Sample Prep using Titania and Zirconia
Publication Abstracts - Sorted Chronologically (Rev. 04/10/08)

1. M. Kawahara, H. Nakamura, and T. Nakajima, "Group Separation of Ribonucleosides and Deoxyribonucleosides on a New Ceramic Titania Column," Analytical Sciences, 5 (6), 763 (1989).

   Abstract: The adsorption properties of new ceramics titania (titanium dioxide, Ti02) as a column packing material for high performance liquid chromatography (HPLC) were investigated in our recent studies. The adsorption mechanism of titania was found to be different from that of silica gel; the titania adsorbs acidic compounds rather than basic compounds, while silica gel adsorbs basic compounds. In addition, titania possessed high resistance to both alkaline and acidic eluents. Therefore, a further attempt has been made to apply this titania column to the separation of various biogenic substances employing hydrophilic solvent systems. Among numerous biogenic substances tested, the titania column showed a peculiar property for nucleosides. This communication deals with the rapid group separation of ribonucleosides and deoxyribonucleosides on a titania column.

2. H. Matsuda, H. Nakamura, T. Nakajima, "New Ceramic Titania: Selective Adsorbent for Organic Phosphates," Analytical Sciences, Vol. 6 (6), 911-912 (1990).

   Abstract: Keywords: Titania, new ceramics, organic phosphate, selective adsorption, high performance liquid chromatography

3. P.A. Connor and A.J. McQuillan, "Phosphate Adsorption onto TiO_2 from Aqueous Solutions: An in Situ Internal Reflection Infrared Spectroscopic Study," Langmuir, 15 (8), 2916 -2921 (1999).

   Abstract: The adsorption of phosphate ions from aqueous solution onto thin films of colloidal TiO_2 has been studied for the first time by in situ internal reflection infrared spectroscopy. Phosphate binds strongly to TiO_2, as evidenced by the large changes in the PO stretching band structure in the infrared spectrum of the adsorbed species compared with the solution species. The Langmuir binding constant for phosphate onto TiO_2 at pH = 2.3 is $(3.8 \pm 0.8) \times 10^4$ dm^3 mol^{-1}, which is similar to the binding constants onto TiO_2 for bidentate ligand species such as oxalate

and catechol. The strength of the binding is also apparent in the kinetics of adsorption, showing fast adsorption and much slower desorption, as expected for a strongly bound species. The kinetics data at pH = 8.3 have indicated coverage-dependent phosphate adsorption and desorption. Experiments with substituted phosphate species have confirmed the bidentate binding of phosphate to Ti(IV) ions at the TiO_2 surface.

4. K. Tani, M. Kitada, M. Tachibana, H. Koizumi and T. Kiba, "Retention Behavior of Monosaccharides and Disaccharides on Titania," Chromatographia, 57 (5-6), 57 (2003).

Abstract: The ability of titania to recognize the position of hydroxyl groups was previously found to result from the formation of a chelate ring between a titanium ion and the two oxygen atoms of the hydroxyl group and carboxylate anion of 2-hydroxycarboxylic acids. We investigated how titania could recognize the position of hydroxyl group of monosaccharides and disaccharides. The retention behavior of monosaccharides and disaccharides on titania was found to be characteristic. Thus, sucrose was eluted much faster than the other sugars, whereas D-ribose, D-fructose and L-sorbose were most strongly retained on titania of all of the sugars tested. It is apparent that the sugars strongly retained on titania have an axial hydroxyl group or neighboring hydroxyl groups of the same conformation.

5. I. Kuroda, Y. Shintani, M. Motokawa, S. Abe, and M. Furuno, " Phosphopeptide-Selective Column-switching RP-HPLC with a Titania Precolumn," Analytical Sciences, 20 (9), 1313-9 (2004).

Abstract: A methodology of phosphopeptide-selective analysis coupled with column-switching HPLC utilizing titania as precolumn media is presented. Phosphopeptides were selectively enriched on titania packing within a protein/peptide mixture without any additional procedure, and analyzed by column-switching high-performance liquid chromatography. First, phospho-compounds were separated from complex mixtures by trapping them under acidic conditions on a titania packing, where non-phosphorylated compounds were effused out of the precolumn. Subsequently, phospho-compounds were desorbed from the titania column under a specific condition and analyzed. The behavior of phospho-compounds on a titania surface, especially adsorption/desorption, was precisely examined and optimized. A phosphoric buffer was successively employed for the elution of phosphopeptides on a titania surface by competition with the free phosphate group. From the successes of a selective concentration/analysis of phosphopeptides with column-switching HPLC with a titania precolumn, a novel phosphopeptide-selective RP-HPLC analysis has been shown to have an application possibility as a tool for phosphoproteomics.

6. A. Sano and H. Nakamura, "Chemo-affinity of Titania for the Column Switching HPLC Analysis of Phosphopeptides," Analytical Sciences, 20 (3), 565 (2004).

Abstract: A column-switching high-performance liquid chromatography (HPLC) system for the determination of phosphopeptides has been developed. The method is based on the selective adsorption of phosphopeptides on a titania (TiO_2) precolumn and successive HPLC separation of the phosphopeptides on an anion-exchange column with a UV detector (215 nm). The recoveries of phosphopeptides were tested using authentic phosphopeptides [Gln-Ile-Ser(p)-Val-Arg, Ile-Ser(p)-Val-Arg and Lys-Gln-Ile-Ser(p)-Val-Arg] at an injection amount of 1 μg. The recoveries were 74.3, 79.6, and 82.6%, respectively, while the corresponding dephosphopeptides were not retained on the titania precolumn.

7. D.E. Koch, R. Isaza, J.W. Carpenter, R.P. Hunter, "Simultaneous Extraction and Quantitation of Fentanyl and Norfentanyl from Primate Plasma with LC/MS Detection" Journal of Pharmaceutical and Biomedical Analysis, 34, 577–584 (2004).

Abstract: The quantitation of both fentanyl and its desalkyl metabolite, norfentanyl, in plasma using LC/MS has not been previously described. The detection and quantitation of fentanyl and norfentanyl was achieved using LC/MS detection. The liquid–liquid extraction used toluene as the organic phase. Chromatography was carried out using a Zirchrom-PBD (50 mm×2.1 mm, 3 μm) column with a mobile phase of acetonitrile–ammonium acetate (10 mM), citrate (0.1 mM, pH 4.4) (45:55, v/v) with a flow rate of 0.3 ml/min. Mass spectroscopy detection was performed using ESI in the positive mode. The LOQ for fentanyl was 25 pg/ml and norfentanyl was 50 pg/ml. For the concentrations of 75, 250, and 750 pg/ml, respectively, fentanyl had inter-day precisions of 6.6, 7.2, and 6.6% with accuracies of 4.0, 5.1, and 5.1% and intra-day precisions of 1.6, 1.9, and 1.9% with accuracies of 11.6, 9.4, and 8.4%, and norfentanyl had inter-day precisions of 7.4, 0.3, and 0.7% with accuracies of 9.1, 8.8, and 12.3% and intra-day precisions of 5.3, 1.4, and 0.1% with accuracies of 10.9, 8.9, and 12.8%. The recoveries of fentanyl were 85, 92, and 75% and of norfentanyl were 40, 49, and 46% at the 75, 250, and 750 pg/ml concentrations, respectively.

8. M. Pinkse, P.M. Uitto, M.J. Hilhorts, B. Ooms, and A.J. Heck, "Selective Isolation at the Femtomole Level of Phosphopeptides from Proteolytic Digests using 2D-nanoLC-ESI-MS/MS and Titanium Dioxide Precolumns," Analytical Chemistry, 76, 3935–3943 (2004).

Abstract: Selective detection of phosphopeptides from proteolytic digests is a challenging and highly relevant task in many proteomics applications. Often phosphopeptides are present in small amounts and need selective isolation or enrichment before identification. Here we report a novel automated method for the enrichment of phosphopeptides from complex mixtures. The method employs a two dimensional column setup, with titanium oxide-based solid-phase material (Titansphere) as the first dimension and reversed-phase material as the second dimension. Phosphopeptides are separated from nonphosphorylated peptides by trapping them under acidic conditions on a TiO2 precolumn. Nonphosphorylated peptides break through and are trapped on a reversed-phase precolumn after which they are analyzed by nanoflow LC-ESI-MS/MS. Subsequently, phosphopeptides are desorbed from the TiO2 column under alkaline conditions, reconcentrated onto the reversed-phase precolumn, and analyzed by nanoflow LC-ESI-MS/MS. The selectivity and practicality of using TiO2 precolumns for trapping phosphopeptides are demonstrated via the analysis of a model peptide RKISASEF, in a 1:1 mixture of a non- and a monophosphorylated form. A sample of 125 fmol of the phosphorylated peptide could easily be isolated from the nonphosphorylated peptide with a recovery above 90%. In addition, proteolytic digests of three different autophosphorylation forms of the 153-kDa homodimeric cGMP-dependent protein kinase are analyzed. From proteolytic digests of the fully autophosphorylated protein at least eight phosphorylation sites are identified, including two previously uncharacterized sites, namely, Ser-26 and Ser-44. Ser-26 is characterized as a minor phosphorylation site in purified PKG samples, while Ser-44 is identified as a novel in vitro autophosphorylation target. These results clearly show that TiO2 has strong affinity for phosphorylated phosphorylated peptides, and thus, we conclude that this material has a high potential in the field of phosphoproteomics.

9. D. R. Craft, K. A. Chisholm, and D. M. Pinto, “Titanium Dioxide Columns for Selective Enrichment and Chromatographic Separation of Phosphopeptides,” Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford St., Halifax, Nova Scotia, CANADA B3H 3Z1 ASMS Poster (2005).

Abstract: To selectively adsorb phosphorylated peptides onto a titanium dioxide support. Methods: Column was prepared using PEEK tubing packed with TiO2 porous beads. Tryptic peptides were loaded on to the column. Phosphorylated peptides were selectively adsorbed onto the TiO2 support. Non-phosphorylated peptides were washed off the column. Phosphopeptides eluted off the TiO2 column in step gradients. Peptides were analyzed by reverse phase LC-ESI-MS/MS on a Qtrap 4000. Results: Successful enrichment of phosphopeptides in the presents of high abundant non-phosphorylated peptides. Separation of acidic peptides

from phosphorylated peptides on the TiO2 column. Substantial increase in the number of *Saccharomyces cerevisiae* phosphoproteins identified with TiO2 clean-up.

10. H. K. Kweon and K. Håkansson, "Characterization of Phosphopeptides by Electron Detachment Dissociation in FT-ICR Mass Spectrometry" Department of Chemistry, University of Michigan, Ann Arbor, MI, USA ASMS Poster (2005).

    Abstract: Overview: Novel Aspect: First demonstration of electron detachment dissociation (EDD) of phosphoserine containing peptides. Purpose: Investigate the utility of EDD for localization of protein phosphorylation sites in negative ion mode FT-ICR mass spectrometry. Methods: Phosphoproteins were enzymatically digested by trypsin or Glu-C. Phosphopeptides from proteolytic peptide mixtures were selectively isolated and enriched by binding to ZrO2 microtips. EDD spectra of phosphopeptides were acquired by irradiating peptide anions trapped in the ICR cell with high-energy electrons (~18 eV). Results: EDD of singly phosphorylated peptides provided *a*•- and *x*- type fragment ions from C - CO bond cleavage. All backbone fragment ions containing phosphoserine retained the labile phosphate group, allowing precise localization of the phosphorylation site. For multiply phosphorylated peptides, only CO2 and phosphate loss was observed in EDD. ZrO2 phosphopeptide enrichment was superior to TiO2 and IMAC methods.

11. P. Kent, L.A. Upton, R. Bobel and K. Nugent, "An Automated MDLC Protocol for Trace Enrichment of Phosphopeptides From Complex Proteome Samples" Michrom Bioresources, Auburn, CA, ASMS Presentation (2005).

    Abstract: Protein phosphorylation is an important post translational modification (PTM), but analysis can be difficult because it often occurs on low abundance proteins, and then only on a small percentage of a protein's total population. Although IMAC is the most popular technique for trace enrichment of phosphopeptides, it suffers from nonselective binding, variable recovery and difficulty in automation. This study looks at a new MDLC protocol for trace enrichment of phosphopeptides that overcomes these limitations. The first dimension of the MDLC system uses a unique titanium oxide (TO) trap column that selectively binds only the phosphopeptides in complex proteome digests, while the non phosphorylated peptides are flushed to waste or eluted to a second dimension nanoRP column for analysis. The phosphopeptides are then quantitatively eluted from the titanium oxide trap column and pH adjusted on RP nanotrap for subsequent analysis by nanoRP LC-MS/MS.

12. M.R. Larsen, T.E. Thingholm, O.N. Jensen, P. Roepstorff, and T.J. Jorgensen,

"Highly Selective Enrichment of Phosphorylated Peptides from Peptide Mixtures Using Titanium Dioxide Microcolumns," Mol. Cell Proteomics 4 (7), 873–886 (2005).

Abstract: Reversible phosphorylation of proteins regulates the majority of all cellular processes, e.g. proliferation, differentiation, and apoptosis. A fundamental understanding of these biological processes at the molecular level requires characterization of the phosphorylated proteins. Phosphorylation is often substoichiometric, and an enrichment procedure of phosphorylated peptides derived from phosphorylated proteins is a necessary prerequisite for the characterization of such peptides by modern mass spectrometric methods. We report a highly selective enrichment procedure for phosphorylated peptides based on TiO_2microcolumns and peptide loading in 2,5-dihydroxybenzoic acid (DHB). The effect of DHB was a very efficient reduction in the binding of nonphosphorylated peptides to TiO_2 while retaining its high binding affinity for phosphorylated peptides. Thus, inclusion of DHB dramatically increased the selectivity of the enrichment of phosphorylated peptides by TiO_2. We demonstrated that this new procedure was more selective for binding phosphorylated peptides than IMAC using MALDI mass spectrometry. In addition, we showed that LC-ESI-MSMS was biased toward monophosphorylated peptides, whereas MALDI MS was not. Other substituted aromatic carboxylic acids were also capable of specifically reducing binding of nonphosphorylated peptides, whereas phosphoric acid reduced binding of both phosphorylated and nonphosphorylated peptides. A putative mechanism for this intriguing effect is presented.

