Identification of post-translational Modifications on Peptide Microarrays

Sandra Mükusch [1,2] and Harald Seitz [1]
1 Fraunhofer Institute for Celltherapy and Immunology Branch Bioanalytics and Bioprocesses Potsdam-Golm, Am Mühlenberg 13, 14476 Potsdam, Germany
2 Free University of Berlin, Institute for Chemistry and Biochemistry, Takustraße 3, 14195 Berlin, Germany

INTRODUCTION

Eukaryotic cells have a highly variable signaling network. Upon external stimulation the protein activity can be modified by post-translational modifications (e.g. phosphorylation). This shift in activity is necessary to react to environmental changes but can also lead to diseases like chronic pain or cancer. Proteins have been selected and the corresponding cytosolic domains were divided into 20-mer peptides, harbouring potential phosphorylation sites for the selected kinases.
A kinase assay using a variety of different recombinant kinases and more complex cell lysates will be performed to verify known phosphorylation sites and identify new ones yielding information about kinase specificity and activity towards the selected peptides. Due to the complex nature of signaling networks, different factors like stimuli, inhibitors, cell conditions and cell types influence the signal output. Therefore two different multiplexing high throughput platforms have been chosen to collect multiple data in one approach (i) Peptide Microarrays, (ii) a Bead based Assay. Working with different platforms, allows us to validate the data. A cross platform comparison will be done by comparing reproducibility, sensitivity, sample volume and so on.

Thin Layer Chromotography (TLC) for Quality Control

TLC seperates molecules through their interaction with the matrix and the running buffer based on their characteristics. We combine classical TLC with modern immunological detection techniques allowing to detect peptides even in a complex mixture and quantify them in a semi-quantitative manner (Fig 1). The limit of detection depends on the used antibodies and peptide. Compared to other techniques the amount of peptide is higher and the multiplexing capacity is limited. TLC combined with immunostaining is a fast and easily methods allowing a quality control of kinases in every lab without any expensive equipment.

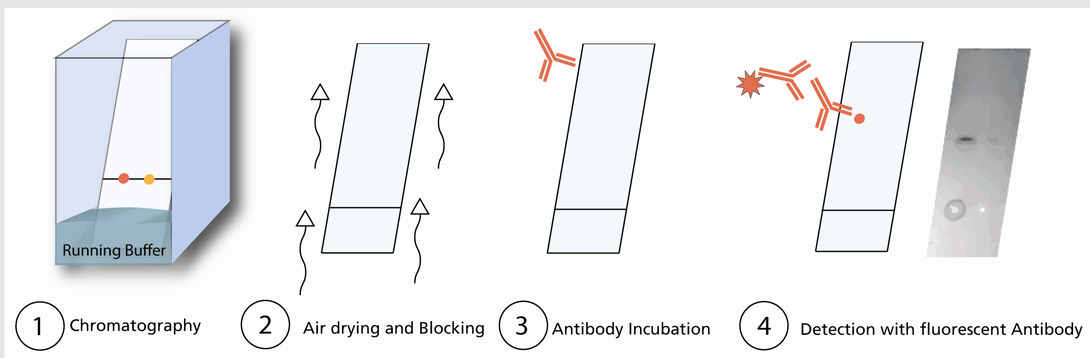

Fig. 1: Workflow of the Immuno-TLC. (1) Chamber with a spotted TLC plate. During the TLC the spotted peptide is transported with the buffer due to its physic-chemical properties. (2) Upon drying and blocking the surface with BSA (3) Incubation of the TLC plate with primary Antibody (4) Detection through a fluorescent labelled Antibody

Bead based Assays

Bead based Assays are very flexible suspension assays. FlexMap3D from Luminex allows the measurement of up to 500 color coded beads with different analytes in one experiment. Multiplexing with beads paves the way to do kinetic studies (Fig. 3), inhibitor or epitope screening. Peptides of interest are coupled with the N-terminus to the bead via EDC-NHS chemistry. After coupling e.g. kinase assays can be performed (Fig. 2). The phosphorylated peptide (Fig. 2) can be measured through antibody detection (Fig. 2). The detection has to be done using a dye in the green spectra while the instrument is able to read 96-Well plates in 20 minutes, fast and reproducible multiplexing is possible.

