Center for Applied Proteomics and Molecular Medicine

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GEORGE MASON UNIVERSITY

INOVA HEALTH SYSTEM

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GMU-US-ITALY Agreement

Istituto Superiore di Sanità – Rome

Enrico Garaci
Ruggero DeMaria
Claudio Belluco

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- **IST Genova**
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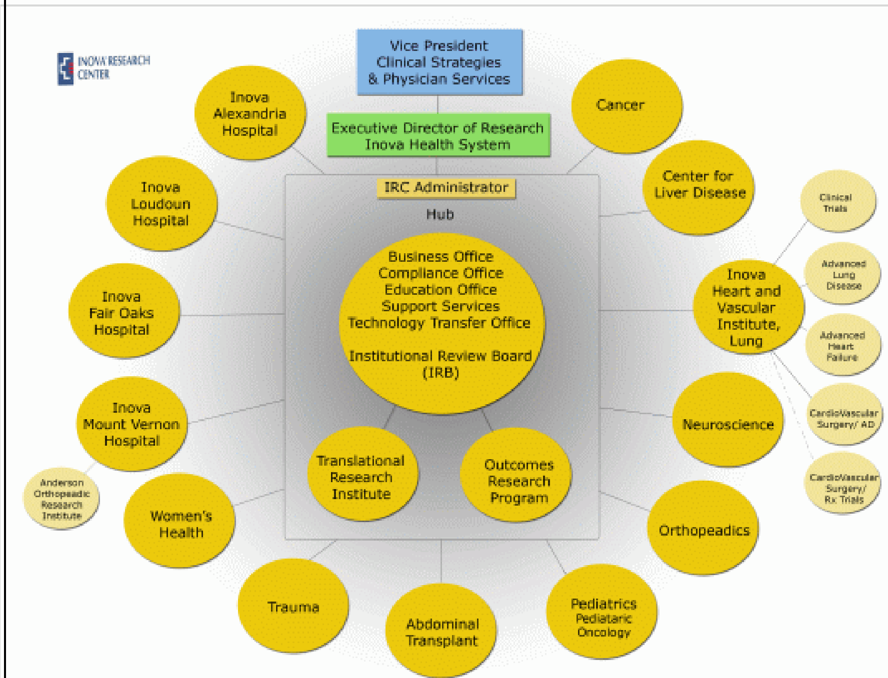
INOVA HEALTH SYSTEM

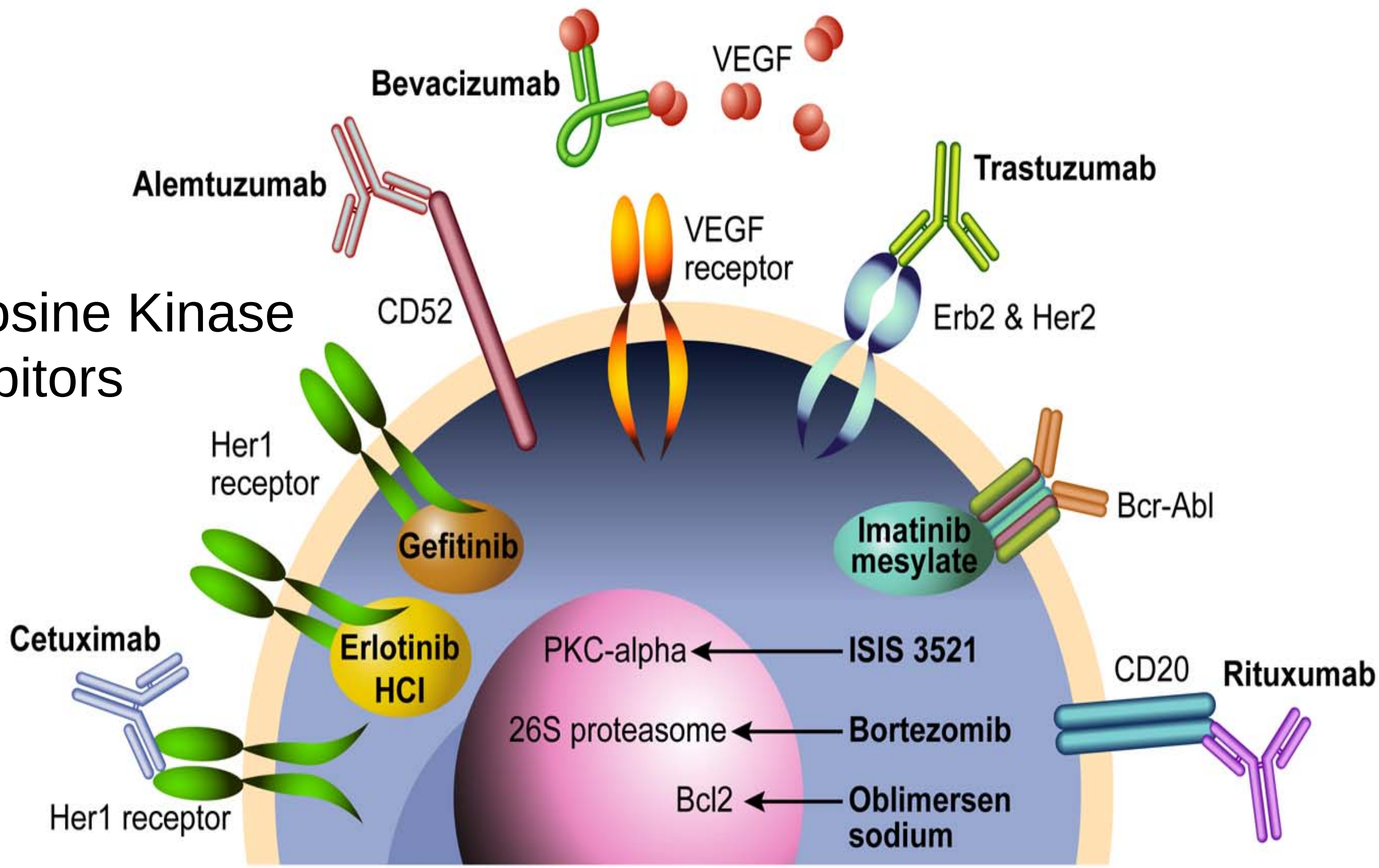
Kirsten Edmiston
Zobair Younossi
Niv Ad



New Class of Tissue Biomarkers: Phosphorylated Proteins

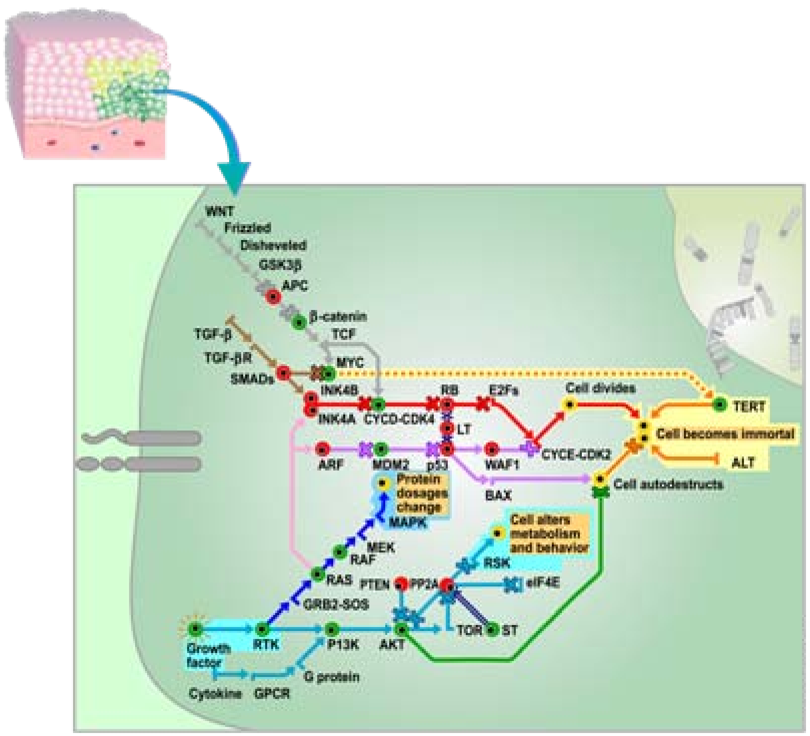
Protein Drug Target Activity Predictors For Molecular Targeted Inhibitors

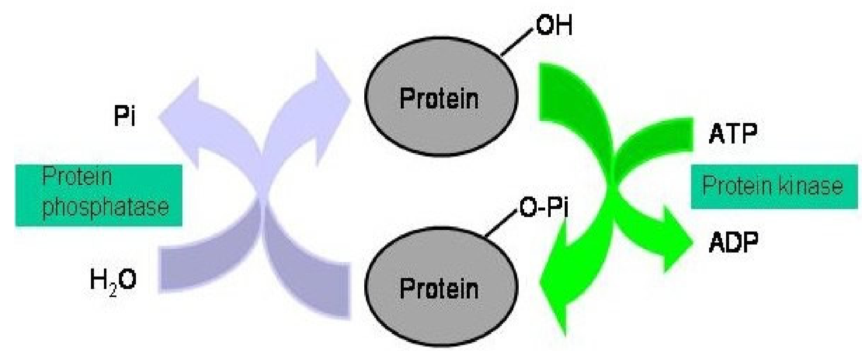
Tyrosine Kinase Inhibitors



Challenge : How do you measure the state of the fluctuating activity of ongoing signal pathways and cellular circuits in lysed tissue cells?

Solution: The state of signal pathways minute to minute is recorded in the phosphorylated or activated state of protein nodes in the signal pathway.





Measure the phosphorylated or activated state of a kinase, a kinase partner, or a pathway

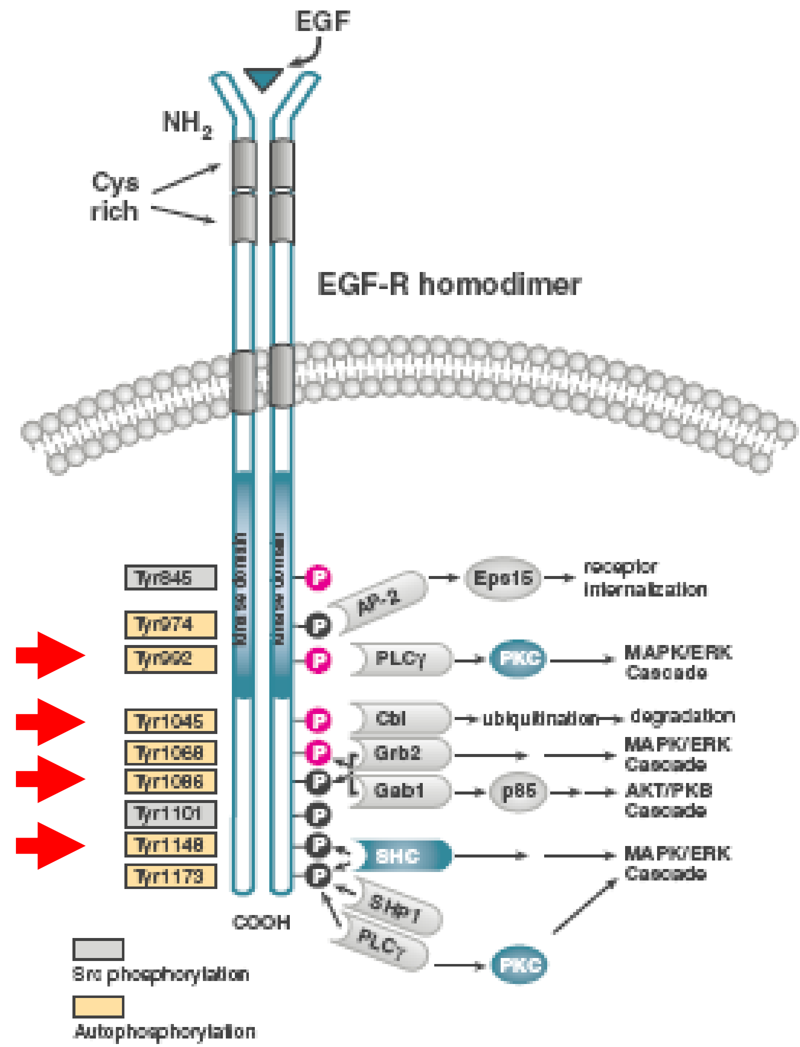
Molecular profiling of tissue proteins: Critical value of phospho-proteins and PTMs

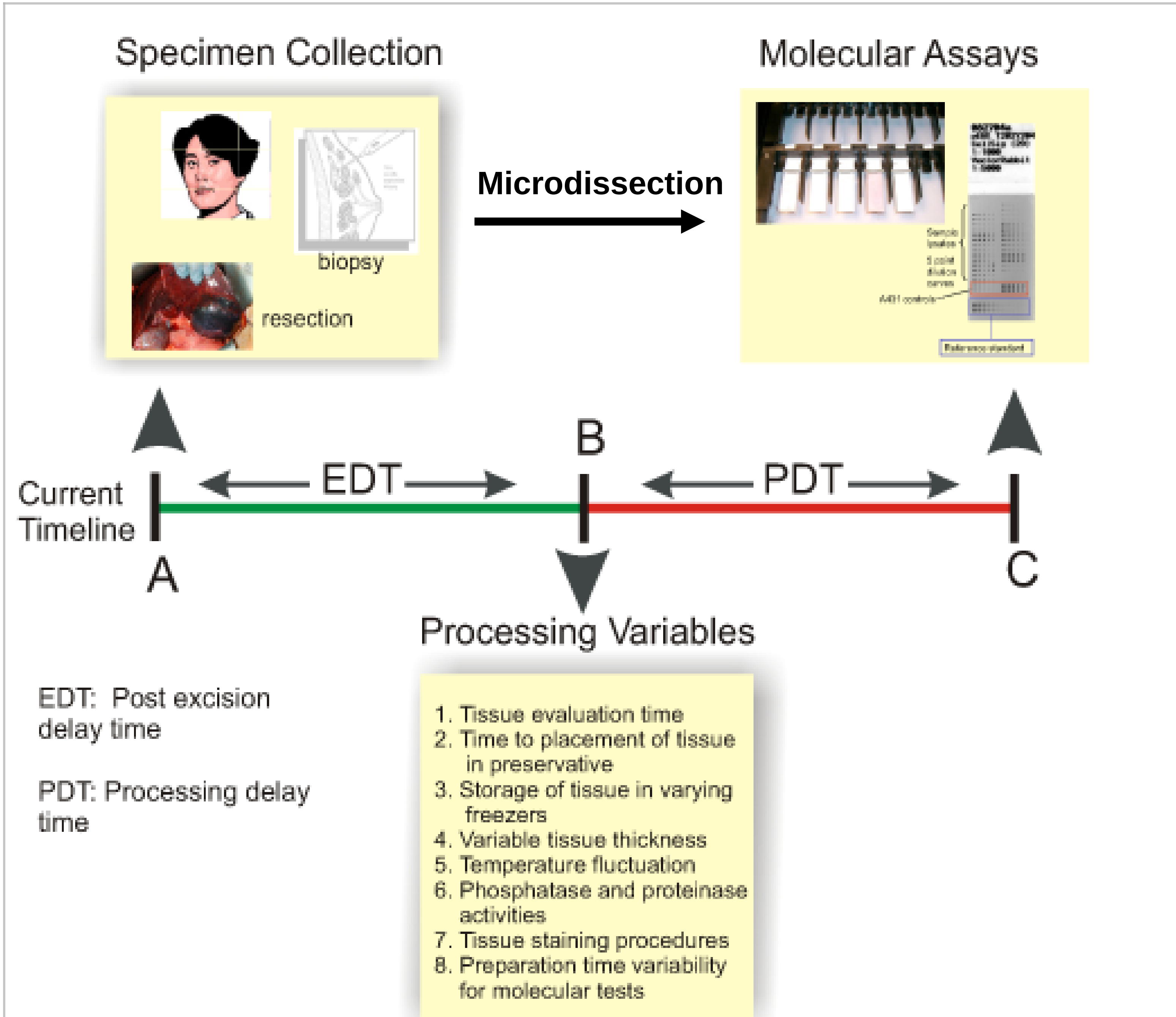
- **Phospho-proteins provide a record of ongoing kinase and kinase substrate activity in key signaling pathways driving pathologic states**

- **Post translational modifications (PTMs) in proteins reflect the state of actual drug targets. This information can not be determined using gene arrays or SNPs**

- **Hundreds of validated antibodies recognize the specific phosphorylated protein residue**

- **Highly sensitive protein array technology for multiplex mapping of tissue and cellular phosphoprotein signal pathways**




Specimen Collection
biopsy
resection
Microdissection
Molecular Assays
B
EDT
PDT
Current
Timeline
A
C
Processing Variables
EDT: Post excision
delay time
PDT: Processing delay
time
1. Tissue evaluation time
2. Time to placement of tissue
in preservative
3. Storage of tissue in varying
freezers
4. Variable tissue thickness
5. Temperature fluctuation
6. Phosphatase and proteinase
activities
7. Tissue staining procedures
8. Preparation time variability
for molecular tests

Tissue handling and morphologic preservation: requirements for medical compliance

- **Pathologic Diagnosis**
  - **Immediate frozen section diagnosis at the time of surgery is used to base surgical decisions and to limit margins of resection. This is limited by poor histology and morphology**
  - **Cutting board examination by the pathologist. This is required for orientation of the specimen, sampling of margins, and procurement of representative sections. This induces further time delays compromising molecular preservation.**
  - **Formalin fixation paraffin embedding permanent slides are used to make the legal diagnosis for the final report. This takes 2-4 days depending on the size of the specimen.**
  - **Tissue procurement for Research /DX may remove a region of tissue essential for diagnosis thus incurring legal liability**
  - **Any new product for tissue handling must support all of the above requirements and be seamlessly integrated into the clinical work flow.**

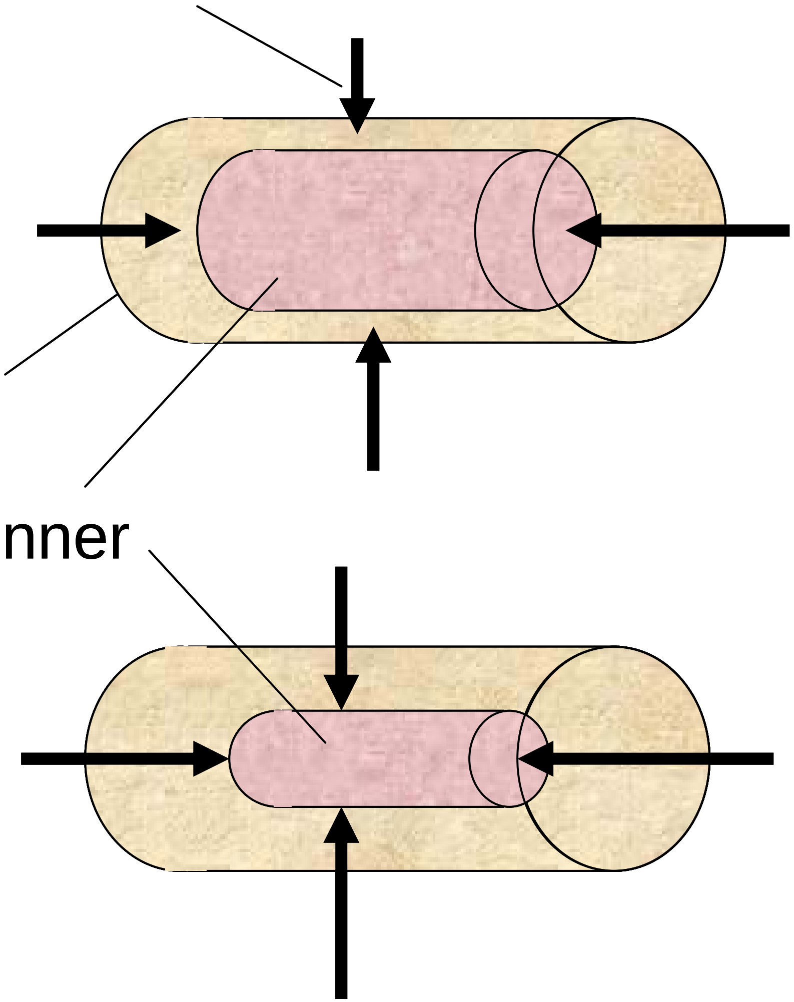
Tissue preservation: deficiencies of conventional practice

- **Formalin fixation**
  - **Formalin fixation /paraffin embedding is 100 years old.**
  - **Cross-linking by formalin masks epitopes and reduces the yield of proteins and RNA from tissues by 10 to 100 fold.**
  - **Slow penetration of tissue by formalin: 0.1cm per hour. The center of the tissue is alive reacting and changing prior to the formalin reaching the internal cells.**
  - **Formalin is a carcinogen.**

- **Direct Freezing**
  - **Liquid Nitrogen (<-75^{o}C) required for long term stabilization.**
  - **Freezing severely compromises histology for accurate pathologic diagnosis and microdissection.**
  - **Unacceptable for routine clinical use in O.R. or Clinic.**
  - **Costly and prone to thawing for shipping of samples.**

Formalin penetration
through surface

Tissue Core
needle biopsy



A. Early Time
3 hours
Formalin
Penetration
Less than 2mm.

Unfixed inner
tissue

B. Late Time
12 hours
Unfixed inner
tissue is still
present

Critical pre-analytical issues for tissue collection

Objectives:

