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Review

Analysing phosphorylation-based signalling networks by phospho flow cytometry

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ABSTRACT

Analysis of signalling events by classical biochemical approaches is limited as the outcome is an averaged readout for protein activation of a single protein within a cell population. This is a clear restriction when addressing signalling events in mixed populations or subpopulations of cells. By combining flow cytometry with a panel of phosphospecific antibodies against several signal molecules simultaneously in a multi-parameter phospho flow cytometry analysis it is possible to obtain a higher level of understanding of the signal transduction dynamics at a single cell level. In addition, analysis of mixed cell populations makes it possible to study cells *ex vivo* in a state more closely resembling the *in vivo* situation. The multimeric analysis yields information on combinations of signals turned on and off in specific settings such as disease (signal nodes) that can be used for biomarker analysis and for development of drug screening strategies. Prostaglandin E_2 (PGE_2) is known to signal through four G-protein coupled transmembrane receptors, EP1–4, activating a multitude of potential signalling pathways. The analysis of the PGE_2 signalling network elicited by activation of the four EP receptors in lymphoid cells revealing several signalling nodes is reviewed as an example.

Contents

1. Introduction

Cellular behaviour is regulated by hormones, neurotransmitters, cytokines, inflammatory mediators, and other extracellular messengers. Signal cues to a cell are transduced across the membrane leading to intracellular signalling events, which elicit a cellular response to a given input. Intracellular signalling events are typically viewed to proceed along linear pathways originating at a specific point in the cell that are then propagated along their path to a specific target at a defined destination. However, transduction of signals from a single extracellular input branch out to multiple pathways and crosstalk is observed between signalling pathways. Furthermore, a cell in a physiological context will at any given time receive multiple parallel signals further complicating the picture compared to an experimental situation. Indeed, the expanding knowledge of signalling resulting from analysis of large data sets that can be collected by high-throughput techniques clearly demonstrate that a model which considers signalling to proceed in a linear fashion along defined pathways is an over-simplistic representation that does not mirror the intricate way signalling proceeds inside a cell. It is increasingly becoming clear that the transmission of signals occurs in networks, consisting of individual nodes and highly connected hubs [1]. Furthermore, it is now evident that signalling networks are highly interlinked with a large degree of crosstalk between individual

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networks [2]. In addition, several branches of independent research have identified the importance of spatial and temporal organisation for the functionality of signalling networks [3–5]. In order to propagate and fine-tune the information conveyed by a specific stimulus and to integrate the resulting response efficiently into a pre-existing dynamic signalling state, signalling networks utilise a number of mechanisms such as post-translational modifications, protein–protein interactions, secondary messengers, radical oxygen and nitrogen species and many more. While it can be expected that many or even all conceivable mechanisms are involved in a selected signalling network, research to date focuses on the identification of mechanism-specific signalling networks such as protein–protein interaction networks or phosphorylation-based networks. Looking at the enormous amounts of data produced for these networks it becomes clear that the task of truly understanding the complexity of individual types of signalling networks is daunting. However, advances in both technology and bioinformatics are now starting to support the study of complex signalling networks that also integrate information on different mechanisms of conveying signals. Here we aim to review recent technological and bioinformatic advances that aid the study of phosphorylation-based signalling networks as well as to highlight important control measures necessary in order to apply these technologies effectively.

2. High-throughput approaches for studying phosphorylation-based signalling networks

In order to study complex and extensive phosphorylation-based signalling networks, high-resolution, high-throughput strategies are necessary as they are able to capture both the magnitude and complexity of the signalling network. In this respect, phosphoproteomic strategies have been the primary choice for studying phosphorylation-based signalling networks as they provide the means to assess global phosphorylation profiles in an unbiased manner. With the development of quantitative mass spectrometry methodologies such as SILAC [6] or stable isotope labelling [7] combined with phosphopeptide enrichment techniques such as TiO_2 [8], changes in phosphorylation-based signalling networks in response to a specific stimulus could be quantitatively assessed on a cell proteome-wide basis. The clear advantage of using phosphoproteomics for the study of signalling networks lies in the unbiased nature of the technology. Phosphopeptides and specific phosphorylated residues can be detected with high confidence and independently of their previous characterisation. Phosphoproteomics therefore is well suited for the discovery of previously unidentified phosphorylation sites and their dynamic changes in response to a stimulus, but such studies are hampered by the low sample number capacity which limits the possibly to conduct multi-parameter studies and by the fact that the technology would average the data gathered from the mixed cell populations analysed.

