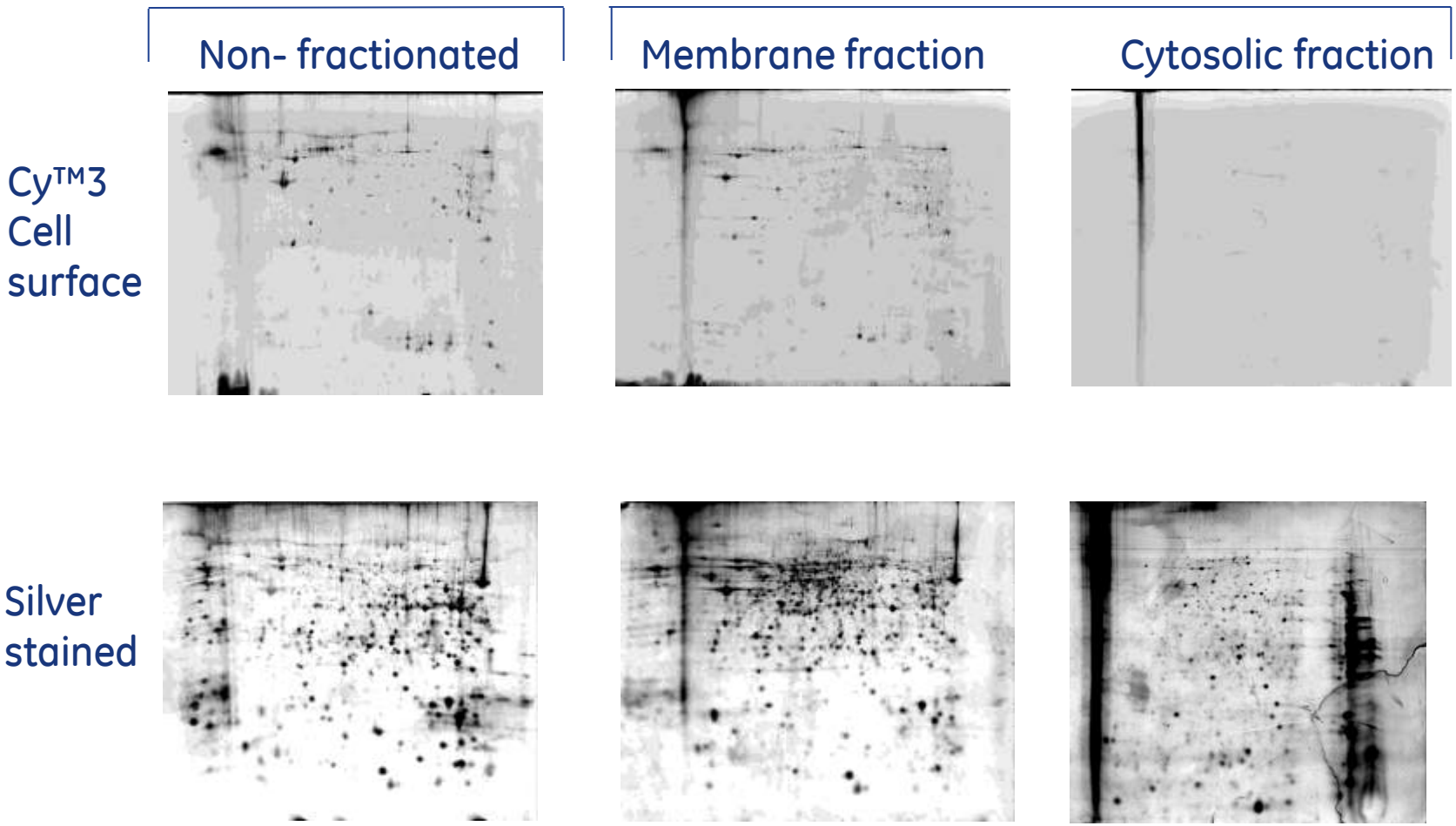
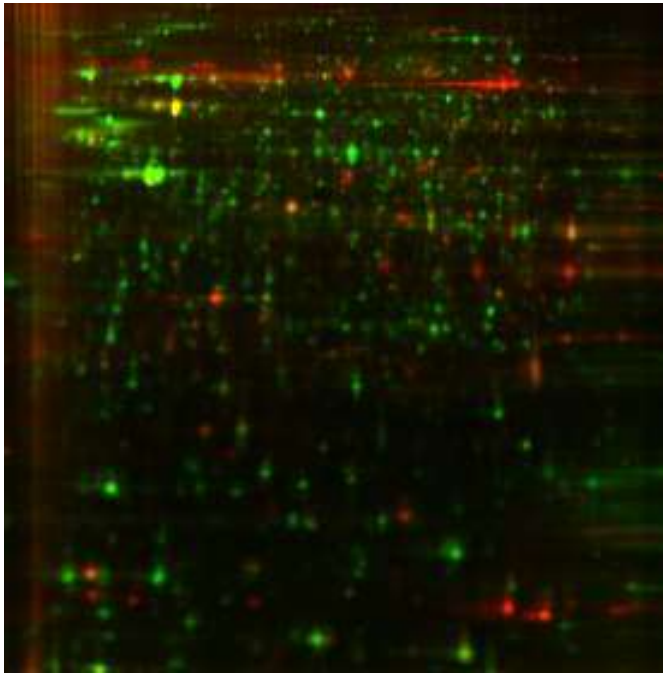
5. Selective labeling of cell surface proteins