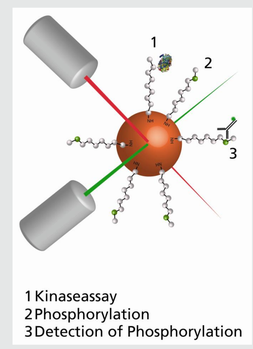

Fig. 2: Bead based Screening for phosphorylation sites. After Kinaseassay (1),Phosphorylation and the detection of Phosphorylation (2, 3), the beadregion will be identified through the red laser, while the detection of the phosphorylation takes place in green laser wave.

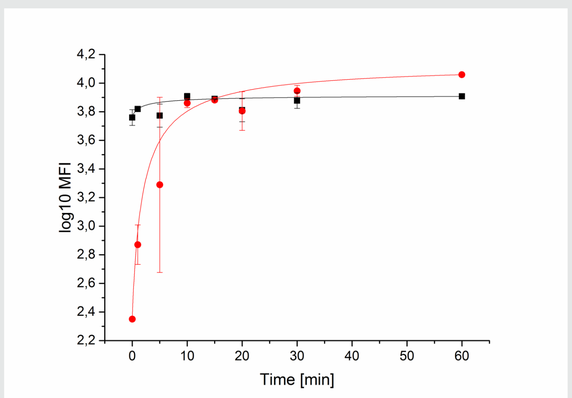

Fig 3. : Multiplexing of two beads showing the phosphorylation over a time of 60 min.

Red dots: A unphosphorylated peptide is coupled to the bead, incubated with a kinase and the phosphorylation event detected with a specific antibody. The signal increases due to a phosphorylation of the peptide. After 10 min the maximal signal intensity (phosphorylation) is reached. The positive control (p-Peptid; phosphorylated peptide) is not influenced by the kinase reaction (black dots).

Peptide Microarray

Thousands of peptides can be spotted onto a modified surface of a standard glass slide with a spot diameter of 100 µm resulting in a so called Peptide Microarray. With covalent immobilized peptides inhibitor screening, signalling analysis, epitope mapping etc. can be done. For a signalling analysis peptides are spotted onto the surface, kinase assay was performed and the detection achieved with specific antibodies (Fig 4 and 5). Peptide Microrarrays have benefits like high degree of multiplexing, low sample volume, low detection limit and can be used to validate the data produced on the bead based assay.

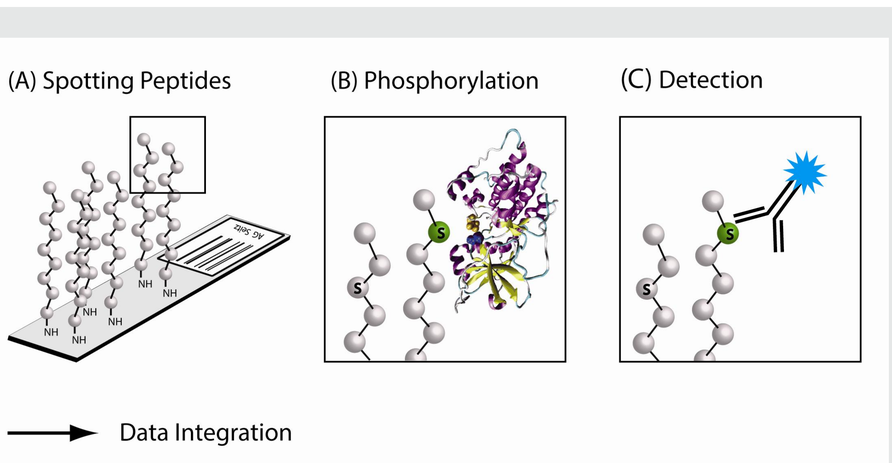

Fig 4.: Workflow Peptidearray.
(A) Peptides are immobilised onto modified glass slides. (B) In the following step a kinase assay wasperformed. (C) Detection of phosphorylated peptides wasachieved, for example, using phospho-specific antibodies or dyes