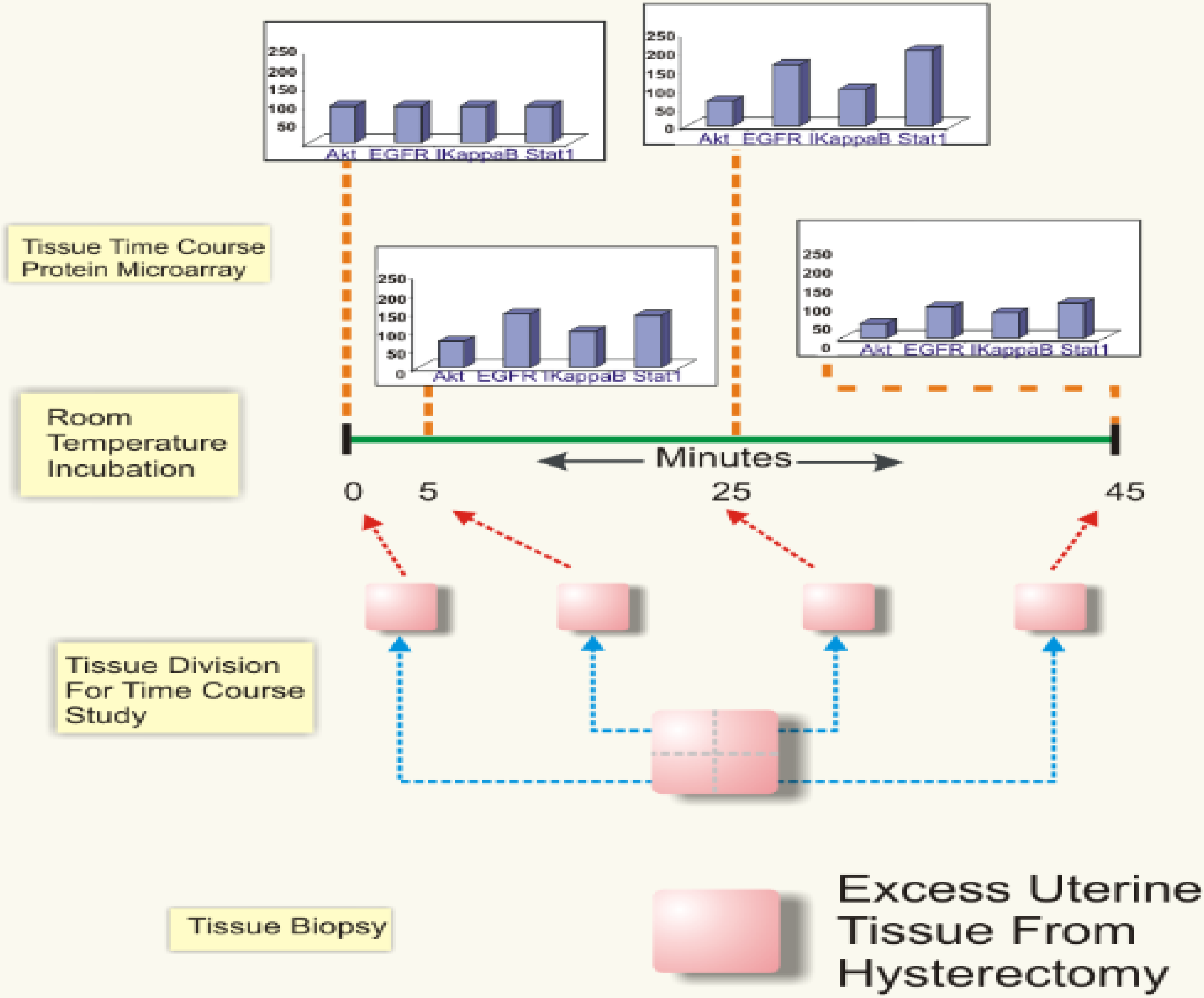
1. Integrate tissue collection with standard of care in real-world, community setting
2. Determine real-world time to acquire tissue
3. Analyze effects of storage temperature – room temp or $4^{o}C$
4. Discover fluctuations in cell signaling over time – use labile endpoints as surrogate markers of stability
5. Compare stability profile of various tissues

Goals:

1. Develop <u>quantitative</u> data for determining rational, data driven basis for collection protocols.
    - Surrogate markers to guide preservation chemistry of the future
    - QC for preservation methods
2. Develop parameters for preservation technology of the future
    - Seamlessly integrated in clinical practice
    - Sentinels/additives to monitor tissue QA/QC process

Post Excision Delay Time Study

Reverse Phase Microarrays Demonstrate Alterations in Signaling Protein Levels At Different Time Points Post-Extraction of Tissue



Tissue Collection Summary

Breast, Colon, Lung, Uterus, Ovary

Average time to cryopreservation: 18.8 minutes

Earliest time: 4 minutes

Longest time: 40 minutes

Mode: 10 minutes

Virginia Espina

50 Protein Endpoints for Stability Studies

Protein/Sub-cellular Location		Protein/Sub-cellular Location	
Cytoplasm	**Function**	**Cytoplasm**	**Function**
4EBP1 (Ser65)	Proliferation/Survival	PDK1 (Ser241)	Proliferation/Survival
Acetyl CoA Carboxylase (Ser79)	Hypoxia/Ischemia	PKC alpha/beta II (T638/641)	Proliferation/Survival
Akt (Ser473)	Proliferation/Survival	SAPK/JNK (Thr183/Y185)	Stress/Inflammation
Akt (Thr308)	Proliferation/Survival	SEK1/MKK4 (Ser80)	Stress/Inflammation
AMPKalpha1 (Ser485)	Hypoxia/Ischemia	Src Family (Y416)	Stress/Inflammation
AMPKBeta1 (Ser108)	Hypoxia/Ischemia	STAT1 (Y701)	Transcription Factor
ASK1 (Ser83)	Stress/Inflammation	STAT3 (Ser727)	Transcription Factor
c-Abl (Thr735)	Proliferation/Survival	STAT3 (Y705)	Transcription Factor
Bad (Ser112)	Apoptosis	Tuberin/TSC2 (Y1571)	Proliferation/Survival
Caspase-3, cleaved (Asp175)	Apoptosis	**Membrane**	**Function**
Caspase-7, cleaved (Asp198)	Apoptosis	Adducin (Ser662)	Adhesion/Cytoskeletal
Caspase-9, cleaved (Asp330)	Apoptosis	Annexin II	Adhesion/Cytoskeletal
E-cadherin	Adhesion/Cytoskeletal	EGFR	Proliferation/Survival
eIF4G (Ser1108)	Proliferation/Survival	EGFR (Y1148)	Proliferation/Survival
eNOS (Ser1177)	Hypoxia/Ischemia	ErbB2	Proliferation/Survival
ERK 1/2 (Thr202/Y204)	Proliferation/Survival	ErbB2/HER2 (Y1248)	Proliferation/Survival
FAK (Y576/577)	Adhesion/Cytoskeletal	Her3 Y1289	Proliferation/Survival
GSK3alpha/beta (Ser21/9)	Proliferation/Survival	VEGF Receptor2 (Y1175)	Hypoxia/Ischemia/Angiogenesis
HIF-1 alpha	Hypoxia/Ischemia	VEGFR 2 (Y951)	Hypoxia/Ischemia/Angiogenesis
HSP 90	Hypoxia/Ischemia	**Nucleus**	**Function**
IkappaB-alpha (Ser32/36)	Stress/Inflammation	ATF-2 (Thr71)	Stress/Inflammation
IRS-1 (Ser612)	Proliferation/Survival	Beta Catenin (Ser33/37/Thr41)	Adhesion/Cytoskeletal
Jak1 (Y1022/1023)	Stress/Inflammation	Chk1 (Ser345)	Cell Cycle
MARCKS (Ser152/156)	Stress/Inflammation	CREB (Ser133)	Transcription Factor
mTOR (Ser2481)	Proliferation/Survival	Cyclin A	Cell Cycle
NF-kappaB p65 (Ser536)	Stress/Inflammation	**Mitochondria**	**Function**
p38 MAPK (Thr180/Y182)	Hypoxia/Ischemia	Bcl-2 (ser70)	Apoptosis
PAK1 (Ser199/204)/PAK2 (Ser192/197)	Adhesion/Cytoskeletal	BAX	Apoptosis

Antibody Validation: **Phosphospecific: 202** **Total protein: 60**

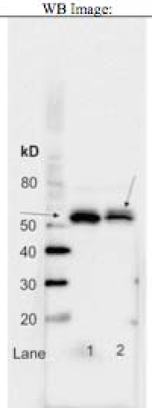
Formalized Process for Antibody acceptance and reporting

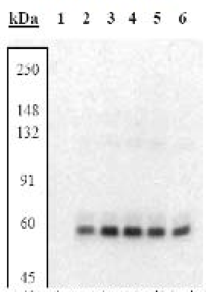
Form Updated on 12/13/05

Antibody Validation Form

Email to Valerie Calvert vcalvert@gmu.edu

Validated by: Julie Wulfkuhle	**Date: 8/16/2006**
PRIMARY ANTIBODY	
Antibody Name:	Phospho-Lck Y505
Company:	Biosource
Catalog # / Lot #:	#44-850G Lot #: 0201
MW:	56kD
Species:	Rabbit
Dilution:	1:1000
SECONDARY ANTIBODY	
Secondary Ab Name:	Goat anti-Rabbit IgG (H+L) HRP-conjugated
Company:	Zymed
Catalog # / Lot #:	#81-6120/ Lot #: 50800159
Dilution:	1:10,000
CONTROL CELL LYSATE	
Control Lysate Name:	Lane 1: Jurkat control; Lane 2: Jurkat +H2O2
Tissue Type:	T-cell leukemia cell line
Company:	Santa Cruz Biotechnology
Catalog # / Lot #:	Jurkat control: #sc-2204 Lot #: E1906; Jurkat + H2O2: #sc-24714 Lot #: C0504
Amount loaded in gel:	20ug each per lane
Other Information:	
WESTERN BLOT	
Type of Development: (i.e. Chemiluminescence, Flourescence, Colorimetric, Radiactive Labeling)	Chemiluminescence
Membrane: (New or Stripped)	New
Best Exposure Time:	6.5 min
WB Image:	Company's WB Image:





Full length untagged recombinant human Lck protein added to background cell extracts were resolved by SDS-PAGE on a 10% Tris-glycine gel and transferred to nitrocellulose. The membrane was blocked with a 5% BSA-TBST buffer for one hour at room temperature and then incubated with the Lck [pY505] antibody in a 1% BSA-TBST buffer for two hours at room temperature, following prior incubation with: the phosphopeptide immunogen (1), the non-phosphopeptide corresponding to the phosphopeptide immunogen (2), the non-phosphopeptide derived from the corresponding region of Src (3), a generic phosphotyrosine-containing peptide (4), no peptide (5) or the phosphopeptide derived from the corresponding region of Src (6). After washing, the membrane was

NOTES / COMMENTS:

Band present at appropriate molecular weight in treated cells.

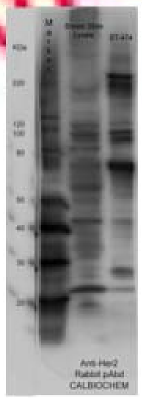
Form Updated on 12/13/05

Antibody NOT Validated Form

Email to *Valerie Calvert* vcalvert@gmu.edu

NOT VALIDATED

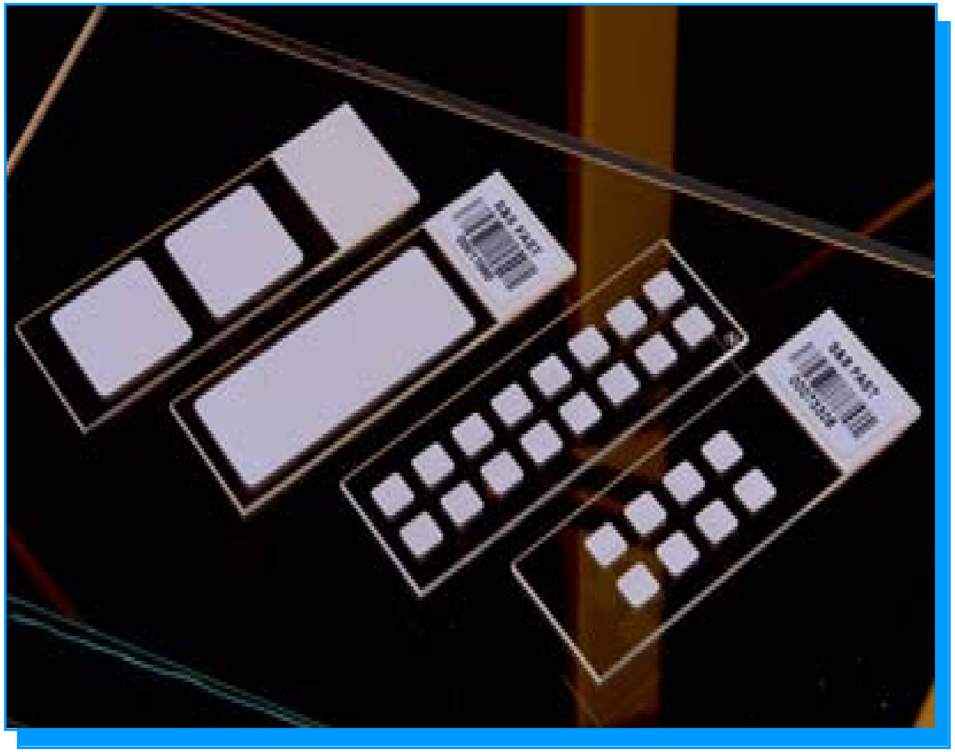
Experiment by: Michele Signore	**Date:** 03.23.07
PRIMARY ANTIBODY	
Antibody Name:	ErbB2
Company:	CALBIOCHEM
Catalog # / Lot #:	PC04/ Lot D35185
MW:	185 kDa
Species:	Rabbit policlonal Abd
Dilution:	1:20 (=5 ug/ml)
SECONDARY ANTIBODY	
Secondary Ab Name:	Goat Anti-rabbit IgG HRP conjugate
Company:	Zymed
Catalog # / Lot #:	816120 / 60706513
Dilution:	1/10,000
CONTROL CELL LYSATE	
Control Lysate Name:	BT-474, breast carcinoma slide lysate
Tissue Type:	Human breast cancer
Company:	Home Brew Lysates
Catalog # / Lot #:	7-10-'06 (BT-474)/ Breast slide lysate from G.E.
Amount loaded in gel:	20ul
Other Information:	
WESTERN BLOT	
Type of Development: (i.e. Chemiluminescence, Flourescence, Colorimetric, Radiactive Labeling)	Chemiluminescence (ECL) Pierce
Membrane: (New/Stripped)	New, PVDF
Best Exposure Time:	20min
WB Image:	



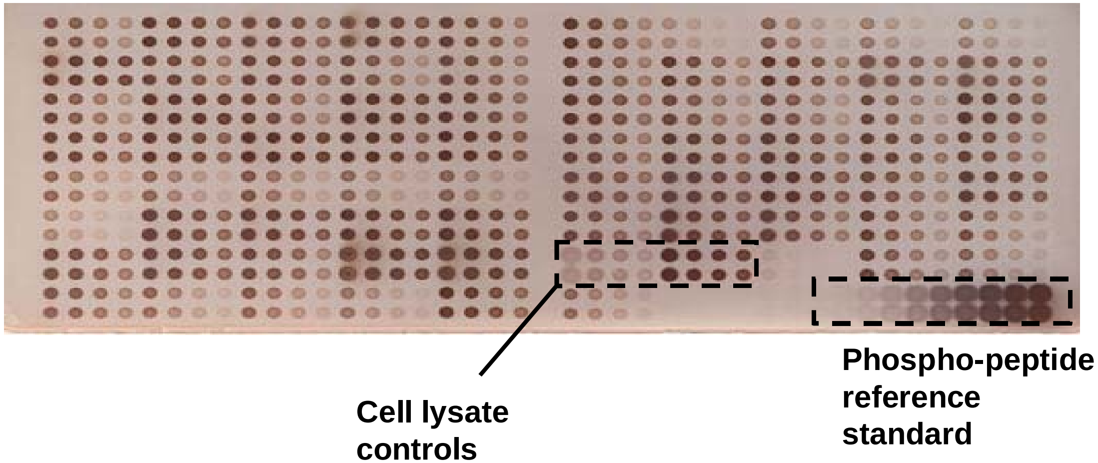
Lane 1. Magic Mark Ladder
Lane 2. BT-474
Lane 3. Breast Slide Lysate

NOTES / COMMENTS:

Aushon BioSystems 2470 arrayer



Whatman FAST Slides



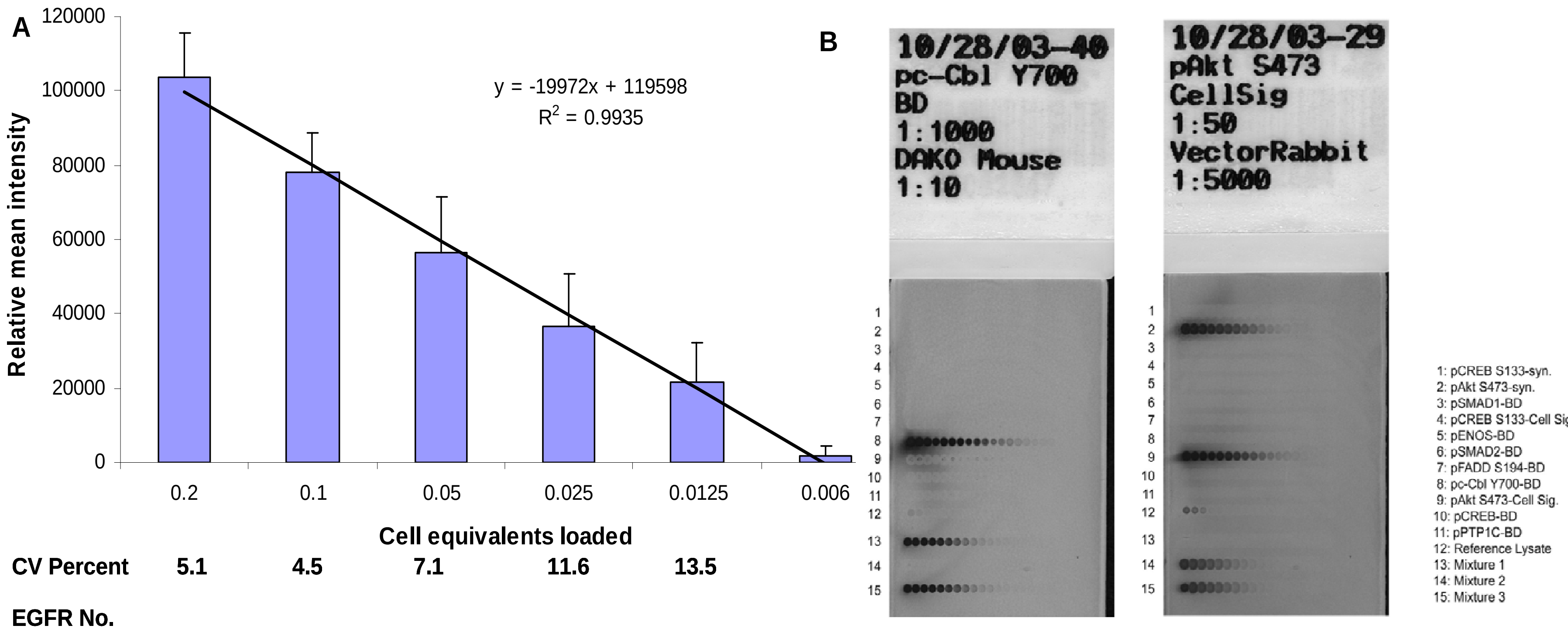
Multiple samples/array

One antibody probe/array

Reverse Phase Protein Microarrays

- Phosphorylated/Activated state of signal proteins
- One antibody per analyte
- High Sensitivity femtomole range
- FNA/core needle Microdissection
- Built-in positive controls and calibrators
- High precision and linearity CAP/CLIA Lab

