Margherita Dell'Aica

Monitoring early signalling events in cells using high-time resolution Phosphoproteomics

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Abbreviations

2-HEC	2-Hydroxyethyl cellulose
96w+R3	96 well plate-based TiO_2 + R3 stage tip desalting
$96w^2$	96 well plate-based TiO_2 + desalting on Thermo Hypersep C18 96 well plates
ACN	Acetonitrile
ADP	Adenosine diphosphate
AGC	Automatic gain control
AKT1	RAC-alpha serine/threonine-protein kinase 1, Protein Kinase B
AR	Amphiregulin
BCA	Binchonic acid colourimetric assay
C18	Octadecyl carbon chain bonded silica for stationary phase column
CASY	Cell counting technology
CID	Collision-induced dissociation
CLH1	Clathrin heavy chain 1
CS	Control Set
DDA	Data-dependent acquisition
DEP	Dielectrophoresis
DIA	Data-independent acquisition
DMEM	Dulbecco's Modified Eagle Medium
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor, also known as ErbB1/Her1
ESI	Electrospray ionization
FA	Formic acid
FASP	Filter Aided Sample Preparation
FCS	Fetal Calf Serum
FEM	Finite Element Method
FFE	Free-flow electrophoresis
FITC	Fluorescein Isothiocyanate
FTMS	Fourier Transform Mass Spectrometry
FWHM	Full Width at Half Maximum
GAB1	GRB2-associated-binding protein 1
GO	Gene Ontology
GuHCl	Guanidine Hydrochloride
H/L	Heavy to Light
HCD	Higher energy collisional dissociation
HEK293	Human embryonic kidney cells 293
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
HS90A	Heat shock protein 90-alpha 1
IAA	2-Iodoacetamide

IEF	Isoelectric focusing
IGF1R	Insulin growth factor receptor
IR	Insulin receptor
iTRAQ	Isobaric tags for relative and absolute quantitation
LC-MS	Liquid Chromatography-Mass Spectrometry
LOC	Lab-on-a-chip
m/z	Mass-to-charge ratio
M^2D	Micromixer Microfluidics Device
MAPK1	Mitogen-activated protein kinase 1
MD	Median
MOAC	Metal oxide affinity chromatography
MS	Mass spectrometry
MSMS/MS2	Tandem mass spectrometry
MWCO	Molecular Weight Cut-Off
NAV	Normalized Abundance Values
NF	Normalized Factors
NI	Normalized Intensities
NR	Normalized Ratio
PBS	Phosphate-buffered saline
PCC	Pearson Correlation Coefficient
PDMS	Polydimethylsiloxane
pI	Isoelectric point
PIK3R1/P85	Phosphatidylinositol 3-kinase regulatory subunit alpha
PKA	Protein kinase A
PLCG1/PLCγ	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1
PRM	Parallel reaction monitoring
PSM	Peptide-Spectrum-Matches
PTM	Post-translational modification
p-value	Probability value
RAF1	RAF (rapidly accelerated fribrosarcoma) proto-oncogene serine/threonine-protein 1 kinase
RP	Reverse phase chromatography
RSD	Relative Standard Deviation
RT	Retention Time
RTK	Receptor tyrosine kinase
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SHC1/Shc	SHC (Src homology 2 domain containing) adaptor protein 1
SIL	Stable isotope labelled
Src	Src (sarcoma) proto-oncogene tyrosine-protein kinase
STAT	Signal transducer and activator of transcription
STE	Stream thinning elements
T+96w	In-tube batch TiO_2 + desalting on Thermo Hypersep C18 96 well plates
T+R3	In-tube batch TiO_2 enrichment + R3 stage tip desalting
TD	Technical Digression Set
TEAB	Tetraethylammonium bicarbonate

TFA	Trifluoroacetic acid
TGF-α	Transforming growth factor alpha
TIC	Total ion current
TiO_2	Titanium dioxide
TMT	Tandem mass tags
T-REx™	Tetracycline-regulated mammalian expression system
UHTRP	Ultra-high temporal resolution phosphoproteomics
z	Charge
ZE	Zone electrophoresis

List of publications, presentations and posters

Publications:

1 Solari FA*, **Dell'Aica M***, Sickmann A, Zahedi RP. Why phosphoproteomics is still a challenge. *Molecular BioSystems*. 2015;11(6):1487-1493.

2 Novo P, **Dell'Aica M**, Janasek D, Zahedi RP. High spatial and temporal resolution cell manipulation techniques in microchannels. *Analyst*. 2016;141(6):1888-1905.

3 Beck F*, Geiger J*, Gambaryan S*, Solari FA, **Dell'Aica M**, Loroch S, Mattheij N, Mindukshev I, Pötz O, Jurk K, Burkhart JM, Fufezan C, Heemskerk JWM, Walter U, Zahedi RP, Sickmann A. Temporal quantitative phosphoproteomics of ADP stimulation reveals novel central nodes in platelet activation and inhibition. *Blood*. 2016

Dell'Aica Margherita contributed to the paper performing the targeted proteomics validation of sites that were found as regulated upon stimulation.