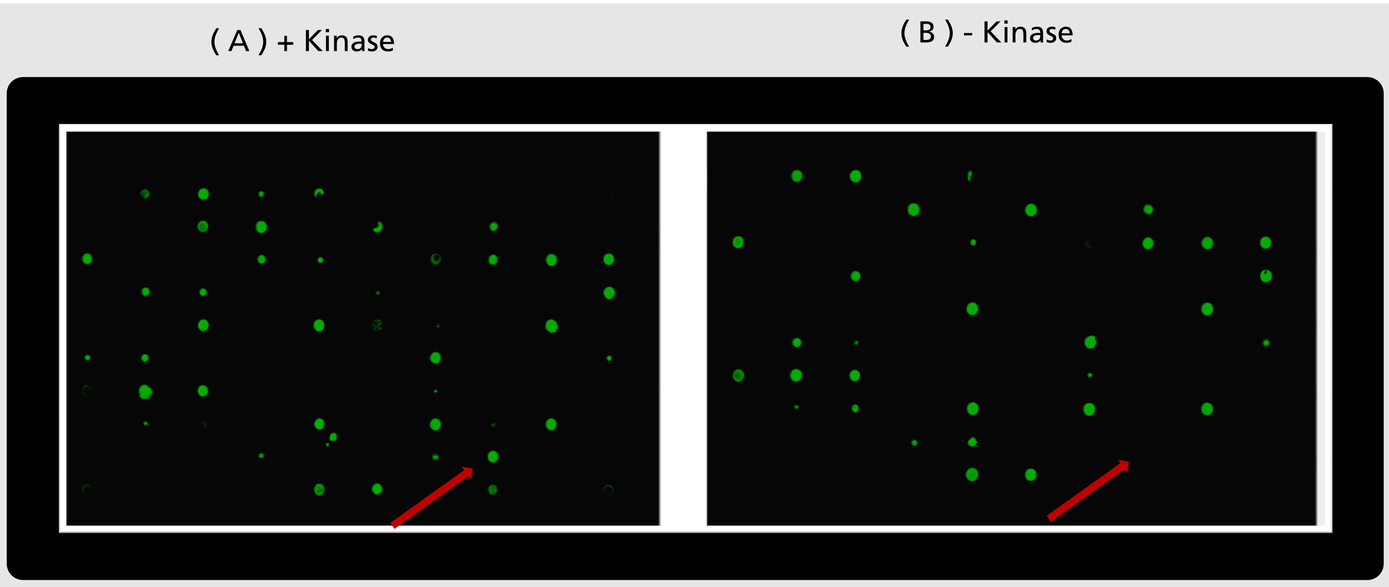

Fig 5. : Exemplary Result of Peptide Microarray.
Every visible dot is a positive signal after incubation with a phosphospecific antibody. The left Array is incubated with a kinase for 30 Minutes at 30°C (A), while the right Array is a control slide. One example for a visible phosphorylation after kinase incubation is pointed out with the red arrow.

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Contact: Sandra Mükusch| Fraunhofer-Institute for Celltherapy and Immunology | Dept. Bioanalytics & Biosensoric | AG Biomarker-Validation & Assay Development | Am Mühlenberg 13 | 14476 Potsdam-Golm |
Telephone +49 331 58187-208| E-mail sandra.muekusch@izi-bb.fraunhofer.de | www.izi.fraunhofer.de