第35回　日本神経科学大会　ランチョンセミナー

蛋白質の翻訳後修飾解析の前処理から FFPEサンプル解析まで

エーエムアール株式会社

1. 脱リン酸化を防ぐ前処理からリン酸化サンプル濃縮技術

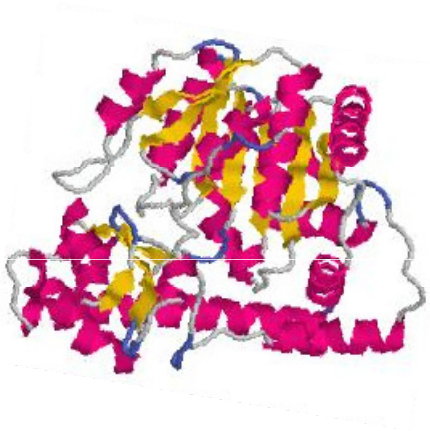
三上紗弥香（エーエムアール株式会社　アプリケーション）

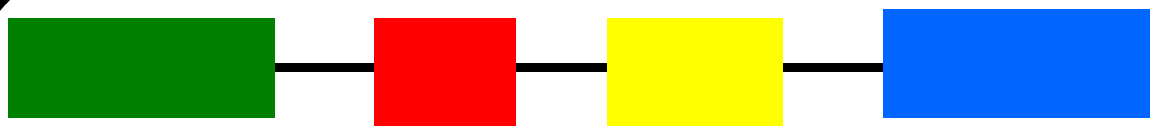
2. ホルマリン固定組織からのプロテオーム解析の実際

福田哲也（株式会社バイオシス・テクノロジーズ ）

サンプルの保存技術について

Protein Variants



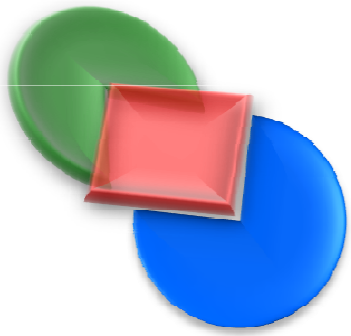


Pre-mRNA

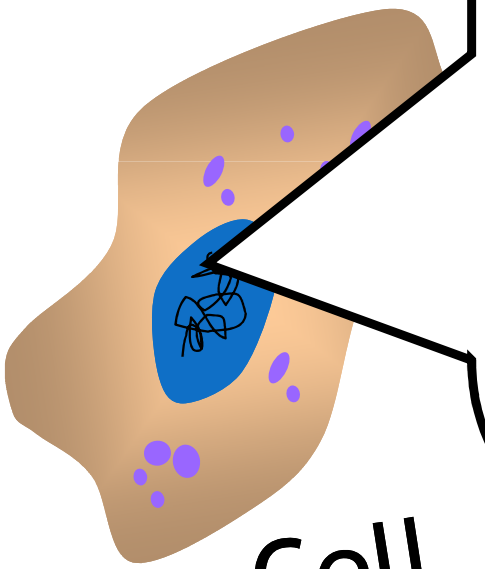
Alternative splicing

mRNA

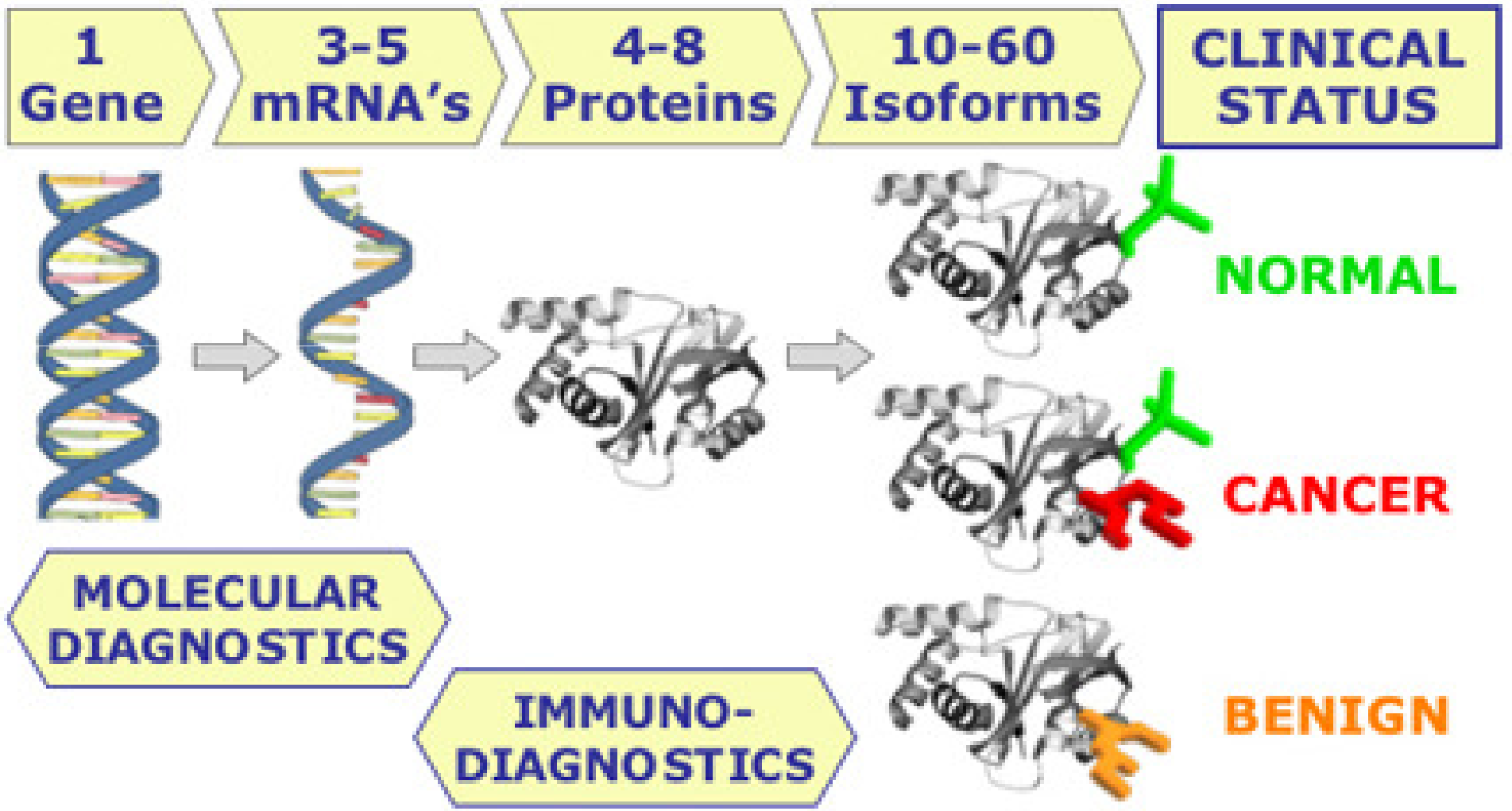
translation



Protein Variants(Isoforms)



Cell



Qualitative and Quantitative LC/MS

ISOFORMS: KEY TO PROTEIN FUNCTION AND DIAGNOSTIC UTILITY

Example of the Clinical Relevance of Protein Isoforms in Cancer

Diagnosis : PSA isoforms

Staging : CD44v3 for breast cancer

Treatment : Specific Tubulin isoform - Ineffective for Chemo therapy

Systemic effects : Myosin Isoforms - Muscular Atrophy in cancer patients

Drug toxicity : Patient specific Drug Toxicity – Cytochrom p-450 isoforms

[1]Mikolajczyk et al. (2004), Clin. Biochem. 37:519-528.
[2]Rys, et al. (2003), Pol. J. Pathol. 54, 243-247.
[3]Dozier et al, (2003), Breast Cancer Res., 5:R157-69.
[4]Nakagawa et al. (1997), J. Urol., 157:1260-1264.
[5]Diffee et al. (2002), Am. J. Physiol. Cell Physiol., 283:C1376-82.
[6]Piver et al. (2004), Biochem. Pharmacol., 68:773-782.

denator

Biological challenge – revealing the true *in vivo* profile

生体サンプルのin vivo プロファイルを得るには？

- Proteins and peptides degrade/change rapidly post-sampling due to endogenous enzymatic activity
- Degradation products interfere with analysis such as 2D-gels, mass spectrometry or Western blot
- Results not representative of *in vivo* state

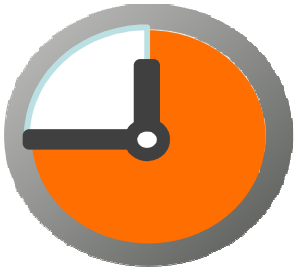
Post sampling changes in samples

生体サンプルのサンプリング後の変化

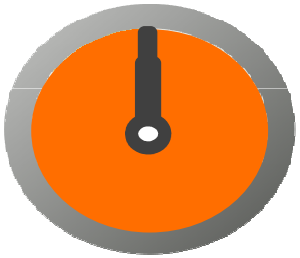
"The sample is alive"

- 15 seconds
  - 25-50% less ATP, ↓
  - 50% less glucose, ↓ 50% more lactate ↑

- 45 seconds
  - 75% less glucose, ↓ 150% more lactate ↑



- 1 min
  - 50% less ATP ↓
  - pH drops due to lactate accumulation ↓
  - 100% less glucose, ↓ 200% more lactate ↑
  - NA/K ATPase stops working
  - K-depolarisation
  - Cytoslic Ca increase



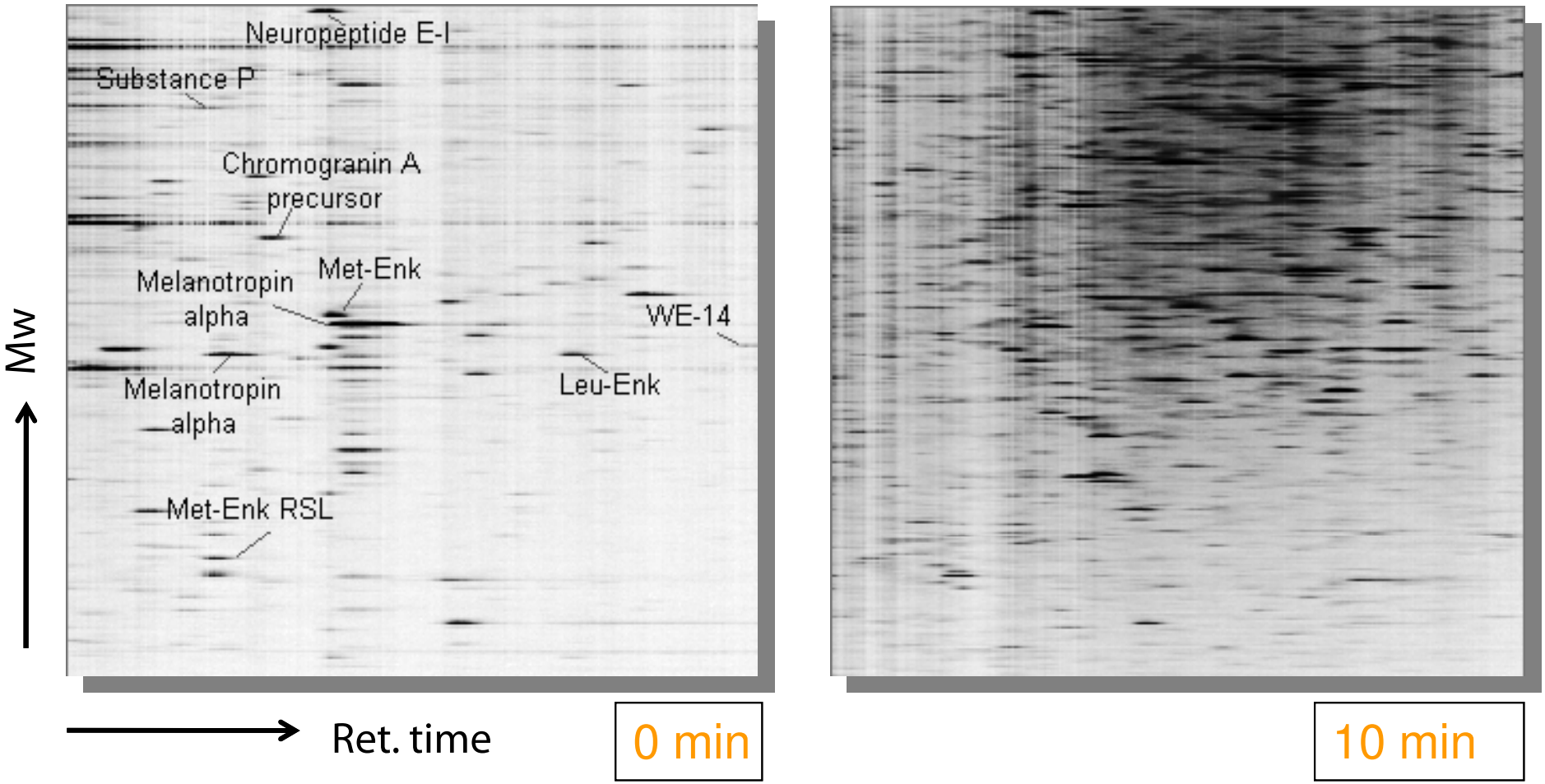
Switch from aerobic to anaerobic metabolism

好気的代謝から嫌気的代謝にスイッチ

denator

Protein degradation -> ***peptides*** *post sampling*

蛋白質の　デグラデーション

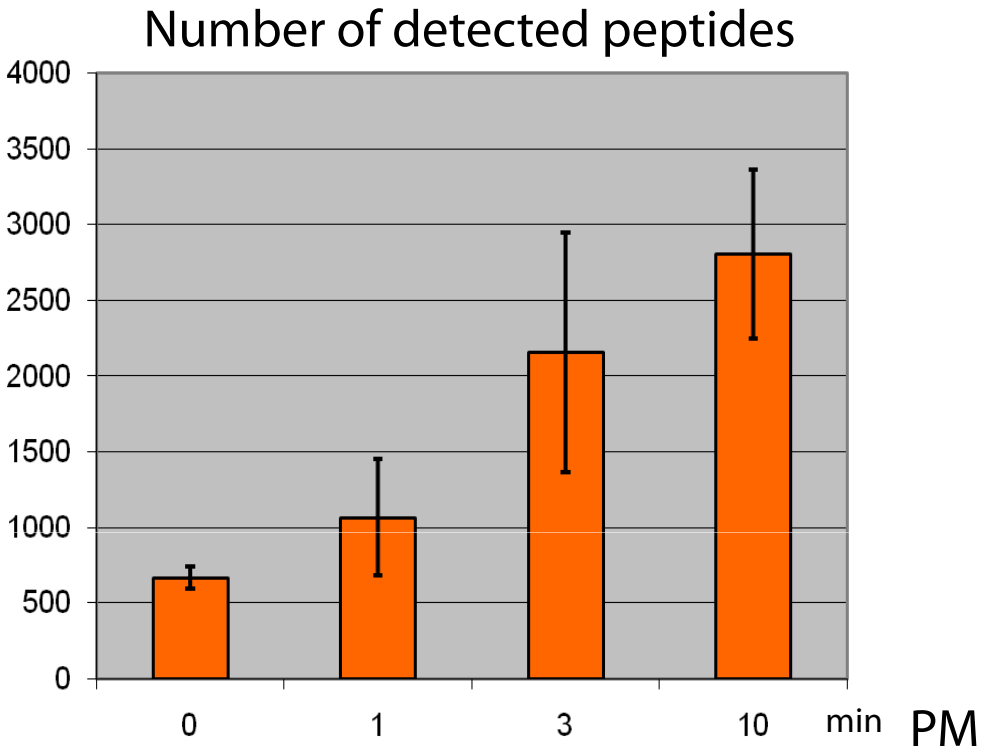


Source: Sköld, et al., *Proteomics 2007, 7, 4445–4456*

denator

Degradation

- Rapid increase of peptides -> the result of degradation
- "New" peptides are fragments from high abundant proteins