Cell surface labeling specificity



Standard DIGE and cell surface labeling – comparison



Filtered for ratio >10:

83 novel proteins present only in the cell surface labeled sample, devoid in the lysate

Film showing experimental procedure

JoVE video

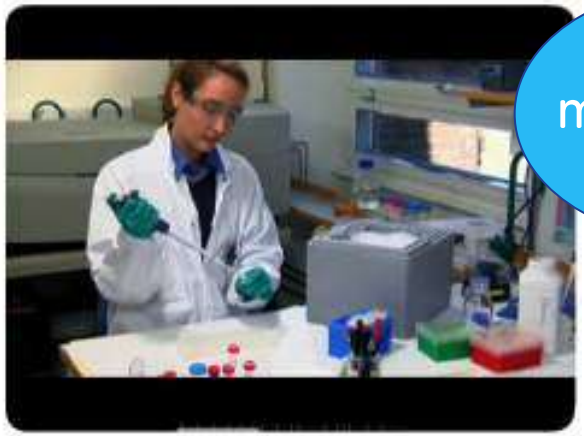
http://www.jove.com/index/Details.stp?ID=945

Title

Selective Labelling of Cell-surface Proteins using CyDye DIGE Fluor Minimal Dyes

Asa Hagner-McWhirter, Maria Winkvist, Stephanie Bourin, Rita Marouga

Research & Development, GE Healthcare Bio-Sciences AB



Top 10 most viewed videos

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5. Summary
Selective labeling of cell surface proteins

- Specific labeling of cell surface proteins
- No need for fractionation or enrichment
- New proteins detected compared to standard labeling protocol