4 Gonczarowska-Jorge H, **Dell'Aica M**, Dickhut C, Zahedi RP. Variable Digestion Strategies for Phosphoproteomics Analysis. In: von Stechow L, ed. Phospho-Proteomics: Methods and Protocols. New York, NY: Springer New York; 2016:225-239.

5 Novo P, **Dell'Aica M**, Jender M, Hoving S, Zahedi RP, Janasek D. Integration of polycarbonate membranes in microfluidic free-flow electrophoresis. *Analyst*. 2017;142(22):4228-39.

6 Wiessner M, Roos A, Munn CJ, Viswanathan R, Whyte T, Cox D, Schoser B, Sewry C, Roper H, Phadke R, Marini Bettolo C, Barresi R, Charlton R, Bönnemann CG, Abath Neto O, Reed UC, Zanoteli E, Araújo Martins Moreno C, Ertl-Wagner B, Stucka R, De Goede C, Borges da Silva T, Hathazi D, **Dell'Aica M**, Zahedi RP, Thiele S, Müller J, Kingston H, Müller S, Curtis E, Walter MC, Strom TM, Straub V, Bushby K, Muntoni F, Swan LE, Lochmüller H, Senderek J. Mutations in INPP5K, Encoding a Phosphoinositide 5-Phosphatase, Cause Congenital Muscular Dystrophy with Cataracts and Mild Cognitive Impairment. *The American Journal of Human Genetics*. 2017;100(3):523-536.

7 Gonczarowska-Jorge H*, Loroch S*, **Dell'Aica M**, Sickmann A, Roos A, Zahedi RP. Quantifying Missing (Phospho)Proteome Regions with the Broad-Specificity Protease Subtilisin. *Anal Chem*. 2017;89(24):13137-45.

Dell'Aica Margherita contributed to the paper helping design the experimental setup and data analysis.

* shared first authorship

Oral Presentations:

1 "Studying protein phosphorylation with ultra-high temporal resolution using a micromixing microfluidic device". Proteomic Forum, 2–5 April 2017, Potsdam, Germany.
2 "Microfluidics and omics: extending the boundaries of knowledge through a multidisciplinary approach". Annual Meeting of the Study Group Bioanalytics of the GBM, 10-11 November 2016, ISAS Dortmund
3 "Studying protein phosphorylation with ultra-high temporal resolution using a micromixing microfluidic device". MicroTAS 2016 Conference, 9-13 October 2016, Dublin, Ireland
4 "Subtilisin - an alternative enzyme for PTM research". EuPA 2015 Congress, 23-28 June 2016, Milan, Italy

Poster Presentations:

1 "Studying protein phosphorylation with ultra-high temporal resolution using a micromixing microfluidic device." HUPO 2016 – 15th Human Proteome Organization World Congress, 18-22 September 2016, Taipei, Taiwan
2 "High temporal resolution study of phosphorylation events in HEK cells using a micromixer microfluidic device." EUROSENSORS 2016, 4-7 September 2016, Budapest, Hungary.
3 "Subtilisin - an alternative enzyme for PTM research". EuPA 2015 Congress, 23-28 June 2016, Milan, Italy
4 "Results As Soon As Possible (rASAP): 2 hours from lysis to label-free quantification of cells and tissues using subtilisin." ASMS 2018, 3-7 June 2018, San Diego, California

Patent:

1 Ultra-fast cell μmixer. Patent No. EP3412764A1.

Awards:

1 Ph.D. abstract competition award, HUPO (Human Proteome Organization conference).
2 CBMS (Chemical and Biological Microsystems Society) Traveling award, MicroTas

Zusammenfassung

Die quantitative Phosphoproteomanalyse wird zur Analyse von Protein-phosphorylierungsereignissen in Zellen und zunehmend auch in Geweben eingesetzt. Ein detailliertes Verständnis der zellulären Signaltransduktion erfordert jedoch individuelle Momentaufnahmen des Phosphoproteoms, die ebenfalls dessen Dynamik widerspiegeln – idealerweise mit einer zeitlichen Auflösung im Sekundenbereich. Die manuelle Probenvorbereitung eignet sich nicht für die Untersuchung solch früher Signalzeitpunkte (<10 s) und mangelt an Auflösung und Reproduzierbarkeit (Fehler >1 s).