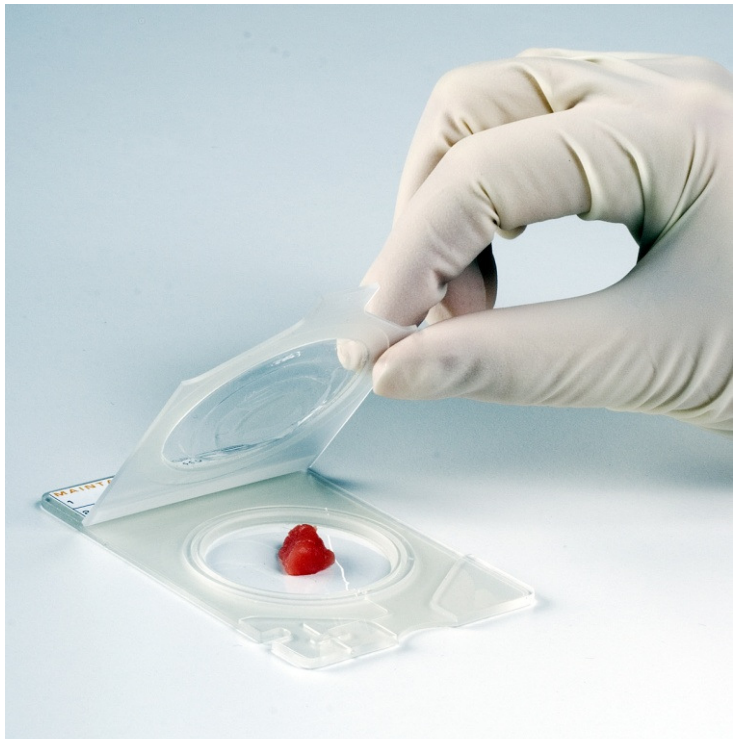


Sköld, et al., Proteomics 2007, 7(24), pp 4445

Stabilization thru Thermal Denaturation

熱による蛋白質の安定化

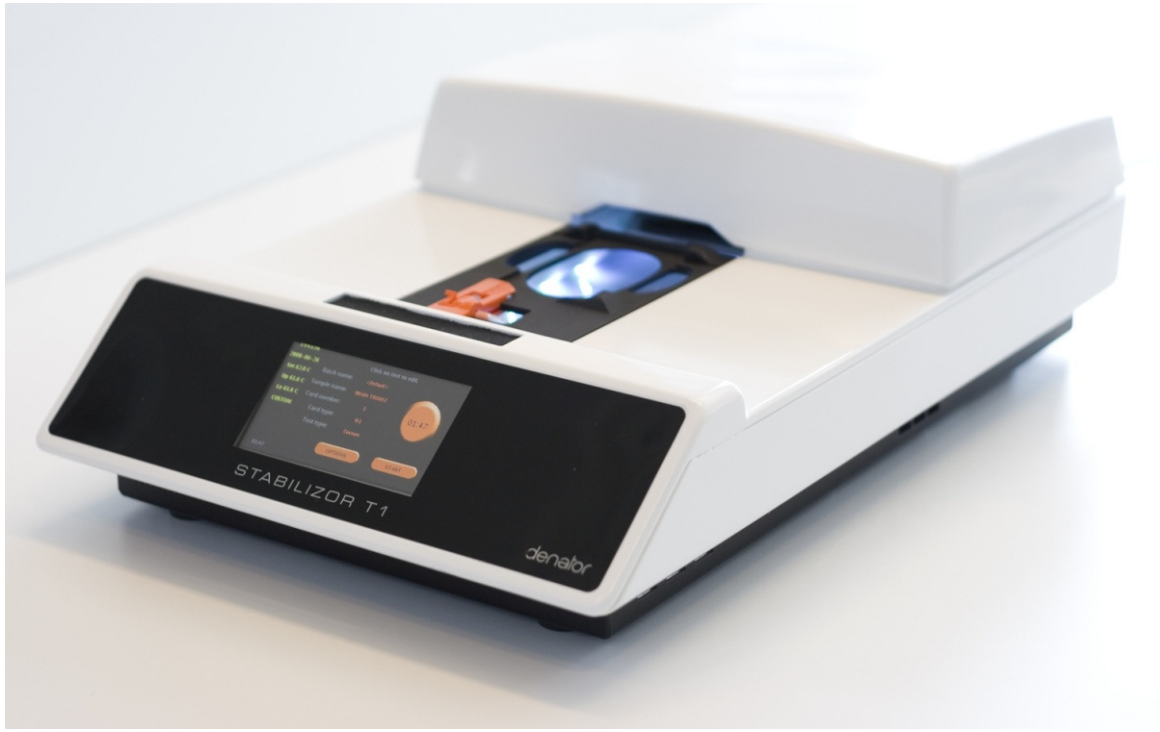
Maintainor®Tissue



Treatment/Storage

処理と保存

Stabilizor®T1

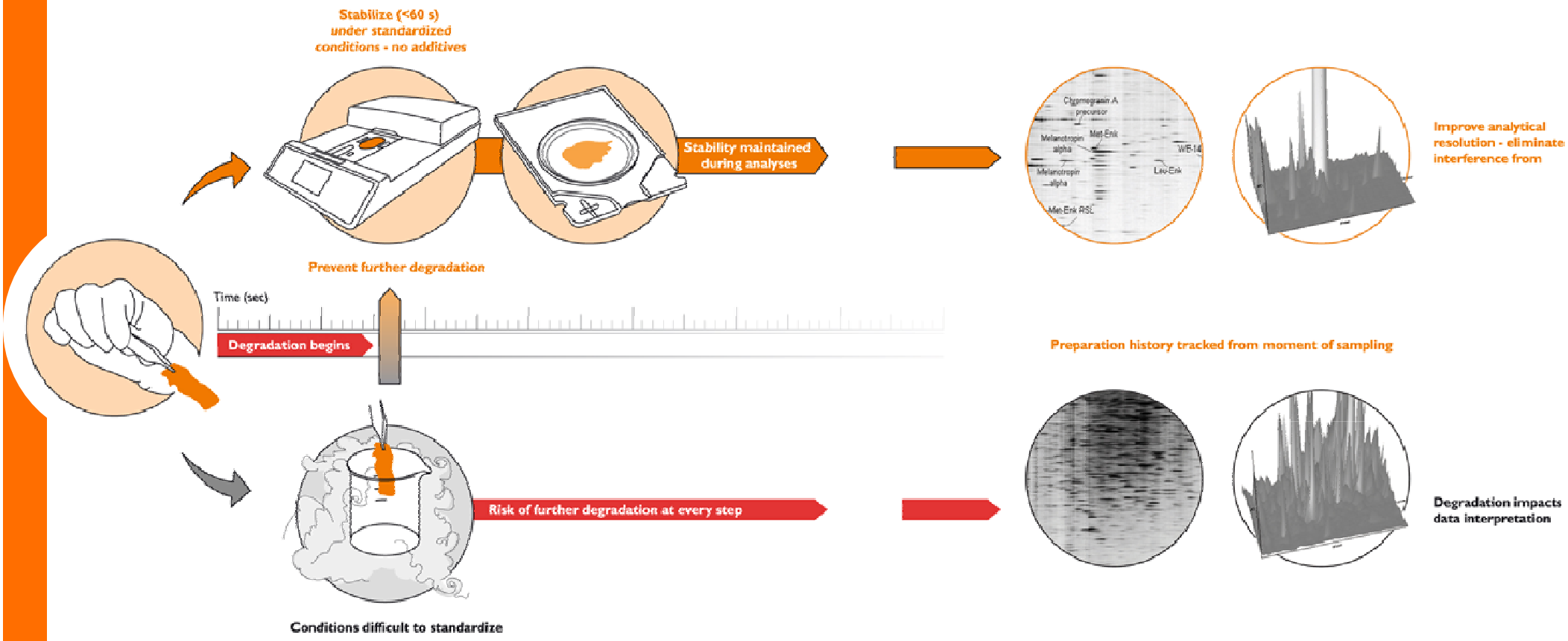


Stabilization

安定化

Stabilizor workflow

安定化処理のワークフロー

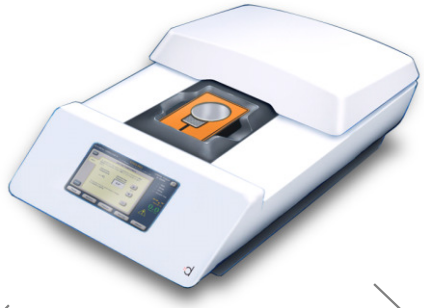


Multiple downstream applications

処理後のアプリケーション

MD LCMS

MALDI MS Imaging



ELISA

2D gels

LCMD

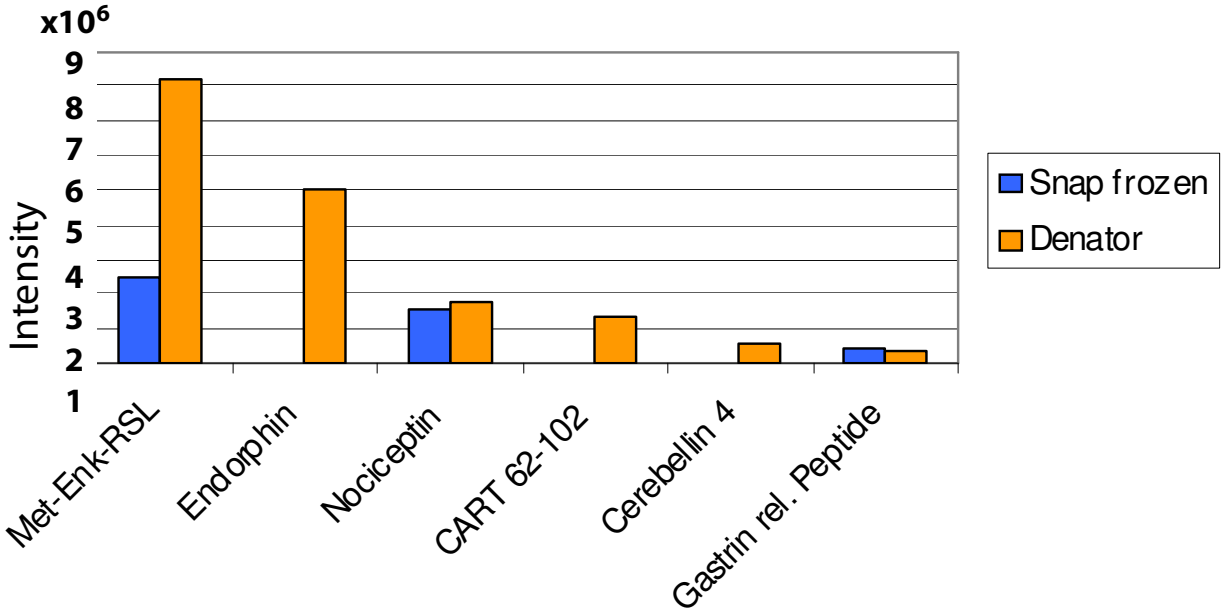
Western blot

LCMD-Laser Capture Micro Dissection

Peptidomics: Discovery and potential drugs

ペプチドミクス

- Bioactive peptides as potential drugs
- Peptides of interest not seen when using conventional sample preparation
- *"Several potentially bioactive peptides where found when Stabilizor was used"*



Data courtesy: Sanofi-Aventis, France

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Post translational modifications

翻訳後修飾

- **Phosphorylation**

  リン酸化修飾

- **Sumolation**

  Sumo化

denator

nature COMMUNICATIONS

OPEN

ARTICLE

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DOI: 10.1038/ncomms1871

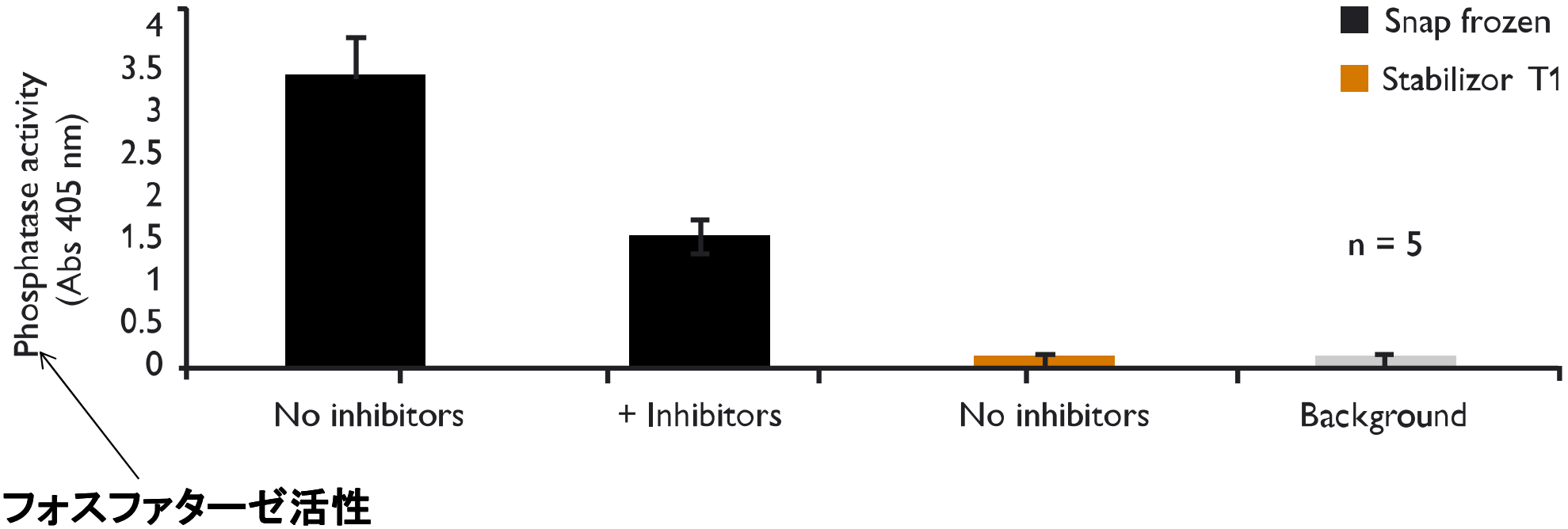
Quantitative maps of protein phosphorylation sites across 14 different rat organs and tissues

Alicia Lundby[1,2,*], Anna Secher[1,3,*], Kasper Lage[1,4,5,6], Nikolai B. Nordsborg[7], Anatoliy Dmytriyev[1], Carsten Lundby[8] & Jesper V. Olsen[1]

Deregulated cellular signalling is a common hallmark of disease, and delineating tissue phosphoproteomes is key to unravelling the underlying mechanisms. Here we present the broadest tissue catalogue of phosphoproteins to date, covering 31,480 phosphorylation sites on 7,280 proteins quantified across 14 rat organs and tissues. We provide the data set as an easily accessible resource via a web-based database, the CPR PTM Resource. A major fraction of the presented phosphorylation sites are tissue-specific and modulate protein interaction networks that are essential for the function of individual organs. For skeletal muscle, we find that phosphotyrosines are over-represented, which is mainly due to proteins involved in glycogenolysis and muscle contraction, a finding we validate in human skeletal muscle biopsies. Tyrosine phosphorylation is involved in both skeletal and cardiac muscle contraction, whereas glycogenolytic enzymes are tyrosine phosphorylated in skeletal muscle but not in the liver. The presented phosphoproteomic method is simple and rapid, making it applicable for screening of diseased tissue samples.

Inactivation of phosphatases

フォスファターゼの不活化



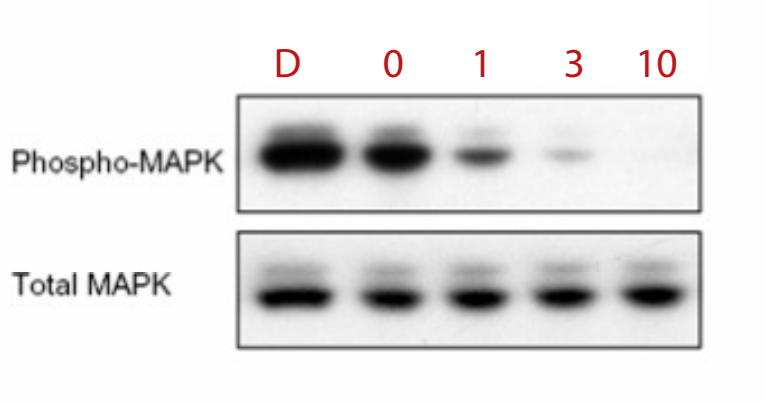
フォスファターゼ活性

Svensson et al., Journal of Proteome Research 2009, 8(2), pp 974–981

Phosphorylations change rapidly

リン酸化の迅速な変化

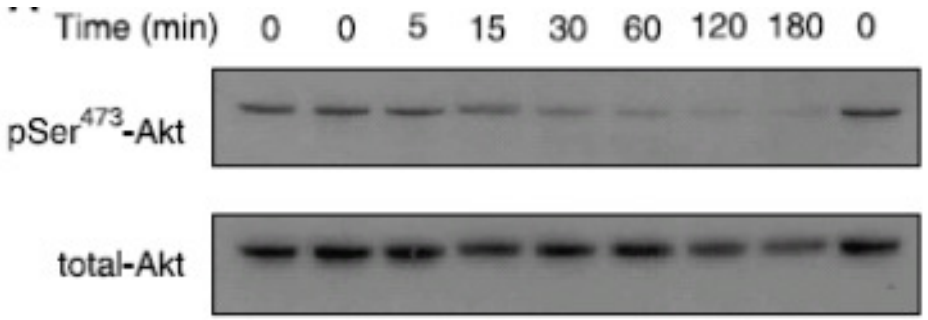
Western Blot – Brain tissue



Western Blot

Source:
Sköld, et al., Proteomics 2007, 7(24), pp 4445

Western Blot – HT29 Xenografts



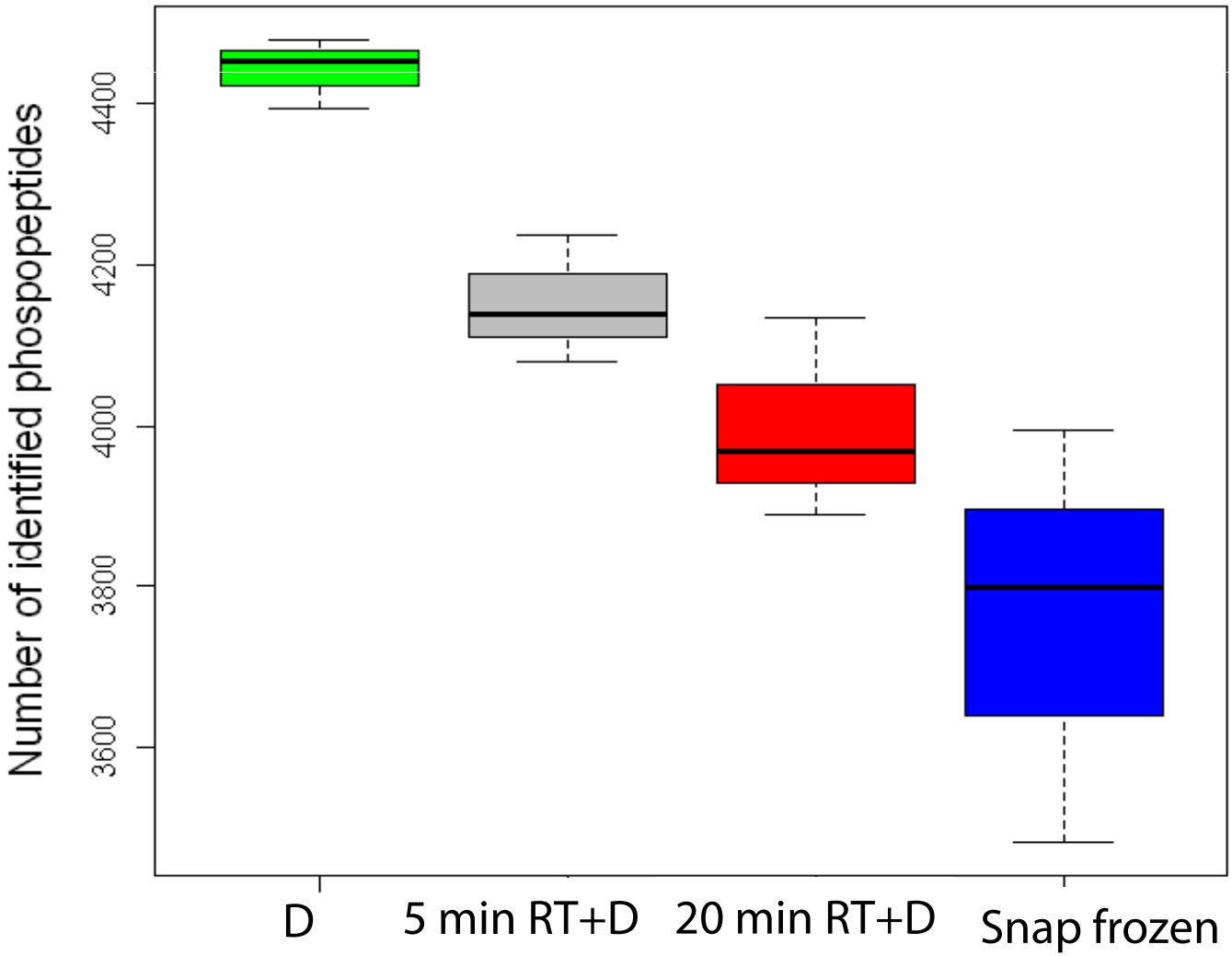
Source:
Baker et al., Clin Cancer Res 2005, 11(12), pp 4339