Agenda

1. 2-D DIGE concepts and benefits
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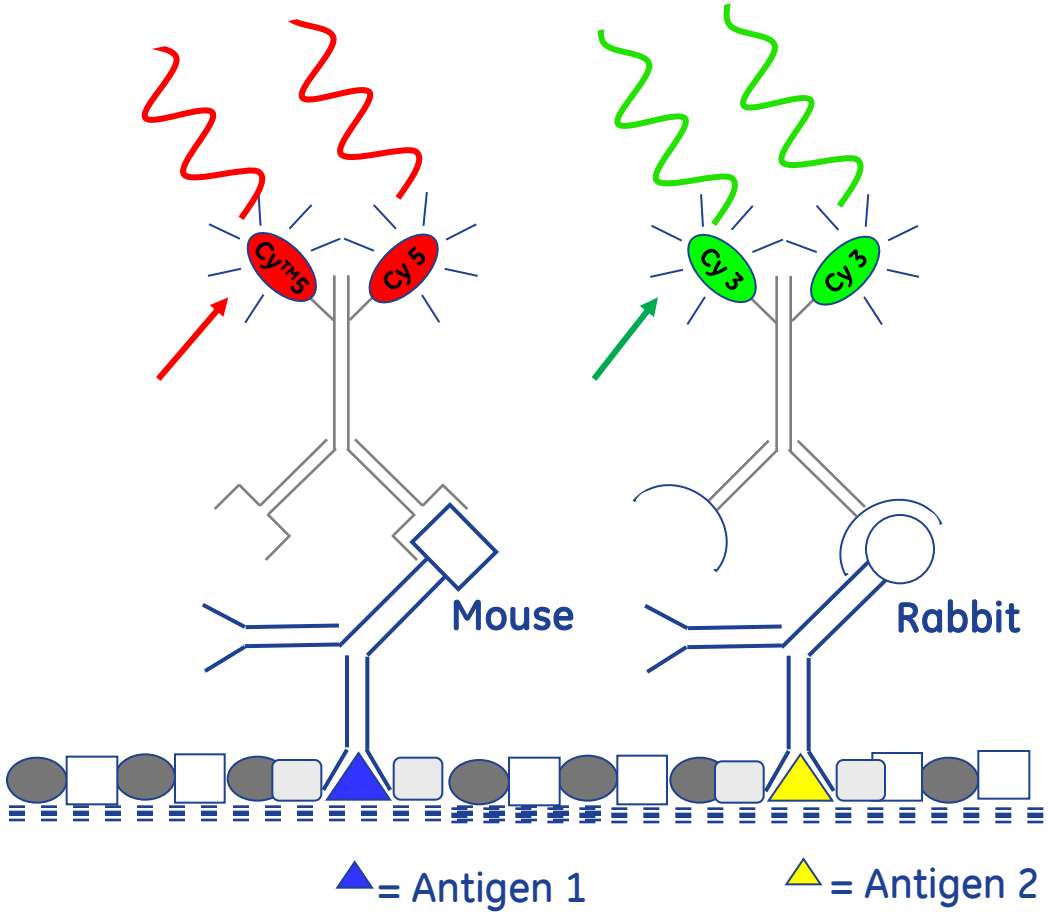
6. Quantitative fluorescent Western blotting



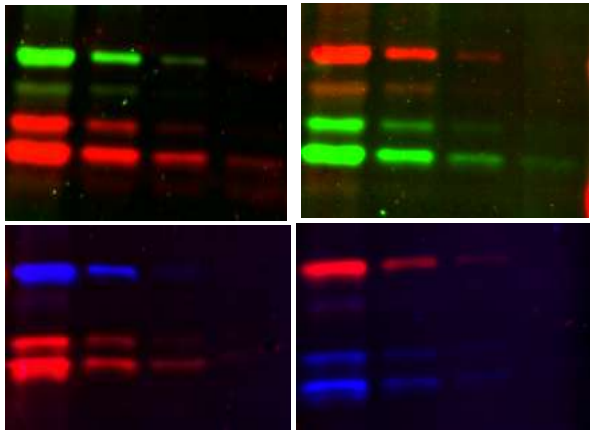
ECL Plex™ secondary antibody CyDye™ conjugate