13. A. Schlosser, J.T. Vanselow, and A. Kramer, "Mapping of Phosphorylation Sites by a Multi-protease Approach with Specific Phosphopeptide Enrichment and NanoLC-MS/MS Analysis," Analytical Chemistry, 77, 5243-5250 (2005).

Abstract: We have developed a multi-protease approach that allows sensitive and comprehensive mapping of protein phosphorylation sites. The combined application of the low-specificity proteases elastase, proteinase K, and thermolysin in addition to trypsin results in high sequence coverage, a prerequisite for comprehensive phosphorylation site mapping. Phosphopeptide enrichment is performed with the recently introduced phosphopeptide affinity material titansphere. We have optimized the selectivity of the phosphopeptide enrichment with titansphere, without compromising the high recovery rate of ~90%. Phosphopeptide-enriched fractions are analyzed with a highly sensitive nanoLC-MS/MS system using a 25-μm-i.d. reversed-phase column, operated at a flow rate of 25 nL/min. The new approach was applied to the murine circadian protein period 2 (mPER2). A total of 21 phosphorylation sites of mPER2 have been detected by the multi-protease approach, whereas only 6 phosphorylation sites were identified using solely trypsin.

Titansphere proved to be well suited for the enrichment of a large variety of phosphopeptides, including peptides carrying two, three, or four phosphorylated residues, as well as phosphopeptides containing more basic than acidic amino acids.

14. H.K. Kweon and K. Håkansson, "Selective Zirconium Dioxide-Based Enrichment of Phosphorylated Peptides for Mass Spectrometric Analysis," Analytical Chemistry, 78, 1743-1749 (2006).

Abstract: Due to the dynamic nature and low stoichiometry of protein phosphorylation, enrichment of phosphorylated peptides from proteolytic mixtures is often necessary prior to their characterization by mass spectrometry. Several phosphopeptide isolation strategies have been presented in the literature, including immobilized metal ion affinity chromatography. However, that technique suffers from poor selectivity and reproducibility. Recently, titanium dioxide-based columns have been successfully employed for phosphopeptide enrichment by several research groups. Here, we present, to our knowledge, the first demonstration of the utility of zirconium dioxide microtips for phosphopeptide isolation prior to mass spectrometric analysis. These microtips display similar overall performance as TiO2 microtips. However, more selective isolation of singly phosphorylated peptides was observed with ZrO2 compared to TiO2 whereas TiO2 preferentially enriched multiply phosphorylated peptides. Thus, these two chromatographic materials possess complementary properties. For r- and *â*-casein, Glu-C digestion provided no evident advantage compared to trypsin digestion when combined with TiO2 or ZrO2 phosphopeptide enrichment.

15. C. Klemm, S. Otto, C. Wolf, R.F. Haseloff, M. Beyermann and E. Krause, "Evaluation of the Titanium Dioxide Approach for MS Analysis of Phosphopeptides," Journal of Mass Spectrometry, 41, 1623–1632 (2006).

Abstract: The affinity of titanium dioxide for phosphate groups has been successfully used for enrichment of phosphopeptides from complex mixtures. This paper reports the relationship between the occurrence of some amino acids and the phospho-specific and nonspecific binding of peptides that occurs during titanium dioxide enrichment. In order to perform a systematic study, two well-characterized peptide mixtures consisting of either 33 or 8 synthetic phosphopeptides and their nonphosphorylated analogs, which differed in charge and hydrophobicity, were synthesized and analyzed by ESI-MS and MALDI-MS. The titanium dioxide procedure was also evaluated for comprehensive detection of phosphopeptides in phosphoproteomics. In summary, our results clearly confirm the high selectivity of titanium dioxide for phosphorylated sequences. Drastically reduced recovery was observed for phosphopeptides with multiple basic amino acids. Nonspecific binding of

nonphosphorylated peptides and sample loss of phosphopeptides must also be taken into account.

16. M. Mazanek, G. Mituloviæ, F. Herzog, C. Stingl, J.R.A. Hutchins, J. Peters and K. Mechtler, "Titanium Dioxide as a Chemo-affinity Solid Phase in Offline Phosphopeptide Chromatography prior to HPLC-MS/MS Analysis" Nature Protocols, 1(4), 1977-1987 (2006).

Abstract: We have developed a new offline chromatographic approach for the selective enrichment of phosphorylated peptides that is directly compatible with subsequent analysis by online nano electrospray ionization tandem mass spectrometry. In this technique, a titanium dioxide (TiO2)-packed pipette tip is used as a phosphopeptide trap that acts as an offline first-dimension separation step in a twodimensional chromatography system. This is followed by online nano reversed-phase high-performance liquid chromatography. Here, we present suitable methods for enrichment, optimized separately for each step: sample loading, washing and elution from the TiO2-filled tips. To increase the trapping selectivity of the TiO2 column, we used the sodium salt of 1-octanesulfonic acid combined with 2,5-dihydroxybenzoic acid as ion-pairing agents and displacers for acidic peptides. These agents also improve the binding of phosphorylated peptides and block the binding of non-phosphorylated ones. This enrichment procedure takes 30 min, followed by a 100-min HPLC program, including washing and an elution gradient.

17. Clementine Klemm, Sebastian Otto, Constanze Wolf, Reiner F. Haseloff, Michael Beyermann and Eberhard Krause "Evaluation of the Titanium Dioxide Approach for MS Analysis of Phosphopeptides," Journal of Mass Spectrometry, 41 (12), 1623 - 1632 (2006).

Abstract: The affinity of titanium dioxide for phosphate groups has been successfully used for enrichment of phosphopeptides from complex mixtures. This paper reports the relationship between the occurrence of some amino acids and the phospho-specific and nonspecific binding of peptides that occurs during titanium dioxide enrichment. In order to perform a systematic study, two well-characterized peptide mixtures consisting of either 33 or 8 synthetic phosphopeptides and their nonphosphorylated analogs, which differed in charge and hydrophobicity, were synthesized and analyzed by ESI-MS and MALDI-MS. The titanium dioxide procedure was also evaluated for comprehensive detection of phosphopeptides in phosphoproteomics. In summary, our results clearly confirm the high selectivity of titanium dioxide for phosphorylated sequences. Drastically reduced recovery was observed for phosphopeptides with multiple basic amino acids. Nonspecific binding of

nonphosphorylated peptides and sample loss of phosphopeptides must also be taken into account.

18. O.N. Jensen, M.R. Larsen, J. Wildgoose, R.H. Bateman, K. Giles, S. Pringle, C. Hughes, J. Langridge, "Separation and Detection of Protein Post-translational Modifications by Liquid Chromatography Coupled with a Novel Ion Mobility Mass Spectrometer," University of Southern Denmark, Odense, Denmark; Waters Corporation. MS Technologies Centre, Manchester, United Kingdom; ASMS Poster (2006).

    Abstract: Here we present the combination of IMS and oa-TOF mass spectrometry for the analysis of post translational modifications. Separation of differentially phosphorylated peptides, based upon ion mobility, has been demonstrated. Fragmentation before and after the IMS separation stage is demonstrated, providing additional specificity

19. J.V. Olsen, B. Blagoev, S. Krüger, F. Gnad, B. Macek, C. Kumar, P. Mortensen and M. Mann, "Dynamics of *in vivo* Tyrosine Phosphorylation Events to Elucidate Growth Factor Induced Pathways by High-accuracy Orbitrap Tandem Mass Spectrometry," Department of Proteomics and Signal Transduction, Max-Planck-Institute of Biochemistry, Martinsried, Germany; ASMS Poster (2006).

    Abstract: Intracellular signal transduction mediated by receptor protein tyrosine kinases, such as the EGF receptor, is initiated by time-ordered tyrosine phosphorylation of recruited adaptors and other signaling molecules (e.g. kinases and phoshophatases). However, when this precise control breaks down, cancer may be the consequence. We have developed an LC-MS based quantitative phosphopeptide-specific approach that combines stable isotope labeling by amino acids in cell culture (SILAC) for quantitation with anti-phosphotyrosine immunopricipitation and titanium dioxide (TiO2) for phosphopeptide enrichment and high-mass accuracy LTQ-Orbitrap tandem mass spectrometry for unambiguous identification and quantitation. We have applied this methodology to study EGF-stimulated HeLa cells using five different activation times and identified several known as well as unknown differentially regulated tyrosine phosphorylation sites in this pathway.

20. B. Maček, I. Mijaković, J. Olsen, F. Gnad, C. Kumar and M. Mann, "Deciphering the Prokaryotic Ser/Thr/Tyr Phosphoproteome: the Case of Bacillus subtilis," 1) Max Planck Institute of Biochemistry, Proteomics and Signal Transduction, Am Klopferspitz 18a, D-82152 Martinsried, Germany; 2) Technical University of Denmark, BioCentrum, Molecular Genetics Group, DK-2800 Lyngby, Denmark; ASMS Poster (2006).

Abstract: *B. subtilis,* a model prokaryiotic organism, is a gram(+) bacterium of significant industrial importance. Its genome consists of about 4100 genes, 2500 of which are estimated to be expressed during growth. Previous MS-based analyses of the proteome have identified in total 1200 gene products in the *log* phase. With its 8 S/T/Y kinases, 11 phosphatases, and about 35 identified phospho-proteins, *B. subtilis* is one of the best-characterized p(S/T/Y) systems in prokaryotes. However, we note that the vast majority of phosphoproteins were identified by peptide mapping or LC-MS/MS after digestion of 32P-labelled 2D gel spots. Only about 10 phosphorylation sites have so far been reported.

21. M. Matis, R. Sack and U.A. Meyer, "Role of Protein Phosphorylations / Dephosphorylations in the Regulation of cytochrome P450 Genes," 1) Division of Pharmacology/Neurobiology, Biozentrum, Basel, 2) Center for Functional Genomics and Bio-Chips, University of Ljubljana, 3) Friedrich Miescher Institute, Novartis Research Foundation, Basel; ASMS Poster (2006).

    Abstract: To study the phosphorylation events involved in the activation of the nuclear receptor CAR by phenobarbital, we first tested our method. We were successful in labeling of peptides with iTRAQ reagent, but not in the enrichment of phosphopeptides. After loading of SCX fractions on TiO2 column we could mostly identify acid unphosphorylated peptides containing D or E residues. To increase the number of identifications, prefractionation of the proteins will be performed and we will also optimize the TiO2 enrichment.

22. C. Pan, J.V. Olsen, B. Macek, F. Gnad and M. Mann "A Phospho-Map of Mouse Liver Cells," Max Planck Institute of Biochemistry, Dept. Proteomics and Signal Transduction, Munich, Germany; ASMS Poster (2006).

    Abstract: A global phosphorylation study on mouse liver cell line Hepa1-6; Phosphatase inhibitors to boost phosphorylation; Strong cation exchange (SCX); Phospho-peptide enrichment by TiO2; 2589 phosphorylation sites on 1244 proteins. We identified 1244 proteins with 2589 phosphorylation sites (>99% confidency) in mouse liver cell line Hepa1-6. Na-pervanadate, the phospho-tyrosine phosphatase inhibitor, serves as a very useful tool to study tyrosine phosphoproteome.

23. C.J. Toher, A.W. Perala, M.M. Shukla, A.K. Shukla, G.A. Valaskovic, "Offline Nano-ESI Phosphopeptide Analysis with Carbon, TiO2, and ZrO2 Wall-Coated Trap'nTips" 1) New Objective, Inc., Woburn, MA, 2) Glygen, Inc., Columbia, MD; ASMS Poster (2006).

    Abstract: Until recently, in-pipette sample preparation for nanobore LC-MS has been impeded by geometric constraints of sample preparation and transfer. Conventional practice has involved sample delivery from a pipette tip to an emitter, pressurized back-loading into a pipette, or

coupling the pipette tip to a larger pipette tip1. The design of carbon, TiO2 and ZrO2 wall-coated pipette tips (Trap'nTips™, New Objective) facilitates phosphopeptide enrichment and manual aspiration through the pipette tip followed by expulsion of purified solution for immediate nanobore column injection or direct loading into an offline nanospray emitter. In addition to concentration, desalting, and MS signal enhancement, Trap'nTips coated with these analyte-specific substrates facilitate offline nanospray analysis by separating phosphopeptides from a tryptic ß-casein digest. For complex digest solutions, Carbon, TiO2, and ZrO2 wall-coated Trap'nTips™ successfully remove phosphopeptides for analysis via offline nanospray GlassTips™ with a multi-layer platinum-coated tip demonstrate robust performance in negative-ion mode. Mass spectra of digest samples purified via carbon, TiO2, and ZrO2 Trap'nTips display exceptional signal-to-noise for phosphopeptide analysis.

24. L. Yu, Z. Zhu, K.C. Chan, T.P. Conrads, H.J. Issaq, D.S. Dimitrov and T.D. Veenstra, "Cell Cycle-defi ned Phosphoproteome Analysis of Cancer Cells by Titanium Dioxide Enrichment and Mass Spectrometry," 1) Laboratory of Proteomics and Analytical Technologies, SAIC-Frederick, Inc., NCI-Frederick; 2)Nanobiology Program, NCI-Frederick, Frederick, MD; ASMS Poster (2006).