denator

PhosphoShotgun

Number of identified phosphopeptides

リン酸化プロテオミクス　ショットガン解析でのリン酸化ペプチドの同定数

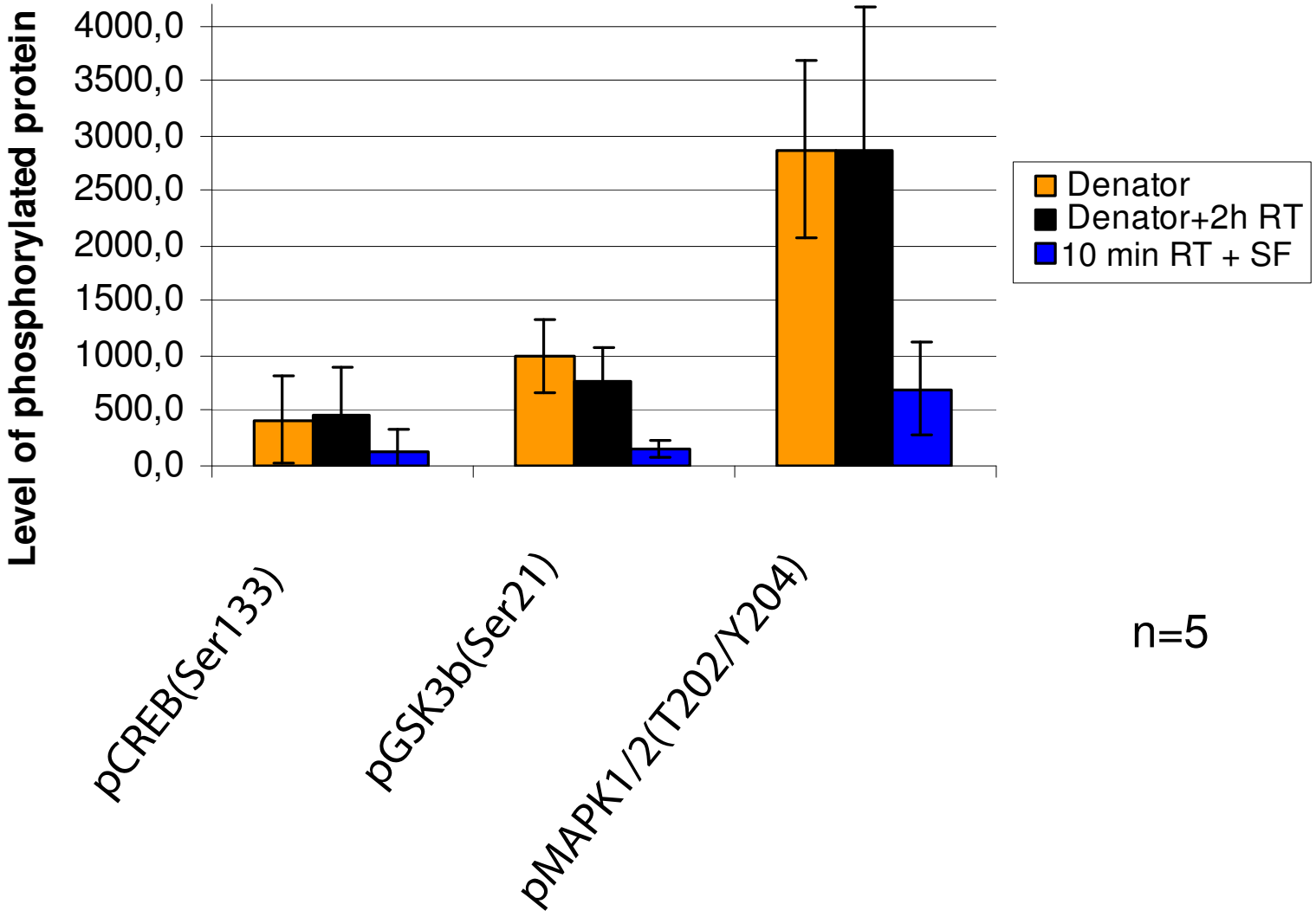


n=3

In collaboration **Jesper Olsson**, Denmark

Phosphorylated proteins – Stability after stabilization

リン酸化蛋白質—処理後の安定性



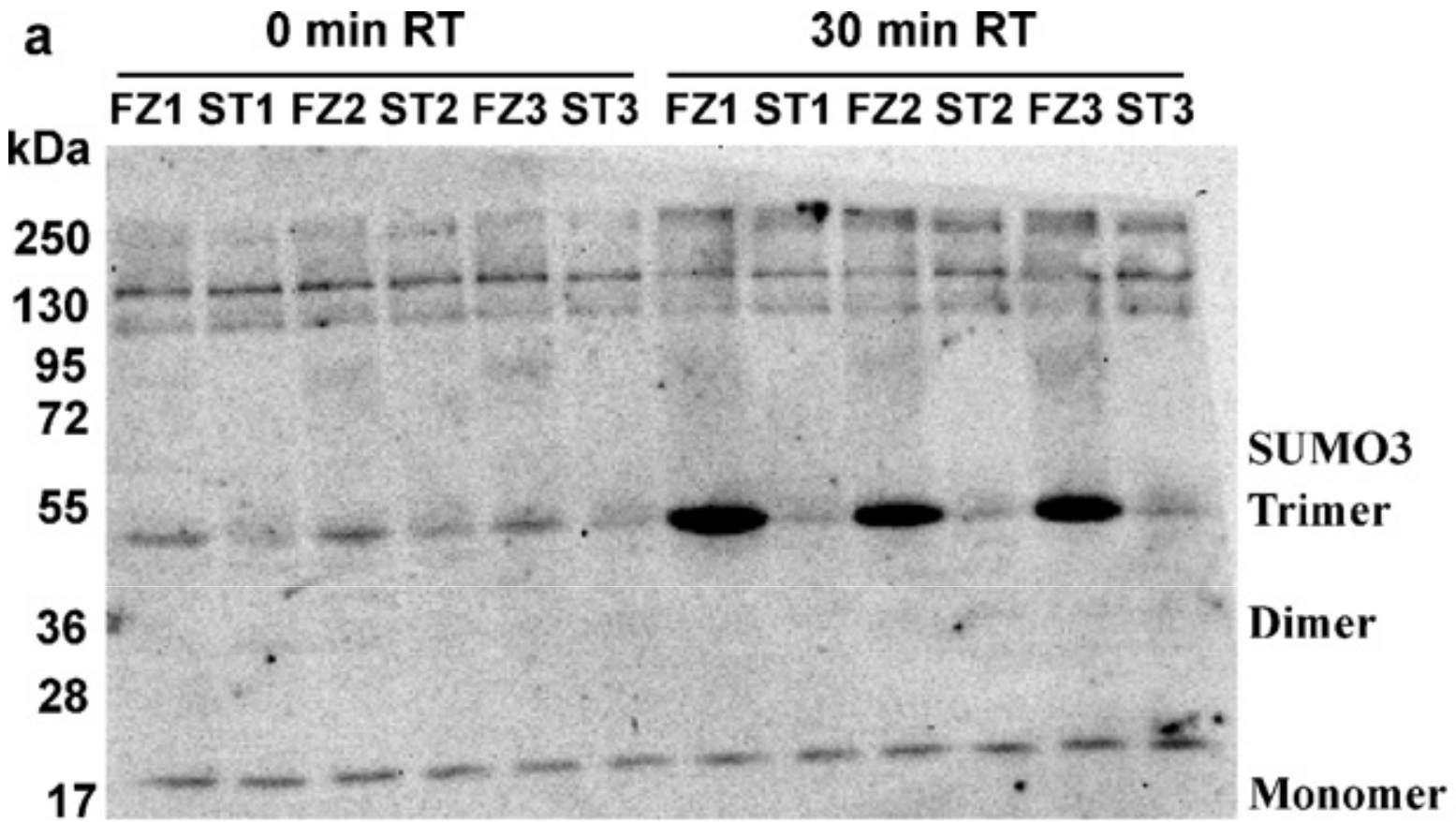
n=5

In collaboration with Prof. Per Svenningsson, Karolinska Institutet

denator

Stabilization of Protein Sumolation

SUMO化蛋白質の安定性

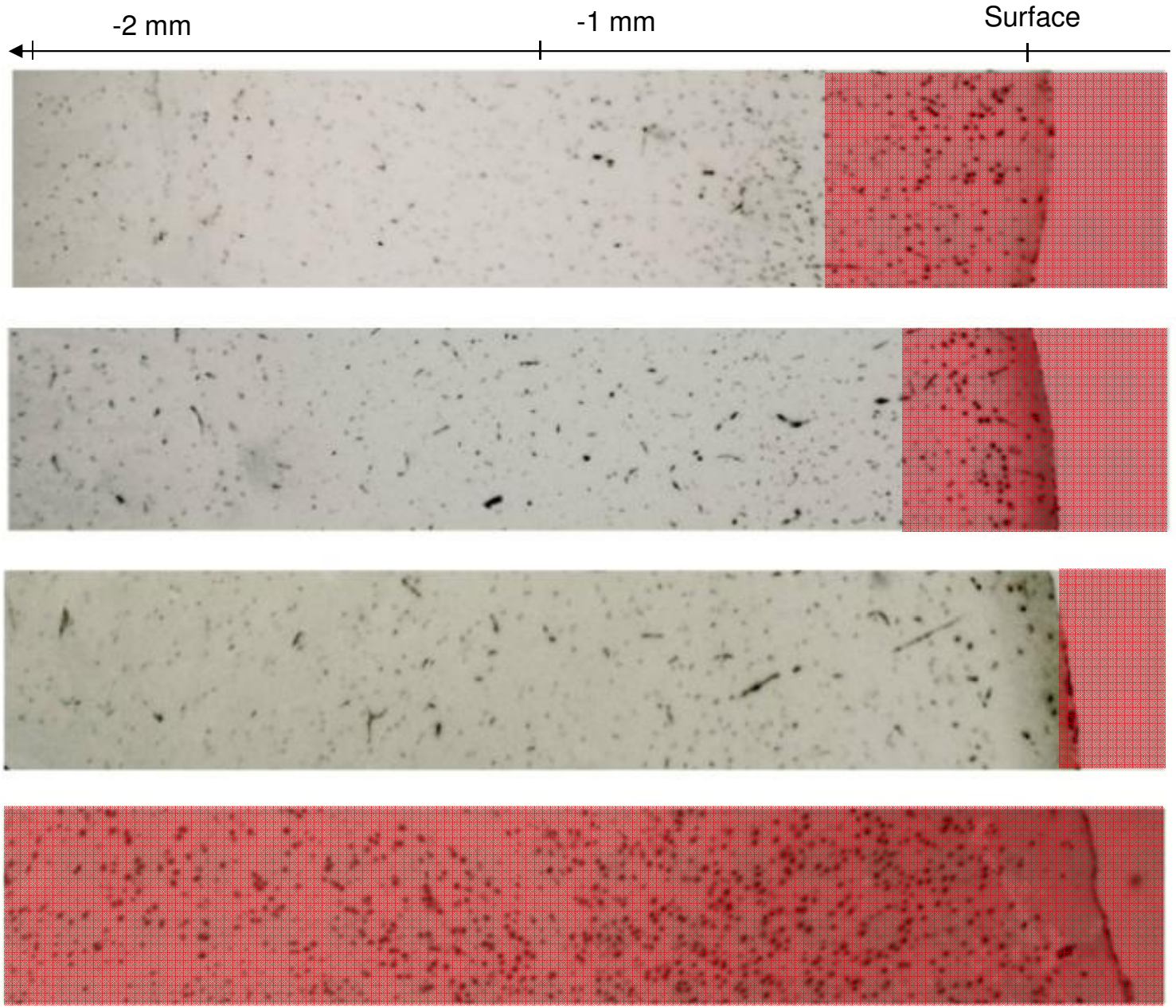


FZ – Snap Frozen ST - Stabilized

Ahmed M, Gardiner KJ. , Journal of Neuroscience Methods. 2011;196:99-106.

denator

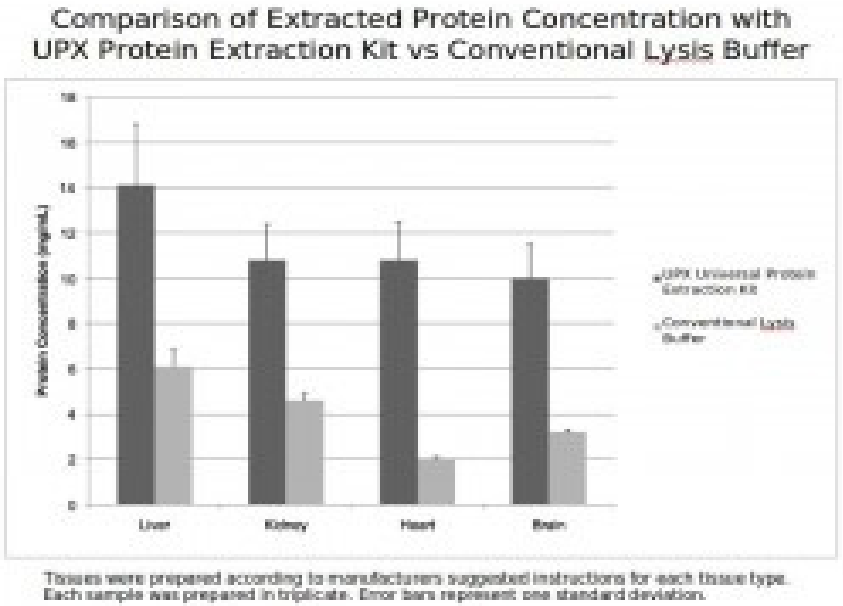
pCREB visualized with IHC from FFPE in coronal sections of mouse brain



サンプルの可溶化

UPX™ Universal Protein Extraction Kit

Extract Membrane Proteins and Soluble Proteins for Mass Spectrometry Analysis



protein discovery

PPS Silent® Surfactant

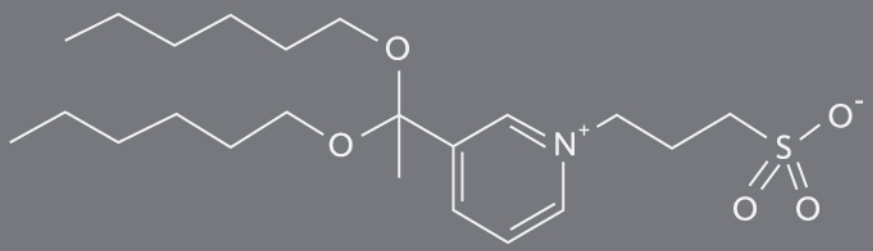
- MS-Compatible Detergent for Solubilizing Membrane Proteins -

PPS SILENT® SURFACTANT

PROTEIN PREP REAGENTS

THE SURFACTANT

Intact PPS Silent Surfactant disrupts cell membranes and solubilizes hydrophobic proteins.