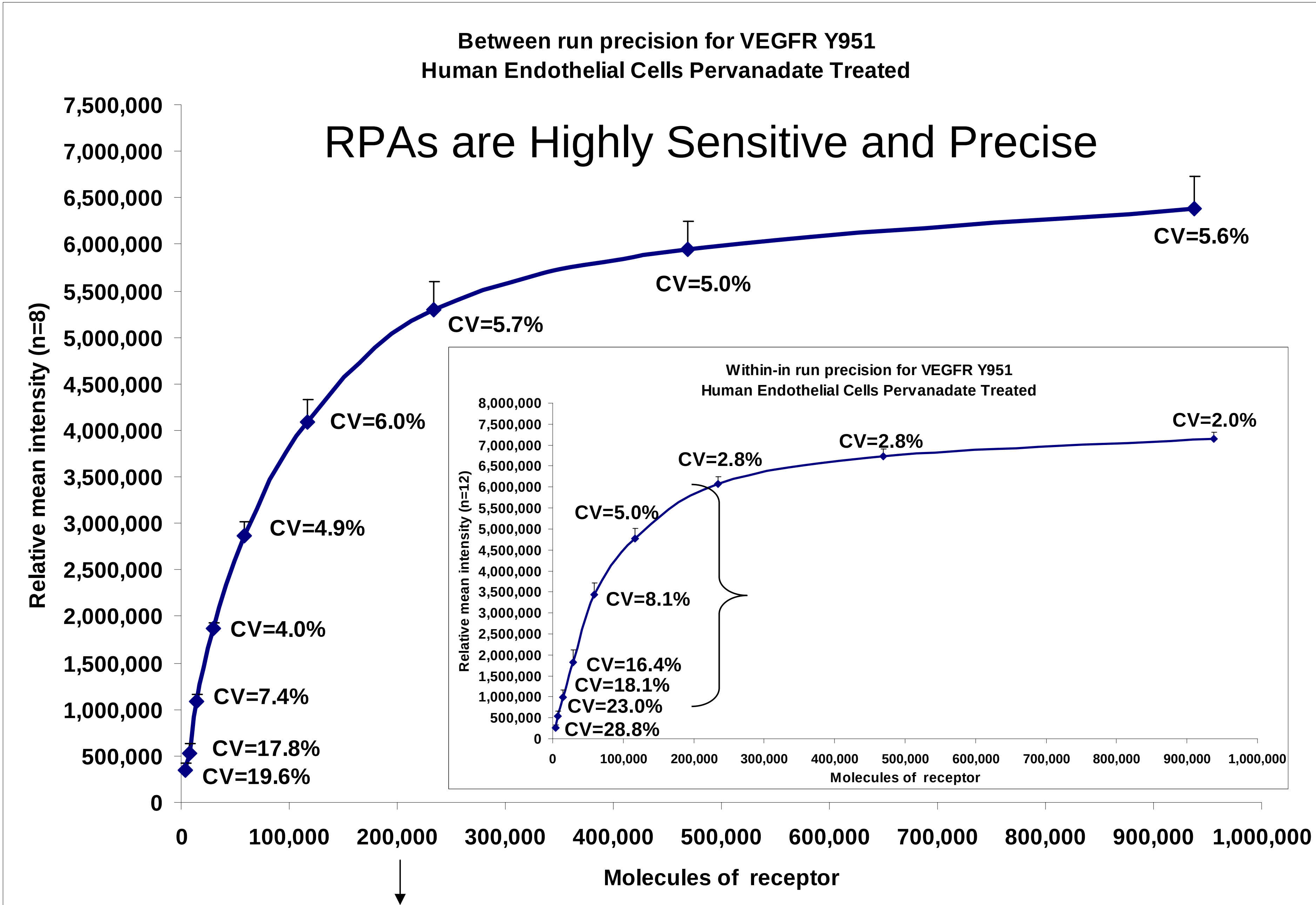
200 validated phos-analytes



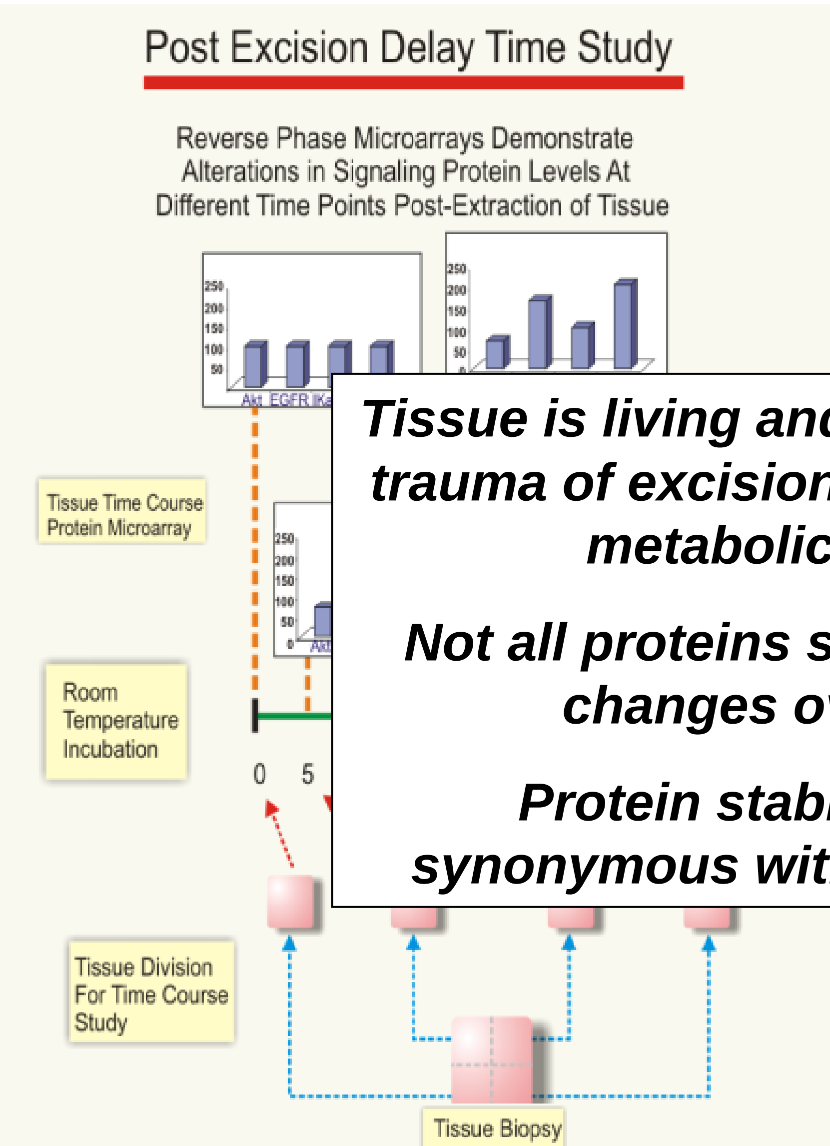
CV Percent	**5.1**	**4.5**	**7.1**	**11.6**	**13.5**
EGFR No. **25,000 receptors/cell**	**5000**	**2500**	**1250**	**750**	**375**

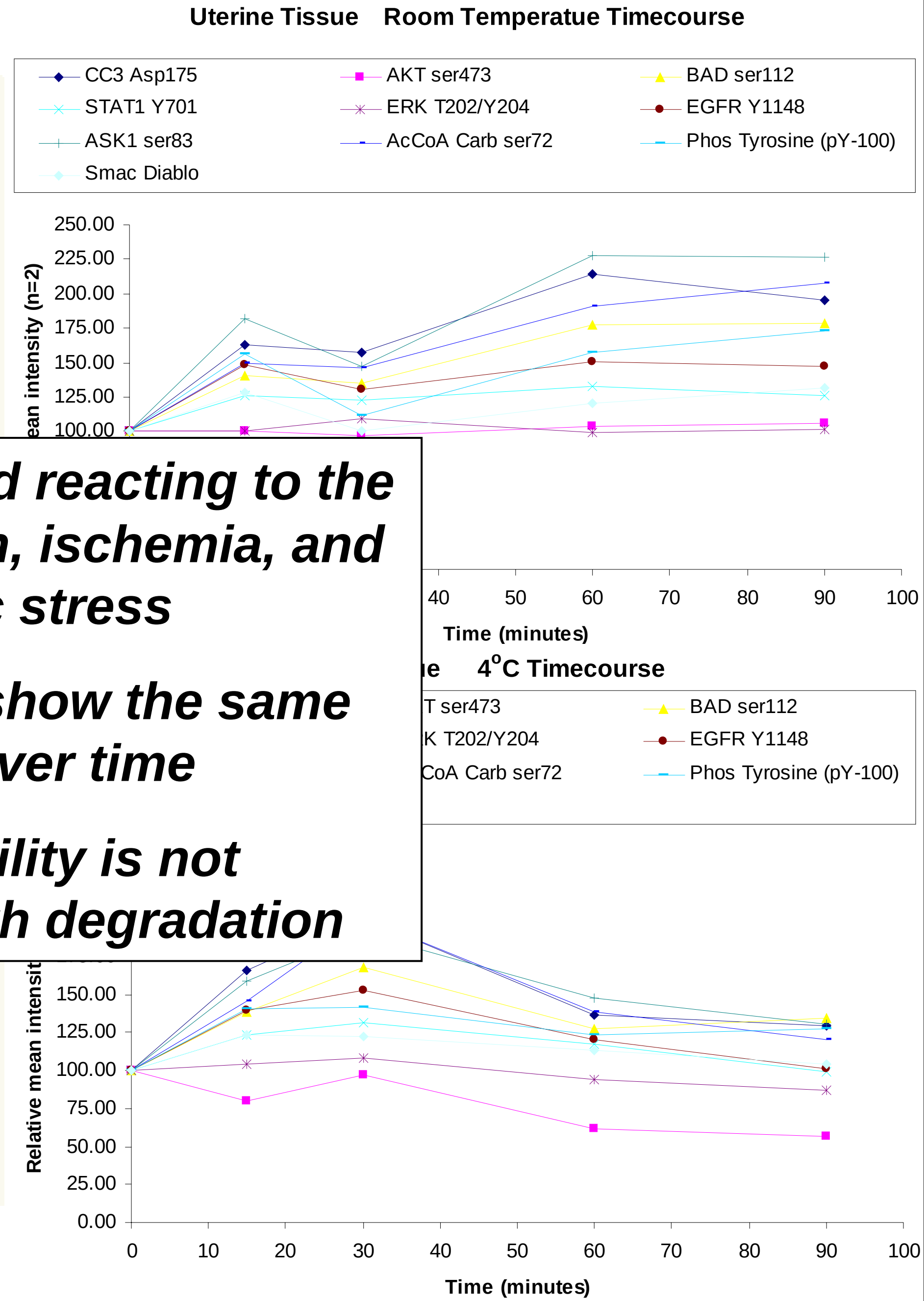
Internal dilution curve assures high dynamic range

RPAs are Highly Sensitive and Precise



20 cell equivalents



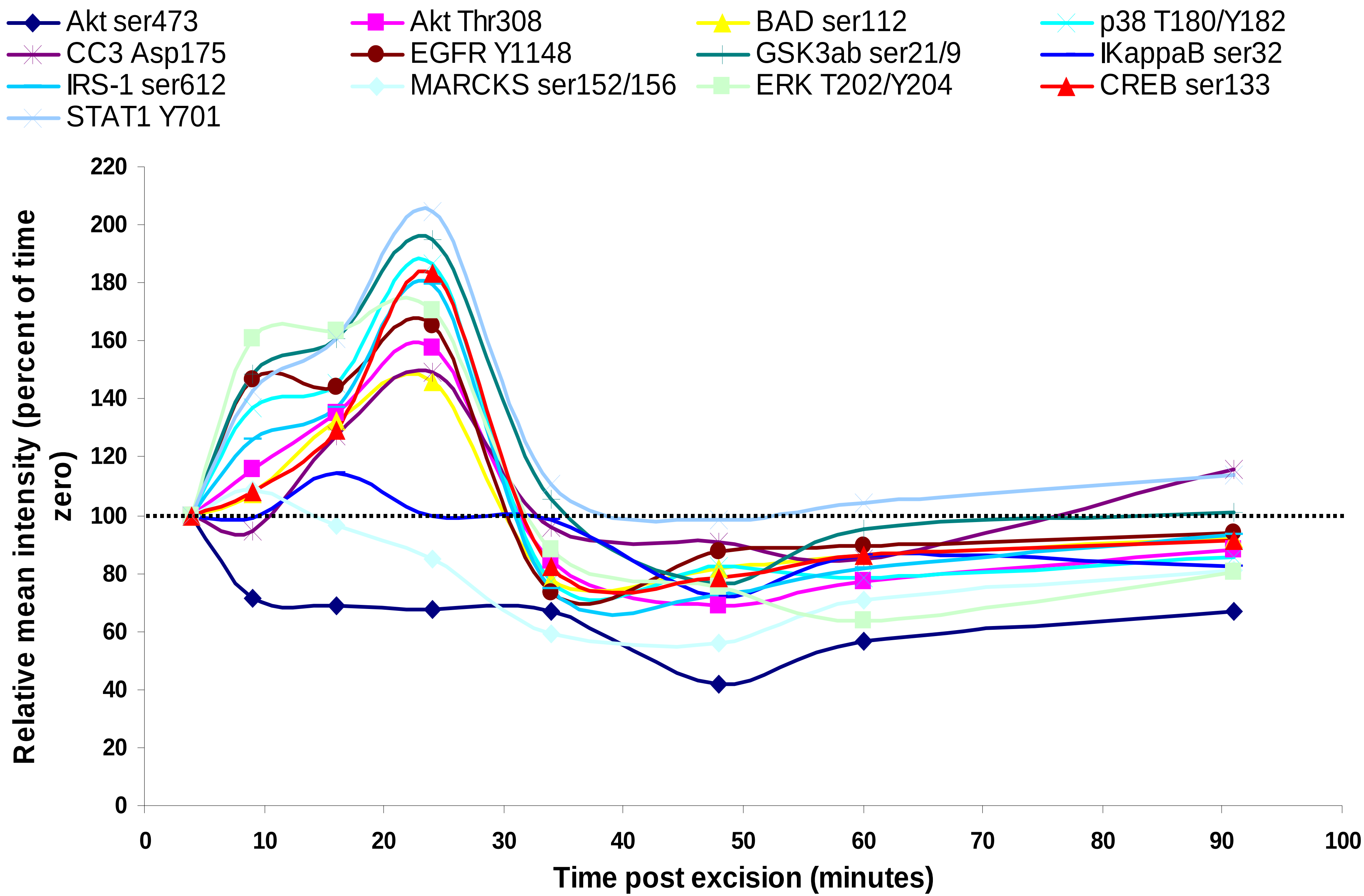


Tissue is living and reacting to the trauma of excision, ischemia, and metabolic stress

Not all proteins show the same changes over time

Protein stability is not synonymous with degradation

Uterus: Room temperature stability timecourse



Reactive Elevation of Signal Pathway Phosphoproteins at RT

		Trial #1 Uterus, benign	Trial #2 Colon, normal	Trial #3 Lung Parenchyma	Trial #4 Lung Squamous Cell Carcinoma	Trial #5 Uterine Leiomyoma	Trial #7 Uterus, benign
		Timecourse range post excision (minutes)					
		4 - 91	40 - 70	10 - 20	10 - 20	10 - 190	30 - 120
Protein	**Function**	Significant changes over time					
Bad (Ser112)	Apoptosis	UE	*	*	*	*	*
Caspase-3, cleaved (Asp175)	Apoptosis	UE	UE/DL	DE	UM	UE	UE
Caspase-7, cleaved (Asp198)	Apoptosis					UE/DL	
Caspase-9, cleaved (Asp330)	Apoptosis					DL	UE
Chk1 (Ser345)	Cell Cycle					UM	
Cyclin A	Cell Cycle					UM	
Acetyl CoA Carboxylase (Ser79)	Hypoxia/Ischemia					*	*
eNOS (Ser1177)	Hypoxia/Ischemia					UE	
HIF-1 alpha	Hypoxia/Ischemia						DE/UL
p38 MAPK (Thr180/Y182)	Hypoxia/Ischemia	UE	DE	UE	UE	*	DE
VEGFR 2 (Y951)	Hypoxia/Ischemia					DL	
Akt (Ser473)	Proliferation/Survival	DE	DE	DE	DE	UE	DE
Akt (Thr308)	Proliferation/Survival	UE	DE	*	UE	UE	*
EGFR (Y1148)	Proliferation/Survival	UE	DE	*	UE		*
eIF4G (Ser1108)	Proliferation/Survival					UE	
ERK 1/2 (Thr202/Y204)	Proliferation/Survival	UE	DE	*	UE	UE	DE
GSK3alpha (Ser21/9)	Proliferation/Survival	UE	*	UE	UE		*
IRS-1 (Ser612)	Proliferation/Survival	UE	DE	DE	UE	UE	*
PDK1 (Ser241)	Proliferation/Survival					UE	
PKC alpha/beta II (Thr638/641)	Proliferation/Survival					UE	
ASK1 (Ser83)	Stress/Inflammation					UE	UE/UM
ATF-2 (Thr71)	Stress/Inflammation					*	
IkappaB-alpha (Ser32/36)	Stress/Inflammation	*	*	DE	UE	UE	DE/UL
Jak1 (Y1022/1023)	Stress/Inflammation						
MARCKS (Ser152/156)	Stress/Inflammation	DE	DE	DE	DE	DL	DE/DM
SAPK/JNK (Thr183/Y185)	Stress/Inflammation					UE	
Src Family (Y416)	Stress/Inflammation					DE	
CREB (Ser133)	Transcription factor	UE	*	*	*	UE	*
STAT1 (Y701)	Transcription factor	UE	DE	UE	UE	UE	*
STAT3 (Y705)	Transcription factor					UE	