Primary antibody

Antigen on membrane

Benefits of fluorescent Western blotting

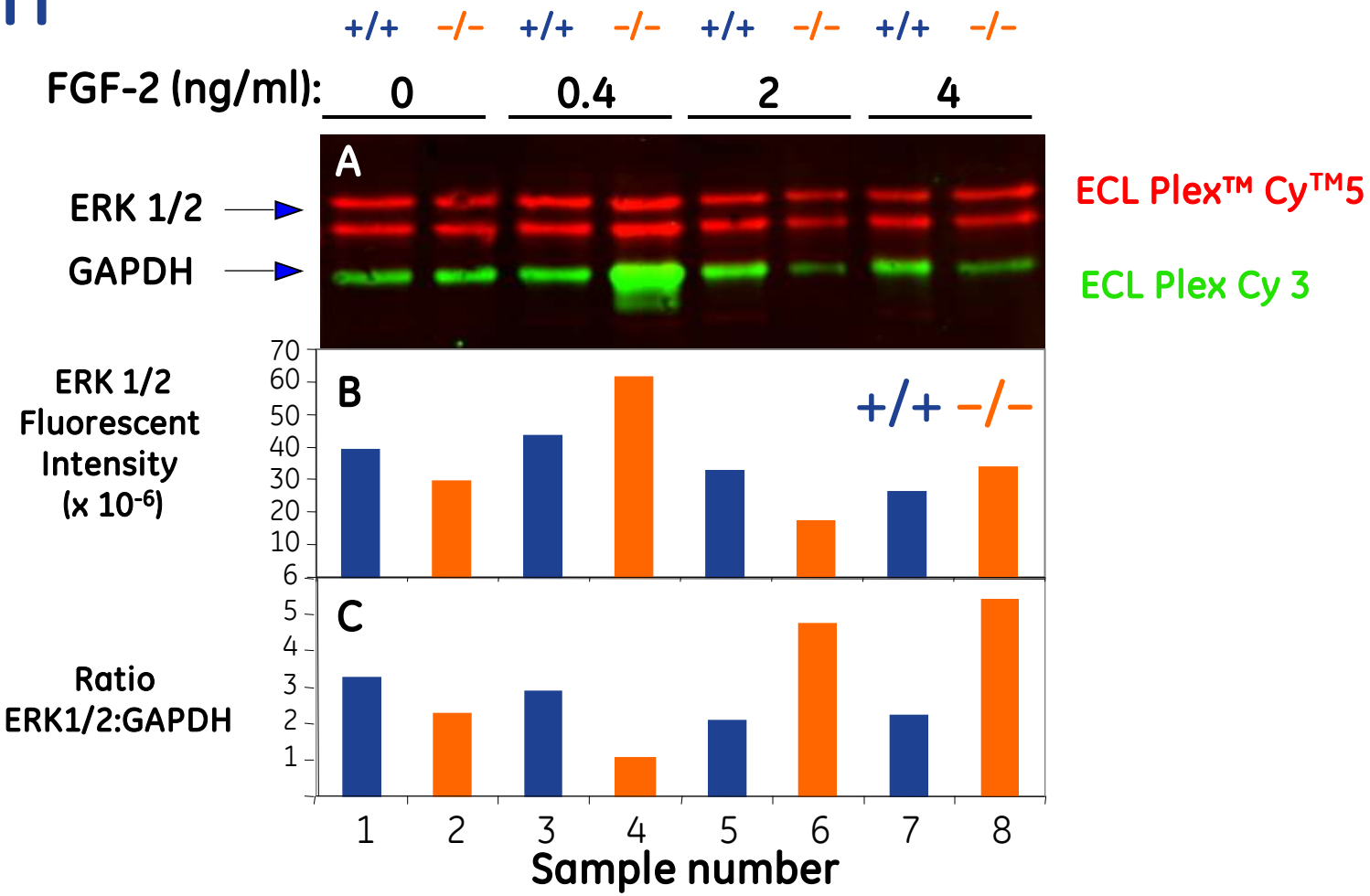
Multiplex detection for better data
-Detect two or more proteins simultaneously
-Normalize against "housekeeping protein" gives reliable quantitation
-Detect targets with similar MW



Highest sensitivity and dynamic range
-Enables quantitation of low and high abundance proteins on the same blot