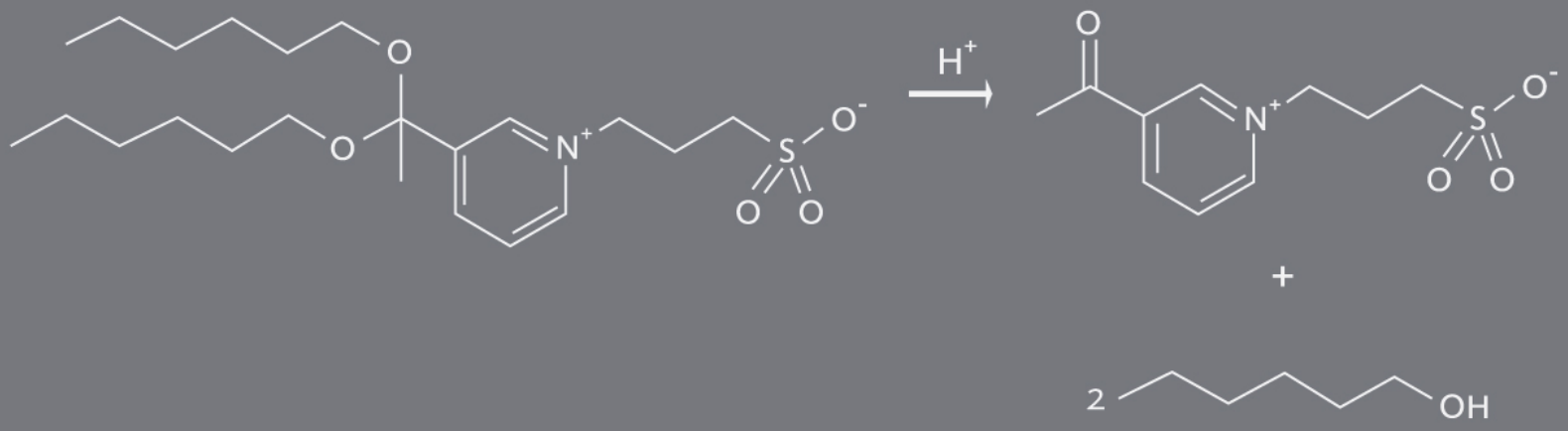


PPS SILENT® SURFACTANT

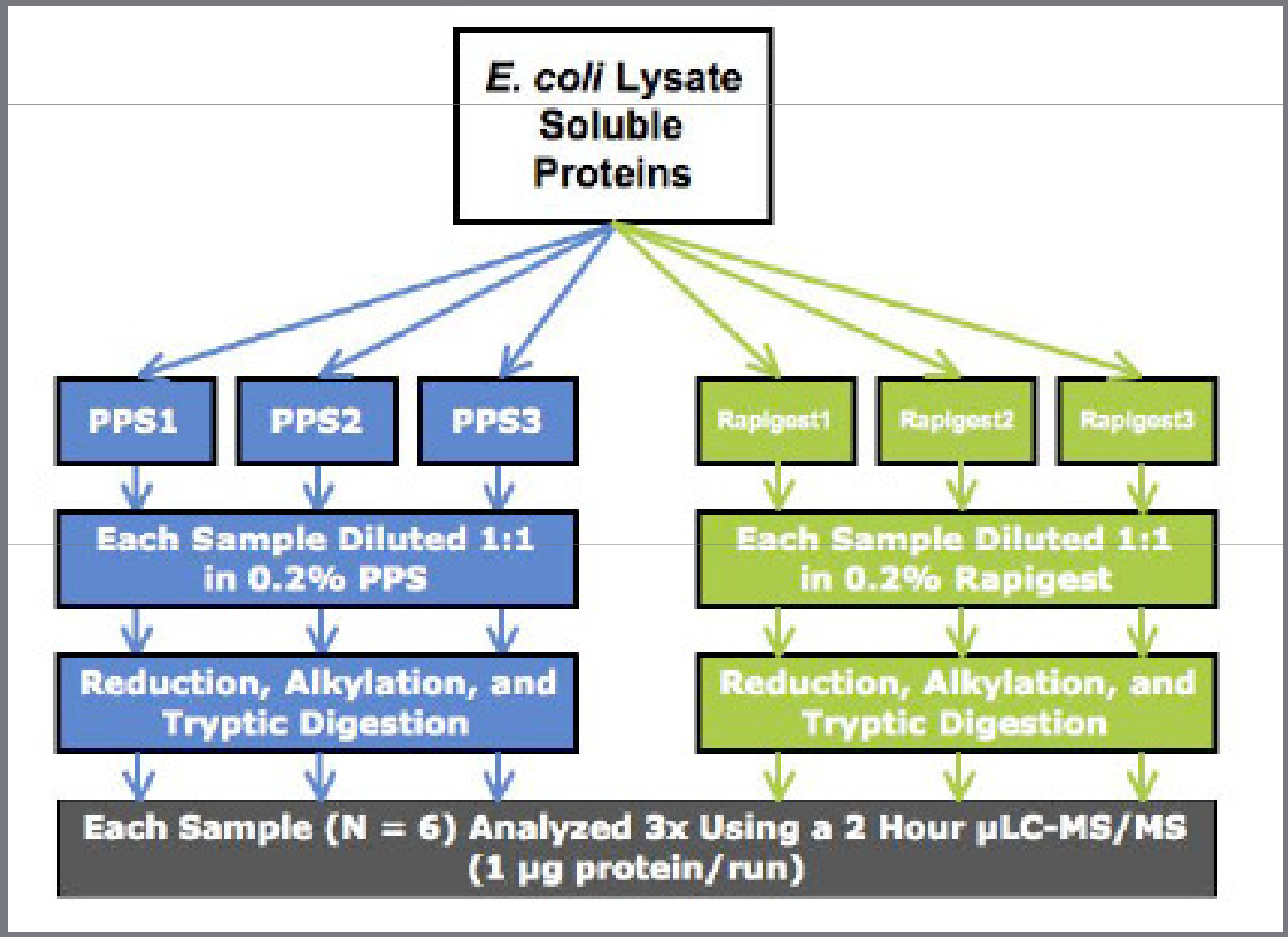
PROTEIN PREP REAGENTS

THE CLEAVAGE

Add acid to reduce the pH and cleave PPS.
The reaction products are soluble and have no surfactant properties.



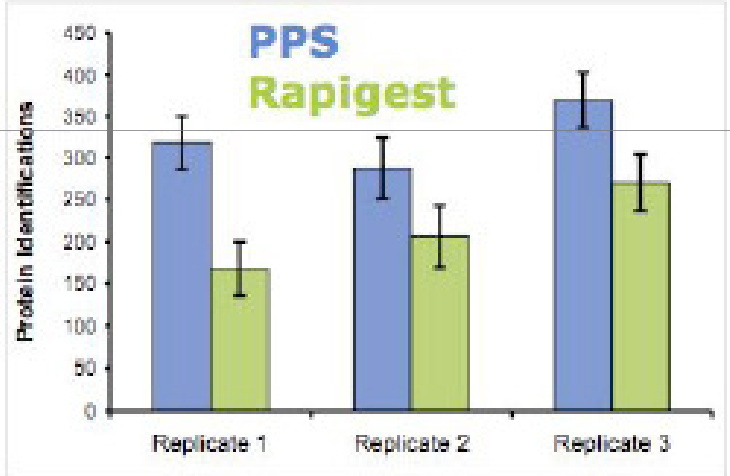
protein discovery



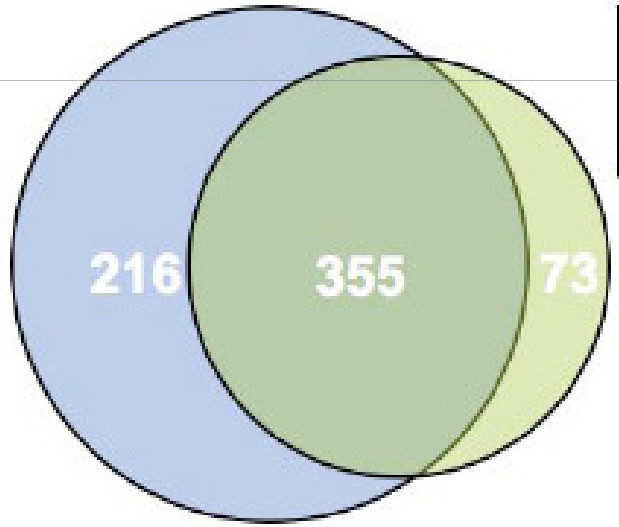
protein discovery

PPS Silent™ Surfactant shows an increase in the overall number of proteins identified from *E. coli*.

Results shown are the number of proteins identified in replicate analyses of *E. coli* extract using LC/MS/MS. Proteins were identified with <1% false discovery rate.



Different Cleavable Detergents Provide Complementary Information:



Nonredundant Protein Identifications from 9 PPS Runs and 9 Rapigest Runs

PPS = 571 Protein IDs
Rapigest = 428 Protein IDs
Total = 644 Protein IDs

PPS SILENT® SURFACTANT

PROTEIN PREP REAGENTS

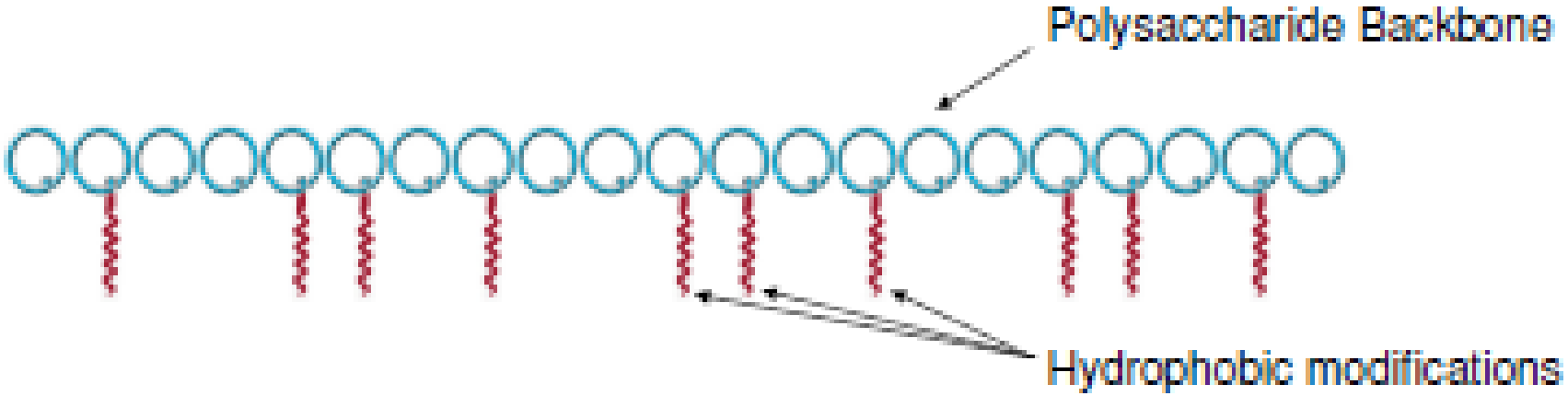
ADVANTAGES

- Improves MS analysis of complex protein mixtures
- Disrupts cell membranes
- Solubilizes hydrophobic proteins
- Improves enzymatic digestion
- Cleaves readily at low pH
- Cleavage products have no detergent properties and stay in solution for easy removal

貫通型膜たんぱく質は？

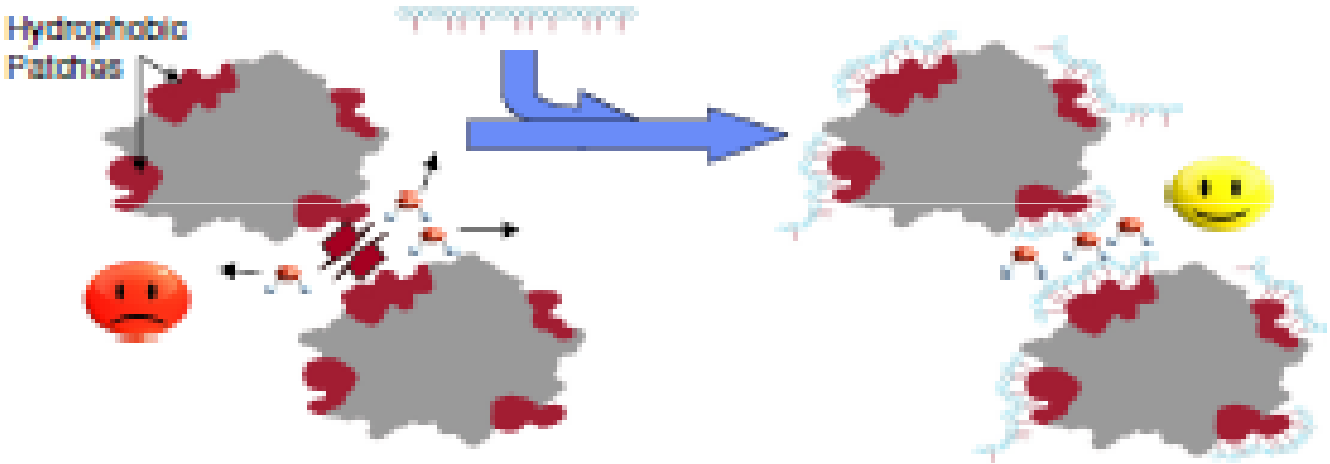
GPCR
トランスポーター

NVoy Technology – What?



- **Specially chosen carbohydrate backbone with**
  - Linear
  - M_w = 5kDa (R_{hyd} ~ 18kDa) => Does not access binding sites
  - Multipoint, regio-specifc modification => Hydrophobic face
  - Uncharged, UV transparent and pH Stable (pH 2 to 11)
- **Dynamic interaction with protein**
  - k_d = 28 µM (GFP) & 12 µM (Hexokinase)
  - is easy to remove from protein sample
  - interaction can be controlled

NVoy Technology – How?

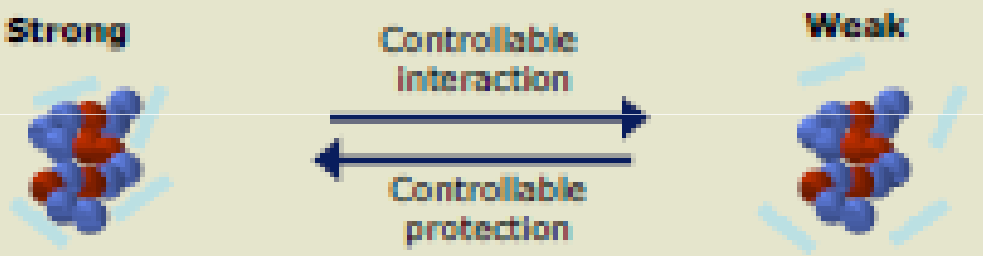


- Hydrophobic interaction: cited as the cause for protein aggregation in ~75% of cases (Key Note presentation at PEGS 2007 meeting)
- NVoy associates with surface exposed hydrophobicity and presents the hydrophobic backbone to the solvent.
- Prevents hydrophobic interaction between the target protein and
  - target proteins => prevents aggregation and promotes a heterogeneous sample
  - process surfaces (resin, membranes, etc.) => increasing yields
  - other proteins => increased purity & yield

NVoy Technology - Overview

Increased protein solubility

- Improved protein stability
- Reduced aggregation
- Process at high concentrations
- Retain protein structure and functionality



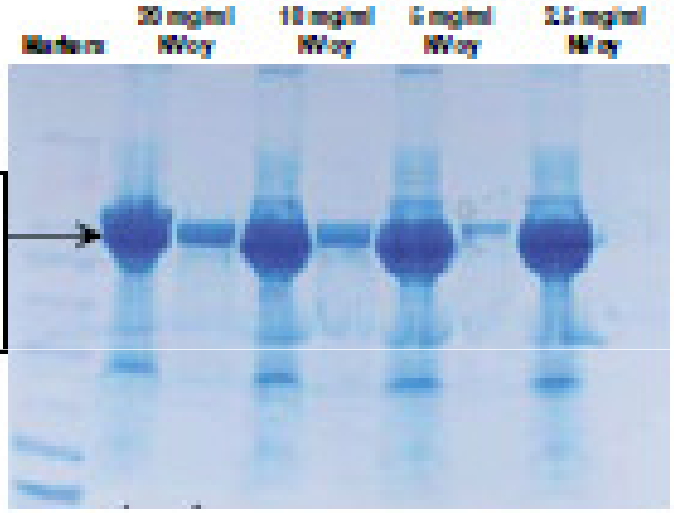
Protein Purification

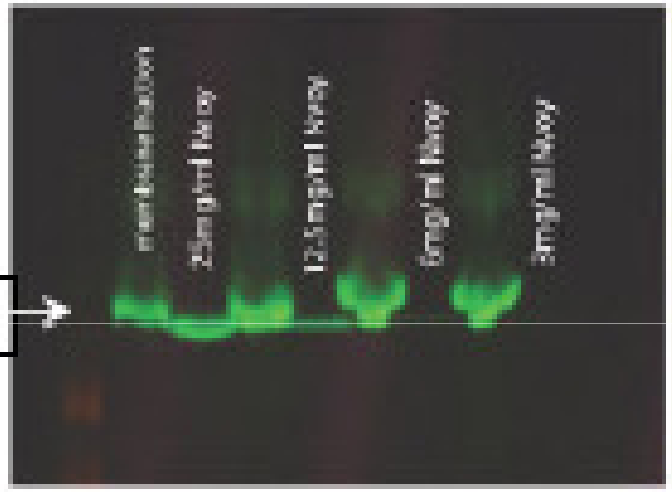
- Reversible binding means polymer is removable
- Higher protein yields due to reduced non-specific binding
- Powerful tool for the removal of endotoxin

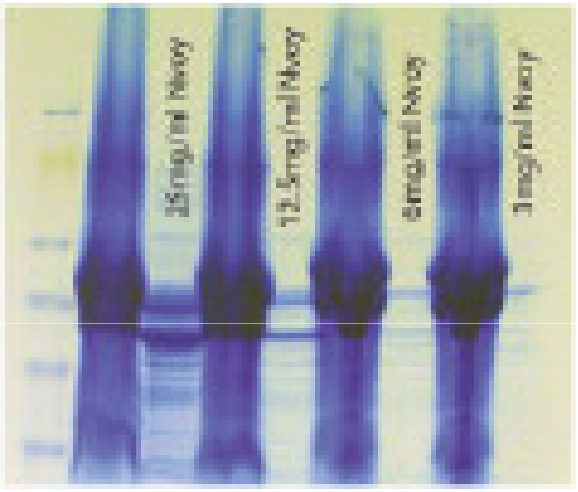
Membrane Proteins

Replacing detergents

- Can be used to completely replace detergents in membrane protein preparation
- Optimal NVoy concentration for extraction is 15 mg/ml although up to 25 mg/ml has been used.