Abstract: Improved performance of TiO2 enrichment for phosphopeptides. Identification of ~30000 phosphosites from >16000 phosphopeptides in Hela cells. Significant coverage of phosphoproteins involved in interphase, M phase, centrosome cycle, spindle organization and cell cycle regulation. TiO2 phosphopeptide enrichment has been optimized to characterize the complex phosphoproteome of HeLa cells at M phase. Column wash with NH4Glu or Glu greatly reduced nonspecific peptide binding to TiO2 columns and thus improved the specificity of phosphopeptide enrichment without significant loss of recovery. More than 16000 phosphopeptides were identified from the HeLa cells at M phase by the multidimensional approach of TiO2 enrichment, LC separation, and mass spectrometry. Approximately 30000 phosphosites were assigned from the identified phosphopeptides. From the large number of phosphoproteins identified, a significant coverage of proteins involved in interphase, M phase, centrosome cycle, spindle organization, and cell cycle regulation was obtained. Many of these cell cycle–related proteins are highly phosphorylated and may provide important information in cell cycle regulation.

25. T.E. Thingholm, M,R. Larsen, L,J. Foster, M, Kassem, O,N. Jensen, "Phosphoproteome Analysis of Human Stem Cell Plasma Membranes using TiO2 Columns and Mass Spectrometry," 1) Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark; 2) Biochemistry and Molecular Biology, BC, Vancouver, Canada; 3) Clinic of Endocrinology Treatment, Odense, Denmark; ASMS Poster (2006).

Abstract: A highly efficient method for purification of plasma membrane proteins is described. According to gene annotation data, 66% of the proteins identified were plasma membrane proteins and ~80% were functionally related to membranes. A very selective enrichment method, employing titanium dioxide (TiO2), is used for purification of phosphorylated peptides from plasma membrane fractions from human mesenchymal stem cells. The use of phosphatase inhibitors improves the level of identified phosphopeptides and a specific Tyrosine phosphatase inhibitor (e.g. Pervanadate) is critical for the identification of Tyrosine phosphopeptides. The combination of sucrose centrifugation and Na2CO3 extraction resulted in an enrichment of membrane and membrane-associated proteins of ~80% 2939 phosphopeptides were identified from a total of 7326 peptides (phosphopeptide enrichment efficiency of 40%). TiO2 is selective and efficient for phosphopeptide enrichment of highly complex biological samples. The use of phosphatase inhibitors leads to improved phosphopeptide recovery. The use of a specific Tyrosine phosphatase inhibitor such as Pervanadate is necessary for the identification of Tyrosine phosphopeptides. For more complete phosphoproteome analysis, further prefractionation or a longer LC gradient is necessary.

26. A.M. Taylor, L. DeSouza and K.W. Michael Siu, "Profiling the Phosphoproteome of Endometrial Carcinoma," Department of Chemistry, Centre for Research in Mass Spectrometry, York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3. ASMS Poster (2006).

Abstract: Phosphorylated proteins from a human endometrial cancer cell line were pre-concentrated using affinity chromatography. After tryptic digestion of the isolated proteins, TiO2 nanocolumns were evaluated for their effectiveness in enriching phosphorylated peptides. C18 nanobore LC-MS/MS with use of the scan modes afforded by a QTRAP instrument enabled the detection of several phosphopeptides.

27. S. Gotta, G.L. Sardone, G.C. Terstappen and R. Raggiaschi, "Comparison Between the Behavior of Different Sorbents Commonly Used for the Selective Isolation of Phosphopeptides from Complex Peptide Mixtures," Siena Biotech S.p.A., Discovery Research; Siena, Italy. ASMS Presentation (2006).

Abstract: This study was performed in order to select in our lab an efficient, simple and rapid method for isolating phosphopeptides from very complex digestion mixtures derived from total lysates, as we're interested in phosphorylation events occurring in rat cortical neurons upon challenging with amyloid-beta(25-35) peptide. We chose four sorbents and compared their yields and selectivity in isolating phosphopeptides from very complex peptides mixtures, as those obtained from rat cortical

neuron lysates, in order to assess their effectiveness on 'real' and difficult samples. We used the Phos-Select™ resin from Sigma on a spin column format, the Ni-NTA spin columns from Qiagen, where Ni was substituted with Fe(III) or Ga(III), and a TiO2 sorbent obtained from Sachtleben, both in batch and on spin columns.

28. Y.Q. Yu, J. Fournier, P. Olivova, M. Gilar, A. Chakraborty, S.J. Berger and J. Gebler, "A Novel Mixed Mode Chromatography Method for the Isolation and Separation of Phosphopeptides," Life Sciences R &D, Waters Corporation, Milford, MA, USA. ASMS Poster (2006).

    Abstract: IMAC and TiO2 type of affinity chromatography is widely used to isolate phosphopeptides. However, some non-phosphopeptides (*e.g.,* acidic peptides) also bind to the affinity sorbent; the degree of non-specific binding increases with increased sample complexity. We present an improved phosphopeptide enrichment/separation method that combines TiO2 and a prototype mixed mode (reversed-phase and strong cation exchange) liquid chromatography media prior to tandem mass spectrometry analysis. Phosphopeptides are weakly retained on the mixed mode LC column due to the negatively charged phosphate. A reversed-phase gradient elutes phosphopeptides according to the number of phosphate groups and their hydrophobicity. Non-phosphorylated peptides retain strongly on the mixed mode LC column due to ionic interaction. A rapid shallow salt gradient is used to elute these peptides. The isolation of phosphopeptides are further improved by processing complex samples using a TiO2 SPE device prior to the mixed mode LC separation.

29. J. Casado-Vela, A. Núñez, P. Alfonso and I. Casal, "Analysis of Phosphopeptides Using Off-line TiO2 Microcolumns Followed by Nano-HPLC Coupled to ESI Ion Trap," Spanish National Cancer Centre, CNIO. MADRID, SPAIN. ASMS Poster (2006).

    Abstract: The combination of high-sensitivity/high-throughput techniques such as nano-HPLC coupled to ESI-ion trap seems the method of choice for the analysis of post-translational modifications, including phosphorylations. The purpose of this study is double: first, to compare the capabilities of the Data Dependent Neutral Loss (DDNL) versus the Single Ion Reaction Monitoring (SIRM) methods for the analysis and detection of phosphorylations using the linear ion trap LTQ (Thermo Finnigan). Second, to carry out a comparative study of two phosphopeptide enrichment procedures using Gallium *immobilized metal affinity chromatography* (IMAC) and TiO2 beads.

30. J. Fournier, Y.Q. Yu, M. Gilar, G.M. Credo, W. Chen and J.C. Gebler,"Phosphopeptide Enrichment for Mass Spectrometry Analysis Using Microscale Titanium Dioxide Solid Phase Extraction," Waters Corporation, Life Sciences R & D, Milford, MA USA. ASMS Poster (2006).

Abstract: The reversible phosphorylation of serine, threonine and tyrosine, is one of the most abundant and significant post-translational modifications involved in a variety of cellular functions. Identification of phosphorylation sites by mass spectrometry is challenging due to the low abundance of phosphopeptides and their low ionization efficiency. Therefore, it is critical to enrich the phosphopeptides prior to MS analysis. One of the most commonly used enrichment techniques is immobilized metal affinity chromatography (IMAC). With this approach the phosphorylated peptides are enriched by the selective chelation with metals, such as Fe3+. With these methods, optimal enrichment and recovery depends largely on the type of metal ion used, packing material, and the loading/elution procedures. Recently, Titanium oxide (TiO2) has been used as an alternative to IMAC (1). IMAC sorbents, along with TiO2 sorbent, were evaluated for phosphopeptide affinity extraction in terms of selectivity and recovery, optimizing various loading and elution solutions using a standard sample containing synthetic phosphopeptides. All three types of phosphorylated amino acids, such as phosphoserine, phosphothreonine and phosphotyrosine are represented in the sample. The synthetic phosphopeptides are spiked into an enolase tryptic digest in various ratios. All sorbents in this study were packed in a 96-well micro Elution plate. Selectivity was evaluated using LC/MS.

31. S. Imanishi, H. Pallari, V. Kochin, and J.E. Eriksson, "Optimization of IMAC and TiO2 Chromatography for Phosphoproteomics," Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, and Department of Biology, University of Turku, Turku, Finland. ASMS Poster (2006).

Abstract: Protein phosphorylation is the most important post-translational modification regulating a vast number of key cellular processes. While immobilized metal affinity chromatography (IMAC) has been widely used for affinity purification of phosphopeptides, the technique still suffers from insufficient specificity. Therefore, there is an urgent need for IMAC optimization to yield the selectivity and sensitivity that is required for more challenging analyses. Recently, titanium dioxide (TiO2) has been shown to be a highly selective material for phosphopeptide enrichment1,2). In this study, these methods were further optimized and compared. The combination of phosphoric acid (PA) and ACN as an IMAC eluant was more efficient than previously known IMAC eluants. However, NH4OH2) was more efficient as an eluant for TiO2 chromatography than PA/ACN and other tested eluants. High concentrations of TFA and ACN were used for washing in TiO2 chromatography. A significant decrease in binding of non-specific peptides was obtained, while phosphopeptides were still retained by the TiO2 column. In contrast, TFA caused poor recovery of phosphopeptides on IMAC. TiO2 chromatography was more selective and sensitive against

tryptic casein phosphopeptides than IMAC. Nine of novel phosphorylation sites, which consisted of 5 of Pro-dependent phosphorylation sites and 4 of the others, were identified by TiO2 chromatography and MALDI-MS/MS analysis from GFP-Nestins phosphorylated *in vivo* by Cdk5/p35.

32. S. Mohammed, M.W.H. Pinkse, J.J. Benschop, Z. Hao, K. Kraiczek and A.J.R. Heck, "Unraveling the Phosphoproteome by Combinations of SCX, Titanium Dioxide Phosphopeptide Enrichment, Chip Based LC and Electron Transfer Induced Dissociation," Utrecht University, Sorbonnelaan 16 Utrecht 3584 CA, The Netherlands, Thermo Electron Corp, San Jose, CA & Agilent Technologies, Waldbronn, Germany. ASMS Poster (2006).

    Abstract: Current phosphopeptide analysis stratedies although sucessful still possess limitations. Ease of use and robustness of enrichment stratedies as well as sequencing of multiply phosphorylated peptides continue to require improvement. In order to help address these issues we evaluate the use of TiO_2 columns in HPLC-Chip systems as well as TiO_2 being coupled to a mass spectrometer equipped with electron transfer induced dissociation (ETD) capabilites.

33. D.R. Craft, K.A. Chisholm, M.B. Gupta, A.A. Doucette and D.M. Pinto, "Using Titanium Dioxide to Selectively Enrich and Fractionate Phosphorylated Peptides Institute for Marine Biosciences," National Research Council of Canada; Child Health Research Institute, U of W. Ontario, Canada; Department of Chemistry, Dalhousie University, Canada. ASMS Poster (2006).

    Abstract: Purpose: To selectively adsorb phosphorylated peptides onto a titanium dioxide support. Methods: Column was prepared using PEEK tubing packed with TiO2 porous beads. Tryptic peptides were loaded on to the column. Phosphorylated peptides were selectively adsorbed onto the TiO2 support. Non-phosphorylated peptides were washed off the column. Phosphopeptides eluted off the TiO2 column in step gradients. Peptides were analyzed by reverse phase LC-ESI-MS/MS on a Qtrap. Result: Successful enrichment of phosphopeptides in the presents of high abundant non-phosphorylated peptides. Separation of acidic peptides from phosphorylated peptides on the TiO2 column. Improve phosphopeptide recovery from TiO2 columns.

34. E. Oliveira, O. Blanco and M.A. Odena, "Comparison of IMAC and TiO2 Methodologies for Phosphopeptide Enrichment before MALDI-TOF Analysis," 1) Proteomics Platform, Barcelona Science Park, University of Barcelona; 2) Department of Biochemistry and Molecular Biology - IRB_Institute for Research in Biomedicine, Barcelona Science Park, University of Barcelona. ASMS Poster (2006).

Abstract: Phosphorylation is a post-translational modification (PTM) of interest because it plays a key role in regulatory pathways at a cellular level. Low stoichiometry of phosphorylated proteins "in vivo" makes enrichment necessary for phosphoproteomic MS-based studies. 2,5-DHB-phosphoric acid (DHB-PA) matrix has been described as optimal for detection of phosphopeptides with MALDI 1, but it fails to produce MS/MS spectra good enough for phosporylation site localization and protein identification even at high laser intensities. IMAC2 and TiO2 microcolumns3 are two methodologies developed for selective enrichment of phosphorylated peptides from phosphorylated and non-phosphorylated sample mixtures. Here we compare IMAC and TiO2 methods and try to improve MSMS fragmentation signal by adding HCCA on DHB-PA spotted phosphopeptide enriched samples. Fragmentation efficiency of phosphopeptides in a MALDI-TOF-TOF instrument can be improved by adding α-cyano-4-hydroxycinnamic acid matrix (HCCA) to the DHB-PA spotted samples. DHB-PA helps detecting phosphopeptides, and then sandwich with HCCA highly improves the quality of MS/MS spectra. TiO2 and IMAC are complementary and sequence dependent. TiO2 seems to have low affinity for positively charged phosphopeptides and it works better than IMAC for enrichment of monophosphorylated peptides. IMAC has a higher efficiency than TiO2 for the recovery of multiphosphorylated peptides.