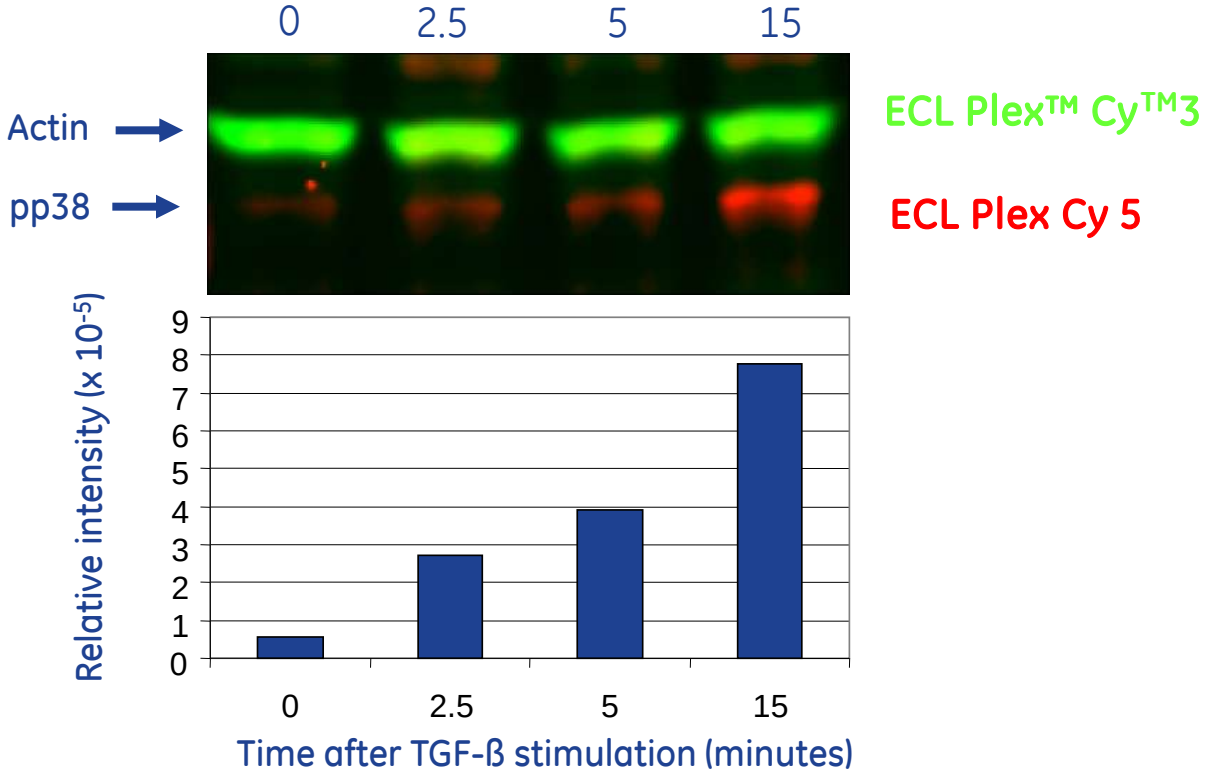
Ease of use
- No need to strip and re-probe
- Saves time and protein
- Eliminates the use of film
- Signal is stable for up to 3 months.

The power of relating to a housekeeping protein



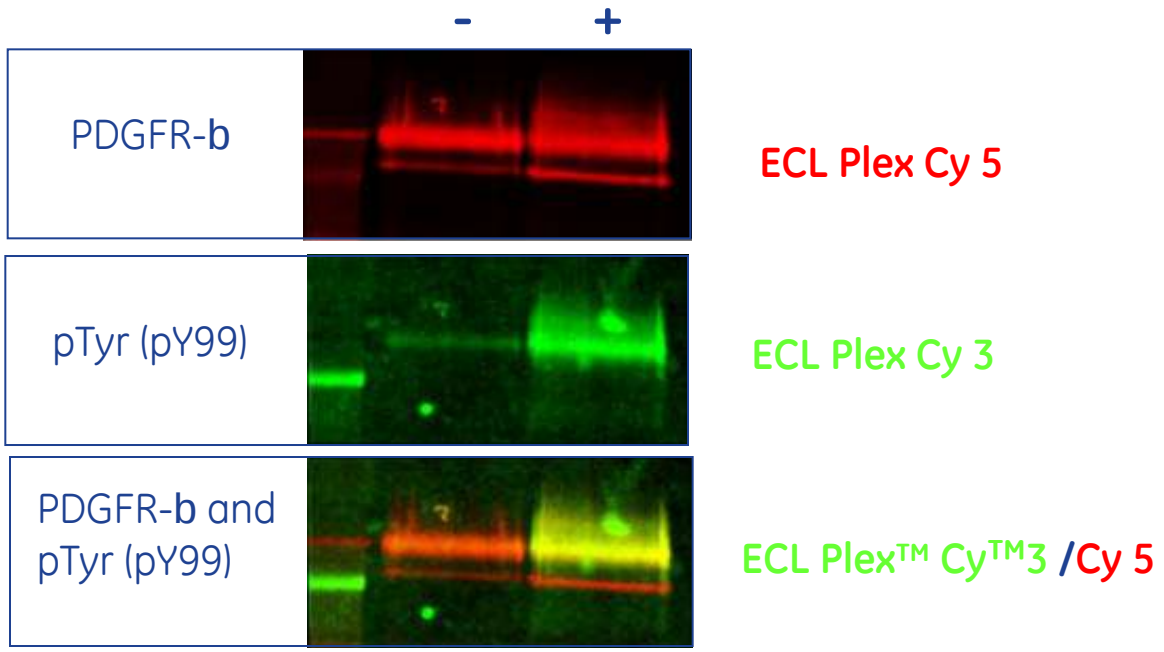
An internal standard leads to the correct biological conclusion