Protein Purification

Increase Purity & Stability

- Large membrane associated protein (>160kDa) that requires co-factors for activity

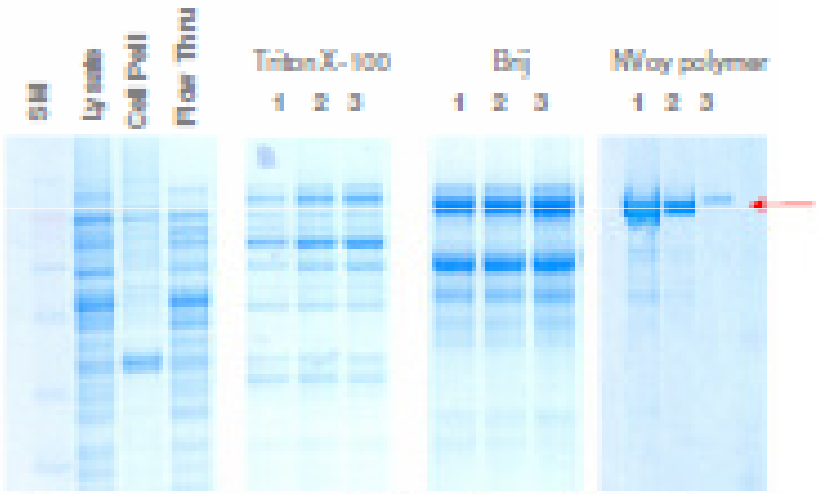
Using Detergents;

results in unstable enzyme preparations containing large number of contaminants

- At 4ºC loss of activity within 24h
- At -80ºC after one freeze/thaw cycle

Using NVoy Polymer;

- Cleaner protein preparations obtained
- Enzyme activity stable at 4ºC for over 4 weeks
- Enzyme activity stable at -80ºC for 4 freeze/thaw cycles

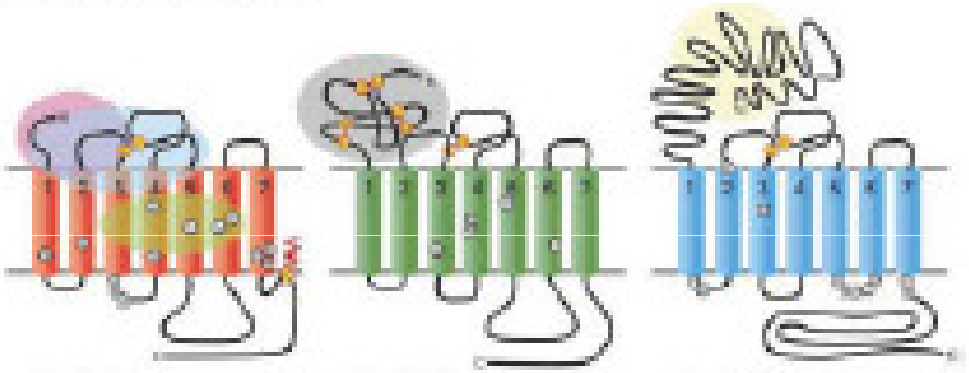


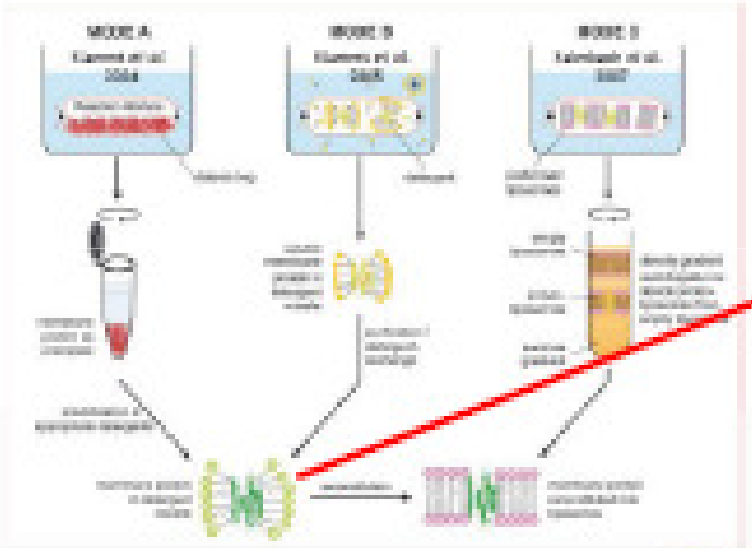
- Enzyme processed from *E. coli* lysates
- Cells cultured at 37ºC, induced with IPTG and cultured for further 72h.
- Cells harvested then lysed with buffer containing co-factors and NVoy polymer / detergents Lysate clarified by centrifugation and purified on ADP Sepharose using NVoy polymer

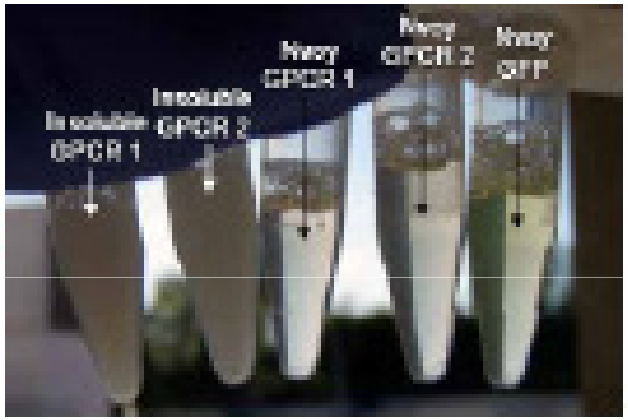
Membrane Proteins

Cell Free Biosynthesis of GPCR's

- Family A and B GPCR's expressed CF Conventionally and with NVoy
- Found that NVoy works as in Mode B
- Concluded that NVoy does not interfere with CF expression
- NVoy enables soluble expression of active GPCR's (>10 nM ligand affinity)



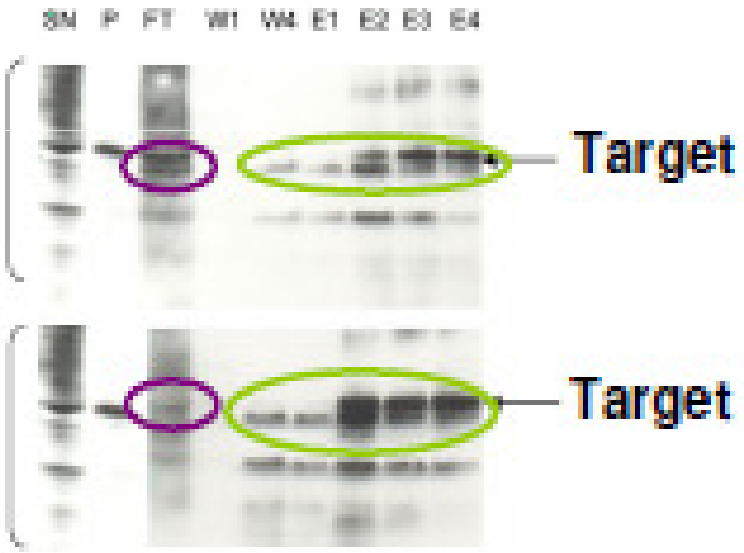




Protein Purification

Increased Yields & Recovery

- Transcription Factor
- Batch Binding to magnetic beads
- Improved binding to the beads
  - less target in Flow Through
  - higher recovey & yields

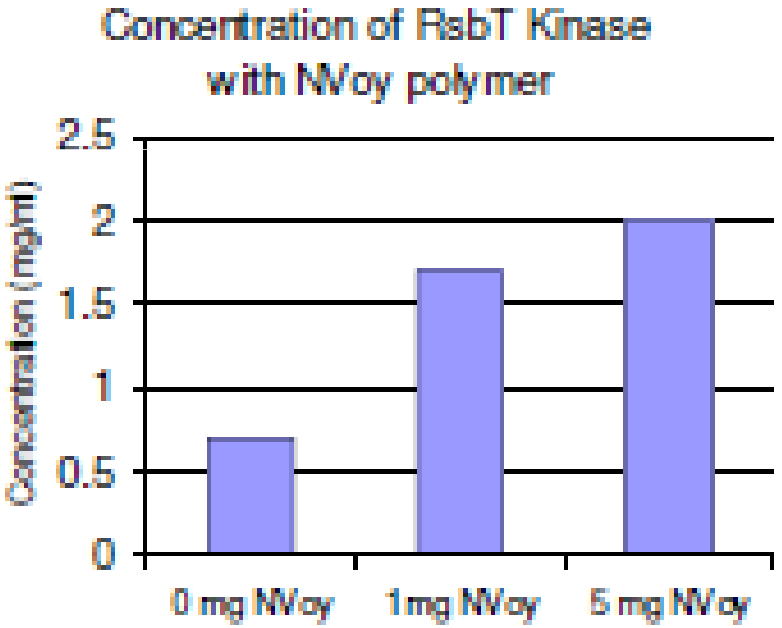


Data by courtesy of Dr Ana Villegas-Mendez, HumProTher Laboratory, France

Ultrafiltration

Improving protein recovery

- 4 kDa RsbT protein kinase
  - Purified 2 mg at 0.2 mg/ml
  - Wanted 10x concentrated (i.e. 10 ml -> 1 ml final)
  - Only soluble to 0.7 mg/ml
- + NVoy
  - Dissolved solid NVoy in protein solution
  - Achieved 10x Concentration
  - Effective at low concentration
  - Optimum ratio: 2:1 – 5:1



Data by courtesy of Institute for Cell and Molecular Biosciences Faculty of Medical Sciences Newcastle University Newcastle-upon-Tyne

翻訳後修飾解析

リン酸化蛋白質

Novel Nanotechnologies for Cancer Research and Drug Discovery

Need for new technologies for phosphorylation detection and identification

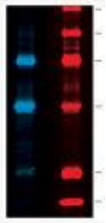
TYMORA

Current phosphoprotein detection methods have limitations

1) Antibodies
   - pTyr are most effective for general detection
   - Difficult to find, expensive

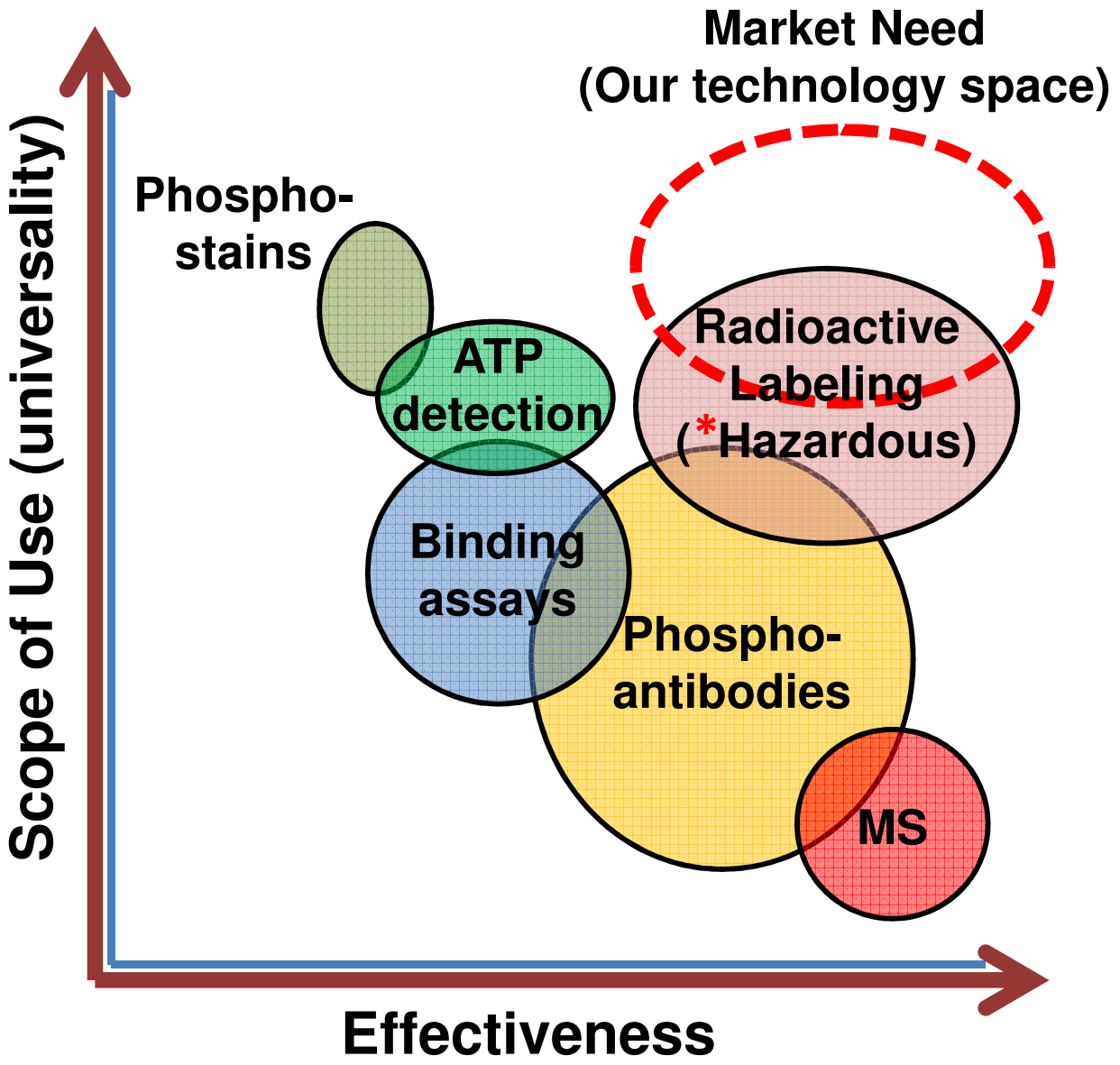
2) ^{32}P labeling
   - Radioactive
   - Not easy to use for *in vivo* phosphorylation

3) Phosphoprotein stains
   - Effective typically for in-gel staining



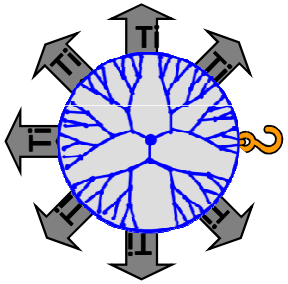
4) Mass spectrometry
   - Low stoichiometry of phosphorylation

Phosphorylation assays category map

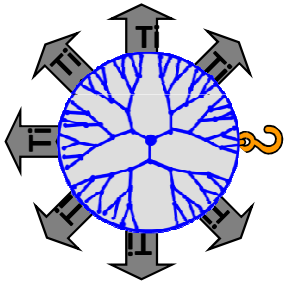


Tymora nanotechnology products

PolyMAC

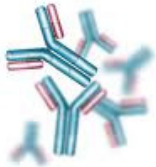


pIMAGO

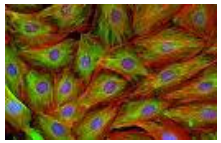


Application

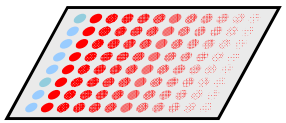
Mass Spec Analysis



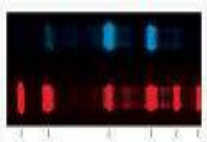
ELISA Assay



Imaging



Kinase Screen



Western Blotting

For enrichment of phosphorylated proteins

For detection and quantitation of phosphorylation

Nanotechnology platform

Foundation

Soluble Nanopolymer (dendrimer)

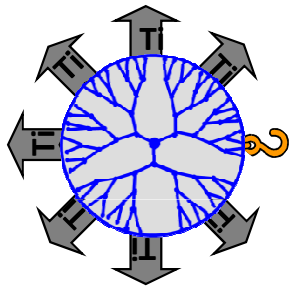
Nanoscale size offers numerous advantages

Greatly reduces test result variability

Multi-functionalized (Multiple groups)

Higher Signal Strength (Sensitivity)

Greatly Reduces Off-target Effects (Selectivity)



PolyMAC
&
pIMAGO

Introduction to two novel technologies

1. PolyMAC – for phosphopeptide enrichment

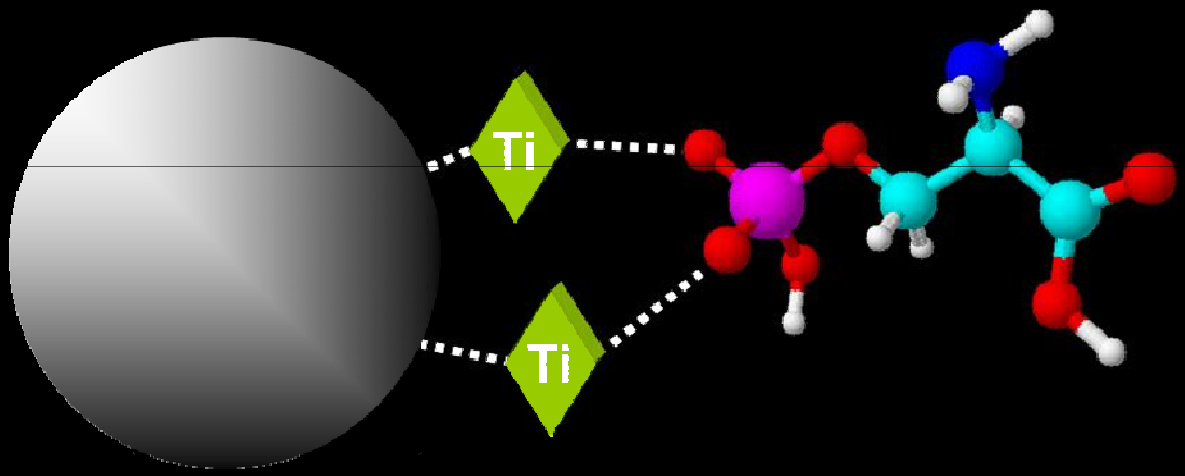
   Goal – To develop a phosphopeptide enrichment method with better selectivity, reproducibility and recovery

2. pIMAGO – for phosphoprotein detection

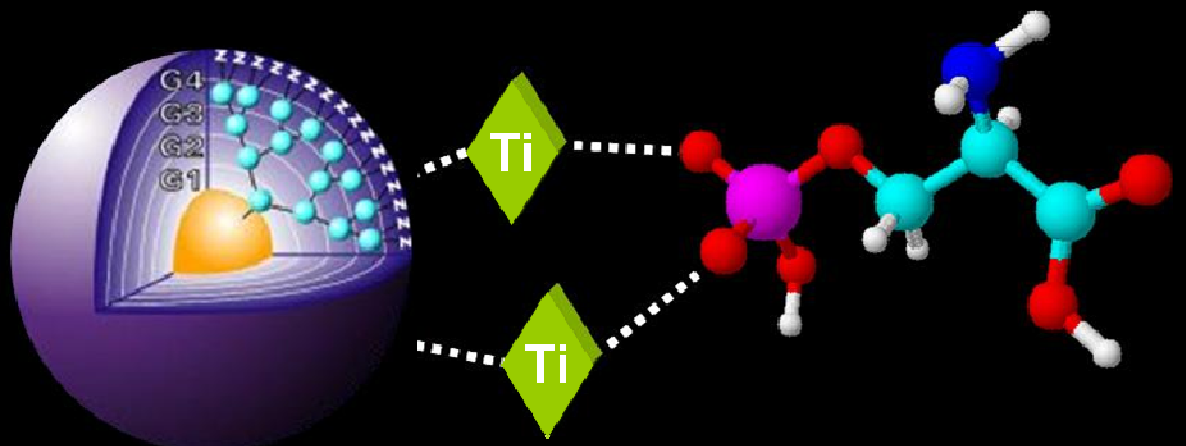
Replaced solid phase with soluble nanopolymer