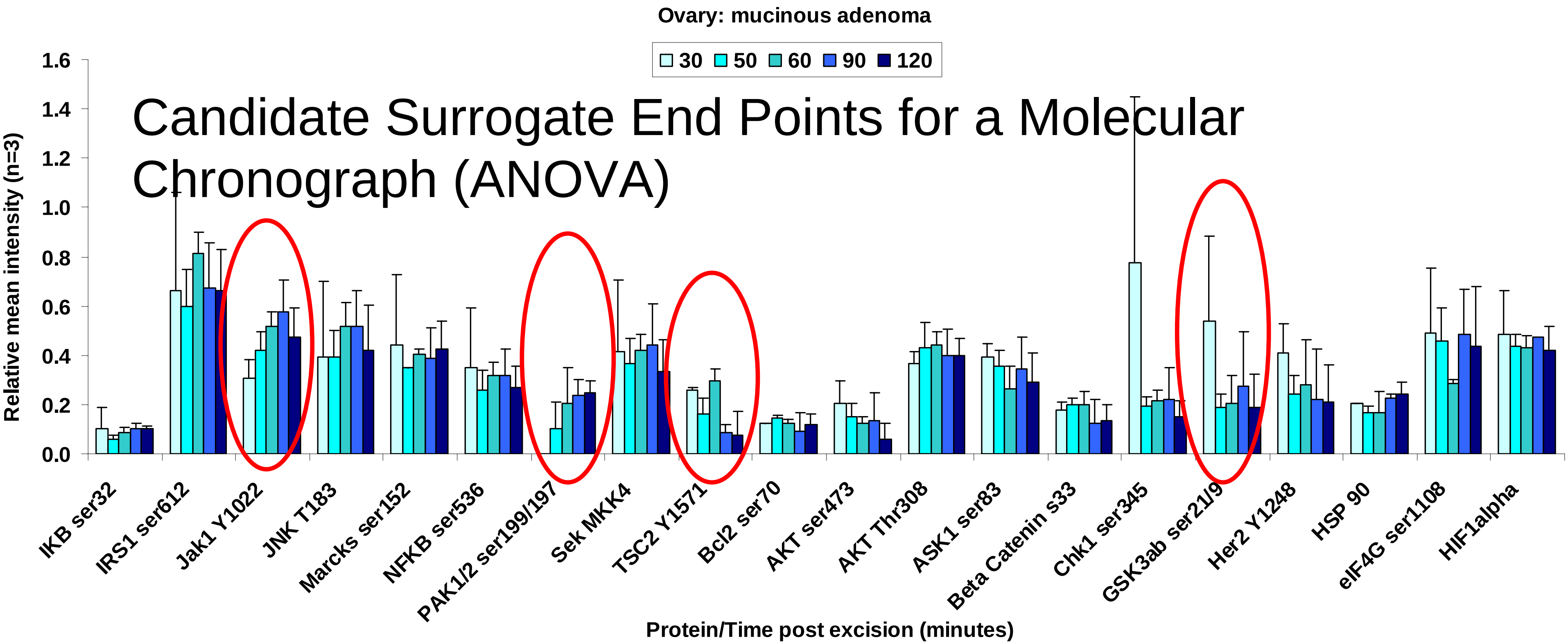
Precision analysis time course

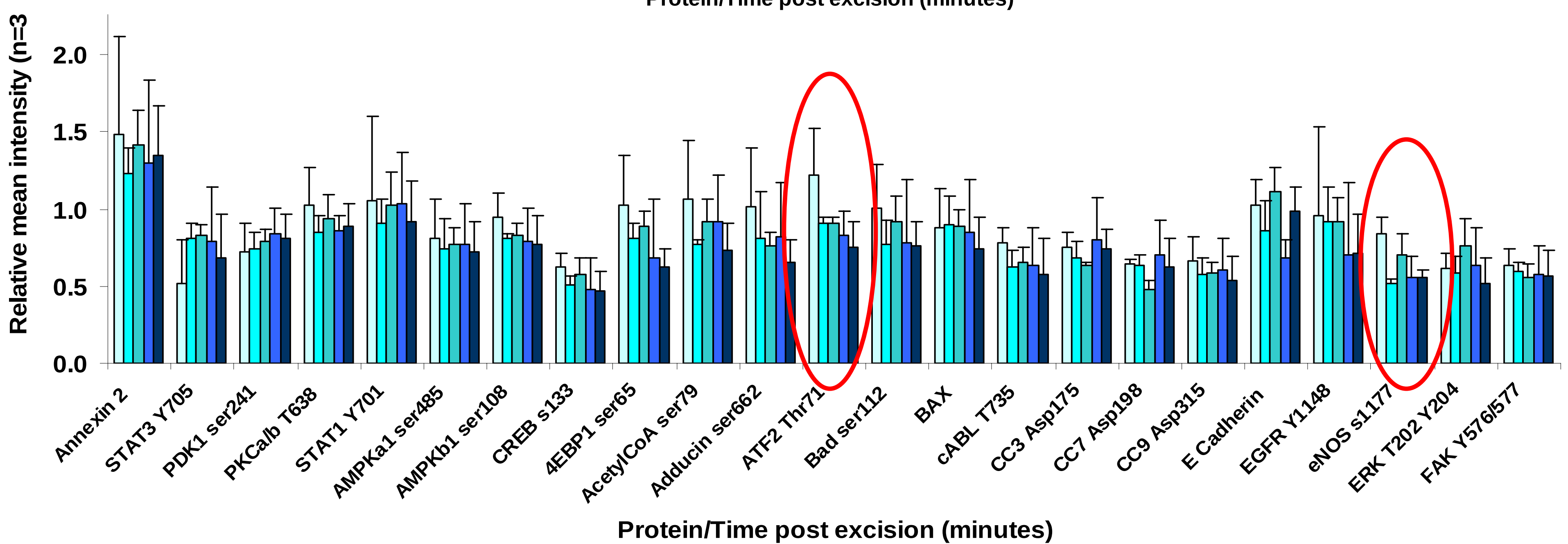
		Trial #8 Uterine Leiomyoma (n=24 pieces)						
		Time post excision (minutes, time zero=26)						
			44	60	119	146	191	949
Protein	Function	Significant changes over time	p value (n=3)	p value (n=3)	p value (n=4)	p value (n=4)	p value (n=4)	p value (n=5)
Adducin (Ser662)	Adhesion/Cytoskeleton	DE/DL		0.0013	0.0437		0.0166	0.002
Annexin II	Adhesion/Cytoskeleton	UL				0.0088	0.0408	
Catenin(beta) (Ser33/37/Thr41)	Adhesion/Cytoskeleton	UL					0.0034	0.0278
E-cadherin	Adhesion/Cytoskeleton	*	*	*	*	*	*	*
FAK (Y576/577)	Adhesion/Cytoskeleton	UM/UL				0.0366	0.0239	
PAK1 (Ser199/204)/PAK2 (Ser192/197)	Adhesion/Cytoskeleton	*	*	*	*	*	*	*
Bad (Ser112)	Apoptosis	UM				0.0071		
BAX	Apoptosis	UM/UL			0.0052	0.0009	<0.0001	0.006
Bcl-2 (Ser70)	Apoptosis	UL					0.0026	0.0359
Caspase-3, cleaved (Asp175)	Apoptosis	*	*	*	*	*	*	*
Caspase-7, cleaved (Asp198)	Apoptosis	UL						0.0029
Caspase-9, cleaved (Asp330)	Apoptosis	DE		0.0462				
Chk1 (Ser345)	Cell Cycle	UM			0.0141	0.0725		
Acetyl CoA Carboxylase (Ser79)	Hypoxia/Ischemia	UE/UM			0.0218	0.0037		
AMPKalpha1 (Ser485)	Hypoxia/Ischemia	UM/UL				0.0064	0.002	
AMPKBeta1 (Ser108)	Hypoxia/Ischemia	UM/UL			0.0212	0.0061	0.0002	0.0161
eNOS (Ser1177)	Hypoxia/Ischemia	*	*	*	*	*	*	*
HIF-1 alpha	Hypoxia/Ischemia	DE/DL	0.0143	0.0029				0.001
HSP 90	Hypoxia/Ischemia	*	*	*	*	*	*	*
4EBP1 (Ser65)	Proliferation/Survival	UL				0.0004	<0.0001	0.0001
Akt (Ser473)	Proliferation/Survival	*	*	*	*	*	*	*
Akt (Thr308)	Proliferation/Survival	UL					0.0245	
c-Abl (Thr735)	Proliferation/Survival	DE/UM/UL		0.0029		0.0103	0.0143	
EGFR (Y1148)	Proliferation/Survival	*	*	*	*	*	*	*
eIF4G (Ser1108)	Proliferation/Survival	*	*	*	*	*	*	*
ErbB2/HER2 (Y1248)	Proliferation/Survival	UL				0.0015		
ERK 1/2 (Thr202/Y204)	Proliferation/Survival	UE/UM/UL		0.0001	0.0002	<0.0001	0.0003	0.0035
GSK3alpha (Ser21/9)	Proliferation/Survival	UL				0.0131		
PDK1 (Ser241)	Proliferation/Survival	DE		0.0355				
ASK1 (Ser83)	Stress/Inflammation	UM/UL			0.0358	0.0016	0.0044	
ATF-2 (Thr71)	Stress/Inflammation	*	*	*	*	*	*	*
SAPK/JNK (Thr183/Y185)	Stress/Inflammation	UE/UM/UL		0.0437	0.0025	<0.0001	<0.0001	0.0004
SEK1/MKK4 (Ser80)	Stress/Inflammation	*	*	*	*	*	*	*
STAT1 (Y701)	Transcription factor	UM/UL				0.0371	0.0106	
STAT3 (Y705)	Transcription factor	UM/UL			<0.0001	<0.0001	0.0002	

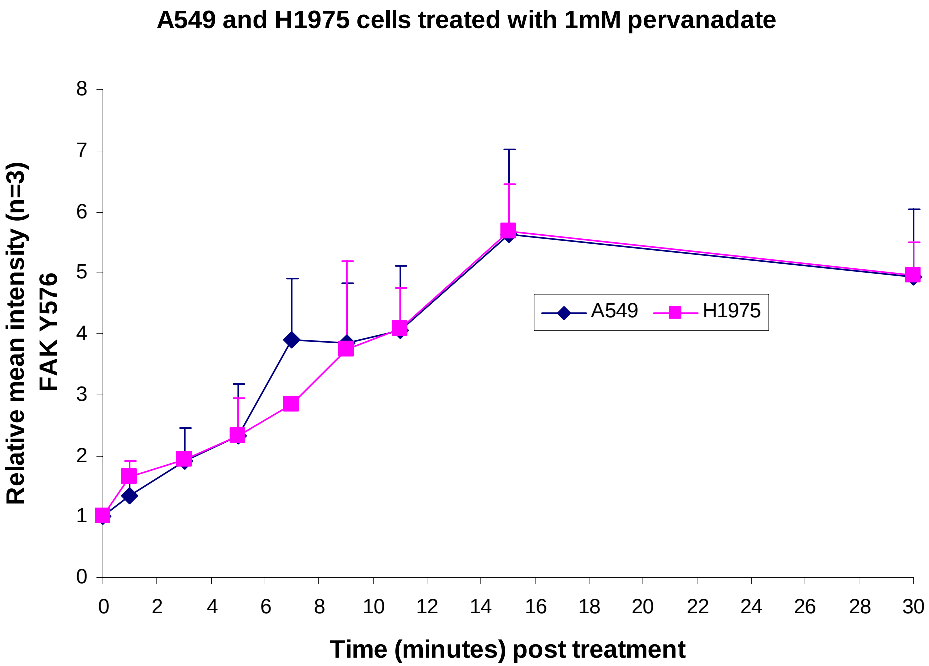
Reactive elevated analytes

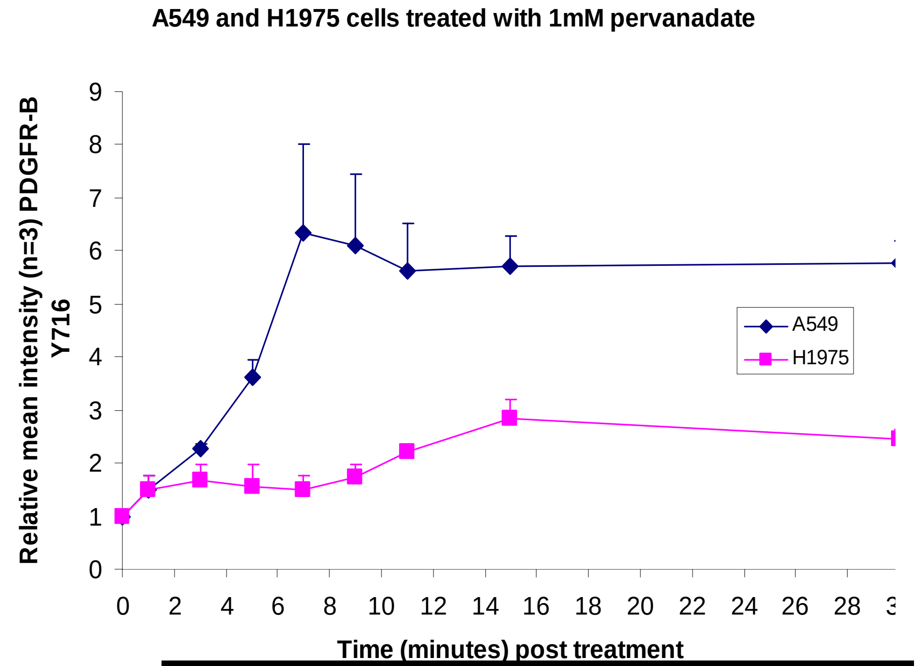
Protein	Function	Significant changes over time	Trial #10 Breast DCIS, Epithelium & Adipose Tissue (n=18) Time post excision (minutes, time zero=20)							
			44	50	80	110	140	230	260	320
			p value (n=2)	p value (n=2)	p value (n=2)	p value (n=2)	p value (n=2)	p value (n=2)	p value (n=2)	p value (n=2)
Catenin(beta) (Ser33/37/Thr41)	Adhesion/Cytoskeleton	DE/DM/DL		0.0013		0.0083	0.0006	0.0085	0.0063	0.0166
E-cadherin	Adhesion/Cytoskeleton	UE/UM/UL	0.0005	0.0001	0.0005	0.0319	0.0001			
PAK1 (Ser199/204)/PAK2 (Ser192/197)	Adhesion/Cytoskeleton	UL					0.0304			
Bad (Ser112)	Apoptosis	DL						0.0132		0.0064
Caspase-3, cleaved (Asp175)	Apoptosis	UE/UM/UL	<0.0001	0.0004	<0.0001		0.0018		0.0211	0.0099
Caspase-7, cleaved (Asp198)	Apoptosis	UE/UM/UL	0.03		0.0139		0.0098			
Caspase-9, cleaved (Asp330)	Apoptosis	UE/UM	0.0033		0.0094					
Chk1 (Ser345)	Cell Cycle	*	*	*	*	*	*	*		
Acetyl CoA Carboxylase (Ser79)	Hypoxia/Ischemia	UE/UL		0.0049			0.0003			0.0484
AMPKalpha1 (Ser485)	Hypoxia/Ischemia	UE/UL/DL		0.011	0.0329		0.011	0.0493		
AMPKBeta1 (Ser108)	Hypoxia/Ischemia	UE/UL/DL		0.005			0.0054	0.032		
eNOS (Ser1177)	Hypoxia/Ischemia	*	*	*	*	*	*	*		
HSP 90	Hypoxia/Ischemia	UL					0.0341			
VEGF Receptor-2 (Y1175)	Hypoxia/Ischemia	UE/UM/UL	0.0188		0.004		0.0024			
Akt (Ser473)	Proliferation/Survival	UE/DL		0.0288		0.0016	0.0089	0.0021		0.0393
Akt (Thr308)	Proliferation/Survival	DM/DL				0.0066		0.0076	0.0166	
c-Abl (Thr735)	Proliferation/Survival	*	*	*	*	*	*	*		
EGFR	Proliferation/Survival	DM/DL				0.0382			0.0297	
EGFR (Y1148)	Proliferation/Survival	DE/DM/DL	0.0139	0.0113	0.0132	0.0041	0.0072	0.0126	0.0034	0.016
eIF4G (Ser1108)	Proliferation/Survival	UE/UM/UL	0.029		0.0111		0.025			
ErbB2	Proliferation/Survival	DM/DL				0.027		0.0471	0.0271	0.0394
ERK 1/2 (Thr202/Y204)	Proliferation/Survival	DE/DM/DL	0.0197		0.027	0.0065		0.0129	0.0116	0.0246
GSK3alpha (Ser21/9)	Proliferation/Survival	DM/DL				0.0203	0.0028	0.0091	0.0309	
Her3 Y1289	Proliferation/Survival	UL					0.0241			
IRS-1 (Ser612)	Proliferation/Survival	UE/UM/UL	0.0221	0.0021	0.0092		0.0003			
mTOR (Ser2481)	Proliferation/Survival	*	*	*	*	*	*	*		
PKC alpha/beta II (Thr638/641)	Proliferation/Survival	DM/DL				0.0402	0.0451	0.0194	0.0464	
ASK1 (Ser83)	Stress/Inflammation	DE/DM/DL		0.0055		0.0085		0.0003	0.0021	0.0055
IkappaB-alpha (Ser32/36)	Stress/Inflammation	DE/DM/DL	0.0266	0.0107	0.0255	0.0042	0.0026	0.0137	0.0019	0.0093
MARCKS (Ser152/156)	Stress/Inflammation	UE/UM/DM/DL	0.0013	0.0001	0.0001	0.0087		0.0023	0.0029	
SAPK/JNK (Thr183/Y185)	Stress/Inflammation	DM/UL				0.0347	0.0031			
CREB (Ser133)	Transcription factor	UM/DL			0.005	0.0109	0.0023	0.0012	0.011	0.0039
STAT1 (Y701)	Transcription factor	UE/UM/UL		0.0079	0.0057	<0.0001	0.0156		0.0068	0.0002
STAT3 (Ser727)	Transcription factor		*	*	*	*	*	*	*	*

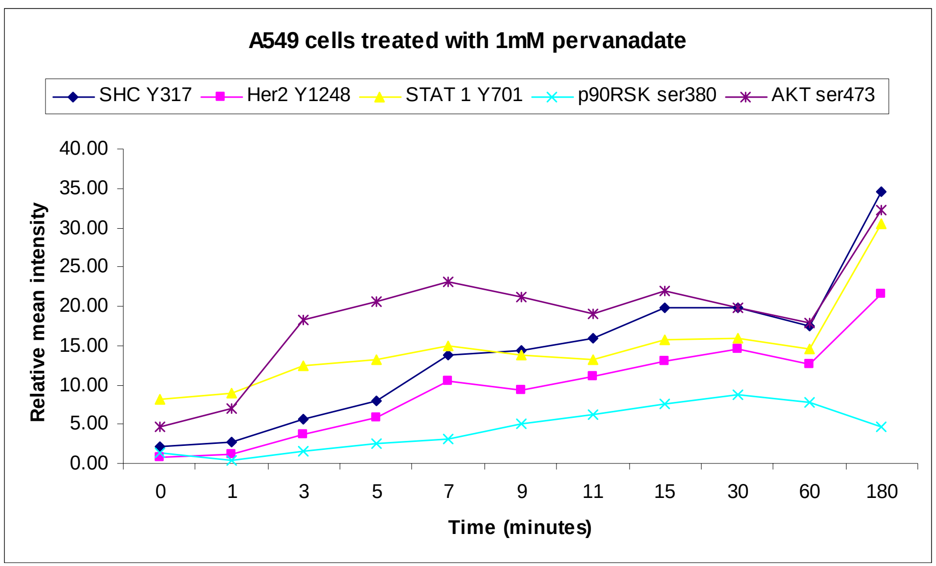
Candidate Surrogate End Points for a Molecular Chronograph (ANOVA)

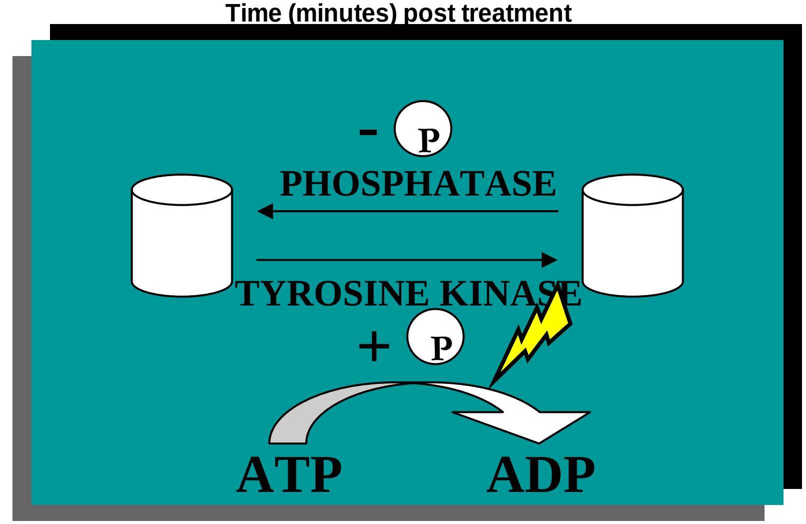








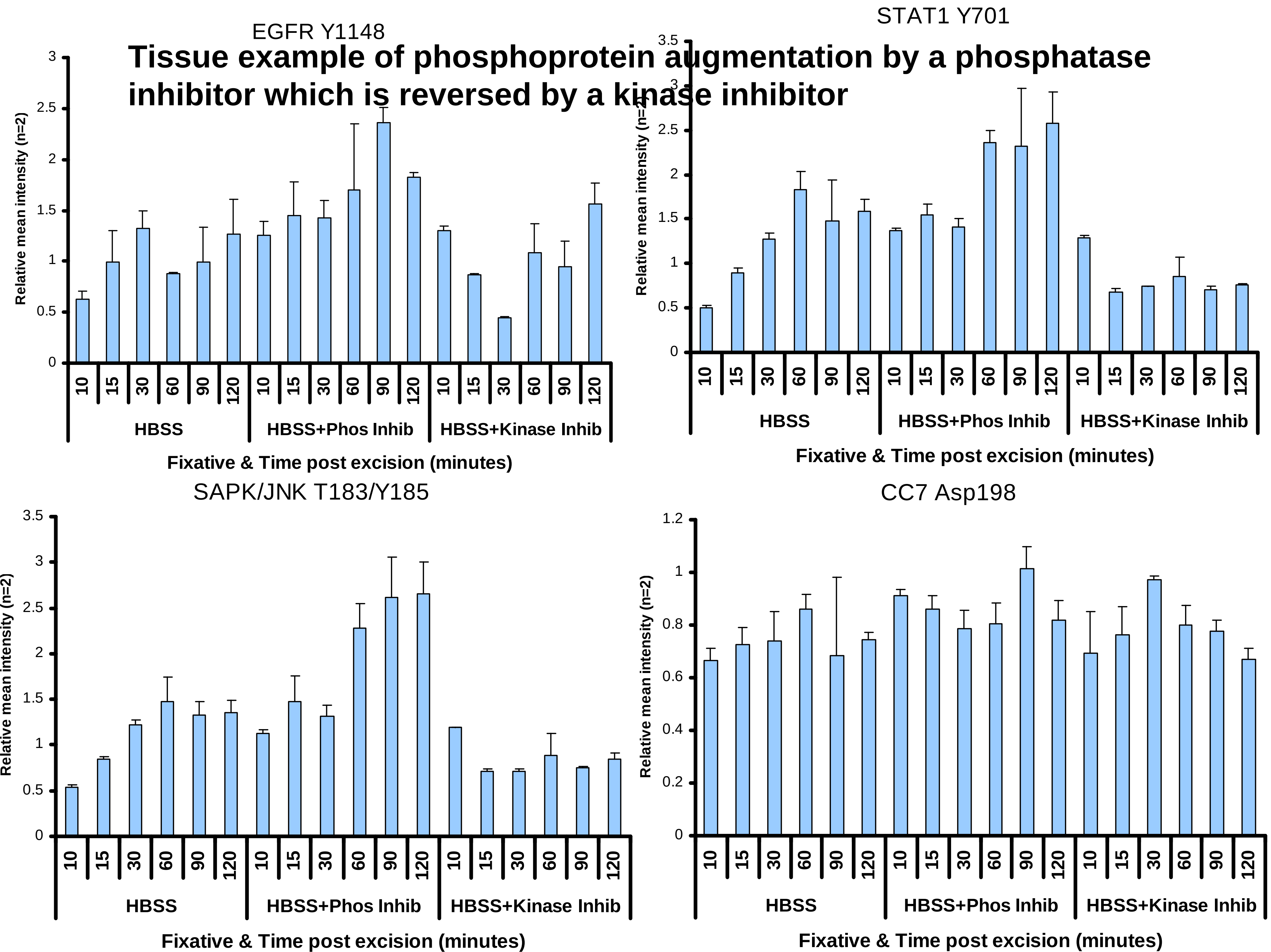


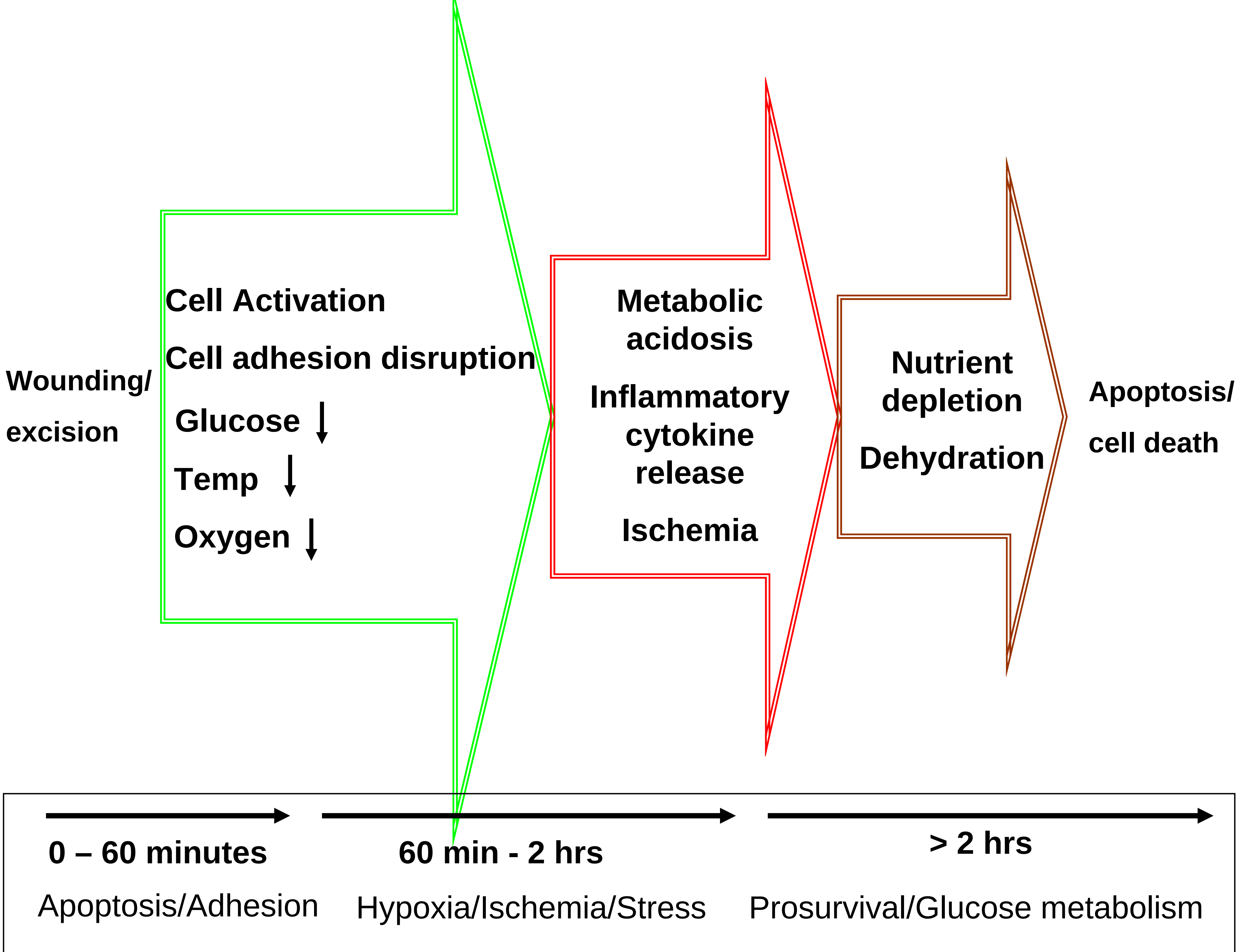


Balance between kinase and phosphatase activity

Accumulation of kinase substrate phosphorylation for living cells in the presence of a phosphatase inhibitor