35. C. Zhang, S. Liu, J. Lenehan, D.W. Litchfield and G. Lajoie "Global Phosphoproteome Analysis by Combining Highly Selective TiO2 Enrichment and EDTA-enhanced Detection by LC-MS," The University of Western Ontario, London, Ontario. ASMS Poster (2006).

Abstract: A global phosphoproteomics analysis of cell protein extracts was performed by high selective TiO2 enrichment and EDTA enhanced LC-MS/MS. 132 phosphopeptides from 54 proteins were identified from TiO2 enriched mitotic cells extracts. The TiO2 enrichment method showed a low recovery for multiply phosphorylated peptides. EDTA had significant effect on the elimination of Fephosphopeptide complex ions for enriched phosphopeptides samples.

36. L.A. Jacobsen, P. Heding, F. Pociot, P. Roepstorff and M.R. Larsen, "Highly Selective Enrichment of Phosphopeptides Involved in the Very Early Cytokine Signaling Event Using an Optimized TiO2 Purification Protocol," 1) Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230, Odense, Denmark; 2) Steno Diabetes Center, Gentofte, Denmark. ASMS Presentation (2006).

Abstract: We have shown an optimized method for highly specific purification of phosphopetides using TiO2 and in addition shown an optimal strategy for studying signal transduction pathways very early after

stimulation. We have found phosphopeptides that are upregulated after stimulation however, attribute the lag of highly regulated phosphopeptides to: 1) the cells were not starved for 12 hours prior to stimulation as pancreatic beta-cells change the response to Il-1β if starved 2) the cells were not synchronized and therefore very short time effects of stimulation might drown in average phosphorylation events 3) the quality of the fragmentation ion spectra using CID was poor, indication an urgent need for ETD in phophoproteomics.

37. E. Kühn-Hölsken, F. Richter, B. Sander, O. Dybkov, C.L. Will, R. Lührmann and H. Urlaub "MALDI-ToF Analysis of Cross-linked Peptide–oligoribonucleotides Derived from Various UV-irradiated Ribonucleoprotein Particles," Bioanalytical Mass Spectrometry Group Max Planck Institute for Biophysical Chemistry, Göttingen, Germany. ASMS Presentation (2006).

Abstract: MS by far most sensitive technique to identify hitherto unknown proteins that contact RNA. State-of-the-art MS allows sequencing of cross-linked protein-RNA heteroconjugates. Detection of protein-RNA contact sites within 17S U2 snRNP and 25S tri-snRNP possible. Miniaturization and enrichment strategies/state-of-the-art MS: pave the way towards the analysis of low abundance protein-RNA complexes. Cross-linking yield.

38. S.S. Jensen, N.H.H. Heegaard, L. Jacobsen and M.R. Larsen, "Exploring the Sialiome Using Titanium Dioxide and Mass Spectrometry; Identification of Sialic Acid Containing Glycopeptides from Glycosylated Proteins," 1) Dept. of Biochemistry and Molecular Biology University of Southern Denmark; 2) State Serum Institute, Copenhagen, Denmark. ASMS Poster (2006).

Abstract: A new method for selective enrichment of SIALIC ACID CONTAINING GLYCOPEPTIDES from complex peptide mixtures using an optimized procedure for TITANIUM DIOXIDE microcolumns is described. Examples of specific isolation of sialic acid containing glycopeptides from FETUIN is shown using O16:O18 labeling. A total of 200 N-LINKED SITES were found in 105 GLYCOPROTEINS from less than 60 μL depleted plasma. Preliminary results on the comparison of sialic acid containing glycopeptides from human plasma from control sample vs. bladdercancer, is shown.

39. T. De Vijlder, K. Laukens, A. Pharazyn, F. Lemière, W. Van Dongen, R. Tuytten, E.L. Esmans, H.A.Van Onckelen, M. Maras, W. De Coen and E.J. Witters, "Biomarker Studies Using the Phosphoproteome," University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium. ASMS Presentation (2006).

Abstract: Phosphorylation and dephosphorylation of proteins in a biological context is a pivotal biochemical event that activates or

deactivates proteins and protein complexes, that in their turn can control the activity status of signal transduction cascades and complete pathways. After perception of biochemical or physicochemical stimuli this cellular reaction is typically in the order of minutes to tens of minutes. Since transcriptomics in general is transparent for both this cellular time frame and any post translational modification, this MS based proteomics strategy offers a unique and generic way to identify biomarkers. In this particular study biomarkers for ecotoxicological events are subject of investigation. Kinetic expression data is gathered for 20 selected model chemicals representing the diversity of known toxicological modes of action, relevant for environmental toxicology. The ultimate aim is to obtain a set of reporter genes with a characteristic "toxicity response profile" (TRP) for each of the 20 selected chemicals using a human breast cancer cell line as biological model. Eventually each TRP will consist out of a selected combination of genes that will be used to construct reporter cell lines for bio-essays.

40. Henrik Molina, David M. Horn, Ning Tang, Suresh Mathivanan and Akhilesh Pandey, "Global Proteomic Profiling of Phosphopeptides Using Electron Transfer Dissociation Tandem Mass Spectrometry, PNAS, 104 (7), 2199-2204 (2007).

Abstract: Electron transfer dissociation (ETD) is a recently introduced mass spectrometric technique that provides a more comprehensive coverage of peptide sequences and posttranslational modifications. Here, we evaluated the use of ETD for a global phosphoproteome analysis. In all, we identified a total of 1,435 phosphorylation sites from human embryonic kidney 293T cells, of which 1,141 (_80%) were not previously described. A detailed comparison of ETD and collision-induced dissociation (CID) modes showed that ETD identified 60% more phosphopeptides than CID, with an average of 40% more fragment ions that facilitated localization of phosphorylation sites. Although our data indicate that ETD is superior to CID for phosphorylation analysis, the two methods can be effectively combined in alternating ETD and CID modes for a more comprehensive analysis. Combining ETD and CID, from this single study, we were able to identify 80% of the known phosphorylation sites in >1,000 phosphorylated peptides analyzed. A hierarchical clustering of the identified phosphorylation sites allowed us to discover 15 phosphorylation motifs that have not been reported previously. Overall, ETD is an excellent method for localization of phosphorylation sites and should be an integral component of any strategy for comprehensive phosphorylation analysis.

41. E. Damoc, C. S. Fraser, M. Zhou, H. Videler, G. L. Mayeur, J. W. B. Hershey, J. A. Doudna, C. V. Robinson and J. A. Leary, "Structural Characterization of the Human Eukaryotic Initiation Factor 3 Protein Complex by Mass Spectrometry," Molecular & Cellular Proteomics, (6), 1135-1146 (2007).

Abstract: Protein synthesis in mammalian cells requires initiation factor eIF3, an approximately 800-kDa protein complex that plays a central role in binding of initiator methionyl-tRNA and mRNA to the 40 S ribosomal subunit to form the 48 S initiation complex. The eIF3 complex also prevents premature association of the 40 and 60 S ribosomal subunits and interacts with other initiation factors involved in start codon selection. The molecular mechanisms by which eIF3 exerts these functions are poorly understood. Since its initial characterization in the 1970s, the exact size, composition, and post-translational modifications of mammalian eIF3 have not been rigorously determined. Two powerful mass spectrometric approaches were used in the present study to determine post-translational modifications that may regulate the activity of eIF3 during the translation initiation process and to characterize the molecular structure of the human eIF3 protein complex purified from HeLa cells. In the first approach, the bottom-up analysis of eIF3 allowed for the identification of a total of 13 protein components (eIF3a-m) with a sequence coverage of approximately 79%. Furthermore 29 phosphorylation sites and several other post-translational modifications were unambiguously identified within the eIF3 complex. The second mass spectrometric approach, involving analysis of intact eIF3, allowed the detection of a complex with each of the 13 subunits present in stoichiometric amounts. Using tandem mass spectrometry four eIF3 subunits (h, i, k, and m) were found to be most easily dissociated and therefore likely to be on the periphery of the complex. It is noteworthy that none of these four subunits were found to be phosphorylated. These data raise interesting questions about the function of phosphorylation as it relates to the core subunits of the complex.

42. S.Y. Imanishi, V. Kochin, S.E. Ferraris, A. de Thonel, H. Pallari, G.L. Corthals and J.E. Eriksson, "Reference-facilitated Phosphoproteomics: Fast and Reliable Phosphopeptide Validation by μLC-ESI-Q-TOF MS/MS," Molecular and Cellular Proteomics, 6, 1380-1391 (2007).

Abstract: Recent advances in instrument control and enrichment procedures have enabled us to quantify large numbers of phosphoproteins and record site-specific phosphorylation events. An intriguing problem that has arisen with these advances is to accurately validate where phosphorylation events occur, if possible in an automated manner. The problem is difficult, as MS/MS spectra of phosphopeptides are generally more complicated than those of unmodified peptides. For large-scale studies, the problem is even more evident, as phosphorylation sites are based on single peptide identifications, in contrast to protein identifications where at least 2 peptides from the same protein are required for identification. To address this problem we have developed an integrated strategy that increases the reliability and ease for

phosphopeptide validation. We have developed an off-line titanium dioxide (TiO2) selective phosphopeptide enrichment procedure for crude cell lysates, Following enrichment, half of the phosphopeptide fractionated sample is enzymatically dephosphorylated, after which both samples are subjected to LC-MS/MS. From the resulting MS/MS analyses, the dephosphorylated peptide is used as a reference spectrum against the original phosphopeptide spectrum, in effect generating two peptide spectra for the same amino acid sequence, thereby enhancing the probability of a correct identification. The integrated procedure is summarised as follows: 1) enrichment for phosphopeptides by TiO2 chromatography, 2) dephosphorylation of half the sample 3) LC-MS/MS-based analysis of phosphopeptides and corresponding dephosphorylated peptides, 4) comparison of peptide elution profiles before and after dephosphorylation, to confirm phosphorylation, 5) comparison of MS/MS spectra before and after dephosphorylation, to validate the phosphopeptide and its phosphorylation site. This phosphopeptide identification represents a major improvement as compared to identifications based only on single MS/MS spectra and probability-based database searches. We investigated an applicability of this method to crude cell lysates and demonstrate its application on the large-scale analysis of phosphorylation sites in differentiating mouse myoblast cells.

43. D. A. Moraga, M. C.Chow, I. Isaac and S. M. Stevens, Jr., "Automated Analysis of Gel-Derived Phosphoproteins Using the Investigator Proteomic System," Journal of Biomolecular Techniques, 18 (1), 26 (2007).

Abstract: The evolution of proteomics-based technologies has led to the development of powerful analytical methods for the analysis of post-translational modifications such as phosphorylation. Chemical derivatization strategies that involve beta-elimination and Michael addition chemistries as well as chromatographic enrichment approaches using immobilized metal affinity chromatography have been successfully applied to global phosphoproteome analysis. Recently, enrichment of phosphorylated peptides using TiO_2 prior to mass spectrometric analysis has been shown to be a selective and robust method for phosphoprotein characterization. We present an automated workflow using TiO_2 microcolumns for the enrichment of phosphorylated peptides derived from in-gel digested proteins in combination with the Investigator Proteomic System (Genomics Solutions, Ann Arbor, MI). A pool of beta casein and ovalbumin (10 μg each) was used as a standard to test the automated phosphoprotein analysis workflow. This same workflow was then successfully applied to characterize in vitro and in vivo phosphorylation events of selected 14-3-3 proteins derived from *Arabidopsis thaliana*. Samples were separated by either 1D or 2D SDS-PAGE, stained with Pro-Q Diamond (phosphoprotein-specific) fluorescent stain, and imaged using either a Typhoon 9400 scanner or the Investigator ProPic (Genomic Solutions). Putative phosphoprotein-containing spots were then excised

and digested in-gel with trypsin using the Investigator ProPic and ProGest, respectively. Tryptic digests from each protein spot were processed with the ProMS workstation using TiO_2 microcolumns (Glygen Corp., Columbia, MD) for phosphopeptide enrichment prior to MALDI-TOF/TOF analysis (ABI 4700 Proteomics Analyzer).

44. J. Zhu and Q. Lin, "Using Titanium Dioxide IMAC for Enrichment of Phosphopeptides Prior to Tandem Mass Spectrometry," Journal of Biomolecular Techniques, 18, 24-25 (2007).

Abstract: Protein phosphorylation plays a significant role in regulating cellular processes such as signal transduction, cell division, cell motility, apoptosis, metabolism, differentiation, gene regulation, and carcinogenesis. Typically, there are 10–20% of proteins which are phosphorylated. Due to the low level of phosphoproteins in the presence of overwhelming amounts of non-phosphorylated proteins, as well as those proteins' wide dynamic variation over time, identification of phosphopeptides is still a formidable task. In addition, phosphopeptides often have poor ionization efficiency in MS analysis. Thus, a highly sensitive detection method plus phosphopeptide enrichment is extremely important for a successful phosphopeptide identification. Currently, immobilized metal affinity chromatography (IMAC) is the method of choice for enriching phosphopeptides from complex biological samples. Typically, nickel, iron, and gallium–based IMAC shows significant binding of non-phosphorylated peptides that have multiple acidic residues. Forest White et al. used a kind of chemistry to put methyl esters onto those acidic groups (D and E) to solve the problem of nonspecific binding to the IMAC beads. However, this approach brings in a lot of side reactions to that chemistry, and raises issues of how complete the modifications are. Recently, several papers and posters have been published demonstrating the unique ability of titanium dioxide and zirconium dioxide to selectively retain phosphopeptides contained in complex biological mixtures. In this application, a TiO_2-based IMAC method was successfully developed to enrich phosphopeptides and adapted to a complex biological sample, *Saccharomyces*. Trapping phosphopeptides are demonstrated via the analysis protein CaO19_4593 (gi|68466366), a family of GTPase-activating proteins which contains multiple kinase-binding domains.