TYMORA

Others:
Metal oxide beads

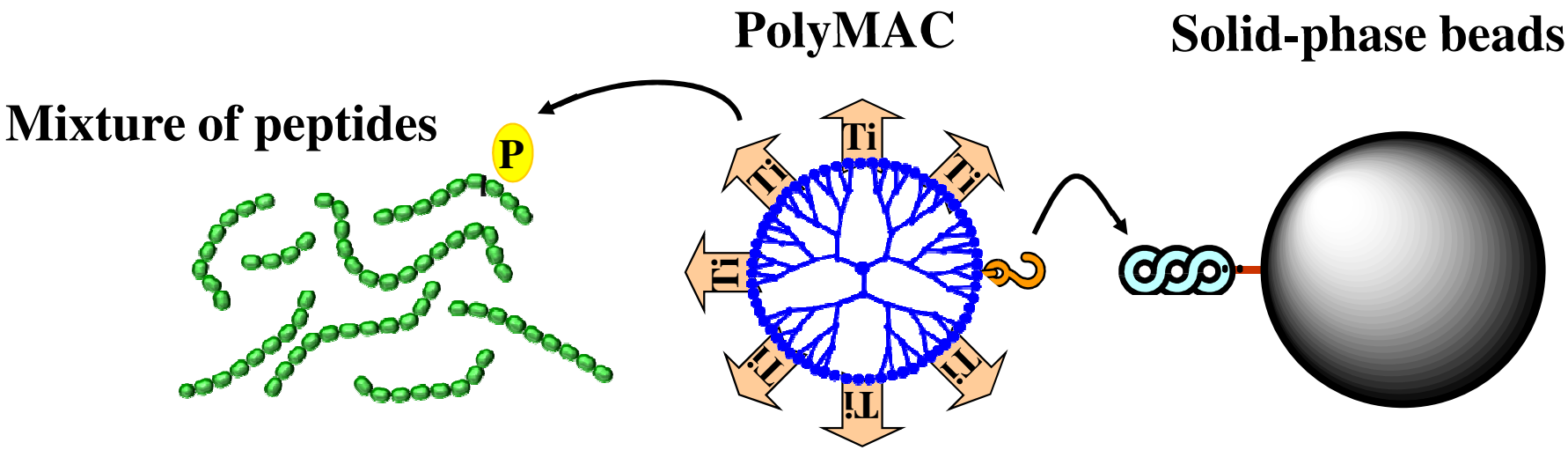


Us:
Dendrimer foundation



Technology 1:
PolyMAC for phosphopeptide enrichment

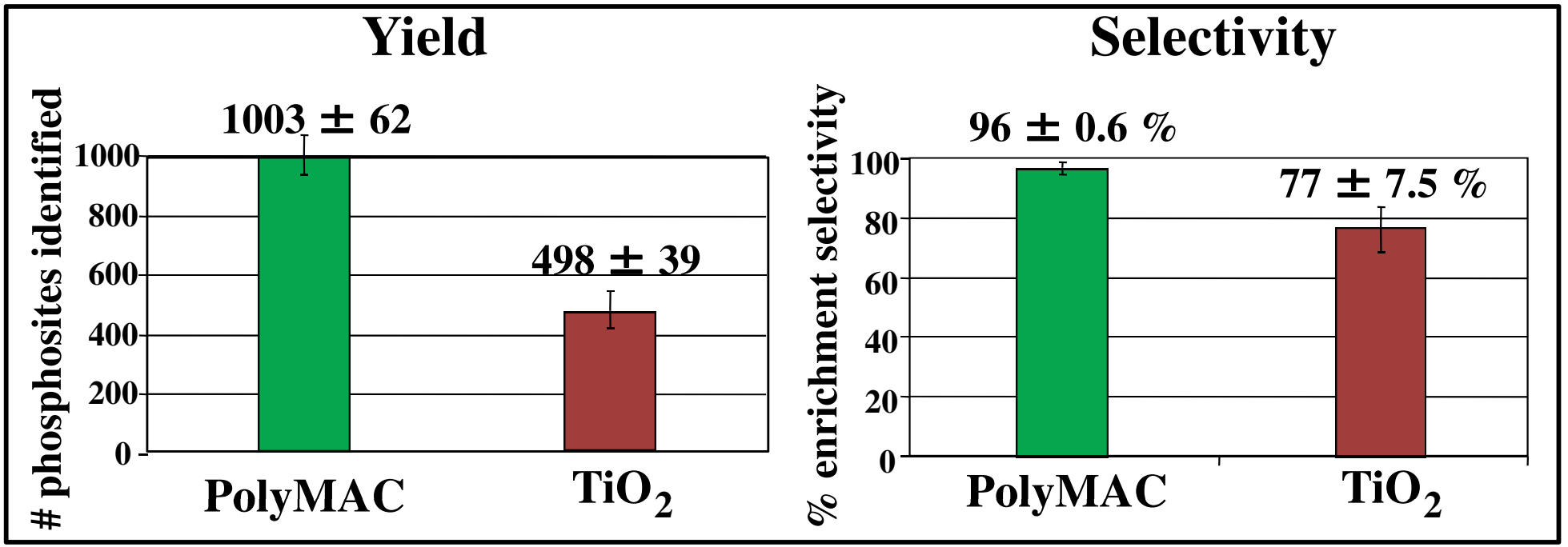
PolyMAC – Polymer-based Metal Affinity Capture



Phosphopeptide enrichment (isolation) is carried out in homogeneous environment using soluble nanopolymer foundation, resulting in improved specificity, higher phosphopeptide recovery, and better sample-to-sample reproducibility.

Iliuk *et.al.* (2010) Mol. Cell. Proteomics **9**: 2162-2172.

PolyMAC-Ti demonstrated superior selectivity and recovery compared to TiO_2

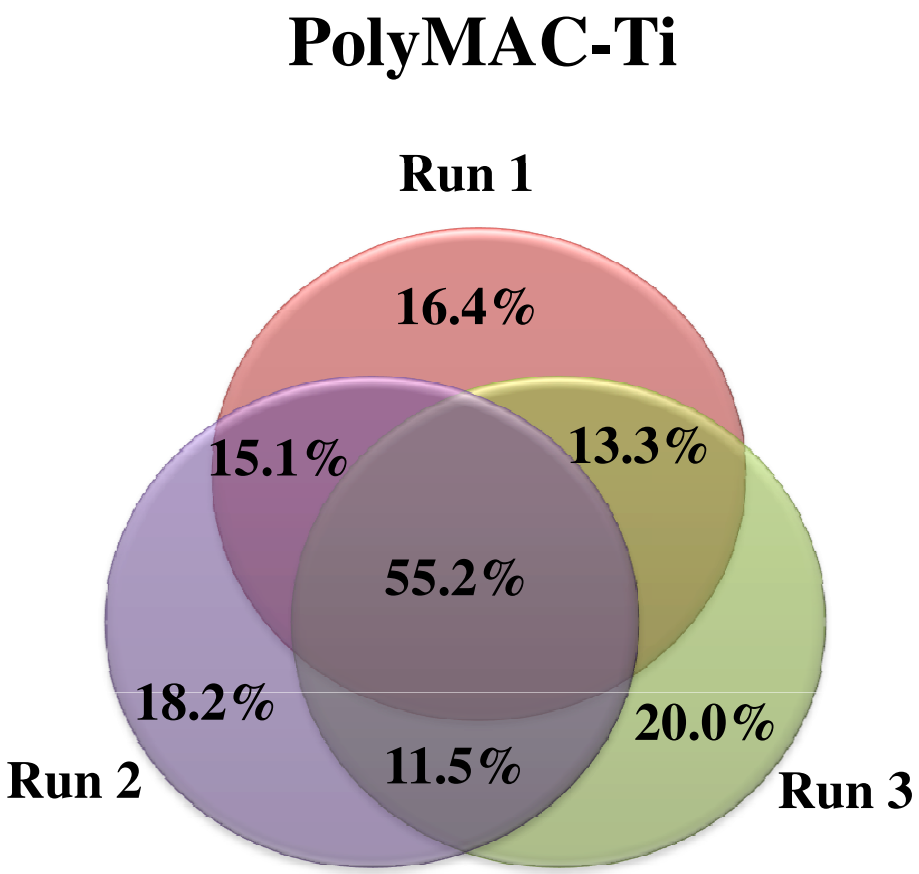


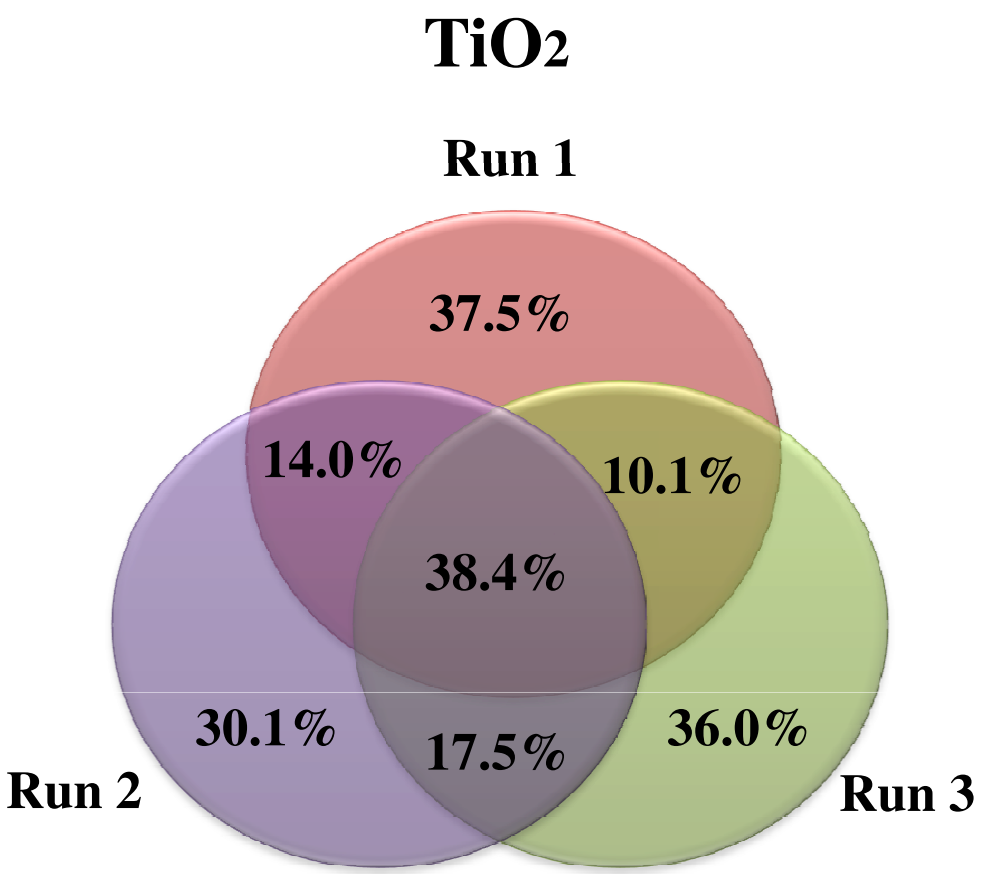
Average of 3 separate experiments

* Error bars represent standard deviation

Data obtained using LC-LTQ-Orbitrap Velos

PolyMAC-Ti demonstrated better reproducibility

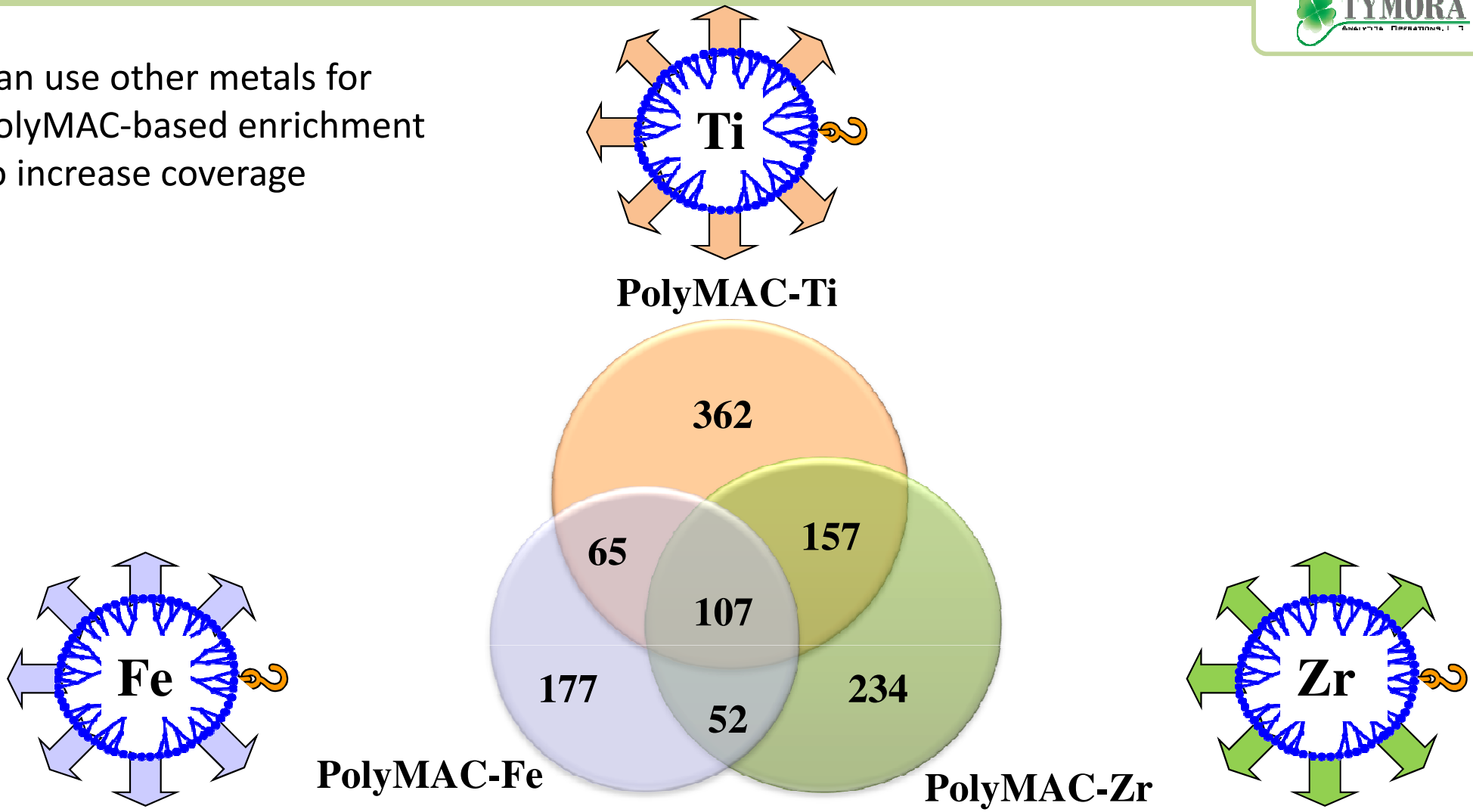




~18% variability of PolyMAC-Ti (other 10% can be attributed to variability of MS)

Increasing phosphopeptide #s with other metals

Can use other metals for PolyMAC-based enrichment to increase coverage



Used a combination of PolyMAC technologies to uncover B cell signaling:

In total, identified 13,009 unique phosphopeptides, containing 18,511 phosphosites

Introduction to two novel technologies

1. PolyMAC – for phosphopeptide enrichment

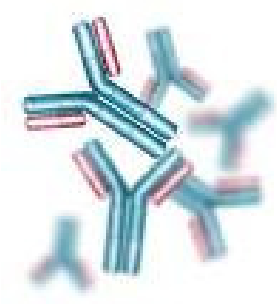
2. pIMAGO – for phosphoprotein detection

Goal – To develop a method for unbiased selective detection of protein phosphorylation independent of sequence microenvironment

Development of kinase inhibitors is expensive

Radioactive labeling:

- Toxic
- Limited usefulness



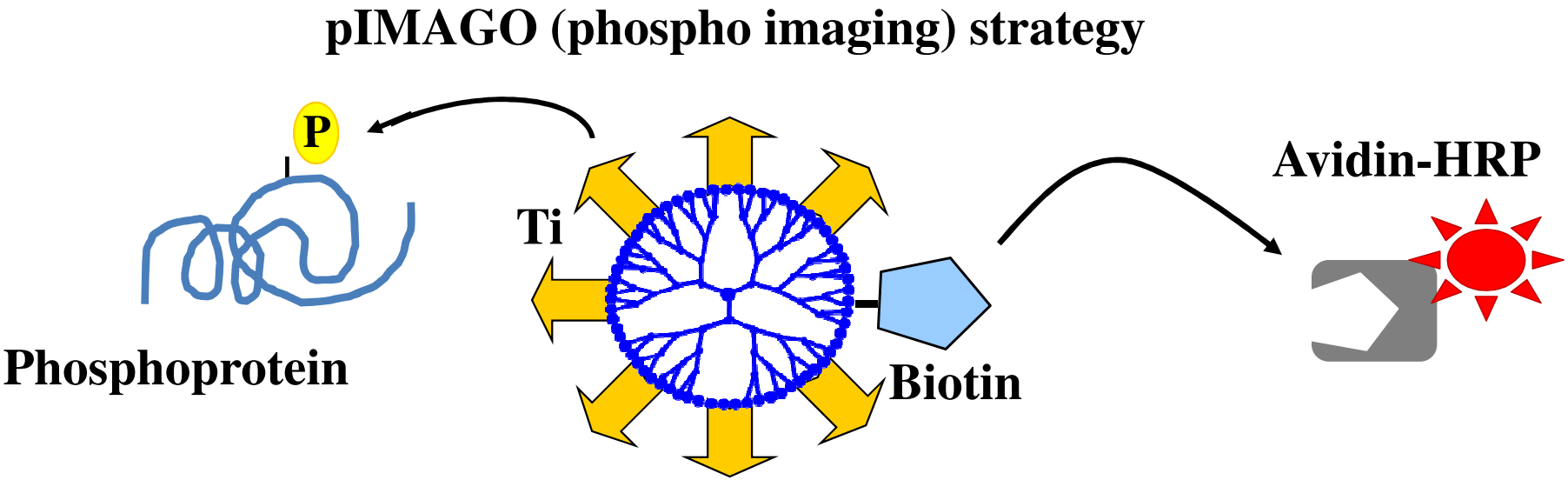
Antibodies:

- Very expensive ($400-600 per site)
- Limited availability

Inhibitor screening:

- Expensive (>$3,000 per compound)
- Artificial results

Technology 2:
pIMAGO for phosphoprotein detection



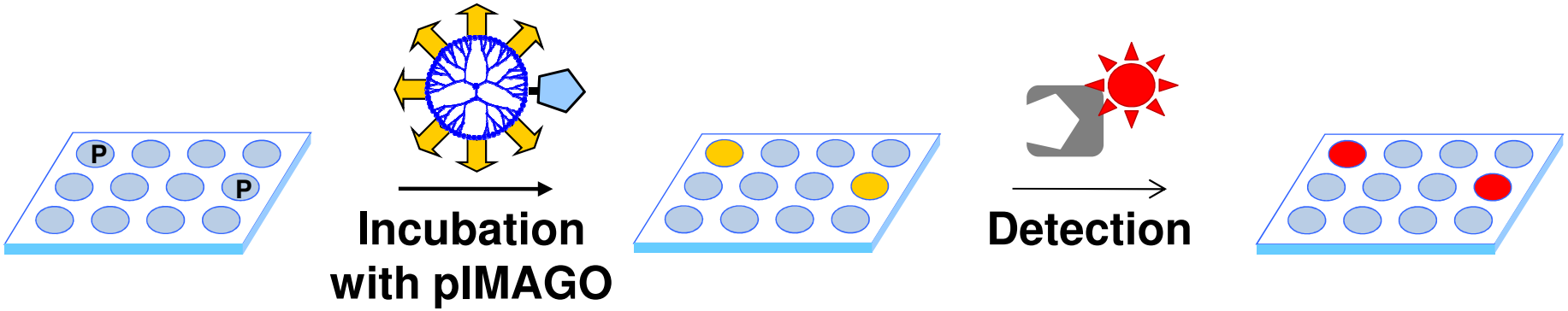
pIMAGO permits highly specific binding to phosphate groups based on titanium metal ion, independent from amino acid sequence.

