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Quantitative proteomic assessment of very early cellular signaling events

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Technical limitations have prevented proteomic analyses of events occurring less than 30 s after signal initiation. We developed an automated, continuous quench-flow system allowing quantitative proteomic assessment of very early cellular signaling events (qPACE) with a time resolution of 1 s. Using this technique, we determined that autophosphorylation of the epidermal growth factor receptor occurs within 1 s after ligand stimulation and is followed rapidly by phosphorylation of the downstream signaling intermediates Src homologous and collagen-like protein and phospholipase C gamma 1.

Quantitative proteomic approaches using mass spectrometry (MS) have become the method of choice for the analysis of phosphorylation events *in vivo*[1]. Stable isotope labeling by amino acids in cell culture (SILAC)[2], combined with treatment of cell populations at many time points, has provided exciting insights into the spatio-temporal dynamics of proteins *in vivo*[3,4], including sites of transient phosphorylation upon growth-factor stimulation[5]. However, as MS-based identification of phosphopeptides from rare and partially phosphorylated signaling molecules often requires large numbers of cells, the scale of such experiments makes precise and uniform initiation and monitoring of cellular signaling technically demanding. Although the earliest reported time points of MS-based proteomics measurements are therefore commonly at least 30 s after signal initiation, fluorescence lifetime measurements and kinetic studies have suggested that autophosphorylation on receptor tyrosine kinases happens within seconds after growth factor stimulation[6–8].

The qPACE approach involves pumping SILAC-labeled cells through a continuous quench-flow system to ensure rapid and reproducible initiation and trapping of time-resolved cellular signaling states. The method permits accurate, MS-based quantitative measurements of signaling events, such as phosphorylation, on a timescale of seconds. Using this system, we have analyzed phosphorylation of the epidermal growth factor (EGF) receptor and its most proximal signaling molecules, Src homologous and collagen-like protein (Shc) and phospholipase C gamma 1 (PLCγ) after 1, 5, 10 and 60 s of EGF treatment.

The qPACE set-up consists of three peristaltic pumps that deliver cells, stimuli and quenching reagent (**Fig. 1a** and **Supplementary Fig. 1** online). An experiment is performed by pumping cells and growth factor into a mixing chamber to initiate stimulation. The duration of stimulation is controlled by adjusting the flow rates and the length of tubing between the mixing chamber and the point of delivery of the quenching reagent, which blocks signaling. Immediate quenching of all cellular activity is crucial for measurements of rapid signaling events. This can be achieved by snap-freezing cells in liquid nitrogen or by the addition of a fixing solution. Although cooling cells with ice-cold solutions and processing them as rapidly as possible is adequate for differential analyses of signaling events on a timescale of minutes, it is not sufficient for a timescale of seconds. The use of 70% ethanol at –20 °C gave the required quenching effect. This is a widely used fixing solution and should trap all signaling events immediately (**Supplementary Methods** online). A drawback of this approach is that, when compared with lysing cells directly, ethanol reduces the efficiency of EGF receptor (EGFR) immunoprecipitation by ~50% (data not shown). As pumping might be harmful to cells, we measured their viability by staining with trypan-blue and using a NucleoCounter before and after pumping, but observed no significant differences (data not shown).

To determine site-specific phosphorylation events, we stimulated HeLa S3 cells labeled with three distinct stable isotope forms of arginine and lysine with 150 ng/ml EGF. We performed two sets of experiments to profile phosphopeptides at five time points (0, 1, 5, 10 and 60 s) using 5 s as a common reference interval (**Fig. 1b**). After quenching, cells were pelleted, lysed and subjected to immunoprecipitation. Anti-phosphotyrosine antibodies were used to characterize tyrosine phosphorylation sites on EGFR and downstream signaling transducers. In a second set of experiments, EGFR was immunoprecipitated with anti-EGFR antibody to determine the profiles of serine and threonine phosphorylation sites on the receptor. Immunoprecipitated proteins were separated by SDS-PAGE and excised gel fragments were digested with trypsin. For anti-EGFR immunoprecipitations, we excised a gel fragment based on the expected migration of EGFR and for anti-phosphotyrosine immunoprecipitations, we excised slices spanning the entire lane. Phosphopeptides were purified by incubation with TiO_2 spheres and analyzed using Linear Ion Trap–Fourier Transform Ion Cyclotron Resonance (LTQ-FTICR) and LTQ-Orbitrap mass spectrometers[5,9] (**Supplementary Methods** online). Labeled peptides appear as triplets that reflect the relative amounts of peptide at three different time points (**Fig. 1c**). Phosphopeptides were validated and quantified by MSQuant (http://msquant.sourceforge.net/). All changes were expressed as fold-change relative to the reference value and normalized to one at time zero