Tissue example of phosphoprotein augmentation by a phosphatase inhibitor which is reversed by a kinase inhibitor





Wounding/
excision
Cell Activation
Cell adhesion disruption
Glucose
Temp
Oxygen
Metabolic
acidosis
Inflammatory
cytokine
release
Ischemia
Nutrient
depletion
Dehydration
Apoptosis/
cell death
0 – 60 minutes
Apoptosis/Adhesion
60 min - 2 hrs
Hypoxia/Ischemia/Stress
> 2 hrs
Prosurvival/Glucose metabolism

Conclusions

Optimal time to preservation – 20 minutes

Real-world tissue collection time – 4 - 40 minutes

False elevation of endpoints: living tissue reacts to trauma of excision ex vivo. Main source of variability is reactive pathways causing false augmentation of phosphoproteins.

Categories of reactive pathways: reflect stages of cell death post excision.

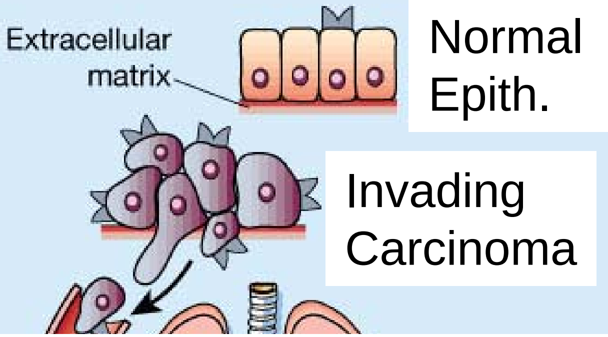
Labile subsets of phosphoproteins: identified labile endpoints for QA/QC of preservation methods.

- Phosphatase inhibitors alone falsely elevate phosphoproteins. Addition of phosphatase inhibitors alone will falsely elevate phospho-endpoints for reactive pathways.
- Kinase inhibitor treatment suppresses the reactive elevation of phosphoproteins verifying that the tissue is alive ex vivo.

Ideal preservation chemistry: maintains morphology and blocks both phosphatase and kinase pathways to prevent fluctuations ex vivo

METASTASIS

Testing the Seed vs. Soil hypothesis using protein signal pathway mapping



38 patients: Matched colorectal cancer and hepatic metastasis

15 patients: Pulmonary metastasis from colorectal cancer

27 patients: Liver metastasis from non colorectal cancer

Mariaelena Pierobon

Question: Is the state of activated signaling pathways in a primary tumor cell different from the metastasis?

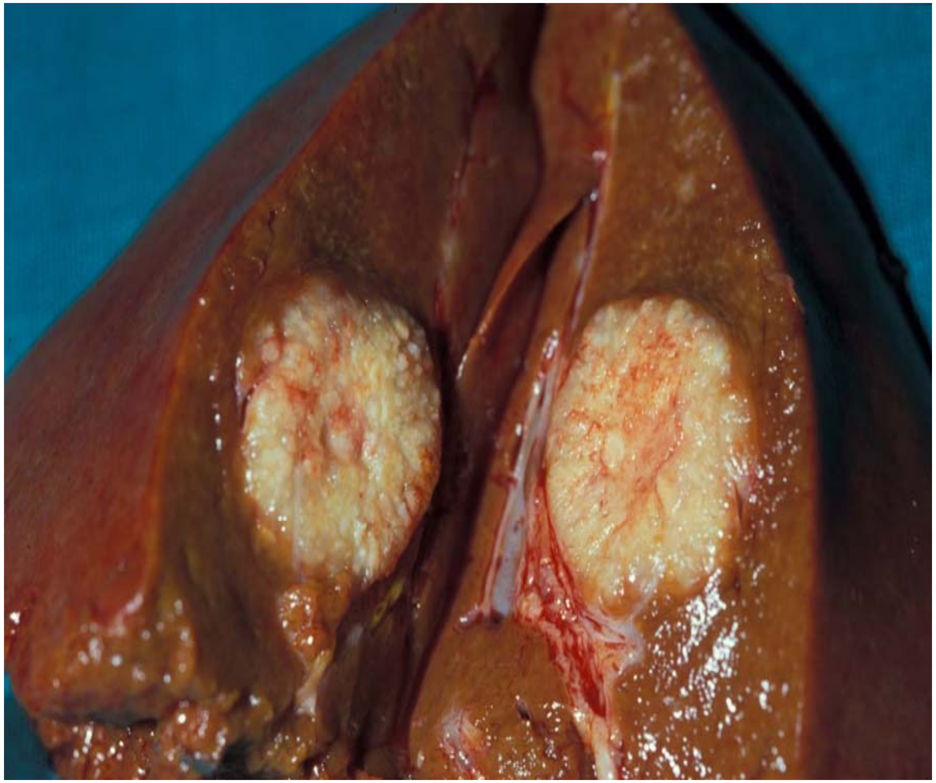
If so: Is the state of activated signal pathways in metastasis dictated by the target organ (soil) or by the primary tumor site (seed)from which the metastasis is derived?

75 kinase and kinase substrates measured

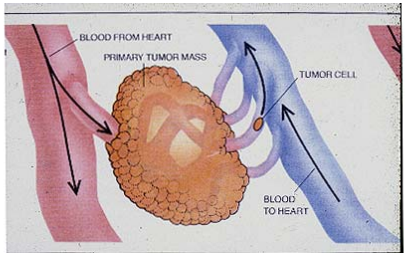
Specimen Procurement: Primary Tumor and Matched Metastasis

- Collected during surgery at the time of vascular clamping and excision.
- Immediately (<20 m) snap frozen and stored at -80C
- Laser Microdissected

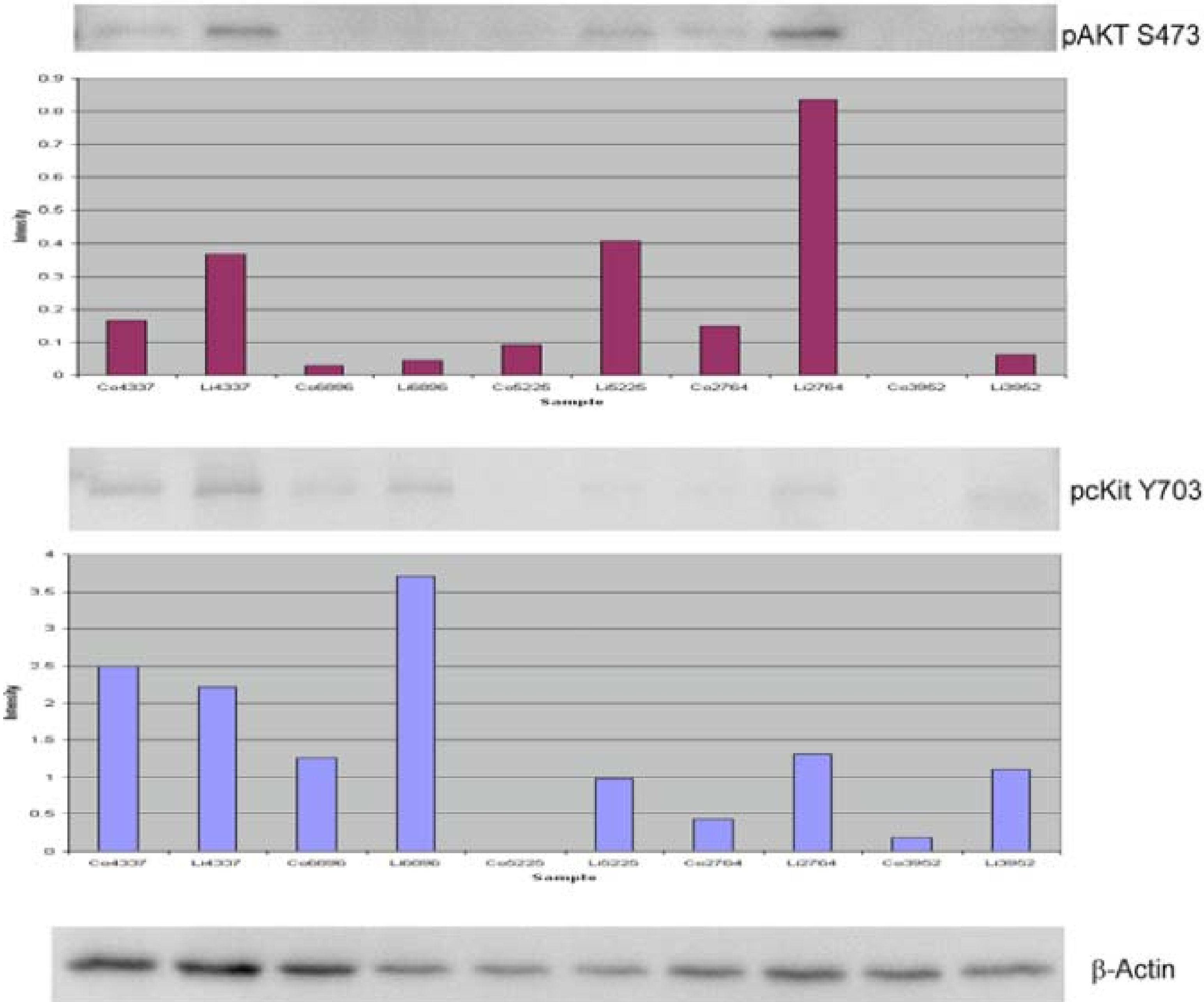
Claudio Belluco



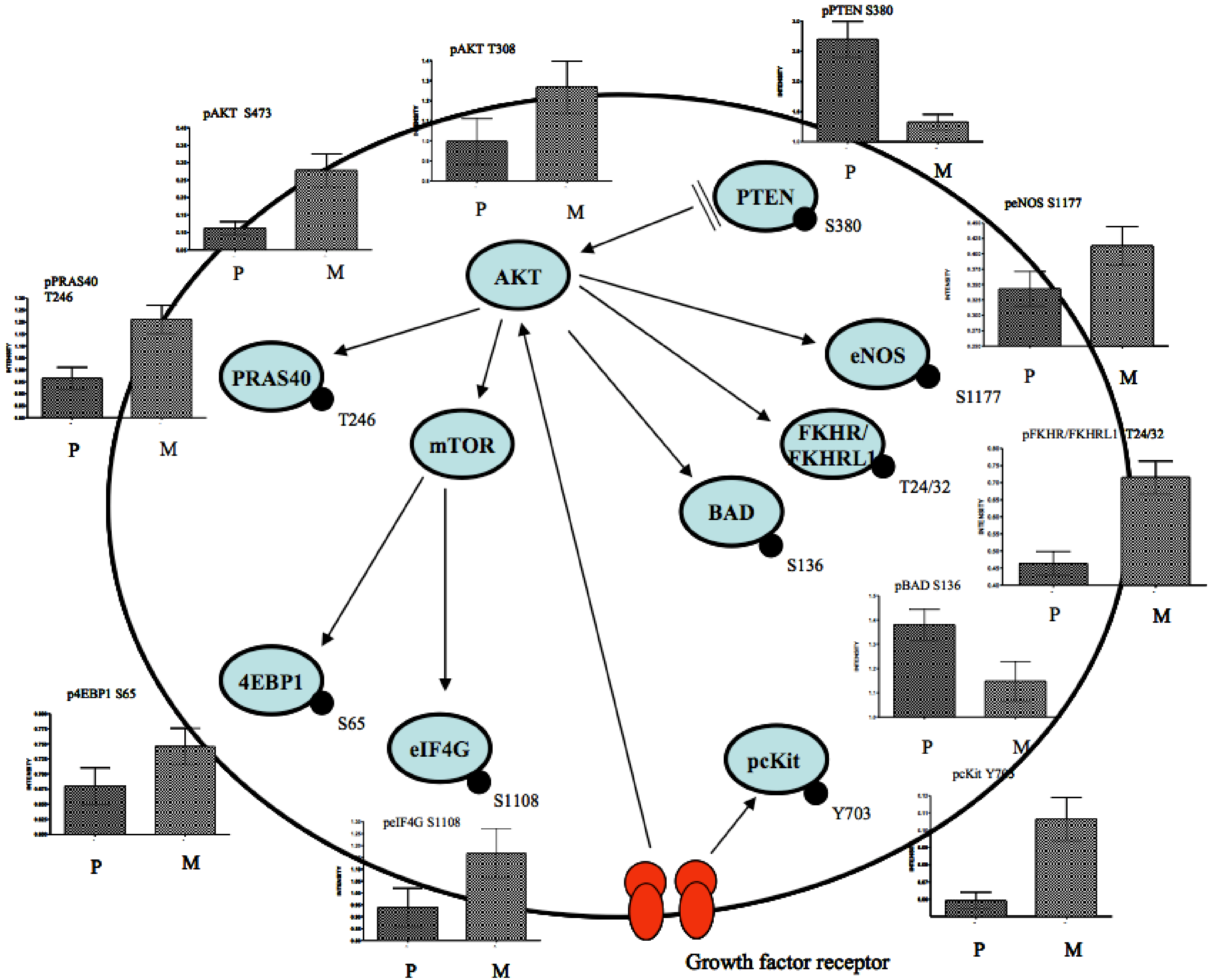
Variable	P value	Regulation in liver metastases
Cl Caspase9 D315	0.0066	↓
EGFR	0.0019	↑
p4EBP1 S65	0.0326	↑
pAbl Y245	0.0037	↑
pAKT S473	0.0001	↑
pAKT T308	0.0163	↑
pBAD S136	0.0087	↓
pcAbl T735	0.0251	↓
pcKit Y703	0.005	↑
pEGFR Y1448	0.0424	↓
peIF4G S1108	0.0416	↑
peNOS S1177	0.0476	↑
pFADD S194	0.0222	↑
pFAK Y576/577	0.0001	↑
pFKHR/FKHRL1 T24/32	0.0001	↑
pIKBa S32	0.0341	↓
pIKBa S32/36	0.0212	↓
pmTOR S2481	0.0436	↓
pP70S6 S371	0.0006	↓
pPDGFRb Y716	0.0262	↓
pPDGFRb Y751	0.0181	↑
pPDK1 S241	0.0051	↓
pPKCa S657	0.0001	↓
pPKCa/BII T638/641	0.0017	↓
pPKC theta T538	0.0182	↑
pPKC zeta/lambda T410/403	0.0001	↓
pPRAS40 S246	0.0003	↑
pPTEN S380	0.0001	↓
pPyk2 Y402	0.0001	↑
pShc Y317	0.0001	↑
pSMAD2 S465/467	0.0043	↑
pSrc Y527	0.0001	↑
pSTAT3 Y705	0.0225	↓
pSTAT5 Y694	0.0159	↑
pVEGFR Y951	0.0001	↑
pVEGFR2 Y1175	0.0481	↑



Matched patient mets and primary : Western Blot Validation

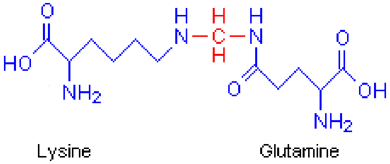


Conclusion: Interconnection of metastasis-associated pathways

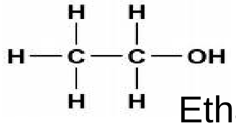


Current Tissue Fixation/Preservation Technology

1. Chemical Crosslinking: formalin, gluteraldehyde



2. Precipitation: alcohol



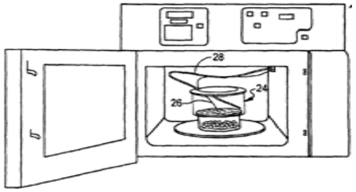
Ethanol

3. Cryopreservation: cryoprotectants, Liquid N_2

4. Thermal inactivation:

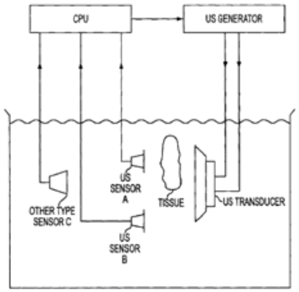
Denator instrument – Skold K et al, Proteomics (2)2007,4445-4456

Microwave radiation - Login GR et al, Methods. 1998 Jun;15(2):107-17



5. Microwave assisted rapid formalin fixation:

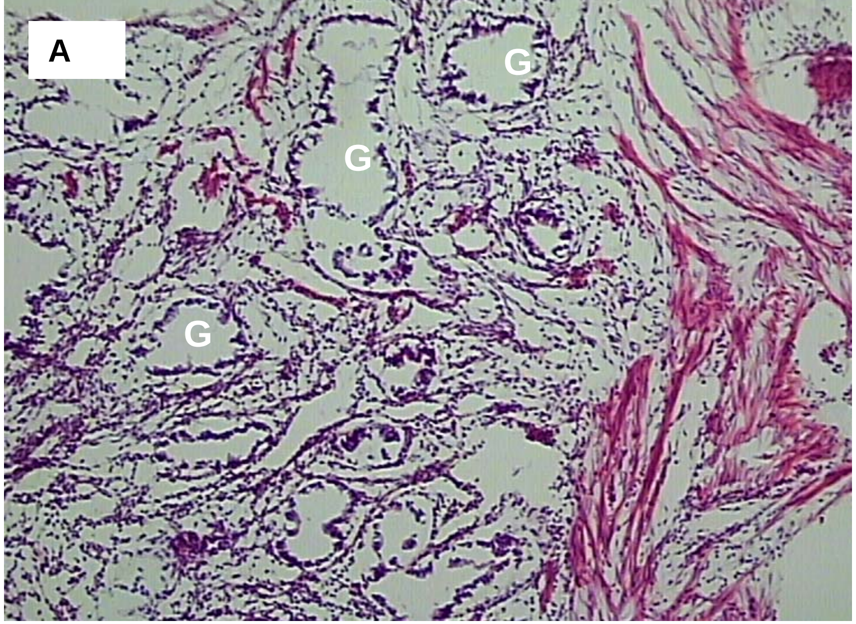
Nadji M et al Appl Immunohistochem Mol Morphol. 2005 (3):277-82

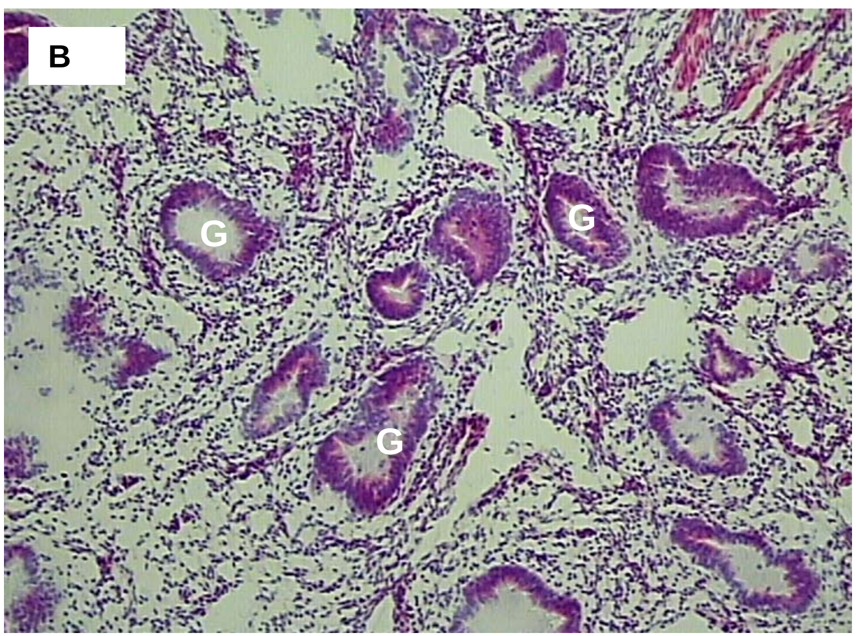


6. Ultra-sound facilitated formalin fixation:

Chu W-S et al, ModernPath (18)2005,850-863.