Detection of a low abundance phospho protein



Changes in phosphoprotein levels can be measured reliably

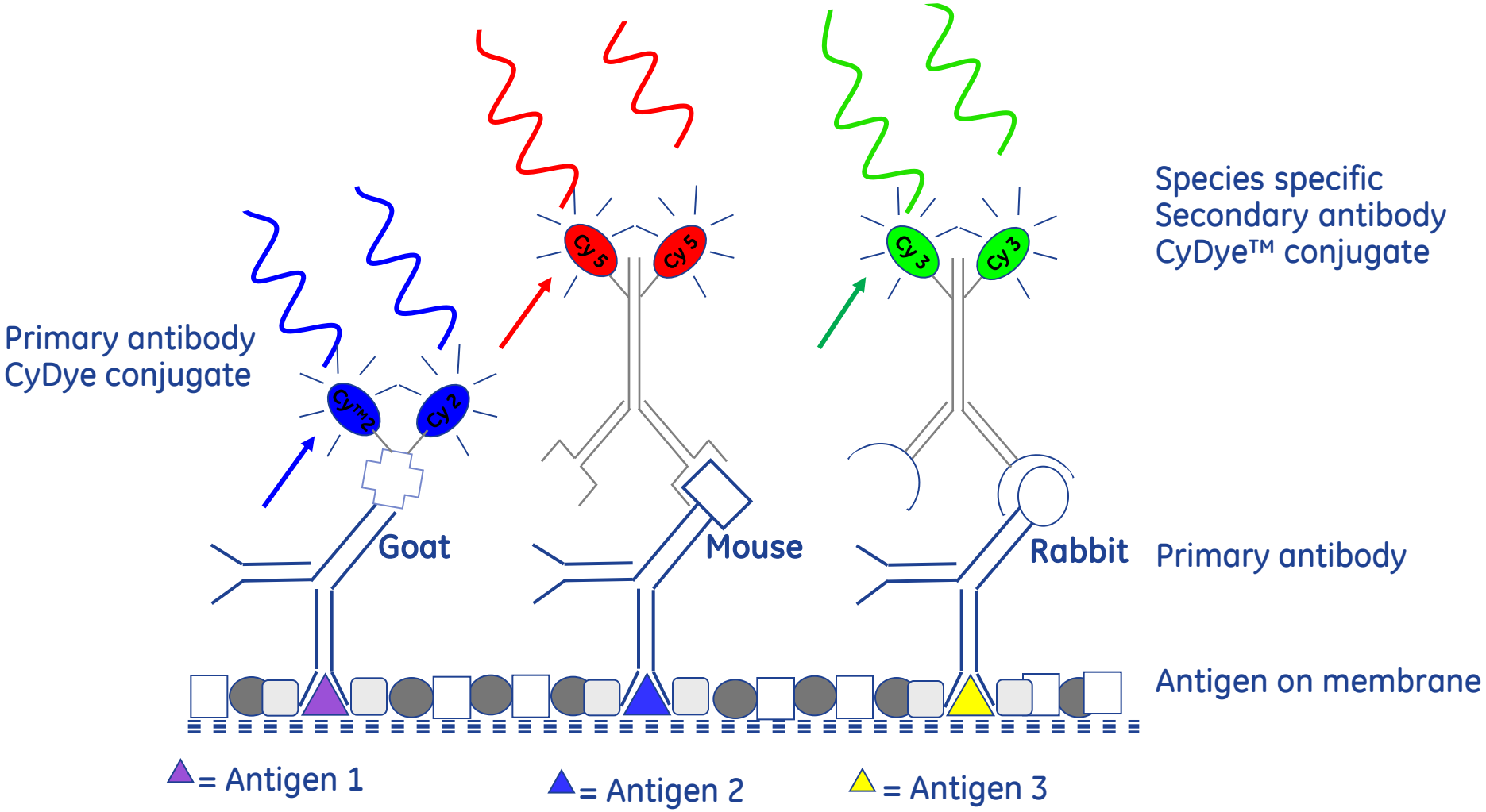
Detection of 2 targets of similar Mw



Data courtesy of Dr Johan Lennartsson,
Ludwig Institute for Cancer Research, Uppsala, Sweden

