# **Enrichment and identification of striatal phosphoproteins from cocaine treated rats**



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# Introduction

The amount of information obtained from a single 2-D Differential Gel Electrophoresis (DIGE) experiment can be greatly enhanced by combining pre-staining of proteins using CyDyes with post-staining of gels with fluorescent dyes<sup>1)</sup>. We used this approach to validate and compare different methods for enrichment of phosphoproteins from rat brain synaptoneurosomes.

We next used phosphoprotein enrichment using Al(OH)<sub>3</sub> to study changes in phosphorylation of synaptic proteins from rat striatum following cocaine treatment.

### Part I. Selecting enrichment method for sample of interest

Four methods for phosphoprotein enrichment were compared for their yield and selectivity, using rat brain synaptoneurosomes as sample:

- 1) Immobilized Metal-Affinity Chromatography (IMAC) using Chelating Sepharose (GE Healthcare) charged with Ga<sup>3+</sup> ions.
- 2) Pro-Q Phosphoprotein Enrichment Kit (Molecular Probes).
- 3) Metal-Oxide Affinity Chromatography (MOAC) using Al(OH)<sub>3</sub>.<sup>2)</sup>
- 4) MOAC using FeTiO<sub>3</sub>, a material demonstrating high selectivity for phosphopeptides.

### Part II. Using phosphoprotein enrichment to study Cocaine-induced changes in protein phosphorylation in rat striatum

Based on the results for selectivity and yield for the four methods in Part I, we chose MOAC using  $AI(OH)_3$  (method 3 from the list above) for a study of phosphorylation changes in striatal synapses in rats following acute cocaine administration (30 mg/kg given i.p.).

For this study, naïve rats were killed by rapid de-cap 15 min following injections of saline or cocaine. Striata were dissected out on ice, and synaptoneurosomes were prepared. To maintain phosphorylation levels of proteins, Phosphatase Inhibitor Cocktail I and II (Sigma) were added to all buffers used for preparating synaptoneurosomes.



Evaluation of phosphoprotein enrichment from synapto-neurosomes by 2-D DIGE. An aliquot of the original sample is labeled with Cy-5, whereas the same amount of protein from an aliquot of the sample after phosphoprotein enrichment is labeled with Cy-2. Following mixing of the two samples and protein separation by 2-D gel electrophoresis, phosphoproteins are stained with Pro-Q Diamond (Molecular Probes), a phosphospecific stain. During scanning of the gel, the Cy-5, Cy-2, and Pro-Q Diamond signals are obtained simultaneously, by employing the Cy-3 channel for the Pro-Q Diamond signal. Simultaneous scanning minimizes errors in quantification of spot intensities and errors introduced during picking of gel spots for identification by MS and MS/MS.

## **Materials and Methods**

# **Results - Part I.**



MOAC using FeTiO<sub>3</sub> generated the largest changes in protein concentration patterns during enrichment. Blue outlines show enriched spots or areas and red spots show depleted protein spots or areas of the gel. The zoomed-in images (bottom left panels) demonstrate the large changes in concentration that were seen for some proteins on the gel. Bottom right panels: 3-dimensional representations of selected protein spots.

## **Selectivity for phosphoproteins**

FeTiO<sub>3</sub> MOAC - Al<sup>3+</sup> MOAC - Ga<sup>3+</sup> IMAC - Pro-Q Kit

Yield of total protein

## References

- 1. Stasyk, T., Morandell, S., Bakry, R., Feuerstein, I., Huck, C.W., Stecher, G., Bonn, G.K., Juber, L.A. (2005). *Electrophoresis*, **26**: 2850-2854.
- 2. Wolschin, F., Wienkoop, S., Weckwerth, W. (2005). *Proteomics*, **5**: 4389-4397.

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Part II.



Synuclein  $\beta$  (=Phosphoneuroprotein :



fied by MALDI-TOF/TOF.

# Conclusions

Using an optimized method for phosphoprotein enrichment, we compared phosphorylation of striatal synaptic proteins isolated from cocaine vs. saline treated rats. 27 known phosphoproteins were identified among a total of 33 proteins. Several of the proteins identified in this study are not readily identified from synaptoneurosomes using 2-D gel electrophoresis-MS without employing phosphoprotein enrichment.

Despite recent advances in methods for phosphoprotein enrichment, researchers attempting to enrich phosphoproteins from complex samples need to recognize the trade-off that often must be made between high yield and high selectivity. We urge researchers attempting to enrich phosphoproteins from complex samples to validate and compare different methods using a relevant sample matrix rather than choosing a method based on tests with mixtures of standard proteins (an approach chosen by many researchers).

To fully appreciate the potential of this approach, the amount of enriched sample loaded on the gel in Part





Comparison of protein phosphorylation in striatum following injections of Cocaine (30 mg/kg, left gel) or saline vehicle (right gel). MOAC using Al(OH)<sub>3</sub> (Method 3, see Introduction) was used for phosphoprotein enrichment, using 3.5 mg of protein from synaptoneurosomes as starting material for each sample ("Cocaine" and "Control"). 50 ug protein of the enriched samples was separated by 2D Gel Electrophoresis. Gels were stained with Pro-Q Diamond phospho stain. Proteins were identi-

# \* NSFL1 (p97 ATPase) cofactor Phosphoneuroprotein 14 Protein kinase Ppk 98 \* Protein phosphatase 1 **Rho GDP dissociation inhibit** \* Spinophilin (= Neurabin-2) ? Tumor rejection antigen

II should ideally be >50  $\mu$ g. Also, experiments should be run in triplicate to ensure the quality of the data.