7. Nitrocellulose cytology imprinting





Ideal Preservative Chemistry Goal for application in the real world of the community hospital or clinic

Tissue submerged in preservative immediately at the time of procurement (e.g. in the OR or biopsy gun)

Room temperature preservation of morphology for gross pathology and immediate frozen section Dx.

Stabilization of phosphoproteins against reactive or degradative fluctuations. Stabilization of RNA

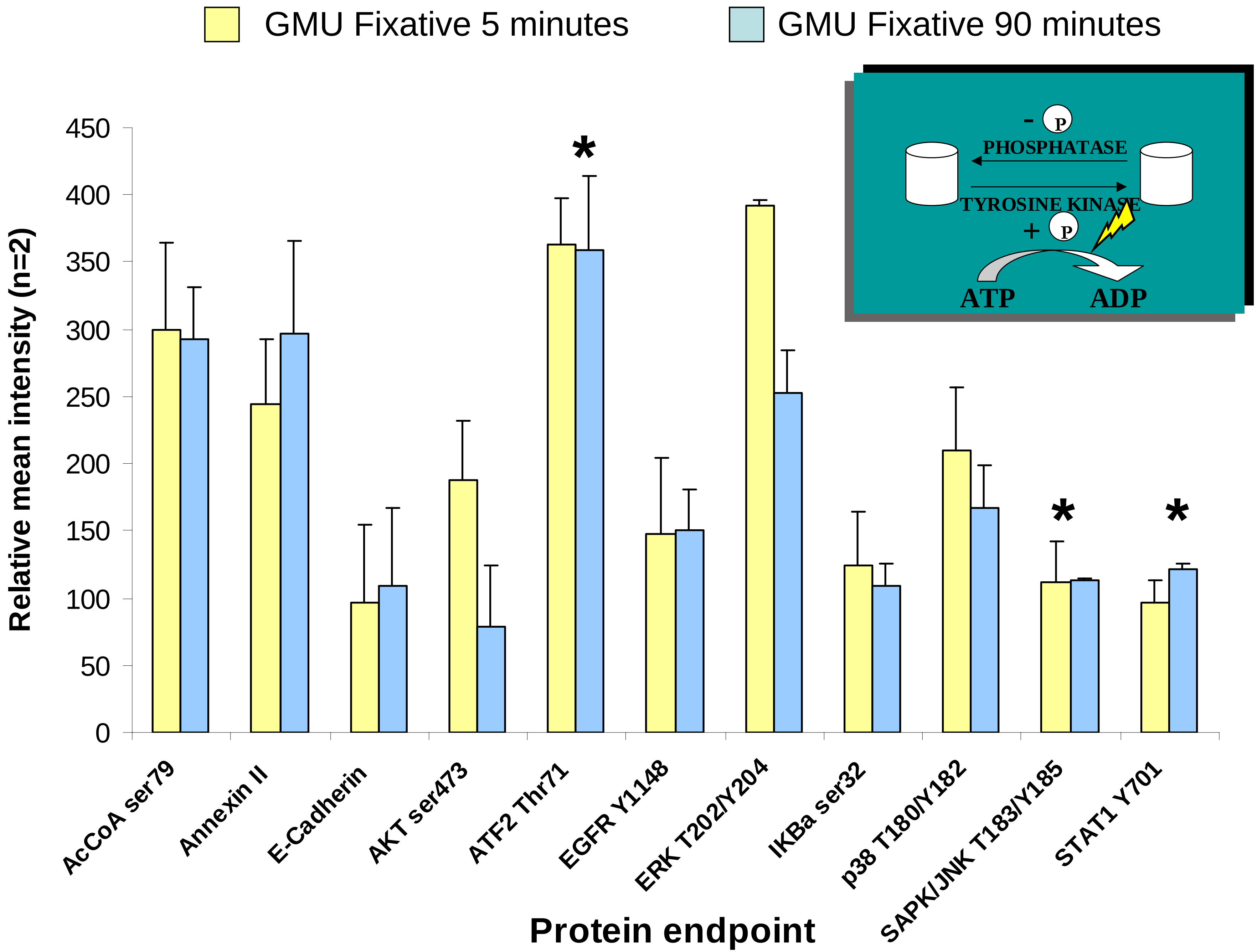
Shipping at room temperature. Paraffin embedding for long term storage, Dx, and I.H.

Development of Ideal Preservative for Phosphoprotein Molecular Analysis

1. **Precipitating Fixative: non-crosslinking (Low concentration to permit FS and reduce shrinkage)**
2. **Reversible lipid soluble and water soluble cross linker chemistry**
3. **Phosphatase Inhibitors: Tyrosine, Serine/Threonine**
4. **Kinase Inhibitors**
5. **Permeation Enhancers**
6. **Preservative base solution**

Alternatives to chemical methods: transient heat induction via microwave, electrical, chemical means

Key facet: preservation of cellular histology and morphology



GMU Fixative 5 minutes
GMU Fixative 90 minutes
- P
PHOSPHATASE
TYROSINE KINASE
+ P
ATP
ADP
Relative mean intensity (n=2)
0
50
100
150
200
250
300
350
400
450
AcCoA ser79
Annexin II
E-Cadherin
AKT ser473
ATF2 Thr71
EGFR Y1148
ERK T202/Y204
IKBa ser32
p38 T180/Y182
SAPK/JNK T183/Y185
STAT1 Y701
Protein endpoint

Clinical Research Trials Gathering Data Toward the Goal of Individualized Therapy.

A. Breast Cancer: (USO, Inova, GSK) Lapatinib Phosphoprotein Stratification: Testing of Room Temperature Fixation Chemistry
Status: Started Sept 2007 Target Completion Dec 2008

B. Multiple Myeloma: (Hem Oncol Assoc, Inova)
Status: Started May 2007 Target Completion May 2009

C. Breast Cancer Carcinoma in Situ: (Inova)
Status: Started Sept 2007 Target Completion Dec 2008

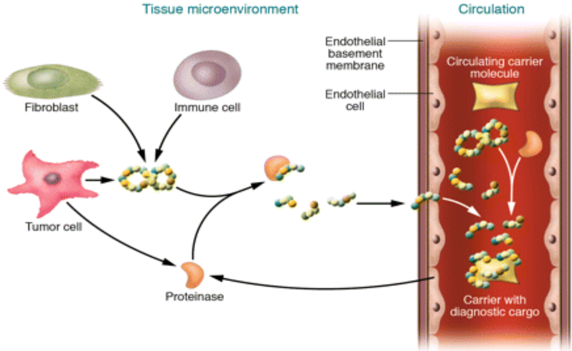
D. Colon Cancer Liver Metastasis: (Novartis)
Status: IRB Review, Target Completion 2009

E. Childhood Rhabdomyosarcoma: (Children's Hospital, Novartis)
Status Not Started: in planning stages, Target Completion 2009

INOVA HEALTH SYSTEM

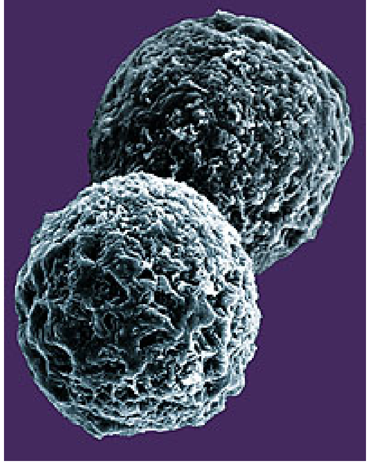
GEORGE MASON UNIVERSITY

Biomarker Challenges



- Biomarkers exist in very low concentration
- Are obscured by abundant resident blood proteins like albumin
- Rapidly degraded by enzymes

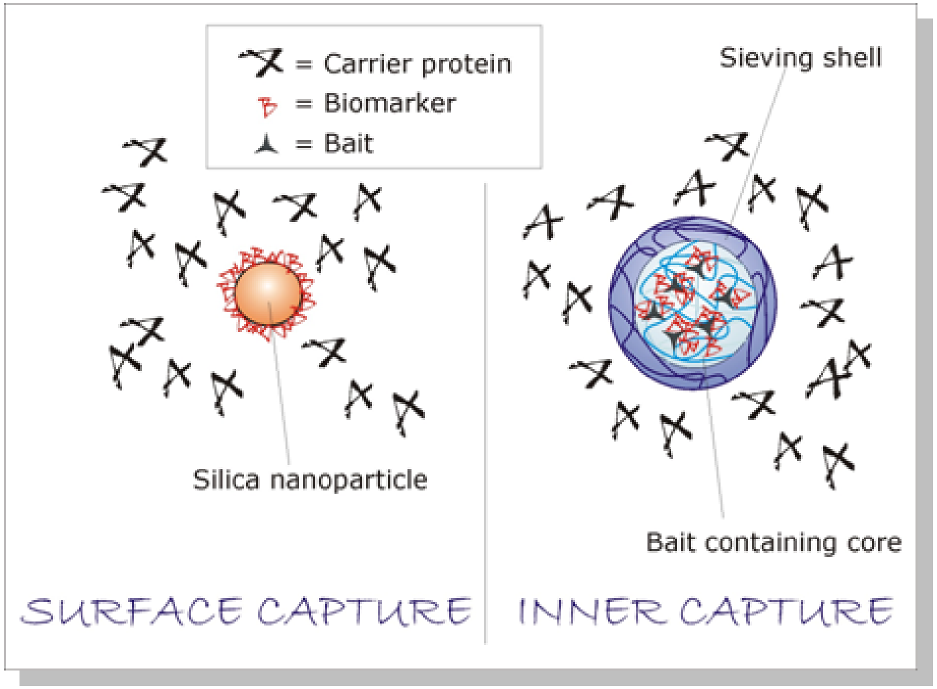
“Smart” Nanoporous Particles

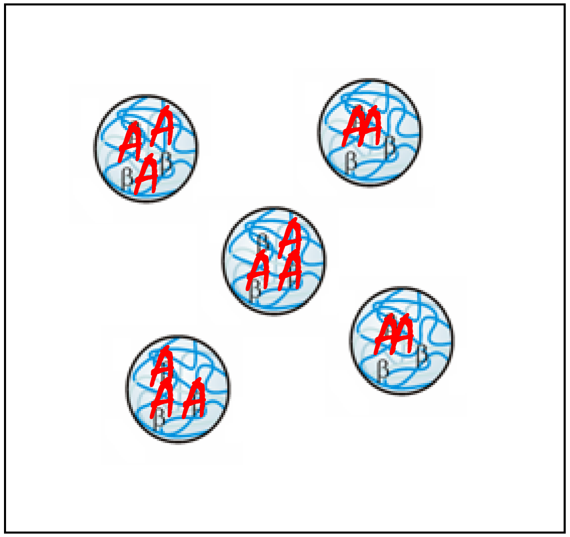


Alessandra Luchini, et al. *Smart Hydrogel Particles: Biomarker Harvesting: One-Step Affinity Purification, Size Exclusion, and Protection against Degradation*. Nanoletters, 2008 Vol. 8, No. 1 350-361

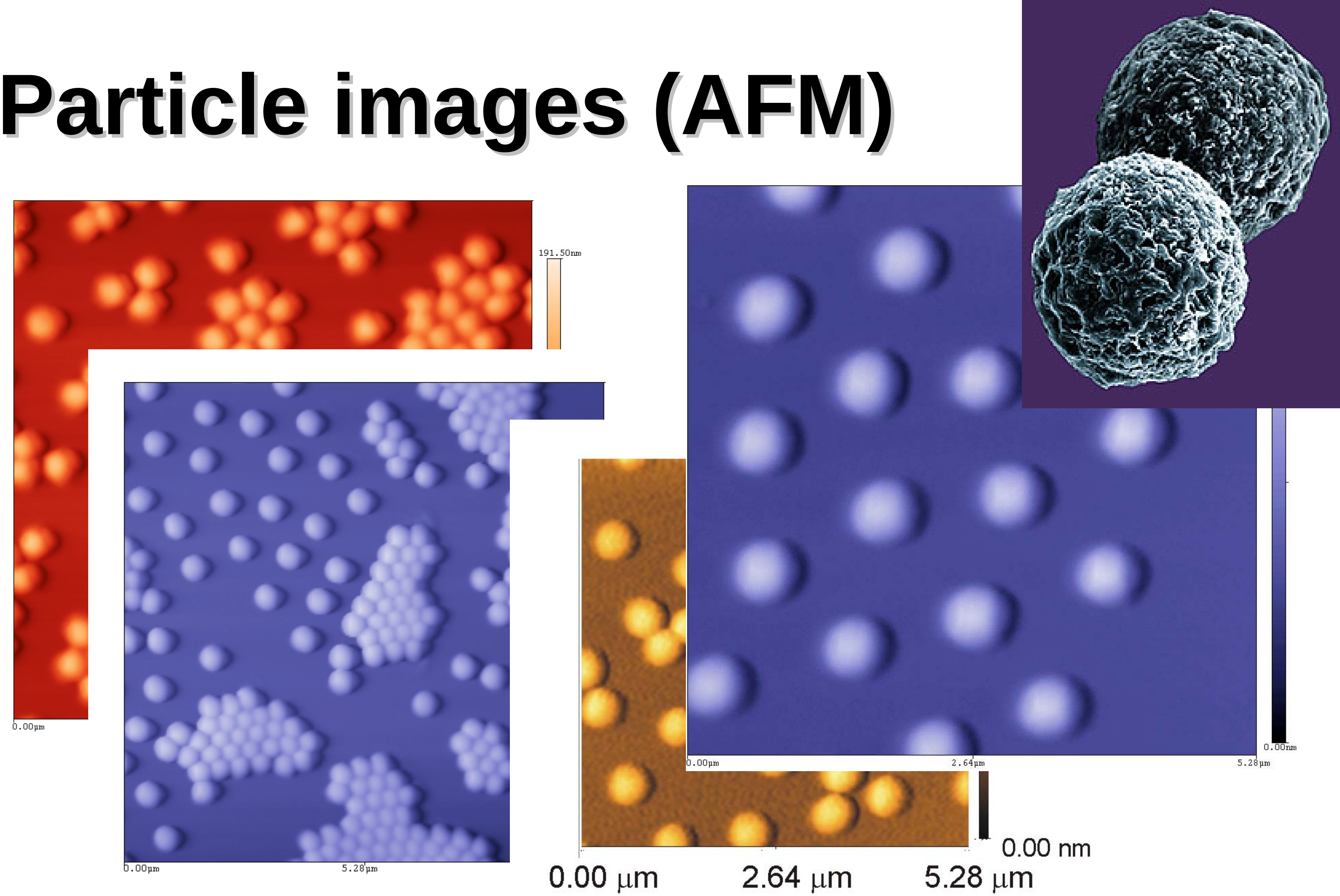
- **Three independent functions within minutes, in one step, in solution:**
  - **a) molecular size sieving**
  - **b) affinity capture of all solution phase target molecules (concentration)**
  - **c) complete protection of harvested proteins from enzymatic degradation**
  - **d) complete exclusion of albumin and high abundance proteins**

Affinity capture - Concentration

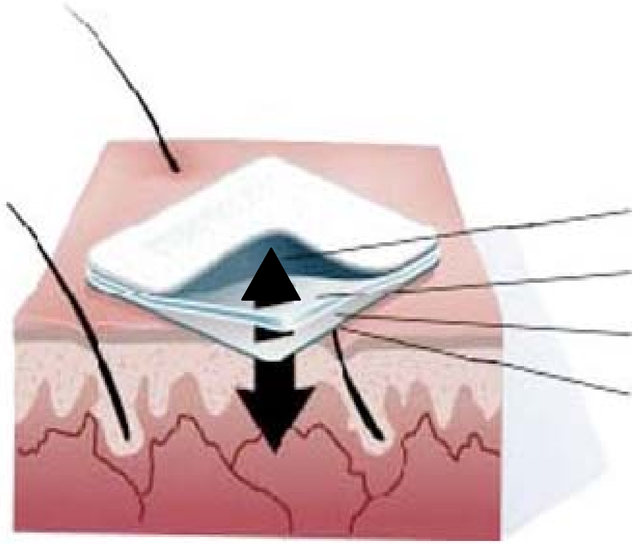




Particle images (AFM)



Smart Nanoparticles for Immediate Room Temperature Biomarker Harvesting, concentration and protection from degradation



Example application to skin patch for diagnostic marker (proteins and metabolites) harvesting

Example Application: nanoparticles in vacutainer immediate concentration and preservation of biomarkers

"Smart" Hydrogel Particles for Biomarker Harvesting: One-step affinity purification, size exclusion, and protection against degradation

Alessandra Luchini, PhD

Center for Applied Proteomics and Molecular Medicine

Co-Directors: Lance Liotta and Emanuel Petricoin

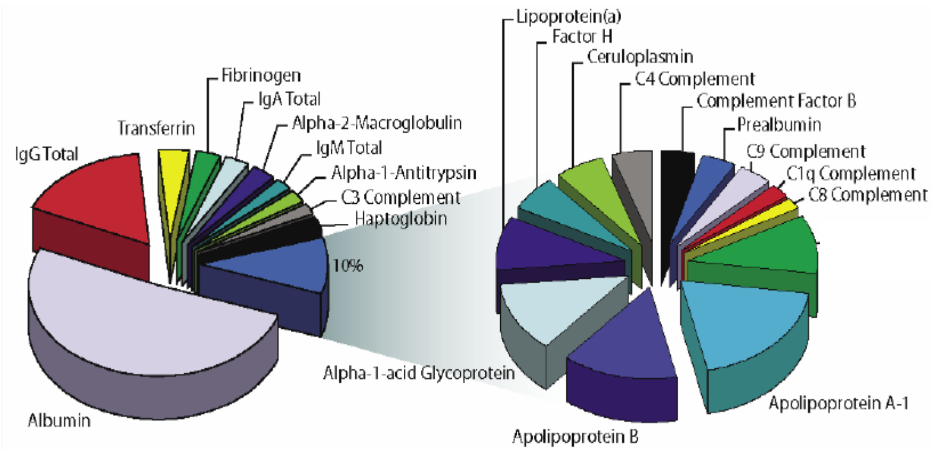
The Center for Applied Proteomics and Molecular Medicine