Detect targets of the same Mw without stripping and reprobing

New approach- triplex



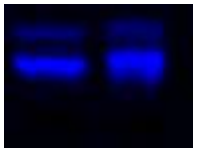
Triplex fluorescent Western blotting

HeLa cell lysate (5 mg)

C = Control (untreated)
T = Treated (UV Irradiated)

C T

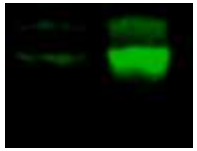
ERK



ECL Plex™ Cy™ 2

C T

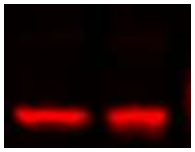
pERK



ECL Plex Cy 3

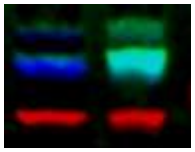
C T

GAPDH



Cy 5 labelled primary antibody

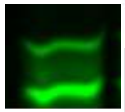
C T



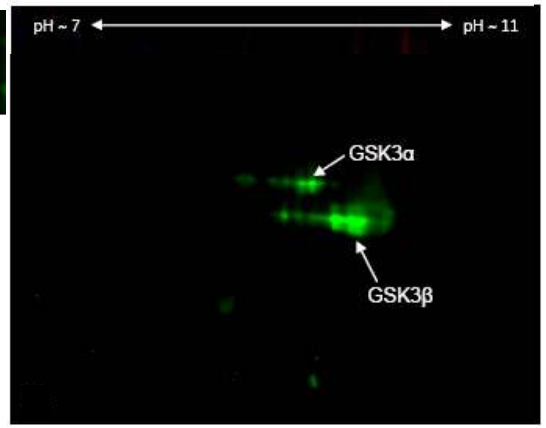
Overlay
Cy 2/Cy 3/Cy 5

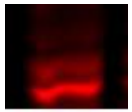
Quantitate targets of the same Mw as well as a standard

Multiplex 2-D Western blotting

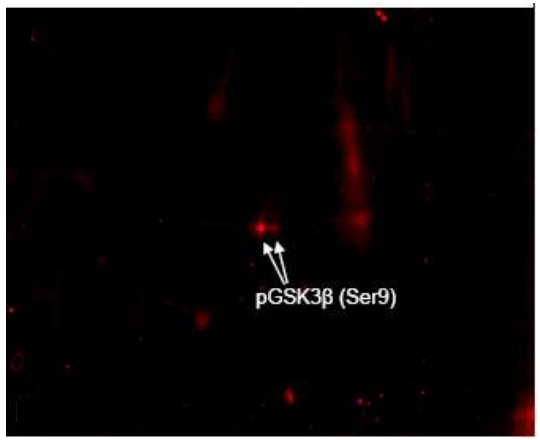


ECL Plex™ Cy™ 3
GSK3b

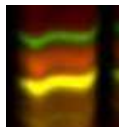




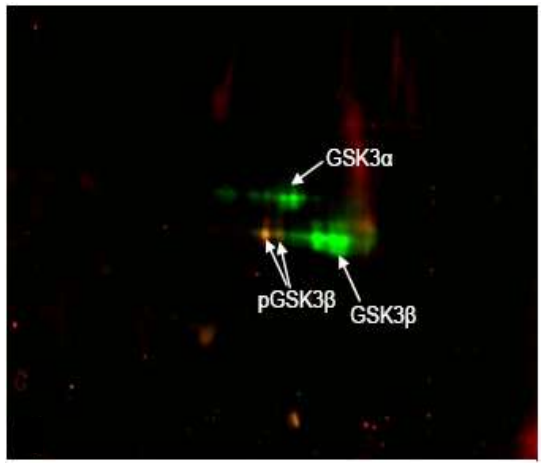
ECL Plex Cy 5
pGSK3b



Study PTMs at high resolution, e.g. phosphoprotein isoforms



Cy 3/Cy 5
overlay



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6. Summary Quantitative fluorescent Western blotting

- Broadest linear dynamic range and sensitivity
- Multiplexing up to 3 targets
- Reliably quantify 2 targets of same Mw
  -**NO** strip and re-probe needed
- Stable signals give reproducible results
  -Comparison of results, ease of use

Thank You for your attention!
Questions?

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Legal statement

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