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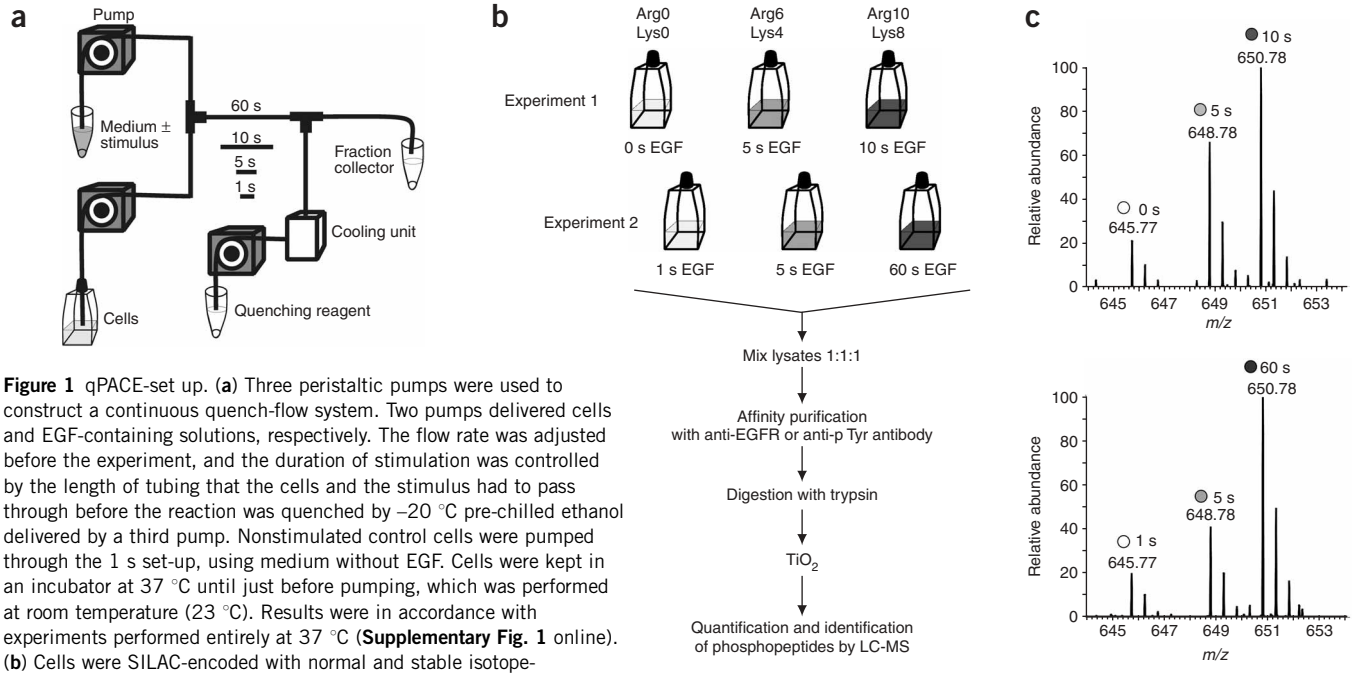

Figure 1 qPACE-set up. (**a**) Three peristaltic pumps were used to construct a continuous quench-flow system. Two pumps delivered cells and EGF-containing solutions, respectively. The flow rate was adjusted before the experiment, and the duration of stimulation was controlled by the length of tubing that the cells and the stimulus had to pass through before the reaction was quenched by –20 °C pre-chilled ethanol delivered by a third pump. Nonstimulated control cells were pumped through the 1 s set-up, using medium without EGF. Cells were kept in an incubator at 37 °C until just before pumping, which was performed at room temperature (23 °C). Results were in accordance with experiments performed entirely at 37 °C (**Supplementary Fig. 1** online). (**b**) Cells were SILAC-encoded with normal and stable isotope-substituted arginine and lysine, resulting in three cell populations distinguishable by mass. Cells were stimulated with EGF for different times and mixed. The experiment was repeated to yield five time points. Combined cells were lysed for affinity-purification using either anti-EGFR or anti-phosphotyrosine antibodies. The resulting protein complexes were separated by SDS-PAGE and digested enzymatically. Phosphopeptides were enriched and analyzed by MS. (**c**) LTQ-FTICR mass spectra of phosphopeptide EGFR1189–1199 GSTAENAEpYLR. Numbering is based on the unprocessed receptor precursor. Different shades of gray correspond to the shades in **Figure 1b**, representing different media used for cell labeling.