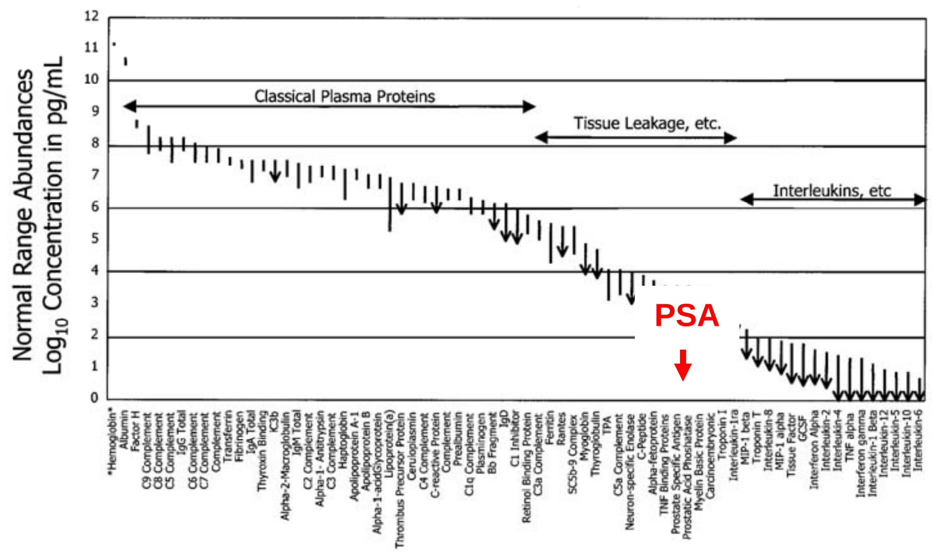
Proteomics Tools for Clinical Medicine

GEORGE MASON UNIVERSITY

Blood Protein Biomarker Discovery and Measurement

An Overwhelming Analytical Challenge

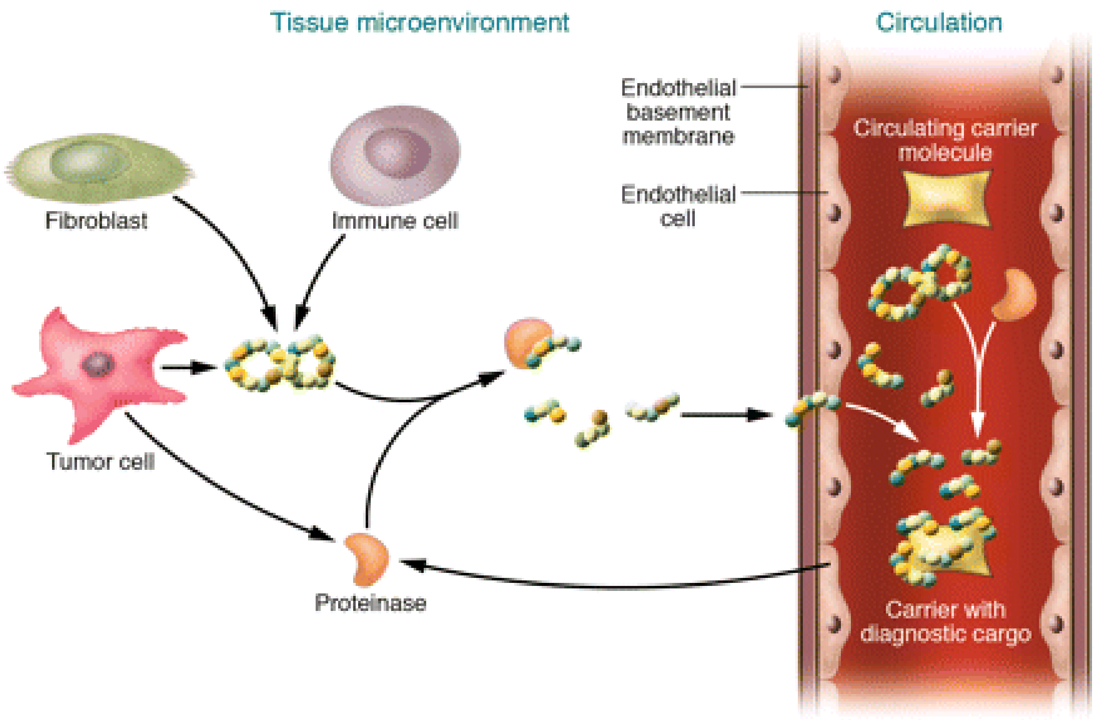




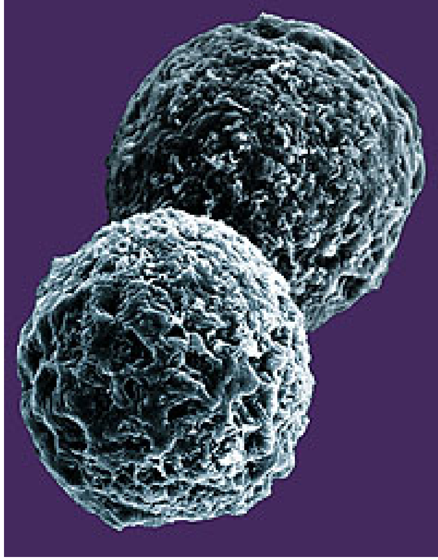
Anderson, N.L., Anderson, N.G. (2002)
Mol. Cell. Proteomics. **1**, 845-867.

- 22 proteins constitute 99% blood protein mass
- High likelihood that biomarkers are low abundance proteins
- No analytical method has sufficient dynamic range

The vast majority of low abundance biomarkers are non-covalently and endogenously *associated with the carrier proteins* that are being removed

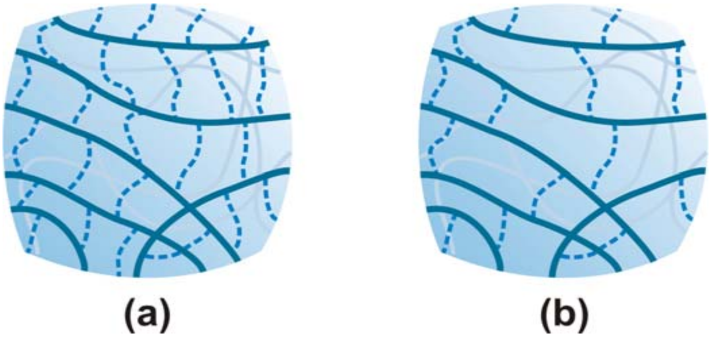


Molecular size sieving

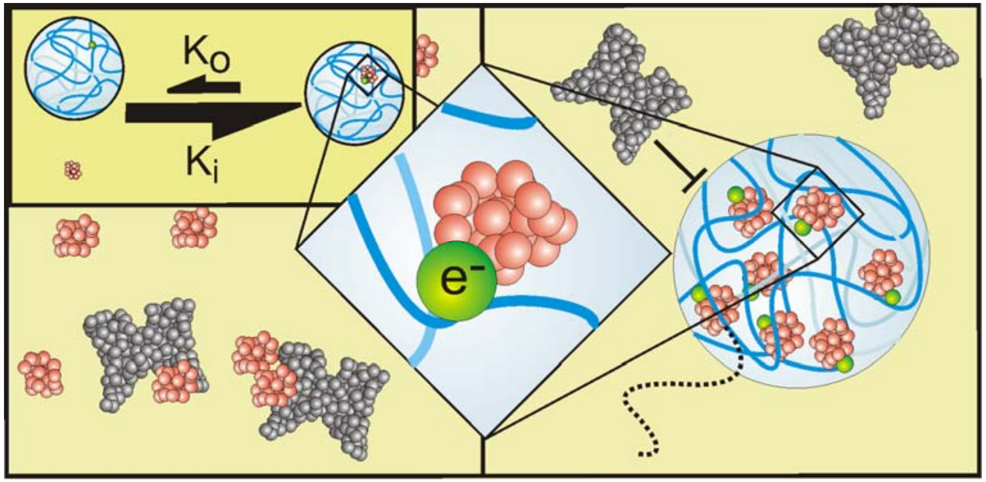


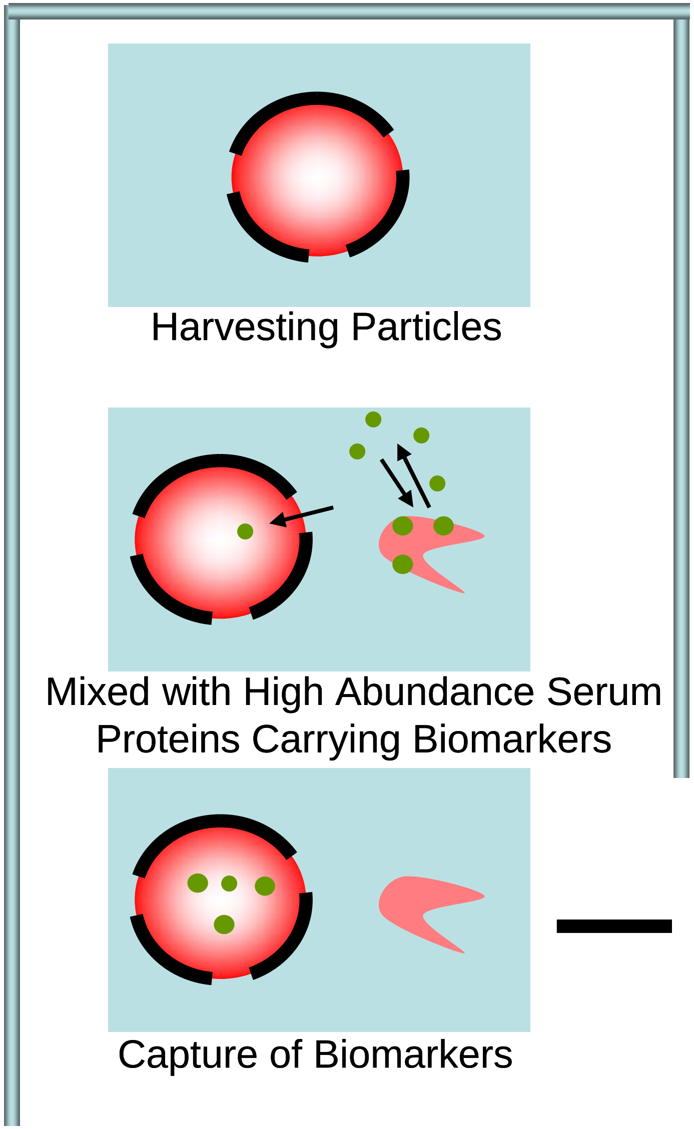
Poly(N-isopropylacrylamide) (pNIPAm)
N,N′-methylenebisacrylamide (BIS)

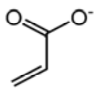
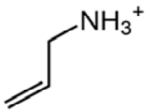
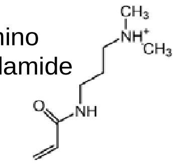
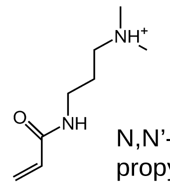
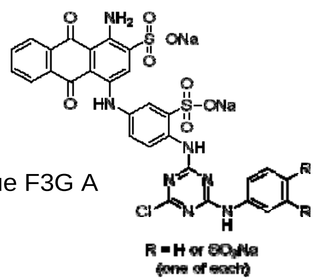
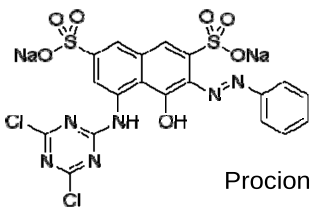
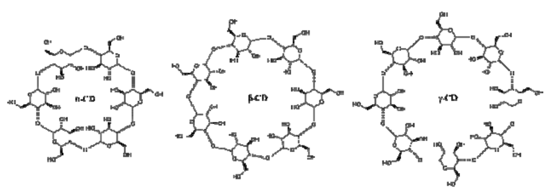
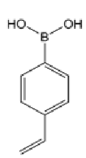
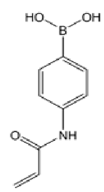
Different concentration of cross linker to alter pore size for size dependent harvesting



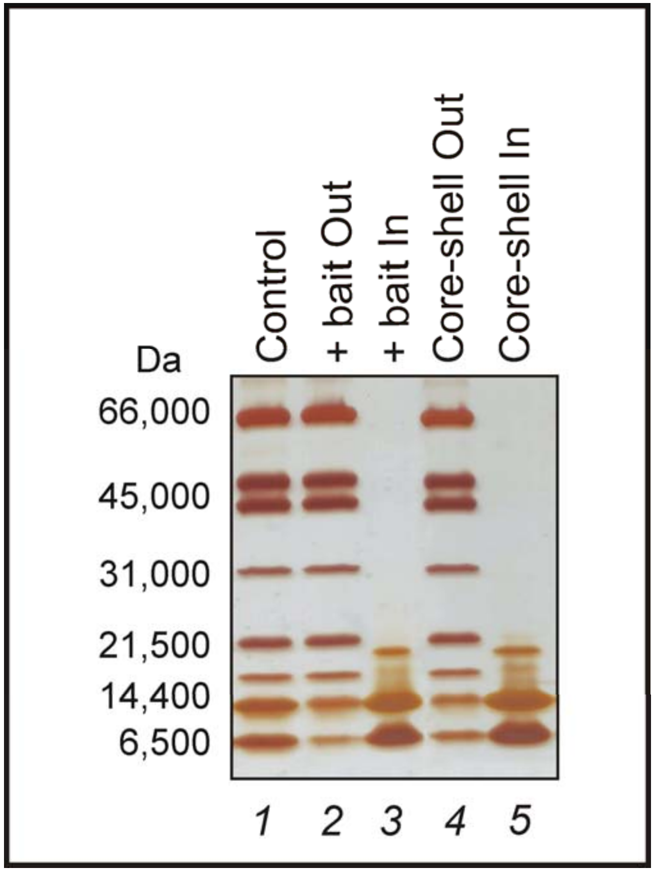
Affinity capture

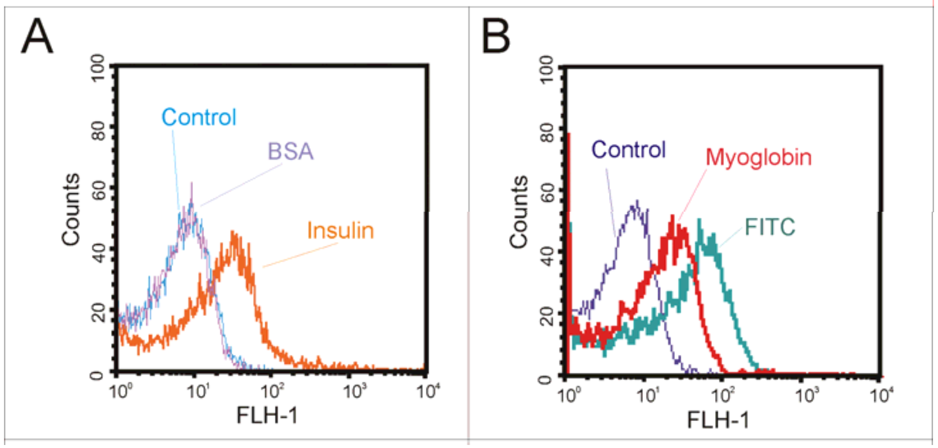




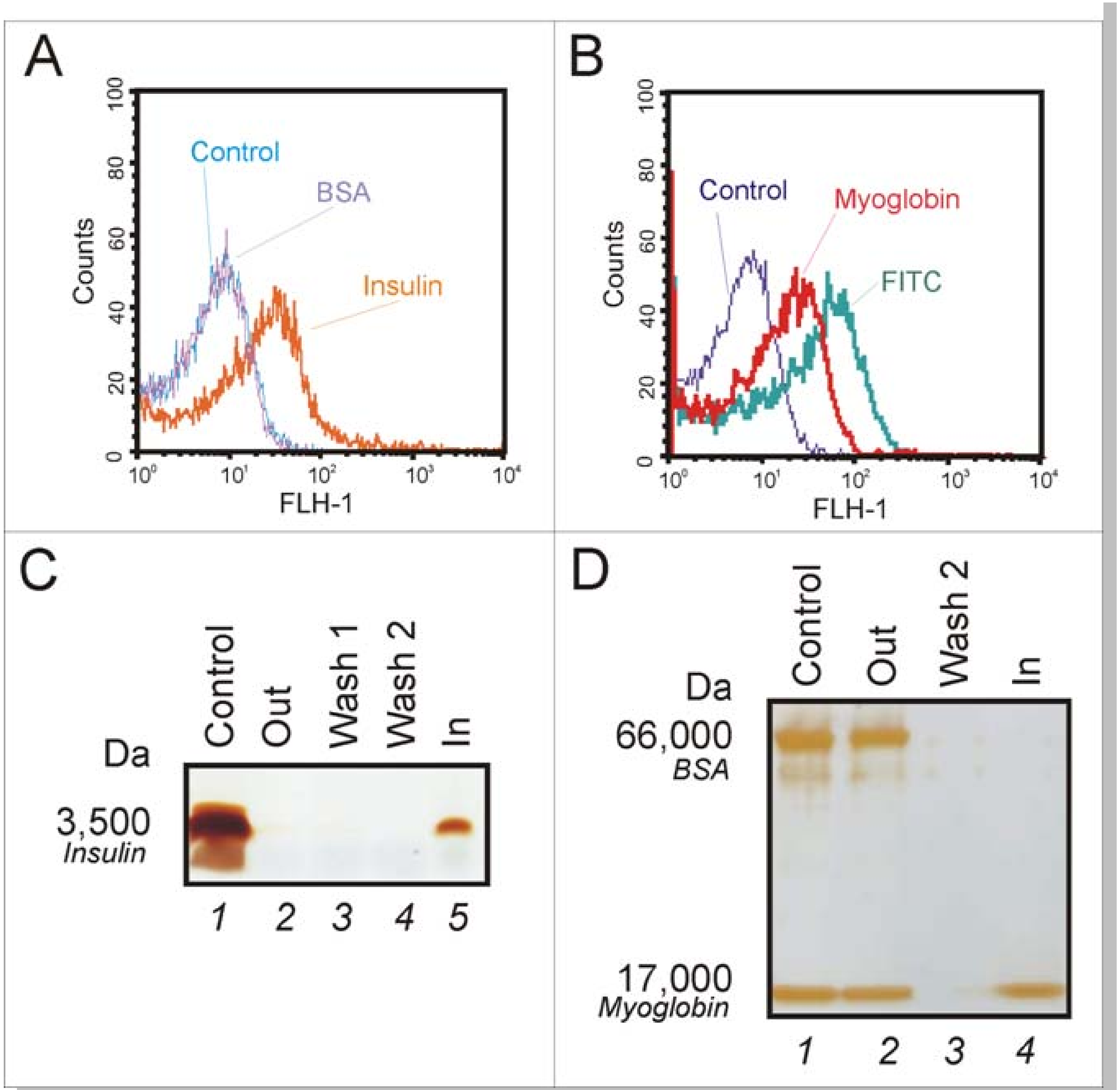
Bait	Target
 Acrylic acid	Cationic proteins and polypeptides
 allylamine  1-vinylimidazole N,N'-dimethylamino propyl]methacrylamide   N,N'-dimethylamino propylscrylamide	Anionic proteins and polypeptides
Cibacron blue F3G A   Procion Red H8BN	Proteins and polypeptides
 Cyclodextrins	Small molecules, cholesterol
p-vinylphenyl boronic acid   N-acryoyl-*m*-aminophenyl boronic acid	Polysaccharides, glycopeptides, RNA
TiO_2 nanoparticles incorporated in NIPAm beads	Phosphopeptides