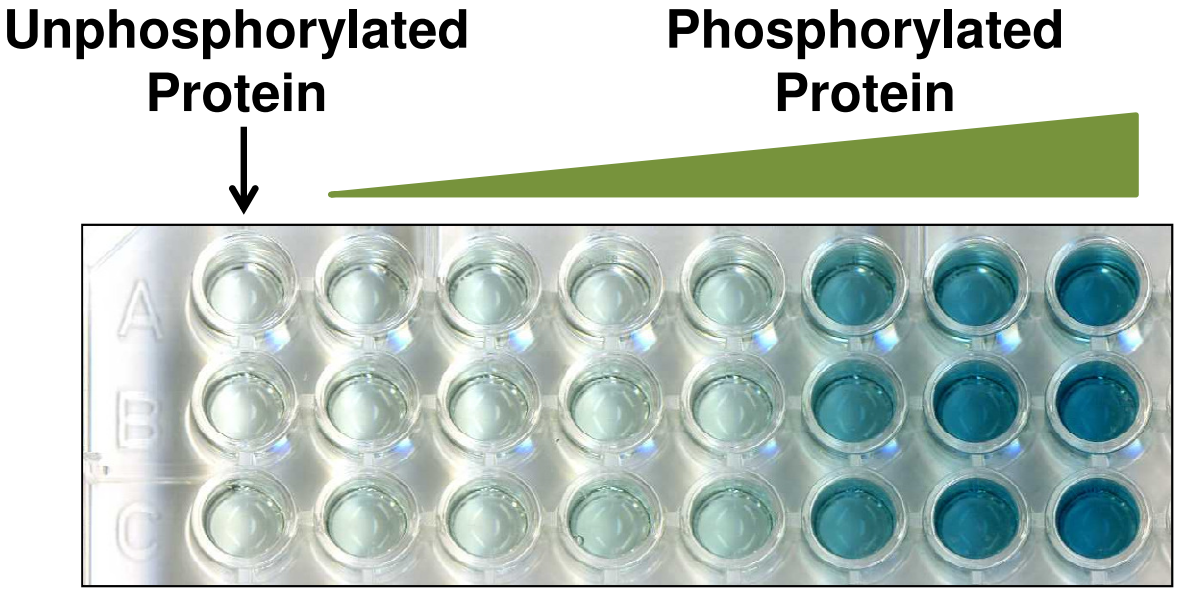
Multiple biotin or fluorescent molecules can be used for detection.

Iliuk *et.al.* (2011) Anal. Chem. **83**: 2767-2774.
Iliuk *et.al.* (2012) Mol. Cel. Proteomics (Epub ahead of print).

pIMAGO detection in microarrays (on plate)

Unbiased detection of any phosphorylation event in array format

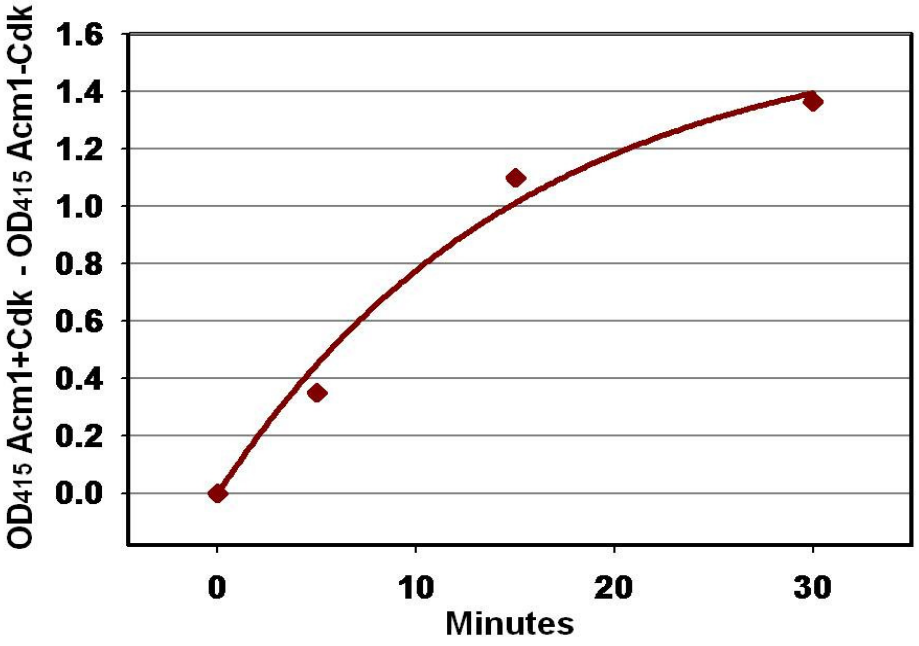




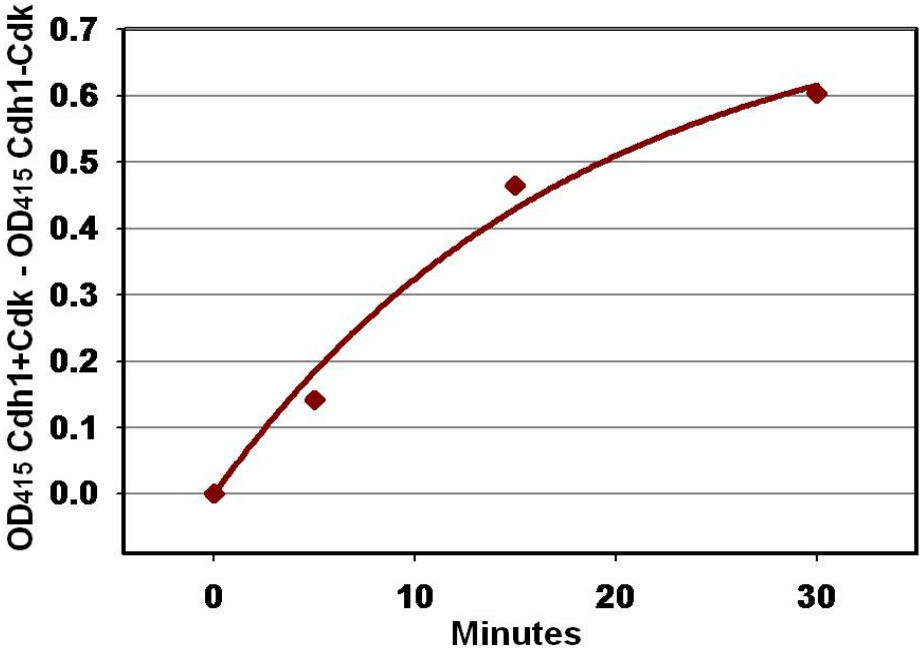
Iliuk *et.al.* (2011) Anal. Chem. **83**: 2767-2774.

pIMAGO utility for *in vitro* Ser/Thr kinase assays

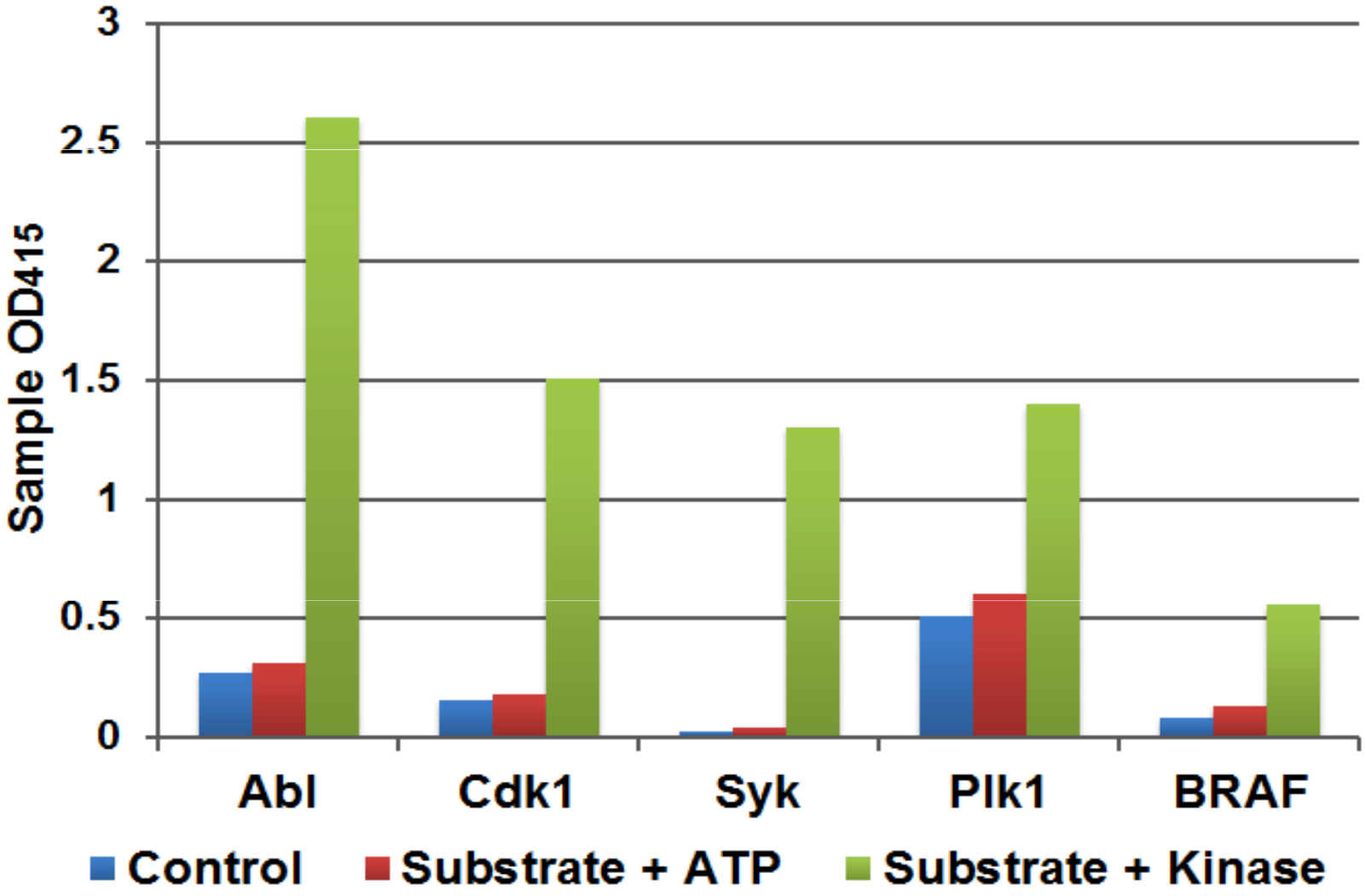
pIMAGO-ELISA
Cdk-Acm1 kinase assay



pIMAGO-ELISA
Cdk-Cdh1 kinase assay

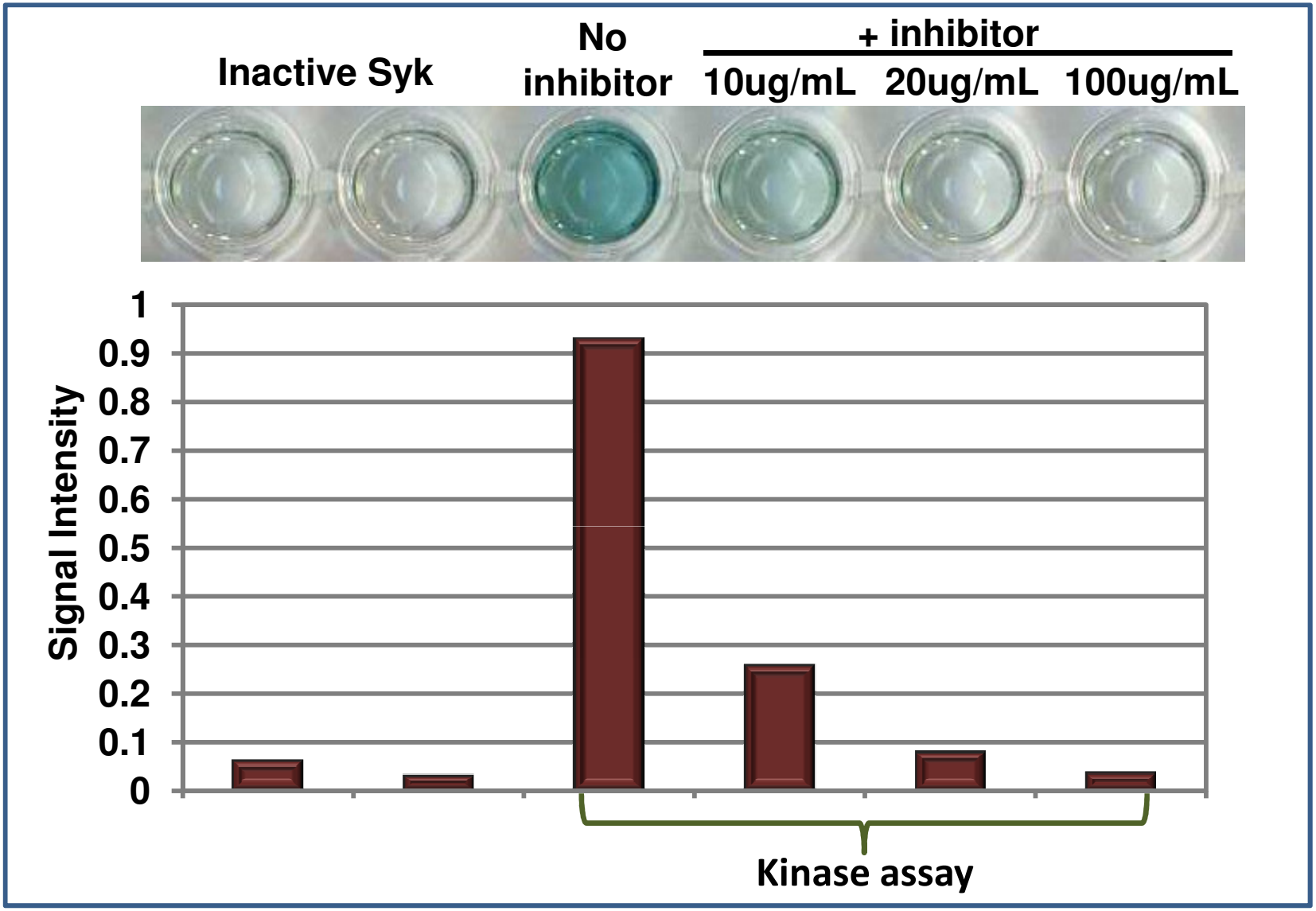


pIMAGO use for kinase profiling



pIMAGO use for kinase inhibitor screening

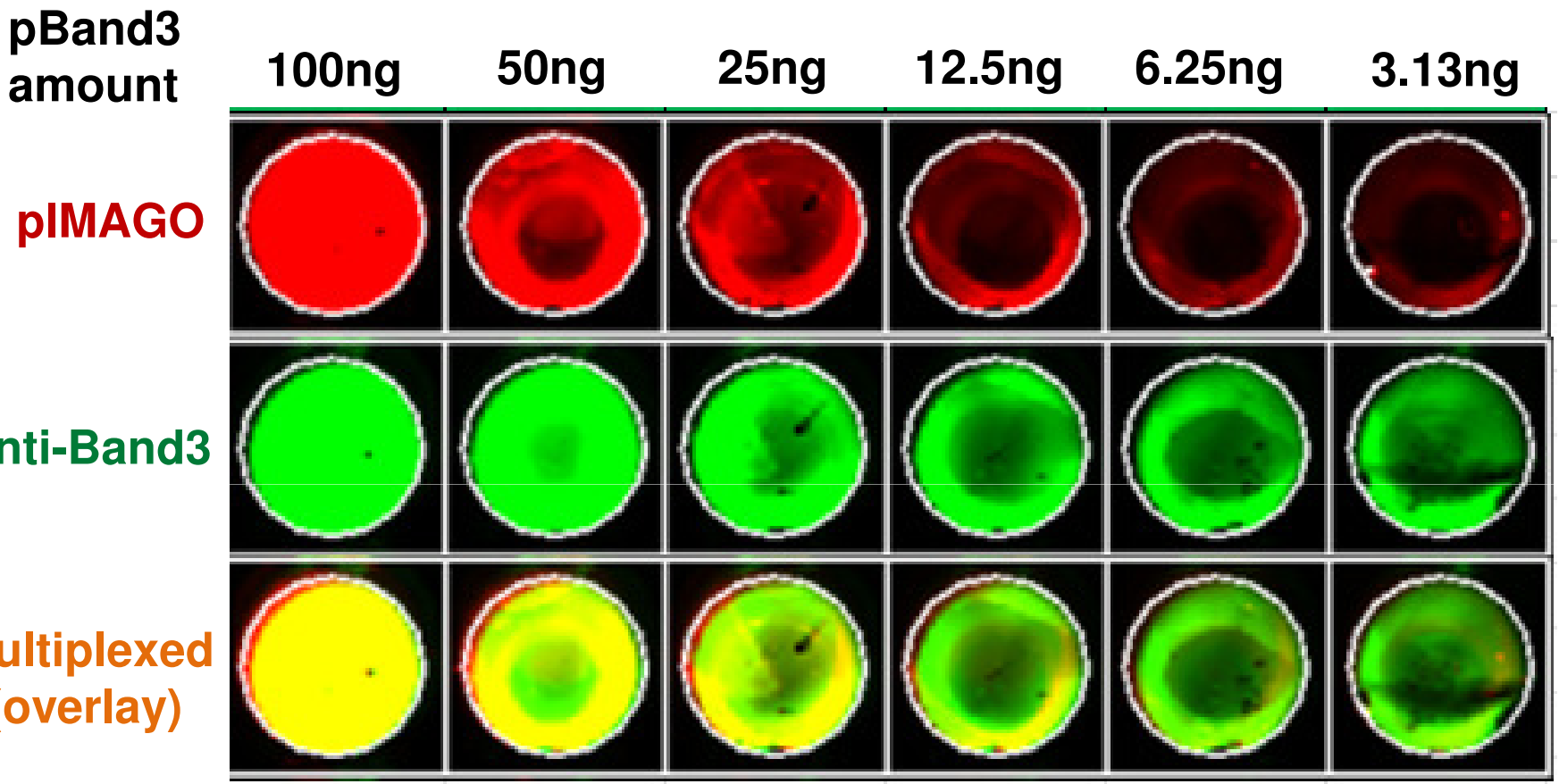
Test of Syk Kinase Inhibitor - Piceatannol



