Quantitative proteomic assessment of very early cellular signaling events: qPACE

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Method Article

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Abstract

Introduction

It has been shown that signaling via phosphorylation happens within seconds after growth factor stimulation in eukaryotic cells. However, these very early signaling events have so far not been analyzed by mass spectrometry \(MS)-based proteomics. Here we report the development of an automated system which allows quantitative proteomic assessment of very early cellular signaling events \(qPACE). Our approach relies on pumping SILAC labeled cells through a continuous quench-flow system to ensure rapid and reproducible initiation and trapping of cellular signaling states. The method permits accurate quantitative measurements by MS of signaling events, like phosphorylation, on a second timescale.

Reagents

\(1) Stable isotope labeling by amino acids in cell culture \(SILAC)-medium \(2) 80% ethanol at -20°C \(3) Growth factors, e.g EGF \(Peprotech) \(4) Suspension cells \(e.g. HELA S3; ATCC Number: CCL-2.2), or adherent cells \(e.g. HELA; ATCC Number: CCL-2) growing on microcarrier beads \(GE Healthcare) \(5) Antibodies for immunoprecipitation, e.g. anti-phosphotyrosine antibody 4G10 \(Upstate, Millipore) \(6) Lysis buffer: 1% NP-40, 0.1% Na deoxycholate, 150 mM NaCl, 1 mM EDTA, 50 mM Tris \(pH 7.5), 1 mM Na orthovanadate, 10 mM beta-glycerophosphate, 10 mM sodium fluoride and protease inhibitors \(e.g. CompleteTM tablets, Roche Diagnostics)

Equipment

\(1) Three pumps, e.g. peristaltic pumps P1 \(GE Healthcare) \(2) Teflon tubing, e.g. 1 or 2 mm i.d. \(3) Two T-connectors for tubing \(4) Cooling aggregates \(5) Equipment for SDS-PAGE \(6) Centrifuge \(7) LC-MS system \(8) Software tools for MS-data analysis, e.g. MASCOT search engine and MSQuant software \(http://msquant.sourceforge.net)

Procedure

\(1) Label your cells with SILAC \(at least 5 population doublings to ensure complete incorporation of isotope-labeled amino acids). \(2) Serum-starve your cells before pumping for approximately 12 h. \(3) Connect pumps with tubing as outlined in **Figure 1**: pump P1 and P2 run with the same speed \(in our case 800 µl/min), P1 delivers medium containing the stimulus, P2 delivers cells \(in suspension or growing on microcarrier beads). Pump P3 delivers the quenching reagent, in our case 80% ethanol prechilled to -20°C and further cooled by -20°C cooling aggregates. To reach proper quenching, pump P3 should run approximately 5 to 10 times faster than pumps P1 and P2 \(in our case 8 ml/min). It is possible to place pumps P1, P2, and the stimulation tube in an incubator to perform experiments at 37°C. \(4) Adjust the tube length determining the time of stimulation according to your pumping speed \(speed of pump P1 + speed of pump P2) and the tubing diameter to reach the desired stimulation time: volume \

(V) = π × r^2 × length \(L). We used 1 mm i.d. Teflon tubes with the following lengths:\(a) 1 s: 3.4 cm, \(b) 5 s: 17 cm, \(c) 10 s: 34 cm, and \(d) 60 s: 204 cm. \(5) Prepare cell suspension and double concentrated stimulation medium. We used 1.5-4×10^8 cells per time point resuspended in 5 or 10 ml of medium and medium containing 300 ng/ml EGF \(150 ng/ml final concentration). \(6) Pump sample through the system. \(7) Pellet your cells by centrifugation. \(8) Discard supernatant, wash pellet with ice-cold PBS, and pellet again. \(9) Lyse cells with lysing buffer containing protease and phosphatase inhibitors. \(10) Immunoprecipitate your complexes of interest, separate them by SDS-PAGE and digest them enzymatically, e.g. with trypsin. We used preconjugated 4G10 antibody to precipitate phosphotyrosine containing proteins. \(11) Analyze resulting peptide mixture by LC-MS. In the case of phosphopeptide analysis, phosphopeptides should be enriched prior LC-MS analysis, e.g. by TiO_2. \(12) Analyse your data and quantitate peptides of interest, e.g. by MSQuant software.

Timing

\(1) Cell culture takes two to three weeks. \(2) One pumping experiment takes approximately 10 minutes depending on cell suspension volume and pumping speed.

Critical Steps

Step \(3): Be aware that ethanol leads to less efficient immunoprecipitation as compared to directly lysing cells \(approximately 50%). Step \(5): Use medium without serum for pumping cells for negative controls. Step \(8): Make sure to wash the cell pellet with PBS as residual ethanol may cause problems regarding the following immunoprecipitation and SDS-PAGE separation.

Troubleshooting

\(1) Pumping speed has to be adjusted to be not harmful for cells. Too high speed may lead to shear forces that destroy cells. \(2) Cell suspension should be kept in motion by a magnetic steer or by shaking in order to prevent aggregation. \(3) Cell density has to be adjusted to prevent clogging of the system.

References

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Figures

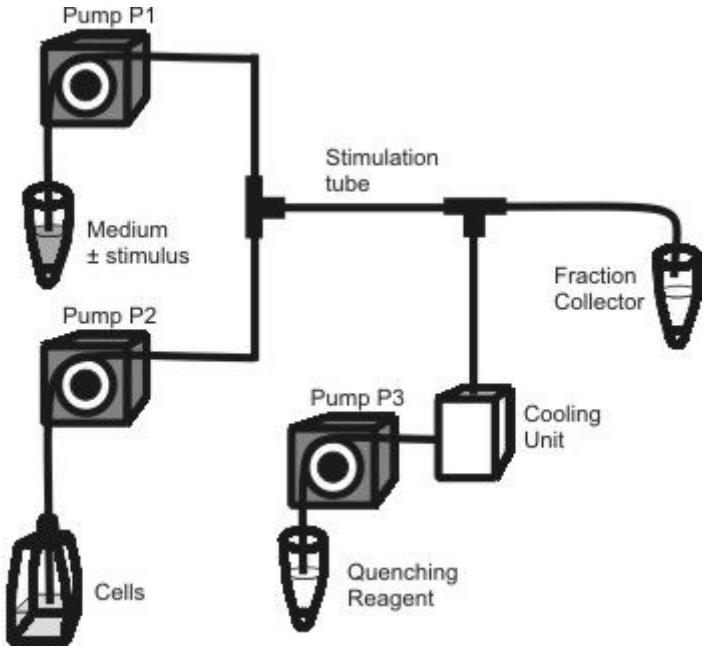

Figure 1

qPACE-set up