45. LiwenWang, Hua Xu, Chen Ren, Shujun Liu, Guido Marcucci and Michael A. Freitas,"(Use of ZrO2 Tips) Enrichment and Characterization of Histone H1 Phosphorylation Isoforms in Chemoprevention of Acute Myeloid Leukemia," 4th Annual Ohio Mass Spectrometry Symposium (March 19-20 2007).

Abstract: Phosphorylation of H1 has been associated with the regulation of gene expression, DNA repair and chromatin remodeling. Characterization of H1 isoform is necessary to understand its role in gene

regulation. Phosphorylated H1 isoforms are altered in human leukemia and respond to chemotherapy agents in vitro. Determining which isoforms are present is of significant clinic importance. Due to the low abundance of phosphorylation in vivo, preconcentration of phosphopeptides is necessary for identification and characterization. Herein we will present the application of zirconium dioxide solid-phase extraction, data-dependent neutral loss MS3 and hierarchical MS3 databases searching for phosphopeptide characterization. By application of this approach the location of phosphorylation sites in histone H1 from Kasumi-1 cells subjected to chemotherapeutic treatment in vitro was determined.

46. Jenny Albanese, Senior Field Applications Specialist - Proteomics, Applied Biosystems, Inc., "IMAC in Combination with LC-Maldi Enabled Femtomole Detection of Phosphopeptides," 2006 Phospho Workshop at Mississippi State University, http://www.lsbi.mafes.msstate.edu/4700_phospho_workshop.ppt

47. C. Collin-Hansen, E.E. Gulcicek, K.R. Williams, C.M. Colangelo, and A.C. Nairn, "Phosphoproteome of Synaptoneurosomes from Cocaine Treated Rats," 1) Yale University, Psychiatry, New Haven, CT; 2) W.M. Keck Foundation, Biotech. Resource Laboratory, New Haven, CT. ASMS Poster #396 (2007).

Abstract: Using casein digests as sample, we performed initial comparisons between different metal oxide materials for phosphopeptide enrichment. Some of these materials were known from the literature (TiO_2, ZrO_2), whereas others (Fe_2TiO_5, $FeTiO_3$) have not been reported used for phosphopeptide enrichment. Notably, most of the metal oxide materials tested provided a quick and robust way to enrich for phosphopeptides from samples of low to moderate complexity. Analysis by LC-MS/MS (Q-TOF) showed that the four materials giving the best relative enrichment in our initial test were TiO_2 Monotip (GL Sciences), TiO_2 TopTip (Glygen), $FeTiO_3$ (Alfa Aesar) loaded in an empty TopTip and TiO_2 Monotip with Fe_2TiO_5 (Alfa Aesar) loaded on top of the TiO_2 monolith crystal provided with these tips. These four top-performing materials from the initial comparison were selected for further testing with a synaptoneurosomal protein digest. Samples were split in four and phosphopeptides were enriched using one of the four metal oxide materials. The results showed that with the synaptoneurosomal protein digest, the TiO_2 affinity materials generally outperformed the other metal oxide materials. An IMAC phosphopeptide enrichment strategy was also compared against the best performing metal oxide resin using synaptoneurosomal proteins as sample. Our preliminary results show that even though TiO_2 metal affinity materials produce higher number of phosphopeptide identifications than IMAC, the two methods produced complimentary results (more than 50 per cent of the phosphopeptides identified by IMAC were not identified by the TiO_2 resins). We will also show results from our preliminary studies comparing enriched

phosphopeptides between synaptoneurosomes isolated from cocaine treated rats versus control using iTRAQ based quantitation. This project has been funded in part with Federal funds from NIDA/NIH grant 1 P30 DA018343, DA10044 and NHLBI/NIH contract N01-HV-28186

48. A. Mikulskis, Y. Wang, A. Bogdanova, E. Golenko, and W.F. Patton**, "**Robust Enrichment of Phosphopeptides from Serum Using Titania-coated Magnetic Beads for Rapid Profiling of Biomarkers by Mass Spectrometry," PerkinElmer, Waltham, MA. ASMS Poster #397 (2007).

Abstract: Direct enrichment of phosphopeptides from serum using titania-coated magnetic beads is presented. Both endogenous serum phosphopeptides and spiked in β-casein phosphopeptides have been successfully enriched using a single step fractionation which was not possible with commonly used IMAC systems. Robust sample processing in less than 30 min using magnetic separation directly in a 96-well plate format is well suited for high throughput profiling of peptide biomarkers in serum. Reproducibility of peak intensities in mass spectra was better than 20%. A set of serum samples from the patients with a congestive heart disorder, a panel of cancer patients, and serum from normal patients were used to enrich phosphopeptides and profile the corresponding mass spectra patterns by MALDI MS analysis. Several unique patterns were identified for each disease type. The patterns in mass spectra of cancer samples resulted in common peaks which were different from the peaks found in the congestive heart disorder and normal serum samples. The representative peptides and corresponding phosphorylation sites were identified by tandem MS analysis. Fibrinogen A phosphopeptide was identified as one of the endogenous phosphopeptides enriched in the analyzed serum samples. Several additional enriched on titania peptides were shown to represent kininogen fragments containing 6xHis-tag-like motifs and likely exhibiting affinity to titania.

49. S. Liu, M. Abu Shehab, M.B. Gupta, V.K.M. Han and G. Lajoie, "Optimized LC-MS/MS for Analysis of Multiply Phosphorylated Peptides of IGFBP1 from Amniotic Fluid of IUGR Patient," 1) University of Western Ontario, London, ON, Canada; 2) Children's Health Research Institute, London, ON, Canada . ASMS Poster #432 (2007).

Abstract: Three phosphorylation sites have been reported. After dual enzyme digestion with Asp-N and trypsin, 3 phosphorylated peptides are generated containing phosphrylated Ser101, Ser119 and Ser169, respectively. Direct analysis of the digest sample allowed the identification of IGFBP1, but failed to detect the phosphorylated peptides. After TiO2 enrichment, 3 phosphorylated peptides were detected and characterized: DASAPHAAEAGSPESPEpSTEITEEELL (P1), 949.74 m/z, DNFHLMAPpSEE (P2), 693.26 m/z, AQETpSGEEISK (P3), 629.76

m/z. We also detected some metal adduct ions in these samples. For example, for peptide P1, ions at 966.06, 967.40 and 968.38 m/z were observed, representing [M+Cr]3+, [M+Fe]3+ and [M+Ni+H]3+, respectively. Metal adduct ions dispersed the signal for the protonated peptide, therefore EDTA was added to the sample to prevent the formation of the metal adduct ions. Upon the addition of EDTA, the signal intensity for the metal adduct ions were suppressed significantly for Cr and Fe adduct ions (below 7% of the protonated peptide ion). However, the intensity for the Ni adduct ion was still very high (40% of the protonated peptide ion). This might be because the acidic conditions in the LC limited the complexation effect of EDTA with Ni which has a lower pKf (18.6) than with Cr (23.4) and Fe (III) (25.1). However, upon addition of EDTA, an additional ion at 976.41 m/z was detected. MS/MS confirmed that the peptide sequence to be DASAPHAAEAGSPESPEpSpTEITEEELL (P4). As previously observed with multi-phosphorylated peptides, the extreme acidic characteristic of the peptide P4 makes it very susceptible to bind metal ions in C18. With the addition of EDTA, this previously undetected phosphorylated peptide became detectable. This result further demonstrates that it is very important to prevent the binding of phosphorylated peptides to C18 especially when multi-phosphorylated peptide or very acidic peptides are analyzed.

50. Y. Ishihama, Y. Kyono, N. Sugiyama, S. Ohnuma, Y. Igarashi, K. Tani and M. Tomita, "Influence of Physico-chemical Properties of Titania on Phosphopeptide Enrichment for Phosphoproteomics" 1) Keio University, Tsuruoka, JAPAN; 2) GL Sciences, Iruma, JAPAN; 3) Human Metabolome Technologies, Tsuruoka, JAPAN; 4) Yamanashi University, Kofu, JAPAN . ASMS Poster #433 (2007).

Abstract: Two commercial titania beads (G-beads from GL science and S-beads from Sachtleben) as well as three calcinated titania beads (200-, 500- and 600-beads) showed the similar results for phosphopeptide enrichment from the standard phosphoprotein digest sample without lactic acid (selectivity, defined as the percentage ratio of the number of phosphopeptides to the number of total peptide identified, was approximately 40% on average). On the other hand, the presence of lactic acid in the sample buffer significantly improved the selectivity for G-beads (77% selectivity), whereas slight increase in selectivity was observed for S-beads (43% selectivity). Calcinated titania (200-, 500- and 600-beads) showed moderate increase in selectivity (65, 63 and 70 % on average, respectively). Comparison of physical properties revealed that both beads have the same crystalline form (anatase form) but the crystalline content is different, i. e., the content is 100% for S-beads whereas G-beads are the mixture of the crystal and amorphous forms. Powder X-ray diffraction (XRD) patterns for three calcinated titania indicated that 200- and 500-beads contain anatase crystal and amorphous forms, and 600-beads contain anatase and rutile crystal forms with some amorphous form. As the calcinations temperature increased, the crystalline

content increased as the peak width became sharper in powder XRD patterns. While the selectivity values for 200-, 500- and 600-beads with lactic acid were similar, the number of phosphopeptides as well as non-phosphopeptides increased as the calcinations temperature increased. These results suggested that the effect of lactic acid on selectivity increase is related to the amorphous form content and the anatase form has affinity both to phosphopeptides and acidic non-phosphopeptides, but less affinity to lactic acid. [1] K. Tani, E. Miyamoto, J. Liq. Chrom. & Rel. Technol., 22(6), 857-871 (1999).

51. J. Rinehart, C.A. Hodson, K.L. Stone, R.P. Lifton and E.E. Gulcicek, "A Phosphoproteomic Approach to Mapping Novel Phosphorylation Sites in a WNK Family Kinase," Yale University, New Haven, CT. ASMS Poster #435 (2007).

Abstract: A simplified enrichment of phospho-peptides with TiO2 is a novel phosphoproteomic approach that has been applied to our study of the WNK family of kinases. Furthermore, traditional mass spectrometry based approaches have yielded little information about the phosphorylation status of WNK kinases. We will report the identification of 11 novel WNK2 phosphorylation sites. This level of resolution has not been achieved for the WNK kinases and provides a new wealth of information into the function and regulation of WNK2 along with a myriad of new functional domains within the protein. We will describe our procedure and application of the TiO2 enrichment technology which is central to our achievement. We will present tables of peptides and phosphopeptides identified and discuss the results of the TiO2 enrichment coupled with the mass spectral analysis. We will also present the first map of the phosporylation sites of WNK2 and present evidence of 5 new regulatory domains within the molecule. We will discuss the implications of our findings to WNK kinase biology in general as well as future applications of TiO2 enrichment in WNK research. These findings have implications for such diverse and fundamental processes as maintenance of cell volume during osmotic stress, dynamic modulation of GABA neurotransmission from inhibitory to excitatory, and blood pressure homeostasis. In addition to WNK kinases, our methodology will provide a template for the study of a great number of human signaling kinases and their substrates. This project has been funded in whole or in part with Federal funds from the National Heart, Lung, and Blood Institute, National Institutes of Health, under contract No. N01-HV-28186 and from NIDA/NIH grant No. 1 P30 DA018343.

52. M. Schirle, M. Bantscheff, M. Raida and B. Kuster, "Direct and Quantitative Comparison of IMAC and Titanium Dioxide for Large-scale Enrichment and Identification of Phosphopeptides," Cellzome AG, Heidelberg, GERMANY . ASMS Poster #437 (2007).

Abstract: Stable isotope labeling using iTRAQ is a powerful tool for method development since it allows a direct comparison of up to four samples in a single MS analysis. Here, we used iTRAQ for a quantitative side-by-side comparison of enrichment of phosphopeptides using IMAC or titanium dioxide. Based on the identification of a large number of distinct kinase-derived phosphopeptides (>200), we found that both methods in general enrich a very similar set of phosphopeptides. However, quantitative analysis by iTRAQ revealed that while overall recovery of phosphorylated species is comparable in both methods, there are significant differences in enrichment efficiency for specific peptides. Differences of up to >5 fold were found reproducibly, with either IMAC or titanium dioxide being superior for a given peptide. Parameters contributing to these biases include number of phosphorylation sites per peptide, net charge and amino acid composition. Based on these findings, an optimized enrichment strategy consisting of consecutive steps of IMAC and titanium dioxide enrichment is suggested. The large body of experimental phosphorylation data available to date opens up the possibility to predict enrichment success based on phosphopeptide properties.

53. A.J. Alpert, S.P. Gygi and A.K. Shukla, "Desalting Phosphopeptides by Solid-Phase Extraction," 1) PolyLC Inc., Columbia, MD; 2) Harvard Medical School, Boston, MA; 3) Glygen Corp., Columbia, MD. ASMS Poster #438 (2007).

Abstract: Titania performed reasonably well with all phosphopeptides, under low- or high-salt conditions. Recovery appears to be somewhat less than quantitative. C-18 silica retained most peptides but failed to retain some phosphopeptides. High levels of salt promoted retention of phosphopeptides by C-18 silica, although some tended to leach out during intermediate water washes prior to elution. HyperCarb was effective at retention of all phosphopeptides from low-salt solvent but retention of phosphopeptides was antagonized by high levels of salt. Accordingly, with the low-salt HeLa cell fraction, HyperCarb retained and released ~ 50% more phosphopeptides than did C-18 silica. With the high-salt HeLa fraction the C-18 material retained more phosphopeptides than did HyperCarb. While the objective was to desalt phosphopeptides and not to enrich them, both HyperCarb and titania proved to be much more selective for phosphopeptides than was C-18 silica under all conditions.

