ANALYSIS OF FAST PROTEIN PHOSPHORYLATION KINETICS IN SINGLE CELLS ON A MICROFLUIDIC CHIP

Matthias Blazek1,2,3, Xuanye Wu1,2, Roland Zengerle2,3 and Matthias Meier1,2

1Microfluidic and Biological Engineering, Department of Microsystems Engineering - IMTEK, University of Freiburg, Georges-Koehler-Allee 103, 79110 Freiburg, GERMANY
2Centre for Biological Signalling Studies - BIOSS, University of Freiburg, GERMANY
3Laboratory for MEMS Applications, Department of Microsystems Engineering - IMTEK, University of Freiburg, Georges-Koehler-Allee 103, 79110 Freiburg, GERMANY

ABSTRACT
We present a microfluidic large-scale integration chip (mLSI) for characterizing the sequential activation of protein kinases within mammalian growth signaling pathways on a single cell level. The polydimethylsiloxane (PDMS) chip integrates automated cell culturing, stimulation and Proximity Ligation Assay (PLA) for protein analytics in 128 individually addressable microchambers. Design and fluidic operations on this chip generation are optimized for fast reagent exchange in order to resolve protein phosphorylation kinetics in a time regime of seconds. In an experimental series we monitored the protein phosphorylation kinetics of the Akt kinase leading to its activation. Long term activated Akt is a pivotal hallmark of several human cancer types. On chip PLA results with fibroblast cells revealed a consistent temporal order for the phosphorylation of Akt at the residues Ser-473 and Thr-308 upon stimulation with platelet derived growth factor (PDGF) and insulin-like growth factor (IGF-1).

INTRODUCTION
Signals from the extracellular microenvironment to the inner compartments of the cell are transmitted through sequential phosphorylation of signaling kinases. Understanding of signal processing requires dynamic information of the phosphorylation reactions, which occur in a time regime of seconds to minutes. Upon growth factor stimulation, Akt kinase is phosphorylated at its residues Ser-473 and Thr-308 [1, 2] from the two different kinases, namely mTORC2 and PDK1, respectively. Full Akt kinase activity is gained, when both residues are phosphorylated. The temporal order of Akt phosphorylation is a long standing research question [2, 3] but required to reveal the signal processing function of Akt. Standard methods, such as Western Blots do not offer sufficient temporal resolution and quantitative information. Here, we use a microfluidic chip in combination with the PLA [4] to quantify phosphorylation responses of the Akt kinase on the single-cell level. In contrast to a previous chip [5], the current design is optimized for fast on-chip fluid exchange to gain a temporal resolution of \( \Delta t = 10s \).

EXPERIMENTAL
The microfluidic large-scale integration chip [6] is shown in figure 1A. 128 cell culture chambers (arrow 1), each holding about 800 fibroblast cells, can be addressed individually with cell media and assay reagents from 22 fluidic inlet ports (arrow 2). Stimulation time series are implemented by allocating the cell chambers with temporal shifts of \( \Delta t = 10s \) between growth-factor stimulation and cell fixation reagents. The PLA setup for targeting the phosphorylation events is depicted in figure 1B. In short, the assay combines two antibodies labeled with different oligonucleotide strands. Upon binding in close proximity the oligonucleotide sequences are complemented, ligated, amplified by rolling circle amplification (RCA) and detected with fluorescence probes. PLA images were acquired on a Zeiss Axio Observer fluorescent microscope with a 20x objective. PLA dot count quantification and single cell segmentation was performed with Matlab image processing toolbox.

The phosphorylation kinetics of the Akt kinase was recorded at the two residues Ser-473 and Thr-308, both upon stimulation with the growth factors PDGF (100 ng/ml) and IGF-1 (200 ng/ml). Stimulation was performed at saturation conditions to ensure comparability of the phosphorylation responses triggered by both growth factors. Before stimulation, the cultured cells on chip were starved with a low-serum media (0.1% FBS) for 12 h. Fixation with 4% formaldehyde at different time points after stimulation conserved the molecular signaling response within the cells for following PLA analysis.
Figure 1. A) Large-scale microfluidic chip platform with integrated cell culturing systems and immune assay. Flow/control channels are highlighted in blue/red. B) Working principle of the immune-assay. Primary (2) and secondary antibody pairs (3) bind to target proteins or phosphorylation sites (1). Oligonucleotide labels on the secondary antibodies can hybridize if both are in close proximity. With help of a DNA probe a close DNA circle is formed (4). The DNA circle is amplified by rolling circle amplification and stained with a fluorescence dye.

Figure 2. Reagent exchange within the cell culture chambers measured with colored aqueous solutions. Flow pressure of 40 kPa results in an exchange rate corresponding to the volume of one cell culture chamber (25nL) per second.

RESULTS AND DISCUSSION
The phosphorylation response of Akt Ser-473 and Thr-308 upon stimulation with PDGF and IGF-1 is shown in figure 3. The results are derived from cell ensembles of at least 500 single cells per stimulation time point (gray dots). Despite the large heterogeneity of the single cell results, the arithmetic mean values (black dots) show a clearly observable activation behavior in response to the stimulus.

For quantification of the phosphorylation rates, the first order rate equation \( N(t) = N_0 \times \left[ 1 - \exp \left( -\frac{t}{t_0} \right) \right] \) was fitted to all single-cell PLA dot count distributions up to 240 s. Here, \( N(t), N_0, t, \) and \( t_0 \) denote for the PLA dot count distribution, a constant, the time point of fixation after stimulation, and the characteristic phosphorylation time, respectively. The fit result of the PLA single cell data revealed that the Akt phosphorylation at both residues is faster in response to the IGF-1 than to PDGF stimulation. Further, we could deduce from the kinetic traces that the characteristic phosphorylation time is consistently faster for the Thr-308 residue than for the Ser-473 residue. This argues that for Akt a precise phosphorylation order for its activation exists.
CONCLUSION
The integrated chip combines a high sensitive protein analytical assay with a microfluidic cell-culturing platform. Temporal control over 128 cell cultures and their microenvironments was used to successfully resolve the consecutive phosphorylation of two residues of the Akt kinase upon different growth factor stimulation. The PLA microfluidic chip is a new method to monitor phosphorylation dynamics for quantitative cell signaling studies, to reveal mechanistic details of signal transduction processes, and to provide higher-throughput data for computational network simulation.

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CONTACT
*M. Meier, Matthias.meier@imtek.uni-freiburg.de

Figure 3. Time traces of Akt phosphorylation at Ser-473 and Thr-308 upon stimulation with 100 ng/ml PDGF or 200 ng/ml IGF-1. Gray dots represent PLA dot count on single cell level. Black dots indicate mean values thereof. Red line indicates the fit function $N(t) = N_0*(1-exp(-t/t_0))$, where $N_0$ represents the full activation signal, $t$ the stimulation time and $t_0$ the characteristic activation time of the phosphorylation event.