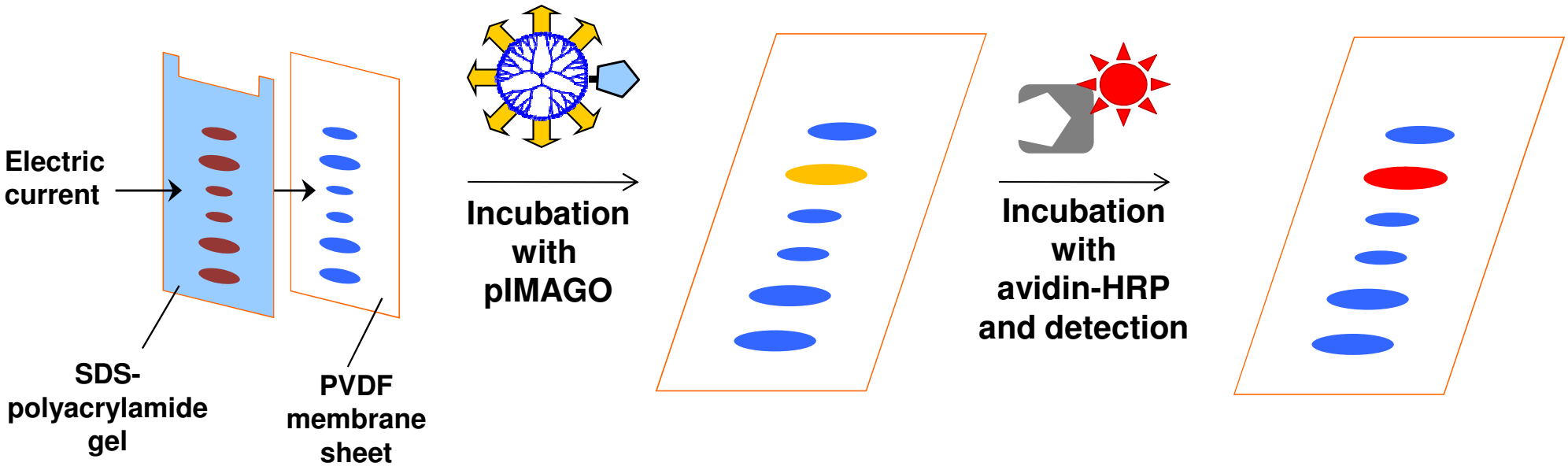
pIMAGO Capability for Multiplexed Detection

TYMORA

Multiplexed detection of phosphorylated protein Band3 using pIMAGO and anti-Band3 antibody



pIMAGO detetion in Western Blots (on-membrane)

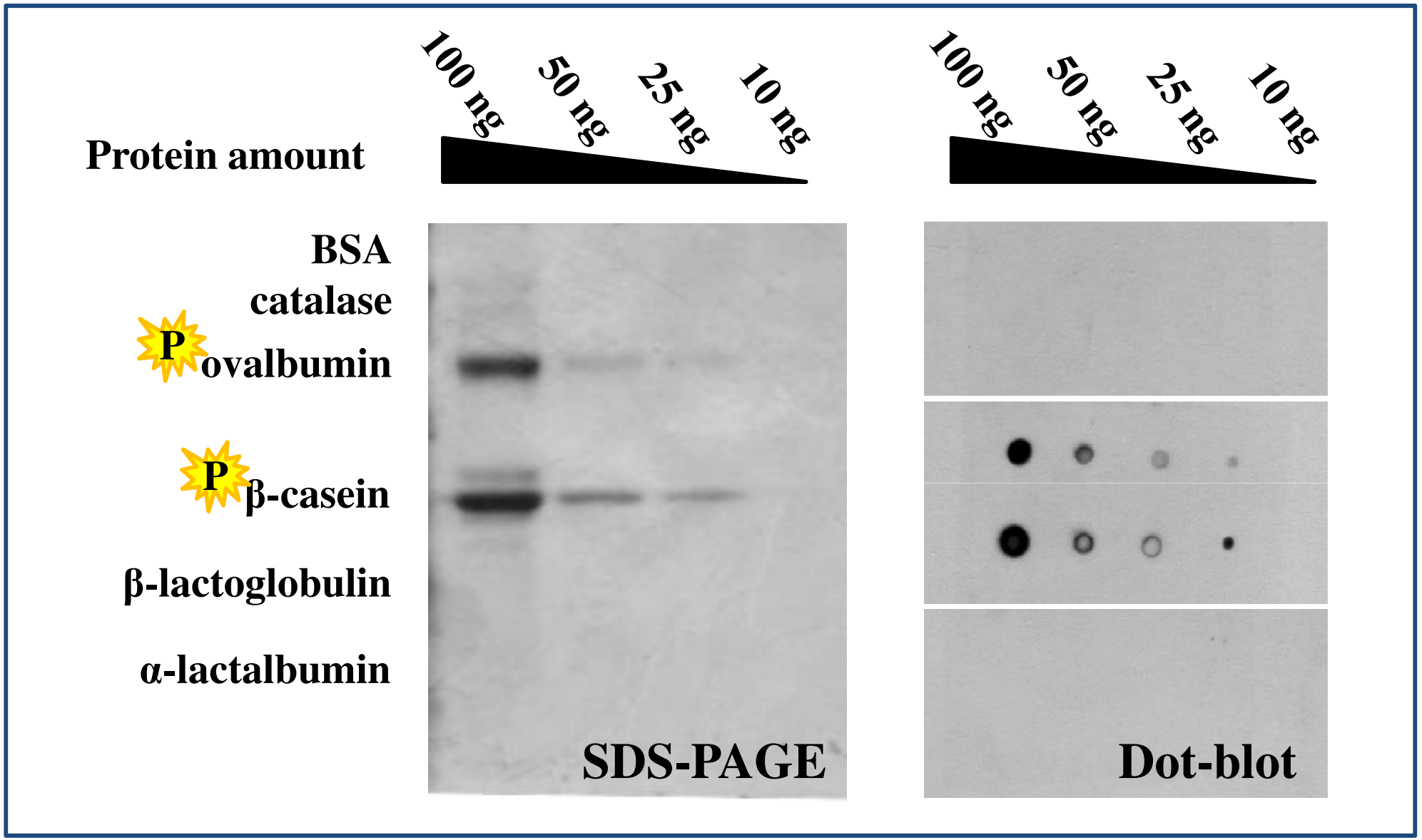


Iliuk *et.al.* (2012) Mol. Cel. Proteomics (Epub ahead of print).

pIMAGO shows good selectivity toward phosphoproteins

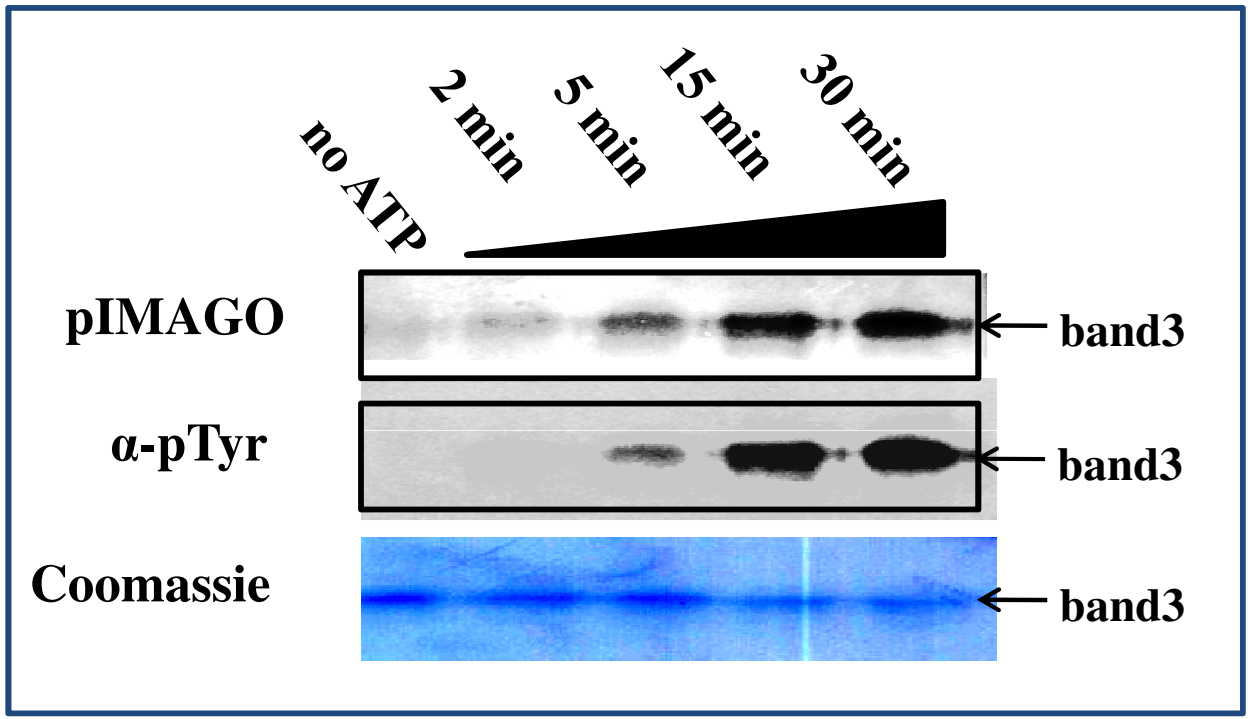
TYMORA

Imaging of 6 proteins (2 phosphorylated)

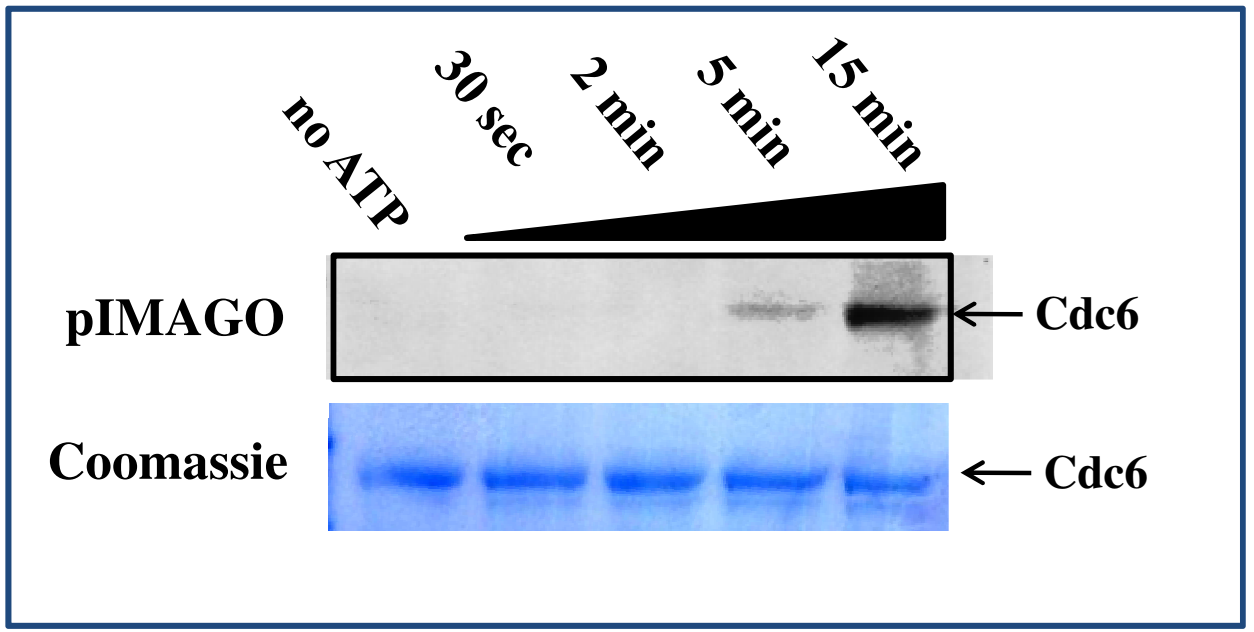


pIMAGO was successfully utilized for imaging of *in vitro* kinase assay

Syk tyrosine kinase with its substrate band 3



Cdk serine kinase with its substrate Cdc6



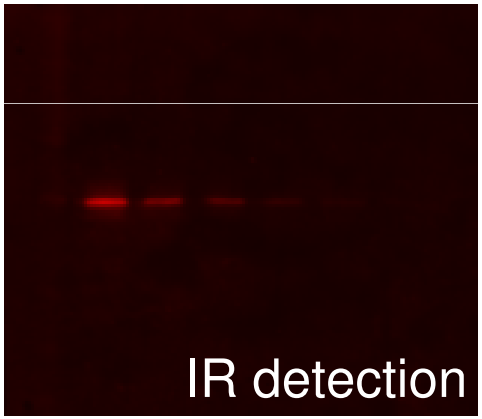
pIMAGO-based multiplexed detection on membrane

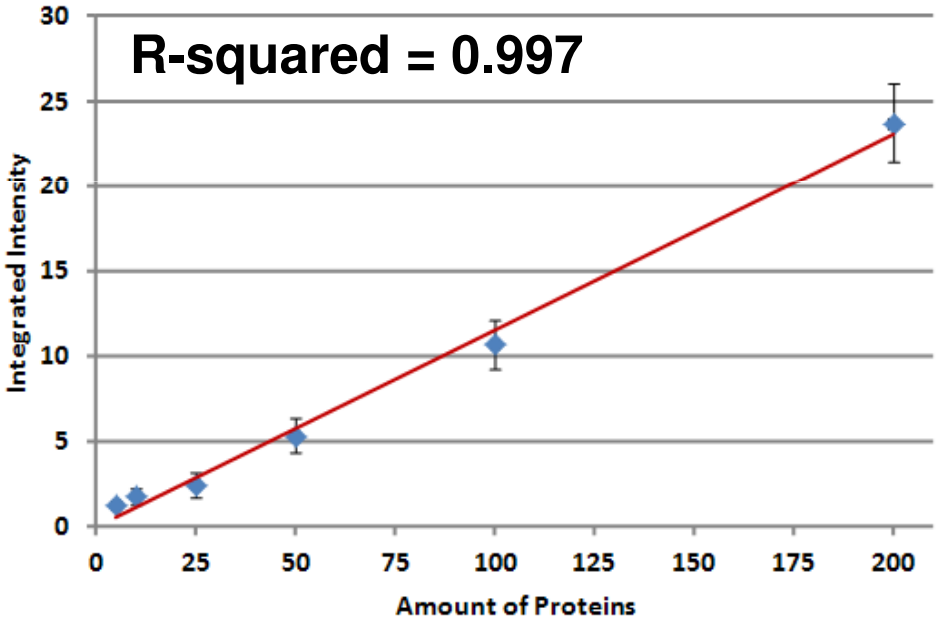
TYMORA

Protein amount

200 ng 100 ng 50 ng 25 ng 10 ng 5 ng

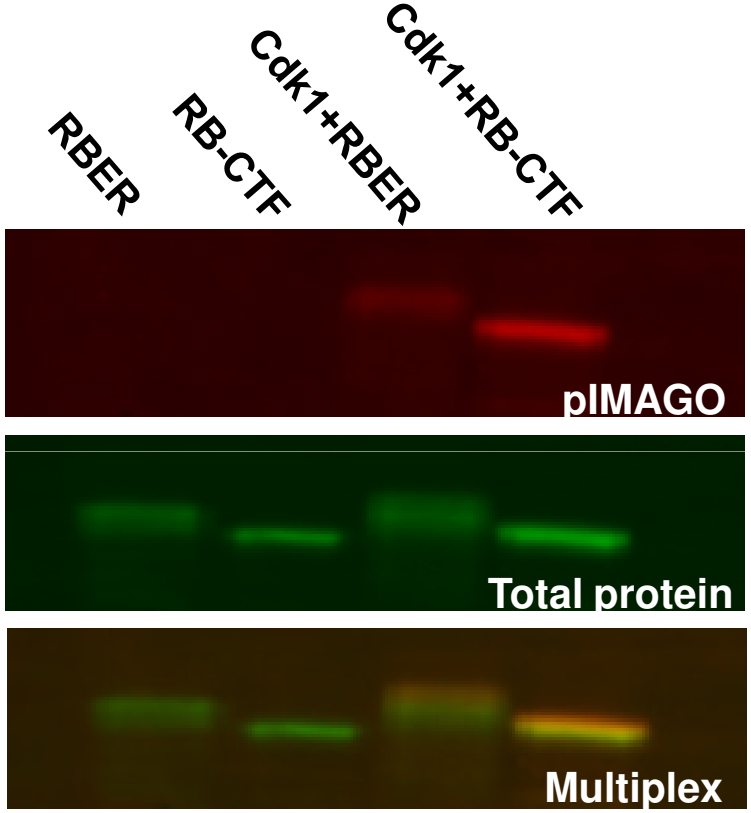
pIMAGO detection of 5 protein mixture (1 phosphoprotein)





Detection in Western Blot format

Multiplex of Cdk1-RBER and Cdk1-RB-CTF kinase assays



Potential applications of pIMAGO

- ***In vitro*** **kinase and phosphatase assays**
- **Kinase/phosphatase profiling and inhibitor screening**
- **Determination and relative quantitation of protein phosphorylation**
- **Analyses of kinase/phosphatase effects on *in vivo* protein phosphorylation**
- **Analyses of stimuli effects on *in vivo* protein phosphorylation**
- **Determination of phosphorylation status of proteins in a complex**

蛋白質の容器への吸着

低吸着チューブの開発