54. H. Flynn and S. Gaskell, "Relative Quantification of Phosphorylated Proteins in SK-MEL-28 Cells, Treated with a Selective Small Molecule Inhibitor of B-Raf kinase, SB-590885," 1) GlaxoSmithKline, Stevenage, U.K.; 2) University of Manchester, Manchester, United Kingdom. ASMS Poster #439 (2007).

Abstract: The efficacy of the Qiagen Phosphoprotein Enrichment Kit was evaluated using lysates from SK-MEL-28 cells following treatment with

SB-590885-R or DMSO vehicle. Protein concentrations were determined post phosphosphoprotein enrichment. The proportion of total cellular protein represented by the phosphoprotein fraction was 8.5% for DMSO vehicle treated cells and 6.8% for SB-590885-R treated cells. Evidence for selective enrichment for phosphoproteins was obtained by 1D gel electrophoretic separations of the whole cell lysates and the phosphoprotein enriched fractions. For both SB590885-R treated and DMSO vehicle control samples, gel images following staining with ProQ Diamond (selective for phosphoproteins) and Sypro Ruby (not selective) were similar for the enriched fractions but markedly different for the total protein samples. We evaluated the performance of titanium dioxide for the enrichment of phosphopeptides from a tryptic digest of a phosphoprotein enriched sample, derived from a cell lysate. Samples were loaded in the presence or absence of a benzoic acid derivative (Waters proprietary enhancer compound). In each case a phosphopeptide enriched fraction was eluted using ammonium hydroxide. Data directed LC-MS/MS analyses were performed with subsequent processing of data and database searching using MASCOT. Considering the top 20 protein hits for each LC-MS/MS analysis we considered the proportion of peptide identifications that corresponded to phosphorylated peptides. This proportion was 55% for the data obtained following analysis of the sample enriched using the benzoic acid derivative and only 5% when this enhancer was omitted. The full paper will describe the combined application of these analytical strategies to the determination of the relative quantities of phosphoprotein in DMSO and SB-590885-R treated SK-MEL-28 cells.

55. M.R. Larsen, K. Engholm-Keller and S.S. Jensen, "Evaluation of Phosphopeptide Enrichment Procedures for Titanium Dioxide and Zirconium Dioxide Chromatography," University of Southern Denmark, Odense, Denmark. ASMS Poster #442 (2007).

Abstract: The TiO2 purification protocol originally published by us (Larsen MR et al Mol. Cell Proteomics, 2005) used 2,5 dihydroxybenzoic acid (DHB) as an additive in the peptide loading buffer to prevent non-specific binding. However, DHB can affect the liquid chromatography and mass spectrometer performance and therefore alternative buffers has been investigated in this study. Here we used a mixture of tryptic peptides originating from 12 proteins (whereof 3 was phosphorylated: alpha and beta casein and ovalbumin) yielding more than 20 phosphopeptides and tryptic peptide mixtures from a cell lysate. Different loading buffers were evaluated using iTRAQ labelling. After labelling peptide aliquots with iTRAQ 114, 115, 116 and 117, respectively, they were loaded onto TiO2 and ZrO2 in different buffers. The eluates were mixed and analyzed by MALDI and ESI MSMS and the iTRAQ ratios were used to find an optimized loading buffer. Preliminary results show that inclusion of 1 M

glycolic acid yield similar results as those obtained using DHB or phthalic acid. In addition, we have found that the level of non-phosphorylated peptides is much higher when only TFA or Formic acid is used in the loading buffer. In our hands TiO2 has proven significant better for phosphopeptide enrichment than ZrO2. To achieve a significant enrichment using ZrO2 it is essential to include an additive such as glycolic acid or phthalic acid. We have also found that TiO2 phosphopeptide enrichment using an optimized method is extremely tolerant towards most detergents, salts and other frequently observed contaminants in biological buffers. Selected examples will be shown.

56. Y. Kyono, N. Sugiyama, S. Ohnuma, T. Masuda, M. Tomita and Y. Ishihama," Effect of Chelating Enhancers on Phosphopeptide Enrichment by Ligand Exchange Metal Oxide Chromatography using Titania and Zirconia," 1) GL Science Inc., Iruma, Japan; 2) Human Metabolome Technologies, Inc., Tsuruoka, Japan; 3) Institute for Advanced Biosciences, Keio Universit, Tsuruoka, Japan. ASMS Poster #443 (2007).

Abstract: The digested phosphoprotein standard mixture was used to evaluate the effect of various modifiers in the loading buffer on phosphopeptide enrichment by MOC tips. Without any enhancer, approximately 10 phosphopeptides as well as several non-phosphorylated peptides were identified with titania, zirconia and $Al(OH)_3 \bullet xH_2O$-MOC tips. Although 2,5-DHB was effective to decrease non-phosphorylated peptides as reported, the number of phosphopeptides were also decreased. We also investigated the effect of the mixture of glutamic acid, aspartic acid and imidazole or OSA in combination with/without 2,5-DHB on the selectivity enhancement and identified several non-phosphopeptides with significant scores in all cases. In addition, we found that some of the enhancers were co-eluted with the phosphopeptides from the MOC tips, which may interfere with the subsequent nanoLC-MS/MS analysis. Based on these findings, small aliphatic hydroxy acids such as lactic acid, glycolic acid, malic acid and HPA were evaluated as the selectivity enhancers. All tested hydroxy acids except for glycolic acid showed higher selectivity than the modifiers mentioned above. Among them, lactic acid and HPA were most effective with titania- and zirconia-MOC tips, respectively, suppressing the identification of non-phosphorylated peptides efficiently. The optimized protocols were applied to the cytoplasmic fraction of HeLa cells and nanoLC-MS/MS analysis successfully identified 20, 68, 60, 147 and 320 phosphopeptides on average with the ratio of the number of phosphopeptides to total peptides = 0.008, 0.047 ,0.114, 0.143 and 0.619 for the eluents from the titania MOC tips with no modifier, 2,5-DHB, OSA, HPA and lactic acid, respectively.

57. A.S. Chien and A.W. Guzzetta, "An Automated Dual Trap & Column Arrangement for Online NanoLC-ESI-MS/MS Enrichment and Analysis of Phosphopeptides," SU Mass Spectrometry, Stanford University, Stanford, CA. ASMS Poster #445 (2007).

Abstract: An automated nanoLC-ESI-MS/MS method involving two traps and two columns is applied to the analysis of proteolytic phosphoprotein digests. The method selectively enriches phosphopeptides while separately retaining non-phosphorylated peptides. The non-phosphorylated peptides are analyzed on a C18 column, while the phosphorylated peptides are analyzed on a graphitic carbon column. The Hypercarb stationary phase has been shown to retain peptides which flow through the C18, including multiply phosphorylated peptides. A proteolytic digest of a known mixture of phosphorylated and non-phosphorylated proteins provides a variety of singly and multiply-phosphorylated peptides against a background of non-phosphorylated peptides, and is used to demonstrate the method. Datasets are analyzed using Sequest, Mascot, and X! Tandem, and collective search results are viewed using Scaffold. Combining the analyses of the fractions provides higher confidence in protein identifications, and often provides evidence of non-stoichiometric phosphorylation by identifying both the phosphorylated and non-phosphorylated versions of a peptide. Preliminary work with TiO2 has shown significant retention of non-phosphorylated peptides along with the desired phosphopeptides. In offline work, extensive washing with assorted solutions, including high organic solvent content and various salts, is effective in decreasing the amount of nonspecific binding. However, the precise wash solution needed is heavily sample dependent, and with most real-world samples, the multiple analyses required to optimize this step are not possible. In addition, many of these wash solutions are not compatible with the online cleanup, and are not used in this work. Instead, the C18 portion of the biphasic trap serves as a "filter" during the TiO2 (or IMAC) elution, to prevent many of these nonspecifically bound peptides from binding to the Hypercarb trap and complicating the analysis of the phosphopeptide fraction. If desired, an additional reverse phase gradient can be run following the Hypercarb elution in order to analyze this set of peptides.

58. L. Wang, C. Ren, S. Liu, H. Xu, G. Marcucci and M.A. Freitas, "Enrichment and Characterization of Histone H1 Phosphorylation Isoforms in Chemoprevention of Acute Myeloid Leukemia," Ohio State University, Columbus, OH. ASMS Presentation (2007).

Abstract: In this study several different chromatin modifying agents were used to treat acute myeloid leukemia patient cells and Kasumi-1 cells ex vivo in order to ascertain their chemotherapeutic potential. The stoichiometric change in phosphorylated H1 variants upon drug treatments

was observed by use of LC-MS. When compared with control samples, the phosphorylated isoforms of H1.5 decreased dramatically after treatment with flavopiridol and Velcade. In contrast, cells treated with 17 AAG, HDAC inhibitor 42 and Aza showed increased phosphorylation. These dramatic changes suggest alteration of the chromatin remodeling signaling pathways. In order to ascertain the specific sites of phosphorylation we developed a proteomic workflow that incorporated phosphopeptide enrichment, LC-MS3 and advanced bioinformatic data analysis. The overall approach was first validated with standard phosphopeptide mixtures obtained from Sigma Chemical Company (St. Louis, MO). Phosphopeptides from the standard mixture were successfully identified using MS2 and hierarchical MS3 database searching. Hierarchical database searching will initially identify (if possible) peptide sequences from the MS2 spectra. Then the corresponding MS3 data will be search using the limited peptide sequences obtained in the MS2. For peptide with poor MS2 quality due to predominant neutral loss, the MS3 data will be search against all possible candidate sequences that correspond to the precursor mass of MS2. In either case, the MS3 sequence and MS2 sequence are used in concert to obtain a peptide match with overall higher confidence. By using this new approach, phosphorylation sites are being identified in ZrO2 enriched H1 digests. Sites determined so far include: 33KApSGPPVSELITK45 and 1Ac-pSETAPAAPAAAPPAEKAPVK20. Ongoing experiment to determine the extent and distribution of phosphorylation of H1 in these leukemic cells along with the improvements in the bioinformatic data reduction will be presented.

59. T.E. Thingholm, O.N. Jensen and M.R. Larsen, **"**A New Phosphoproteomic Strategy – Separation of Mono- from Multi-phosphorylated Peptides Combined with Optimized MS3 Mass Spectrometric Analysis," University of Southern Denmark, Odense, Denmark**.** ASMS Presentation (2007).

Abstract: Human mesenchymal stem cell (hMSC) extract was used to demonstrate the performance of the new analytical strategy. Using an optimized TiO2 method (Thingholm et al, Nature Protocols, 2006) a total of 327 unique phosphopeptides (383 phosphorylation sites) were identified from 120 microgram hMSC protein. Of these 71.0% were mono-phosphorylated, 17.1% were multi-phosphorylated and 11.9% were non-phosphorylated. Using the new strategy for separation of mono-phosphorylated from multi-phosphorylated peptides combined with different MS fragmentation methods, more than 500 unique phosphopeptides were identified from the same amount of material. In the mono-phosphopeptide fraction enriched by IMAC and TiO2, 244 phosphopeptides were identified. Of these 94.7% were mono-phosphorylated, 4.5% were multi-phosphorylated and 0.8% were non-phosphorylated. In the multi-phosphopeptide fraction recovered from the

IMAC resin, 263 phosphopeptides were identified of which 67.3% were multi-phosphorylated, 31.2% were mono-phosphorylated and 1.5% were non-phosphorylated. By combining the phosphopeptides identified from each fraction with the phosphopeptides identified from the flow-through from the IMAC column after TiO2 enrichment, a total of 515 unique phosphopeptides (692 phosphorylation sites) were identified from 120 microgram hMSC protein. Almost 200 unique multi-phosphorylated peptides were identified. This is to our knowledge the highest number of multi-phosphorylated peptides identified from such low amount of starting material. In one single protein, Serine/arginine repetitive matrix protein 1, 22 phosphopeptides were identified. Of these 16 were multi-phosphorylated, resulting in a total of 34 phosphorylation sites. In comparison, a previous study identified 35 phosphorylation sites in this protein using 8 mg of HeLa cell nuclear preparation as starting material (Beausoleil et al., PNAS, 2004). The presented method will be combined with pre-fractionation methods e.g. isoelectric focusing, and electron transfer dissociation (ETD) mass spectrometry to increase the number of phosphorylation sites identified from low amount of starting material

60. J.V. Olsen, M. Vermeulen, A. Santamaria, F. Gnad, C. Kumar, J. Cox, E.A. Nigg, and M. Mann, "The Cell Cycle Dependent Phosphoproteome and Proteome Analyzed by Quantitative Proteomics," Max-Planck-Institute for Biochemistry, Martinsried (near Munich), Germany. ASMS Presentation (2007).

Abstract: Although mass spectrometry-based proteomics is particularly well-suited for unbiased and large-scale analysis of phosphorylation sites, global quantitative analysis of phosphopeptides by MS is by no means trivial. Extremely confident phosphopeptide identification was achieved by the SILAC encoded information combined with the high mass accuracy of the LTQ-Orbitrap using the automatic real-time internal lock-mass recalibration (average absolute mass accuracy of 0.5 ppm). The cell cycle experiment discussed here is performed by collecting SILAC-encoded HeLa S3 cells at multiple time points over the cell cycle, mixing them three by three using an asynchronous cell population as the internal standard, in-solution digestion, phosphopeptide-enrichment via titanium dioxide (TiO2) chromatography and subsequent identification and quantitation by online LC-MS/MS. Cells were pre-synchronized in G1/S by a double thymidine-block, released and collected at different time points across the cell cycle. In addition, we measured and quantified the proteome over the cell cycle. In addition to defining expression changes at the whole proteome level, this dataset is also used to correct observed phosphosite dynamics by a given protein expression change across the cell cycle. So far, cell cycle dynamic profiles for more than five thousand unique phosphorylation sites on more than two thousand different proteins have been determined. Cell cycle stage regulated phosphorylation sites were identified from many known cell cycle dependent kinases and their

substrates; cyclins, lamins, as well as many others. Additionally, we have identified and quantified hundreds of novel cell cycle regulated phosphorylation sites. Our quantitative phosphoproteomics strategy provides a powerful tool for large-scale studies of in vivo phosphorylation dynamics on a systems biology level. We expect this data set to be invaluable for the cell cycle and cancer communities as it directly connects gene expression changes with protein regulatory information on a whole proteome level.