Molecular size sieving

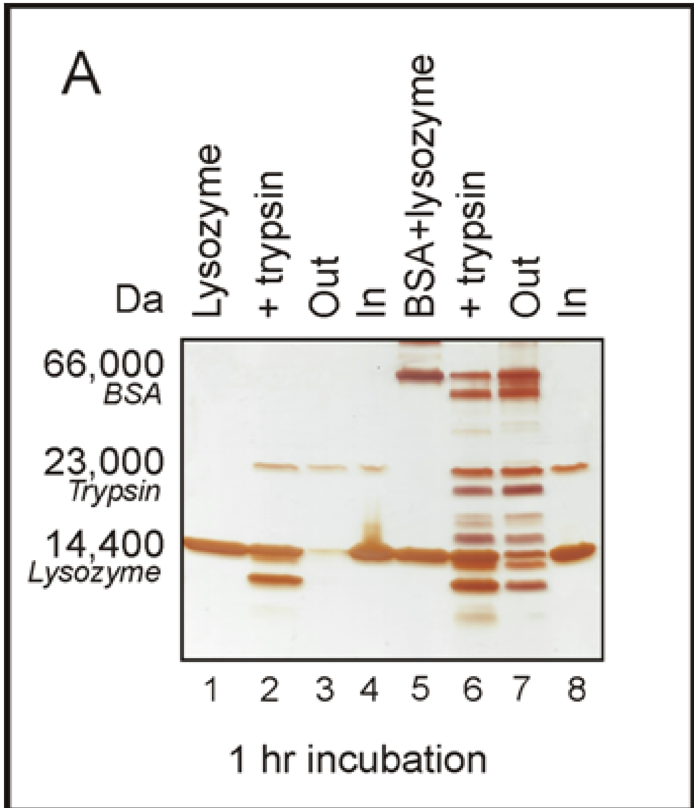


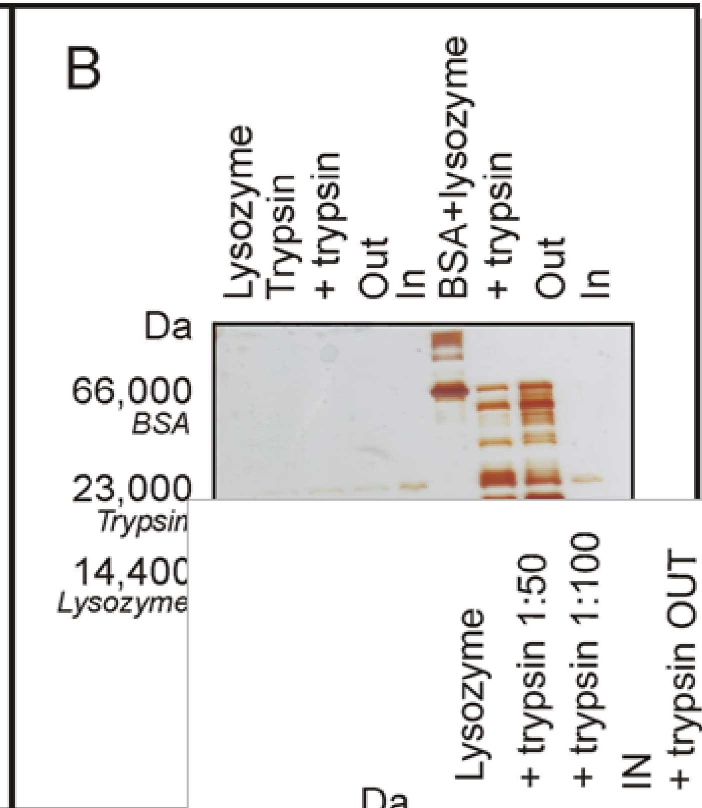


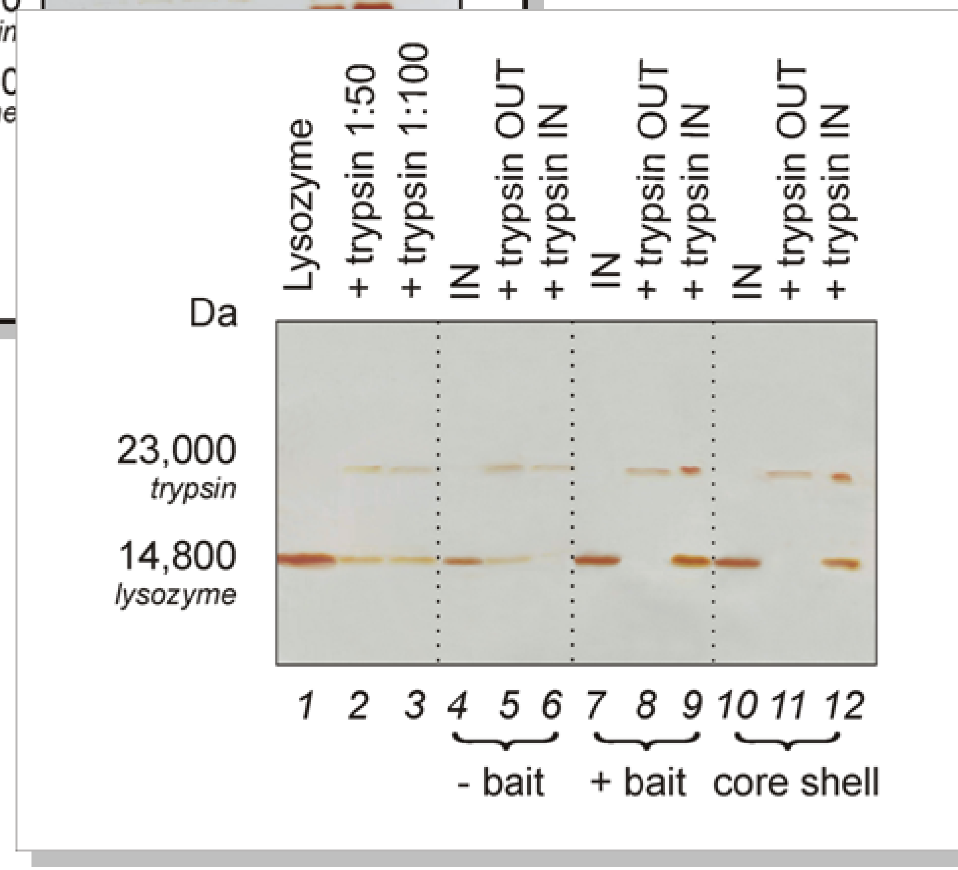
Complete Exclusion from Albumin

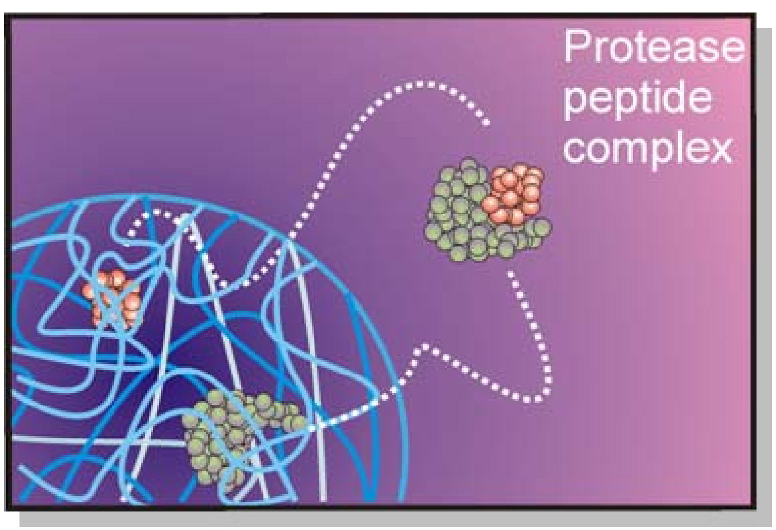


Protection From Degradation

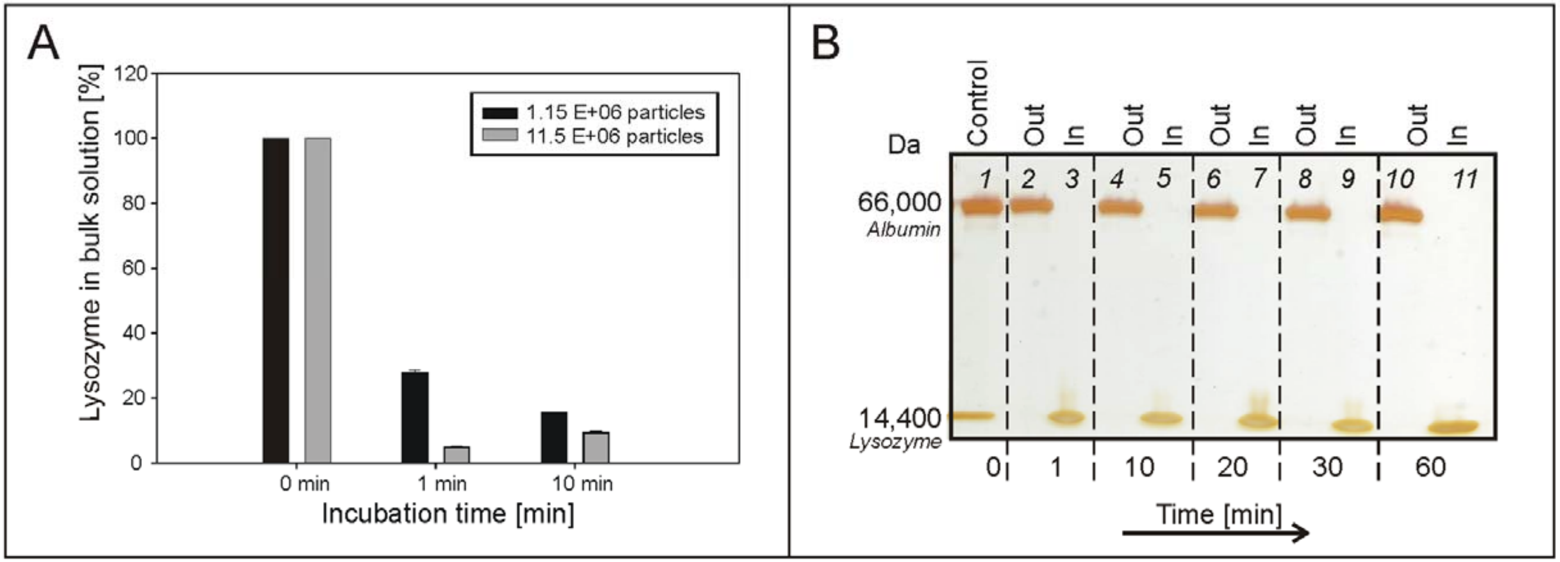




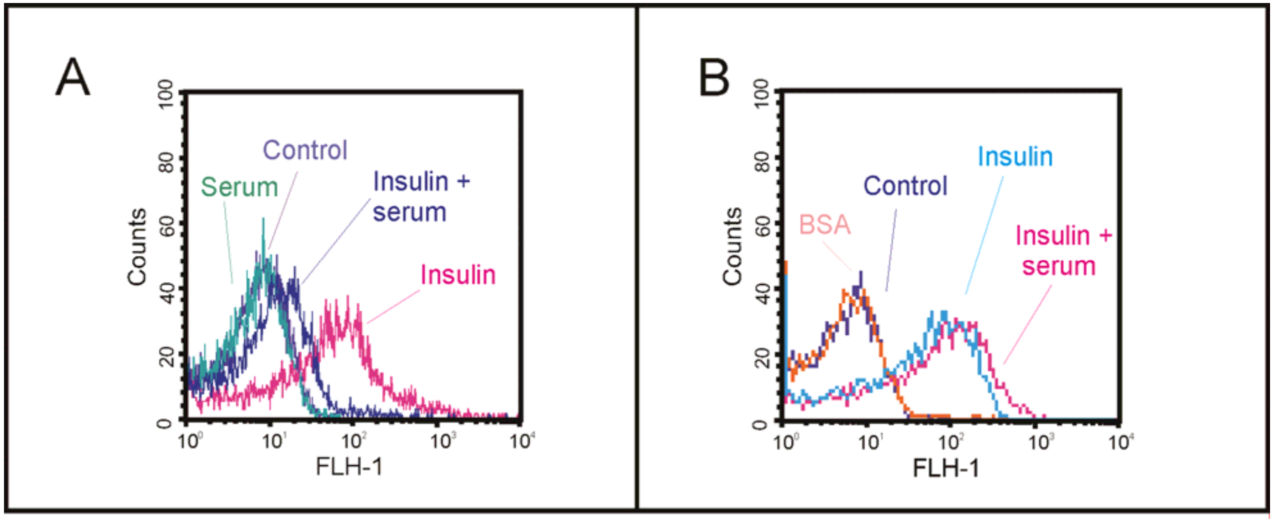


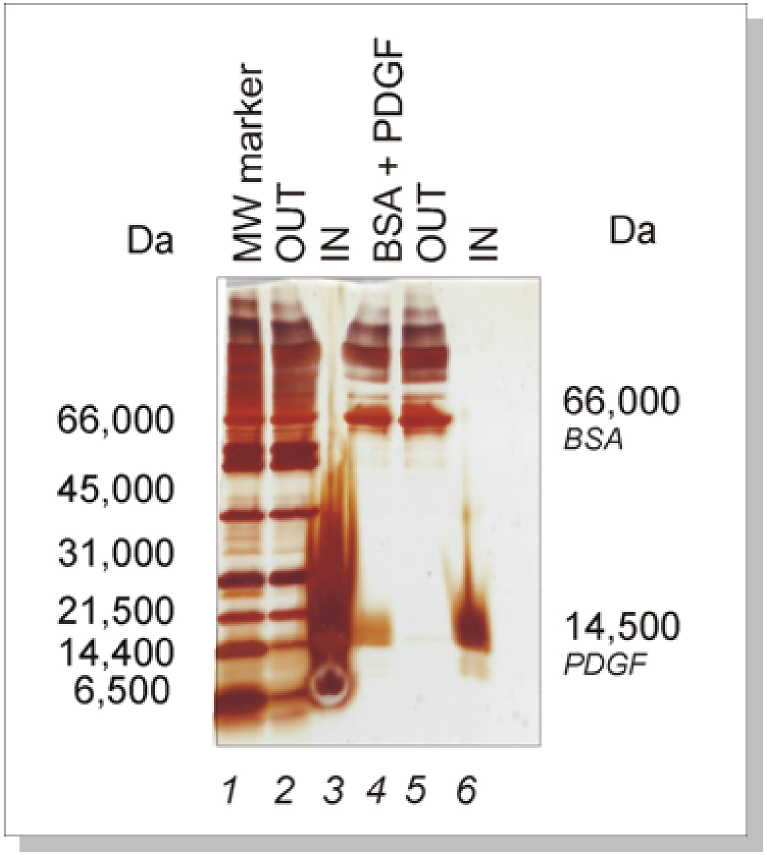


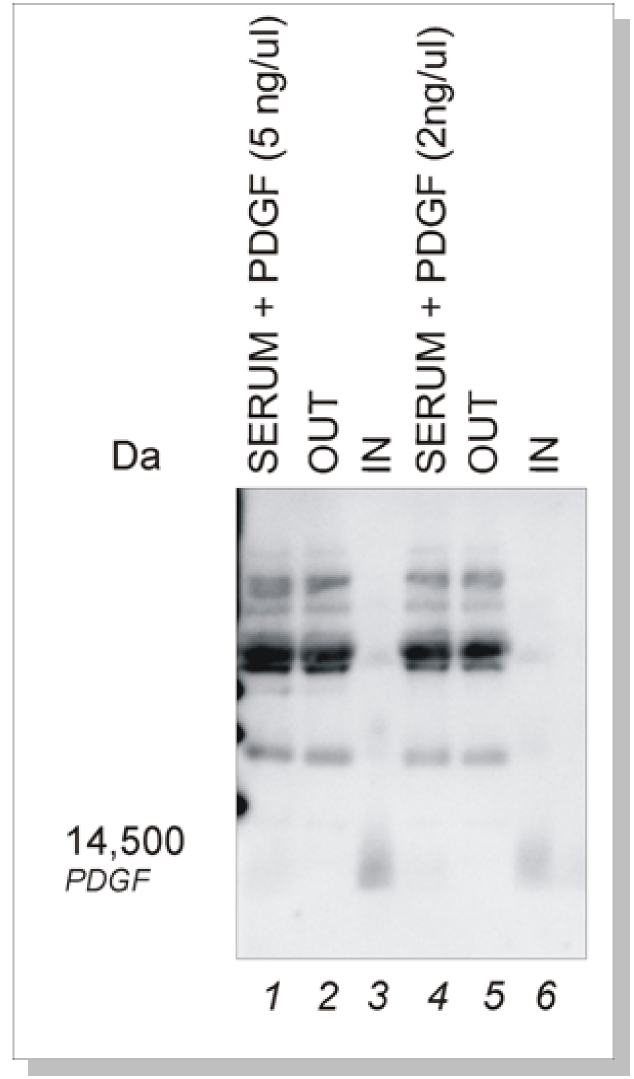
Kinetics of protein uptake – complete with a few minutes



Protein concentration



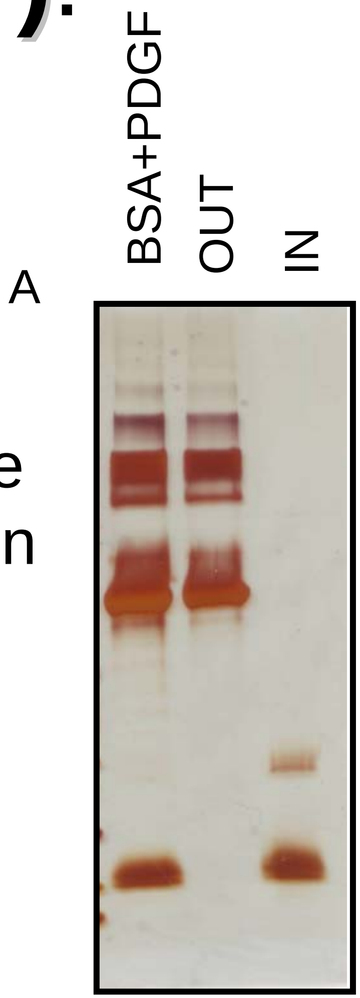


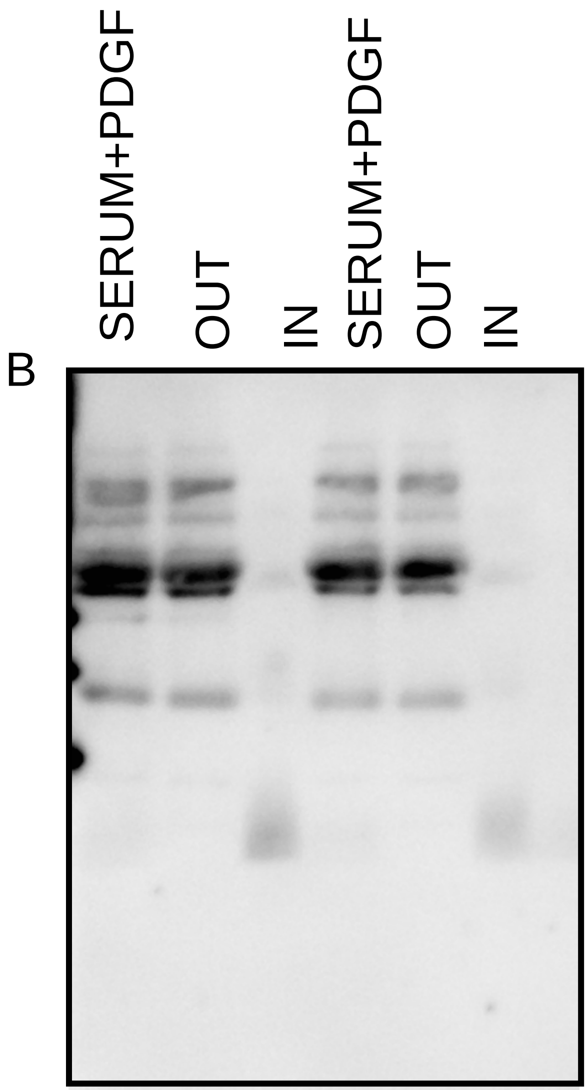


Sequestration and concentration

Platelet derived growth factor (PDGF):

Complete separation from albumin





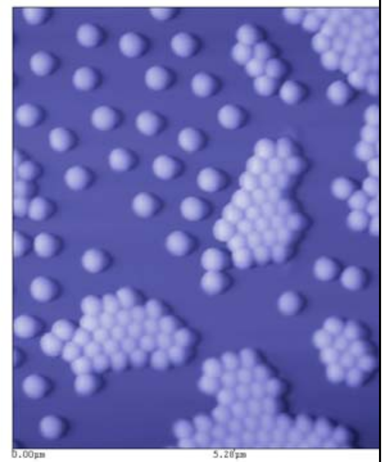
Particles make PDGF detectable in human serum by Western Blot

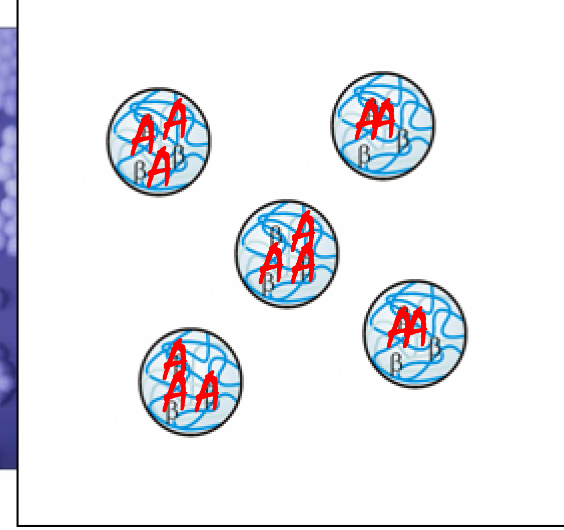
Conclusions

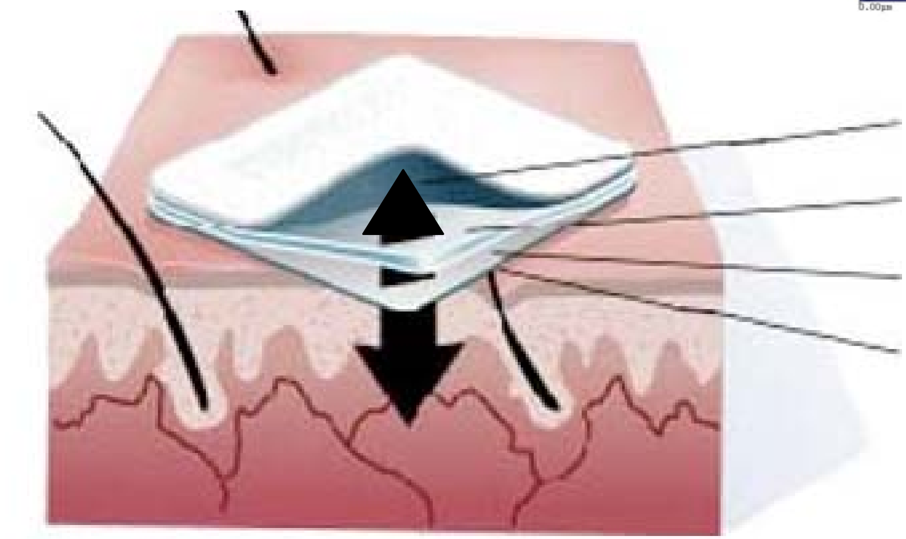
- Smart hydrogel perform in solution molecular sieving based on protein size
- Uptake is very fast (less than 10 minutes)
- Using a charge bait (acrylic acid) increases the concentration of sequestered low molecular weight proteins
- Particles protect harvested proteins from enzymatic degradation
- Particles remove PDGF from albumin carrier and concentrate recombinant PDGF spiked in human serum

Smart Nanoparticles for Biomarker Harvesting

Example application to skin patch for diagnostic marker (proteins and metabolites) harvesting







- User friendly non invasive
- Amplifies low abundant markers over time of patch duration
- Protects biomarkers from degradation
- Mail-in room temperature shipping

Example BrCa Phospho-protein Markers

- **J Clin Oncol** 2008 Jan 22: Johnston et al. Phase II study of predictive biomarker profiles for response to HER-2 in advanced inflammatory breast cancer with Lapatinib monotherapy.
  - **Finding: Tumors expressing pHER-2 and pHER-3 were more likely to respond to lapatinib (9/10 versus 4/14)**
- **In Vivo** 2007 (21(6): 967-72 Magkou et al. A IH evaluation of phosphorylated Akt at threonine 308 in invasive breast cancer. N=152
  - **Finding: Tumors expressing p308Akt were positively associated with HER-2 ($p<0.005$), Apoptosis (p53) ($p<0.020$), proliferation Ki-67 ($p<0.013$)**

Breast Tissue: Labile endpoints stabilized by kinase plus phosphatase inhibitor