(**Supplementary Methods** online). We were able to detect eight phosphorylation sites on EGFR, three of which displayed more than a twofold increase in phosphorylation within 5 s exposure to EGF (**Fig. 2a**). Tyrosine phosphorylation is the hallmark of EGFR activation[10–12] and accordingly, these three sites are tyrosine residues Tyr1092, Tyr1172, and Tyr1197. The latter two are known binding sites for Shc, a signal initiator in EGFR signaling[5,12]. The three sites showing increased phosphorylation in <5 s indicate that almost immediate autophosphorylation of the receptor occurred. These findings were also confirmed by western blot analysis (**Fig. 2b**). The observation that the abundances of corresponding nonphosphorylated peptides did not change substantially suggests that only a small subset of receptor molecules are involved in signal transduction at this early stage (**Supplementary Fig. 2** online). The nonregulated phosphorylation sites comprise serine and threonine residues known to participate in negative feedback starting minutes after stimulation[5,12–14].

Analysis of downstream signal components revealed a regulated phosphotyrosine site in Shc and two such sites in PLCγ (**Fig. 2a**,**b**). The phosphorylation of Shc at Tyr427 follows immediately after phosphorylation of EGFR, indicating that Shc and EGFR have to be in close spatial proximity before stimulation. Phosphorylation of the two regulated sites of PLCγ lagged behind; the phosphorylation of Tyr1253 was first detected after 5 s and phosphorylation of Tyr771 was only detected after 60 s. Two additional phosphotyrosine sites, Tyr16 in cell division cycle 2 protein and Tyr707 in CUB domain–containing protein 1, were not affected by EGFR stimulation even after 60 s (data not shown).

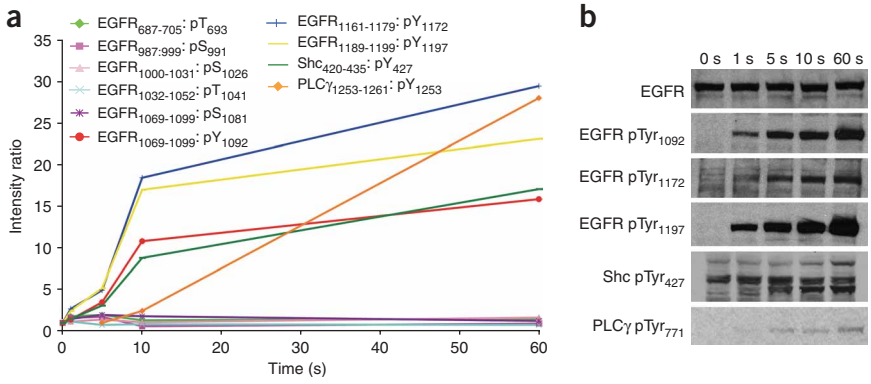

Figure 2 Activation profiles of phosphopeptides. (**a**) Normalized time profiles of tyrosine, threonine and serine phosphorylation sites, as detected by MS. Profiles of regulated phosphopeptides were recorded in at least two biological replicates. The 5 s value was used as a common time point to integrate the time series 0-5-10 s and 1-5-60 s. For normalization, the value at 0 s was set to 1 and the other values adjusted accordingly. In the case of PLCγ site Tyr1253, it was only detected after 5 s; therefore this value was used for normalization. As PLCγ site Tyr771 (PLCγ764–778) was only detected after 60 s, this prevented determination of an activation profile. (**b**) Regulated tyrosine phosphorylation sites detected by qPACE were monitored by western blot analysis using site-specific antibodies. The three isoforms of Shc (p66, p52 and p46) showed different activation profiles that could not be discriminated at the peptide level as all three forms share the regulated tyrosine site.

To our knowledge, this is the first report on a quantitative proteomic assessment of very early signaling events in eukaryotic cells. We demonstrated previously how MS-based proteomics enables endogenous signaling molecules and their individual phosphorylation sites to be profiled[5] to discriminate between closely related signaling pathways[15] and to identify novel interaction partners[16]. The qPACE approach enables similar experiments to be performed on a timescale of seconds to better distinguish molecules acting as sensors from downstream transducers. This will enhance understanding of how these molecules integrate signals to decide cell fate. The system can also be used to identify genuine ligand-receptor or drug-receptor complexes by excluding receptor candidates activated indirectly at a later time point. We have analyzed the consequences of EGFR stimulation for 1–60 s, but in principle it is possible to perform reproducible measurements on a timescale of <1 s. Although our results were obtained using suspension cells, use of microcarrier beads extends the method to adherent cells (**Supplementary Fig. 3** online). Thus, qPACE is a generic method applicable to the study of early signaling events in many cell culture systems.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

J.D. and V.A.: **Figures 1** and **2**, **Supplementary Figures 1, 2** and **3**, cell culture, stimulation experiments, western blot analyses and MS analyses. J.O. and J.B.: **Figure 2a** and MS analyses. M.M., B.B. and J.S.A.: experiment design, result analysis and writing of the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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