In addition to phosphoproteomic approaches, phospho flow cytometry is emerging as a high-throughput methodology that can be used to study phosphorylation-based signalling networks in a highly efficient and reliable manner [9–14]. Phospho flow cytometry relies on well-characterised phospho-epitope specific antibodies and allows the analysis of intracellular phosphorylation events on a single cell level in mixed cell population where differences in signalling profiles in different cell subsets can be identified. Furthermore, the introduction of fluorescent cell barcoding allows for the concomitant analysis of multi-sample, multi-perturbation experiments in one experimental run [15]. In this approach, cells from different samples are labelled with serial dilutions of one or more fluorescent dyes prior to combining all samples in one experimental tube. Fluorescent cell barcoding allows the analysis of cells from many samples in one experimental run and enables the identification of the cells originating from each individual initial sample after flow cytometry analysis.

Below we will review approaches to study phosphorylation-based signalling networks and we will further discuss important issues relating to the use of phospho-epitope specific antibodies in such analyses.

3. Phospho flow cytometry

High-throughput phospho flow cytometry was first described in 2002 [16]. It utilises fluorescently labelled phospho-epitope specific antibodies on a standard flow cytometry setup. In order to facilitate recognition of short-lived intracellular phospho-epitopes, cells need to be formaldehyde-fixed as well as methanol-permeabilised. Following this procedure, cells can be stained with a variety of additional fluorescently labelled antibodies raised against proteins of choice, such as cell phenotype markers, cell activation markers, transcription factors, etc. In such a setup, fluorescently labelled antibodies against cell phenotype markers can be used in addition to phospho-epitope specific antibodies and fluorescence cell barcoding (FCB) dyes. By using phenotype markers, phosphorylation-based signalling networks in individual cell subpopulations present in a complicated cell mixture can be studied. This feature of high-throughput phospho flow cytometry provides a suitable tool for the study of complex signalling networks in different cell subpopulations. While it has been possible to study signalling in cell subpopulations for some time by means of specific cell isolation kits or flow cytometry-based cell sorting, the phospho flow methodology combined with cell surface markers allows for signalling events in cell subpopulations to be studied in the presence of other cell types. For example, we could in a recent report look at parallel signalling events in T cell subsets such as naïve and memory CD4 and CD8 cells in the context of other circulating cells [14]. Because a given stimulus can trigger a variety of different responses dependent on cell type that can lead to the production of an array of cytokines and other intercellular communication molecules, it is critical to study signalling networks in a more physiological context.

4. PGE_2 regulated signalling networks

In a recently published study, we utilized an extensive triple approach to investigate PGE_2 signalling in T cells where we combined high-resolution phosphoproteomics as an unbiased approach, kinase prediction and bioinformatic network analysis, and phospho flow analysis (Fig. 1) [14]. By using phospho flow cytometry to study phosphorylation-based networks, one can not only track the phosphorylation status of a specific site in high kinetic resolution, but one can also observe the activation status of suspected signalling hubs downstream of the investigated receptor. For example, when studying prostaglandin E_2 (PGE_2)-induced signalling networks, the activity of known signalling hubs downstream of the four possible PGE_2 receptors (EP1–4) could be observed (Fig. 2) [14]. In this case, the activities of the major kinases known to be involved in EP receptor signalling have been assessed. Protein kinase A (PKA; downstream of EP2 and 4) activity was observed by a PKA substrate specific antibody; calmodulin-dependent protein kinase II (CAMKII; downstream of EP1) and protein kinase B (PKB/Akt; downstream of EP3) activity was determined using phospho-epitope specific antibodies against the auto-phosphorylation site of the respective kinases. By utilising this strategy, activity of kinase hubs, which per definition are crucial in phosphorylation-based signalling networks, can be assessed and in addition can be confirmed by measuring changes in the phosphorylation status of known substrates of the investigated kinase. Furthermore, in this study, differences in the basal activation status of the PKA signalling node could be identified between different T cell subsets with CD4 and CD8 memory T cells ($CD45RO^+$) exhibiting significantly higher basal activity compared to other CD3 T cell populations [14]. This again highlights the power of this technology to study signalling networks in the context of other cell types present *in vivo*.