In dieser Arbeit wurden Mikrofluidik und Massenspektrometrie basierte Phosphoproteomanalyse kombiniert, um neue Einsichten in die Dynamik des zellulären Signalings nach spezifischer Zellstimulation mit beispielloser zeitlicher Auflösung (im Millisekunden- bis Sekundenbereich) zu erhalten. Das Projekt basierte auf der Idee, eine Mikrofluidiks Apparatur, welche usprünglich am ISAS wurde um die ultraschnelle Zellaktivierung bei EGFR-Stimulation mittels Fluoreszenzbildgebung aufzuklären, für die Proteomanalyse anzupassen. Dieses System verwendete eine integrierte Pinched-Flow-Architektur. Obwohl es in der Lage war, die erforderliche Zeitauflösung zu erreichen, erwies sich das Design als inkompatibel zu phosphoproteomischen Arbeitsabläufen. Daher wurde ein neuartiges Mikrofluidisches Mikromischsystem entwickelt: das M^2D, eine geeignete Alternative basierend auf der Zellstimulation aufgrund von chaotischem Mischen. Die Vorrichtung umfaßt zwei in Reihe geschaltete Mischsegmente: das erste zum Steuern des Mischens von Zellen mit einer Ligandenlösung und das zweite zum Steuern der Lyse von Zellen, welche essentiell ist, um den momentanen zellulären Zustand einzufrieren. Verschiedene Stimulationszeitpunkte können untersucht werden, indem das Totvolumen zwischen den beiden Segmenten bei konstanter Flussrate variiert wird. Das neuartige Design erlaubt die vollständig kontrollierte zeitliche Stimulation und Lyse von Zellen, um Signalereignisse bereits 100 ms nach Stimulation mit einer zeitlichen Auflösung von 0,10 ± 0,08 s zu untersuchen. Mit Hilfe von EGFR-überexprimierenden HEK293-Zellen konnte gezeigt werden, dass die M^2D-Technologie selbst keine zellulären Signalwege induziert (kein artifizielles Signaling) und dass technische Replikate von 0,5, 1,0, 3,0 und 5,0 sekündiger EGFR-Stimulation hohe Reproduzierbarkeit und konsistente Ergebnisse lieferten. Als nächstes wurden nahezu 28.000 Phosphopeptide über verschiedene Zeitpunkte der EGFR-Stimulation (“Discovery”) quantifiziert. Die Daten erlauben es, die Signalweiterleitung über die Zeit zu verfolgen: Der EGFR-Signalweg zeigt eine Regulation bereits nach 3 s, während MAPK- und mTOR-Signalwege selbst nach 10 s EGF-Stimulation keine signifikante Regulation aufwiesen. Ein “targeted” PRM-Assay unter Verwendung von stabilisotopenmarkierten Standardpeptiden wurde für mehr als 40 phosphorylierte und nicht-phosphorylierte Peptide des EGFR-Signalwegs entwickelt, um deren Konzentration(sänderung) zwischen 0,1 s und 10,0 s EGFR-Stimulation mit höherer Präzision als im Discovery-Ansatz zu verfolgen. So konnten signifikante Veränderungen an EGFR pTyr-Stellen bereits nach 0,5 und 1,0 s Stimulation nachgewiesen werden.

Summary

Quantitative phosphoproteomics is applied for the in-depth analysis of protein phosphorylation events in cells and increasingly also in tissues. A precise understanding of cellular signalling, however, requires individual snapshots of the phosphoproteome that also reflect its dynamics, ideally with the potential to provide temporal resolution down to the seconds time range. Manual sample preparation is not suited for studying early time points of signalling (< 10 s) and lacks resolution and reproducibility (errors > 1 s).

In this work, microfluidics and mass spectrometry-based phosphoproteomics are combined to obtain novel insights into signalling dynamics upon specific cell stimulation with unprecedented temporal resolution (in the milliseconds to the second range). The project started based on the idea of adapting a microfluidics device, previously developed in our institute[1] to elucidate ultrafast cell activation upon EGFR stimulation via fluorescent imaging. This device was designed with integrated pinched-flow architecture. Although capable of providing the time resolution needed for studying cell-ligand interaction, the device turned out to be impractical for well-established proteomics workflows. Therefore, a novel microfluidic micromixing device was developed: the M^2D, as a suitable alternative strategy for cell stimulation based on chaotic mixing. The device comprises two mixing segments connected in series: the first for controlling the mixing of cells with a ligand solution, and the second for controlling the quenching and lysis of cells, fundamental to freeze the cellular state. Different stimulation time points can be achieved by varying the dead volume between the two segments at a constant flow rate. The original design allows conducting fully controlled temporal stimulation and quenching/lysis of cells to study signalling events as early as 100 ms after stimulation, with a temporal resolution of 0.10 ± 0.08 s. Using EGFR-overexpressing HEK293 cells, it was possible to demonstrate that the M^2D technology itself does not induce cellular signalling (no artefacts) and that technical replicates of 0.5, 1.0, 3.0 and 5.0 second EGFR stimulation showed high reproducibility and consistent results. Next, around 28,000 phosphopeptides were quantified across different time points of EGFR stimulation (discovery). The data allows following signal propagation over time: The EGFR pathway shows regulation already after 3 s, whereas MAPK and mTOR signalling pathways showed no significant regulation even after 10 s of EGF stimulation. A targeted PRM assays using stable isotope labelled standards peptides was developed for more than 40 phosphorylated and non-phosphorylated peptides of the EGFR pathway to follow their concentrations between 0.1 s and 10 s of EGFR stimulation with higher precision compared to the discovery approach. Thus, significant changes on EGFR pTyr sites could be detected already after 0.5 and 1.0 s of stimulation.