61. M. Trost, M. Marcantonio, M. Courcelles, M. Desjardins and P. Thibault," System-biology Analysis of Interferon-&gamma Activated Mouse Macrophages: from the Cytosol to the Phagosome," 1) Institute for Research in Immunology and Cancer, Montréal, Canada; 2) Dept. Patho. & Cell Biol., Université de Montréal, Montréal, Canada. ASMS Presentation (2007).

Abstract: The kinetic profiles of phosphopeptide abundances were monitored over different time periods and lead to the identification of early signaling and regulatory events including the enhanced phosphorylation of members of ROS complex (p43, p67, etc…) in the first 5 min following IFN-&gamma administration. Long lasting cytotoxic effects of IFN-&gamma were also noted in macrophages for exposure periods extending to 24h. Specific signaling pathways were revealed from phagosome protein extracts. SDS-PAGE separation and LC-MS analyses of phagosome proteins identified 2,881 proteins, providing the by far most thorough analysis of this important organelle. By combining TiO_2-phosphopeptide enrichment with sensitive LC-MS and 2D-LC-MS analyses we identified more than 2,500 different phosphorylation sites on 903 phagosomal proteins, most of them were unreported. Identified proteins comprised more than 50 kinases including 7 members of the MAP kinase transduction pathway. We also identified several phosphorylation sites on different receptors including all known receptors involved in phagocytosis such as Complement and Fc-Receptor, and many GTPases. IFN-&gamma activation lead to a significant increase in the phosphorylation of cytoskeleton proteins such as CDC42 effector, Ena/VASP or Zyxin and other proteins such as Nuclear Co-activators, Kinase Fyn and the anti-apoptotic proteins SON and BAX-inhibitor, linking the phagosome to other signaling pathways. In contrast, we observed dephosphorylation of key regulatory proteins including vesicle-associated membrane proteins (VAMPs) and other vacuolar sorting proteins together with Rab- and Rab-interacting proteins, suggesting changes in vesicle trafficking and membrane fusion events through IFN-&gamma activation. Decrease in phosphorylation was also observed for members of the MAP-kinase pathway, as well as different Protein Kinase C-isoforms and several serine/threonine kinases. Detailed phosphoproteome analyses provided valuable insights into the mechanism of IFN-&gamma activation, highlighting important signaling events

contributing to the phagosome maturation and the recruitment of key cellular effectors involved in the internalization and degradation of pathogens.

62. A.Y. Kehasse, D.H. Perlman, M.E. McComb, I. Boucher, V.T. Randall and C.E. Costello," Optimized Enrichment and Detection Methodologies for the Study of Phosphopeptides of the Epidermal Growth Factor Receptor," 1) BUSM, Center for Biological Mass Spectrometry, Boston, MA; 2) BUSM, Cardiovascular Proteomics Center, Boston, MA; 3) BUSM, Department of Biochemistry, Boston, MA. ASMS Presentation (2007).

Abstract: We have recovered EGFR from cells in culture with high yield and purity using immunoprecipitation followed by SDS-PAGE. We have explored the use of multiple proteases for in-gel digestion, to target, in particular large tyrosine-containing tryptic peptides that may have been unrepresented in previous MS analyses. Using optimized procedures for recovery from gel, we have subjected the EGFR peptides to various forms of chromatographic enrichment and separation techniques including titanium dioxide chromatography to exploit the differential binding and elution of EGFR phosphopeptides. A panel of matrices and matrix additives are being explored for their capacity to enhance ionization of EGFR phosphopeptides in the positive and negative ion mode of the MALDI-TOF MS. Initial results suggest that significant improvements can be made in sample preparation, enrichment and ionization methodologies in order to maximize the detection of EGFR phosphopeptides.

63. F. Richter, E. Kühn-Hölsken, M. Gronborg, M. Raabe, U. Plessmann and H. Urlaub, "2D-nanoLC Approach using TiO2 Columns for the Enrichment of Protein-RNA Cross-links and Phosphopeptides Derived from Ribonucleoprotein Particles for MS-based Identification," Max Planck Institute for Biophysical Chemistry, Goettingen, Germany. ASMS Poster (2007).

Abstract: In recent years we have established purification strategies for the mass-spectrometric analysis of protein-RNA contact sites in ribonucleoprotein particles. Using capillary-LC and immobilized metal-ion affinity chromatography to concentrate peptide–RNA cross-links selectively, we have reduced the amount of starting material to 50–100 pmol, so that cross-links derived from native RNPs, i.e. isolated from cells, can be analyzed by MALDI-MS. Further reduction for analysis of low-abundance RNPs requires a significant increase in the yield of UV-induced cross-linking and/or an LC set-up coupled to MS similar to the state-of-the-art protein-analytical approach. For the latter purpose, we have established a chromatographic system that allows us to concentrate peptide–RNA cross-links from amounts of UV-irradiated RNP in the low pmol range. After digestion of the protein and RNA moieties with

endoproteinases and ribonucleases, cross-links are concentrated on TiO2 columns within a multidimensional chromatography system and subsequently separated on nanoLC analytical columns. Our chromatographic set-up has several advantages. (i) The bulk of hydrolyzed RNA, which would bind strongly to TiO2 and prevent enrichment of cross-linked species, is removed on-line before the enrichment step. (ii) In contrast to our previous studies, off-line enrichment on TiO2 beads is not required, as the TiO2 columns are integrated within the system, reducing loss of enriched species. (iii) Our system can be coupled either directly to ESI-MS or to a MALDI target spotter. In feasibility studies with U1 snRNPs and [15.5K-61K-U4atac snRNA], we isolated and sequenced peptide-RNA cross-links derived from 1–10 pmol of UV-irradiated (sub)-spliceosomal complexes by both MS/MS methods. Ribonucleoproteins, in particular those in complexes that are involved in (alternative) splicing, are highly phosphorylated. We therefore also applied our strategy to native non-cross-linked RNPs for the selective enrichment of phosphopeptides. We identified phosphopeptides derived from native (sub-)spliceosomal U1 and [U4/U6.U5] tri-snRNP complexes.

64. H. Piechura, J. Barbour, E. Neuhaus, H. Hatt, H.E. Meyer and B. Warscheid, "Application of Phosphoproteomic Strategies and Mass Spectrometry to Study the Molecular Processes Underlying Odor Perception in Mouse," 1) Medical Proteome Center, Bochum, Germany; 2) Cellphysiology, Bochum, Germany. ASMS Poster (2007).

Abstract: Due to the low abundance of phosphopeptides and the limited amount of protein that can be extracted from OE (150-300 μg), different strategies to selectively enrich and identify phosphoproteins were evaluated. In a first approach, proteins were first separated by 2-D PAGE and phosphoproteins were subsequently visualized using the ProQ Diamond® stain specific for phospho-Tyr, phospho-Ser, and phospho-Thr. However, the staining was not reproducible and exhibited a high background. In addition, phosphorylation sites could not be detected in ProQ Diamond-stained proteins by MS. We therefore sought alternative approaches facilitating sensitive MS-analysis of phosphoproteins. We immunoprecipitated phosphoproteins from protein extracts derived from control and odorant-treated OE using phospho-Tyr-specific antibodies. Immunoprecipitated proteins were then separated by SDS-PAGE and peptide samples were generated by in-gel tryptic digestion for subsequent MS analysis. We additionally established SCX chromatography in combination with titanium dioxide microcolumns as alternative technique for global and gel-free phosphoprotein analysis. Efficiency and specificity of phosphopeptide enrichment using this LC/MS-based method was tested using a complex protein digest to which different amounts of a standard peptide mixture composed of mono and multiple phosphorylated peptides were added. For peptide identification and protein assembly as well as

detection and characterization of the location of phosphorylation sites, peptide mixtures from immunoprecipation or titanium dioxide experiments were analyzed by nano-HPLC/ESI-MS/MS on a QTOF and/or an ion trap instrument. In ongoing work, we will combine the enrichment protocols described here with chemical labeling techniques using isotope-encoded tags. This will provide us with quantitative information about stimulus-induced changes in the phosphoproteome of the OE from odorant-treated compared to control mice.

65. K. Lohrig, B. Müller, D. Leister and D. Wolters, "Optimizing Phosphoprotein Analysis for Arabidopsis Thaliana," 1) Ruhr Universität Bochum, Bochum, Germany; 2) Ludwig Maximilian Universität, München, Germany. ASMS Poster (2007).

Abstract: In general, phosphopeptides could be detected with reproducible retention times and with comparable intensities in the base peak chromatogram for titanium dioxide enrichment. However, during the evaluation of titanium dioxide as an enrichment method for low complex phosphopeptide mixtures, only minor differences were observed between the employed loading buffer systems. In contrast to simple phosphopeptide mixtures, more complex peptides mixtures simulated with synthetic phosphopeptides spiked in Corynebacterium Glutamicum show significant increase of nonspecific binding of non-phosphopeptides due to different loading buffer conditions. Therefore, loading buffers containing DHB as modifier proved to be most effective for background reduction for complex samples. Using an online titanium dioxide enrichment system shows to be problematic due to basic elution conditions. Base stabile HPLC resins are mandatory since decreased stability of reversed phase material resulted in inferior separation quality. For further complementary analysis of phosphorylated peptides, IMAC enrichment was used. Therefore, a commercially available IMAC resin (PhosSelect, Sigma) was tested with the same samples as used in titanium dioxide approach. In contrast to titanium dioxide, significant reduction of non-phosphorylated peptides was observed in simple as well as complex peptide mixtures. Detection of phosphopeptides was performed by an LTQ System with a MSA (multistage activation) based scanning method. Pseudo MS^3 scanning provides better sequence coverage of phosphorylated peptides, since fragmentation of phosphorylated peptide and dephosphorylated ones occurs simultaneously. Adaptation of most suitable enrichment methods to Arabidopsis thaliana samples is currently progressing. Minor problems regarding sample compatibility with the developed system are already solved, e.g. by initial precipitations steps. Furthermore, additional enrichment methods, such as SCX prefractionation or phosphoprotein enrichment by IMAC will be established in the near future. Moreover, a combination of these prefractionation methods with titanium dioxide is a

possible strategy to obtain a comprehensive overview about phosphoproteins in Arabidopsis thaliana.

66. S.M. Frawley and J.J. Tepe, "A Covalent Solid-phase Enrichment Technique Used in the Isolation and Analysis of Phosphorylated Proteins." Michigan State University, East Lansing, MI. ASMS Poster (2007).

Abstract: The problems associated with phosphorylated protein/peptide analysis are well documented.(3) These obstacles require the enrichment of phosphopeptide/proteins prior to their introduction into a mass spectrometer in order to obtain accurate modification site identification. In recent years, various techniques have been employed to enrich phosphopeptides. The most widespread method used is immobilized metal affinity chromatography (IMAC).(2,4) The observation of coordination between titanium dioxide to phosphate has also initiated the implementation of titanium dioxide columns to purify phosphopeptides.(5) Unlike IMAC or titanium dioxide, which utilizes metal ion-phosphate affinity, we have developed a diazo-functionalized resin that covalently binds to the phosphate moiety. The diazo resin was synthesized from various immobilized hydroxyl or amino solid supports and introduced to a proteolytic digestion of commercially available phosphorylated protein, β-casein. To prevent non-specific binding, the carboxylic acid residues were methyl esterified prior to their introduction to the resin. The formation of the covalent phosphoester bond was followed by successive washings to remove unbound peptides from the mixture. The isolated phosphopeptides were then collected upon the hydrolysis of the phosphoester, which cleaved the peptide from the solid support. Subsequent MALDI ion trap MS/MS sequence analysis of the enriched β-casein samples confirmed the identity of both the monophosphorylated fragment, containing residues 48-63 (FQsEEQQQTEDELQDK) and the tetraphosphorylated fragment containing residues 16-40 (RELEELNVPGEIVEsLsssEESITR). No other peptides were present in the enriched samples, confirming the proof of principle of the technique to isolate phosphorylated peptides. Work towards the enrichment and analysis of other phosphorylated proteins, including intracellular protein, NF-κB, will be discussed. (1) *Trends Biochem Sci*. 1993, *18*, 172-177. (2) *Anal. Chem*. 2002, *74*, 3429-3433. (3) *Nat. Biotechnol*. 2003, *21*, 1047-1054. (4) *Anal. Chem*. 1999, *71*, 2883-2892. (5) *Anal. Chem*. 2004, *76*, 3935-3943.

67. N. Sugiyama, S. Ohnuma, Y. Kyono, Y. Igarashi, K. Shinoda, T. Masuda, A. Nakamura, M. Tomita, Y. Ishihama, "Toward Phosphoproteome Profiling Using Hydroxy Acid-Modified Metal Oxide Chromatography Coupled with NanoLC-MS/MS," 1) Human Metabolome Technologies, Inc., Tsuruoka, Japan; 2) Institute for Advanced Biosciences, Keio Universit, Tsuruoka, Japan; 3) GL Sciences Inc., Iruma, Japan. ASMS POSTER (2007).

Abstract: The cytoplasmic fraction of HeLa cells, which were cultured without special stimulation, were applied to lactic acid-modified titania tip, followed by data-dependent nanoLC-MS/MS analysis. Approximately 350 phosphopeptides were obtained in a single 2-hour nanoLC-MS/MS gradient run, whereas only a few phosphopeptides were identified without lactic acid. The lactic acid-modified titania tip gave high selectivity (the ratio of the number of phosphopeptides to that of all peptides = 0.619) in comparison with the ratio without lactic acid (ratio = 0.008). Similar results were also obtained for HPA-modified zirconia tip. In total, 1151 and 1220 phosphopeptides were identified by four replicated experiments using hydroxy acid-modified titania and zirconia tips, respectively. Out of 1730 distinct identified phosphopeptides, 641 were found by both methods and the others were uniquely identified by a single method. To increase the coverage of phosphoproteome, we applied the sample enriched with lactic acid-modified titania tip to an alternative approach, namely neutral loss-triggered MS3 analysis with the use of multistage activation. The number of identified phosphopeptides and the selectivity were almost equal to those obtained by the conventional MS/MS analysis, whereas approximately only 20% of identified phosphopeptides were identified by both approaches. Thus, the complementary usage of titania/zirconia MOC tips and the conventional MS2/neutral loss multistage MS3 approaches allowed covering the wider range of phosphoproteome based on the phosphopeptide profiling. This approach was applied to the rodent malaria parasite *Plasmdium berghei* toward elucidation of signal transduction in regulating gametogenesis. Quantitative phosphoproteome profiling for gametocytes treated with/without xanthurenic acid (XA) at the blood stage during the malaria parasite life cycle was carried out and 1670 and 1489 phosphopeptides were identified, respectively. We also quantified them based on the ion counts and found that the XA treatment significantly increased 22 phosphopeptides including MAPK-2 as reported and decreased 5 phosphopeptides.

68. E. Simon, M.A. Young and P.C. Andrews "Methyl Esterification of Peptides Improves the Isolation of Phosphorylated Peptides from Titanium Dioxide," University of Michigan, Ann Arbor, MI. ASMS Poster (2007).

Abstract: The influence of peptide methyl esterification on phosphopeptide enrichment was assessed by comparison of MALDI-TOF/TOF mass spectra of methyl esterified versus non-methyl esterified digests. General observations from the casein mixture included a remarkable reduction in spectral complexity of the methyl esterified samples relative to the corresponding non-methyl esterified samples. The non-methyl esterified spectra were littered with an abundance of non-phosphorylated peptide signals, including the two most intense peaks in the spectrum. In contrast, the eight most intense peaks in the corresponding methyl esterified casein spectrum represented

phosphopeptides. Overall, the number of phosphopeptides detected increased from eight to ten when the casein mixture was methyl esterified. The procedure was repeated for CDK2. First, the unmethylated sample digest (~0.5 – 1 μg) was passed through a TiO2 column and analyzed. The base peak of this spectrum represented a phosphopeptide that included a previously known phosphorylation site at residue T160. As in the case of the non-methyl esterified casein mixture, this spectrum was littered with an abundance of non-phosphorylated peptides. Arbitrarily, the twelve most abundant peaks were analyzed and sequence verified by tandem mass spectrometry. Only the base peak was determined to represent a phosphopeptide. Another aliquot of the CDK2 digest (~0.5 – 1 μg) was subjected to methyl esterification followed by enrichment with TiO2. Again, the complexity of the corresponding mass spectrum was significantly reduced. The twelve most abundant peaks were analyzed by tandem mass spectrometry. The two most abundant peaks represented phosphopeptides. The base peak corresponded to the phosphopeptide described above and the second most abundant peak represented a phosphopeptide with phosphorylation determined to reside at S48. The significance of this second phosphorylation site to CDK2 function and regulation is being investigated. Selective elution by altering the pH of the elution buffer provided an additional improvement in selectivity.

69. J. Zhao, S.M. Camp, E.T. Chiang, A. Schilling, S.M. Dudek and J.G.N. Garcia, "Novel Protein Kinase A-mediated Endothelial Cell Myosin Light Chain Kinase Phosphorylation Sites Using Data Dependent Nano-LC/MS/MS Mass Spectrometry Method," 1) University of Chicago, Chicago, IL; 2) University of Illinois Chicago, Chicago, IL ASMS Poster (2007).

Abstract: For phosphopeptide enrichment from mixture peptides of digested h\His-tagged protein, we found T\titanium dioxide to be a more efficient method than IMAC. Furthermore, the efficiency of sample production and recovery are increased using on-bead digestion. The protein sequence coverage reached 70% on each single nano-LC/MS/MS analysis. With high mass accuracy mass spectrometry and optimized biochemical protocols, 55 novel PKA phosphorylation sites were identified on nmMLCK1. We confirmed well-known PKA-mediated smooth muscle MLCK (smMLCK) phosphorylation sites that correspond to sites on nmMLCK (S1208, S1760, S1773, S1776). In addition, we identified two PKA phosphorylation sites (T694, T1309) located in a putative nmMLCK SH2 binding domain, and four phosphorylation sites (T335, S618, T978, S1020) located in a putative nmMLCK SH3 binding domain. Further structure/function analysis of the functional effects of these novel phosphorylation events will provide important insights into the regulation of nmMLCK activity and barrier regulation.

70. M.E. Graham, G.E. Craft, N. Bache, M.R. Larsen and P.J. Robinson, "AP180 is Multiply Phosphorylated and Has a Novel Post-translational Modification: N-acetylglucosamine Phosphorylation," 1) Children's Medical Research Institute, Westmead, Australia; 2) University of Southern Denmark, Odense, Denmark ASMS Poster (2007).

Abstract: Phosphorylation sites were detected at Ser-248, Ser-296, Ser-300, Ser-306, Ser-313, Ser-325, Ser-594, Ser-600, Ser-621, Ser-627, Ser-868 and Ser-877. The results suggest that interaction of AP180 with clathrin and AP-2 will be affected by phosphorylation at serine residues that are mainly clustered together near interaction motifs. Since 32P-labelling was used, we were able to compare AP180 from control synaptosomes and KCl stimulated synaptosomes and identify the sites which had a high turn-over of phosphate and/or were responsive to the KCl induced depolarization. The stimulus-dependent ranking of the sites will facilitate future work on the phospho-regulation of AP180. The titanium dioxide also enriched for a novel post-translational modification. The mass spectral data indicated that Thr-310 was modified by O-linked N-acetylglucosamine which was itself phosphorylated. This modification was confirmed using phosphatase treatment and beta-elimination.

71. S. Mohammed, M. Pinkse, J. Benschop, K. Kraiczek, G. Gauthier and A. Heck, "Evaluation of Chip Designs for Titanium Dioxide Based Phosphopeptide Enrichment, Separation and Mass Spectrometric Detection," 1) University of Utrecht, Utrecht, Netherlands; 2) Agilent Technologies, Waldbronn, Germany ASMS Poster (2007).

Abstract: Titanium dioxide has proven to be a highly facile material for phosphopeptide enrichment. Initial experiments focused on a simple 2 part TiO2-RP pre-column where the entire sample was introduced over both sections during loading. All bound material was analyzed on a 15cm analytical column which is also integrated on the chip. Such a design allowed analysis of what does not bind to the TiO2 phosphopeptide enrichment column, the flow-through, as well as the analysis of the bound phosphopeptides, the elution. Although the design was successful, phosphopeptide breakthrough and incomplete elution increased over time. To overcome such issues different pre column designs with better defined packing material reservoirs were investigated. Evaluation of different configurations will be presented including designs that incorporate multiple switching valves that allow reversed flow creating a smaller footprint for the pre-column with the benefit of lower dead volume times and better separation. Such designs proved to be reliable and highly reusable. Examples of 'real world' experiments will also be shown.

72. S.Y. Imanishi, V. Kochin, S.E. Ferraris, A. de Thonel, H. Pallari, G.L. Corthals and J.E. Eriksson, "Reference-facilitated Phosphoproteomics: Fast and Reliable

Phosphopeptide Validation by microLC-ESI-Q-TOF MS/MS," Turku Centre for Biotechnology, Turku, Finland. ASMS Poster (2007).

Abstract: We have optimized an off-line TiO2 chromatography as selective phosphopeptide enrichment procedure. Following enrichment, half of the phosphopeptide fractionated sample is enzymatically dephosphorylated, after which both samples are subjected to LC-MS/MS procedure, with a tandem-in-space mass spectrometer. From the resulting MS/MS analyses, the dephosphorylated peptide is used as a reference spectrum against the original phosphopeptide spectrum, in effect generating two peptide spectra for the same amino acid sequence, thereby enhancing the probability of a correct identification. This phosphopeptide identification represents a major improvement as compared to identifications based only on single MS/MS spectra and probability-based database searches. We investigated an applicability of this method to crude cell lysates and demonstrate its application on the large-scale analysis of phosphorylation sites in differentiating mouse myoblast cells.

73. K. Hakansson, J.T. Adamson, Y. Kong, H.K. Kweon and H. Liu, "Development of Novel Tools for Mass Spectrometry-based Carbohydrate Analysis," University of Michigan, Ann Arbor, MI. ASMS Presentation (2007).

Abstract: Preliminary data for an O-sulfated peptide in a proteolytic digest show selective sulfate binding to both zirconia and titania although competition from acidic peptides with multiple carboxylic acids is observed. Thus, we believe these materials are promising for enriching sulfated carbohydrates. We have found that utilization of divalent metal adducts as charge carriers in electrospray ionization allows ECD (which requires at least two charges) of neutral and acidic species, including neutral and sulfated oligosaccharides. Previous ECD of protonated aminosugars [1] did not result in any cross-ring cleavages. By contrast, we have shown that ECD of metal-adducted linear and branched oligosaccharides provides extensive, sometimes dominant, cross-ring cleavage [2]. Cross-ring fragments are also observed in IRMPD depending on metal ion type, however, glycosidic cleavages dominate in that fragmentation technique. For all metal-adducted oligosaccharides investigated, complementary structural information was obtained with ECD as compared to IRMPD. We and others have also found that EDD, which operates in negative ion mode, is a valuable technique for characterization of sulfated carbohydrates. For example, enhanced cross-ring fragmentation is observed compared to IRMPD and collisional activation [3]. 1. Budnik et al. Anal. Chem. 2003;75:5994 2. Adamson and Hakansson. Anal. Chem. 2007; accepted. 3. Wolff et al. J. Am. Soc. Mass Spectrom. 2007;18:234.

74. A.J. Ytterberg, R.R. Ogorzalek Loo, P. Boontheung and J.A. Loo, "Isolation and Identification of Phosphopeptides by Combining Strong Cationic Exchange and Hydrophilic Interaction Chromatography," UCLA, Los Angeles, CA ASMS Poster (2007).

Abstract: When total digests of saliva proteins are fractionated on SCX microcolumns, the majority of the phosphopeptides are not retained by the column and end up in the flow through. To fractionate the phosphopeptides further and separate them from non-phosphopeptides we utilized HILIC chromatography. As expected, most of the non-phosphorylated peptides elute early in the step gradient, while phosphopeptides elute late. Furthermore, peptides which had a variable number of phosphorylated amino acids could also be separated into different fractions. Columns are relatively inexpensive since only a small amount of stationary phase are used for each column and do not need to be reused, preventing cross-contamination. The microcolumns also made it possible to separate the peptides using 2D-LC without HPLC, and the fractionation itself is relatively fast. After lyophilization, the different fractions are fully compatible with ESI. In preliminary experiments using approximately 12 ug of saliva, we mapped out phosphorylation sites from proteins such as the salivary acid proline-rich phosphoprotein 1/2, the salivary alpha-amylase and statherin. A comparison between isolation of phosphopeptides using either TiO2 or a combination of SCX and HILIC will be presented.

75. C. Xu and T.A. Neubert, "Fractionation of Phosphopeptides Using Titanium Dioxide Chromatography with pH Gradient Elution," NYU Medical Center, New York, NY. ASMS Poster (2007).

Abstract: When using 50 mM Tris-HCl with increased pH (7.2, 7.6, 8.0, 8.4 and 8.8) as sequential elution buffers, the elution of phosphopeptides from the TiO2 material under these pH conditions didn't differ significantly. Phosphopeptides began to elute from the TiO2 tip at pH 7.2. Phosphopeptides with mass 1927 (2 phosphorylations) and 2061 (1 phosphorylation) appeared in 5 different pH fractions. The efficiency of separation was very low. We made new serial elution buffers by mixing an equal volume of AcN with the above Tris-HCl pH buffers. The new buffers containing 50% AcN seemed to largely increase the binding strength of phosphopeptides to the TiO2 tips. At pH 7.2, 7.6 and 8.0 fractions, few phosphopeptides eluted. Phosphopeptides started to elute from the tips at pH 8.4. Monophosphopeptides were eluted at pH 8.4 and 8.8, however, the peptides with mass 1411(2 phosphorylations), 1927 (2 phosphorylations), 3008 (4 phosphorylations), 3122 (4 phosphorylations) and 2722(5 phosphorylations) were not observed with significant abundances in these fractions. However, in the last elution with 500 mM ammonium, these five multiply phosphorylated peptides were eluted. These results suggested that AcN was an import buffer additive for

strengthening the binding of phosphopeptides to the TiO2 tips, and increasing the selectivity of pH-based separation. Based on the above experiment, we propose to pack a titanium dioxide separation column for the enrichment and fractionation of phosphopeptide using pH gradient elution. And we are also going to test different kind of pH buffers to optimize the separation of phosphopeptides using titanium dioxide chromatography.