The use of a four-color multiplex antibody-based assay with laser-scanning high content screening to examine kinase inhibitor effects on cellular signaling

Jessica A. Cherry¹, Paul Wylie², Christopher A. Manning², Diana Caracino², Randall K. Wetzel¹

Cell Signaling Technology Inc¹, Danvers, MA 01923, USA • TTP LabTech Ltd², Melbourne Science Park, Melbourn, Royston, Herts SG8 6EE, UK

Abstract

Traditional biochemical and house-based assays (e.g., western blot, immunoprecipitation, ELISAs) have been integral in the analysis of individual signaling events, however they are limited in their ability to monitor the phosphorylation of multiple proteins on a per cell basis. The use of multiple endpoints in a single well can often yield more information than individual single endpoint assays, which is especially important in the analysis of complex cellular signaling. To address this need, a panel of high-quality antibodies was generated using novel antibody development technologies to simultaneously monitor signaling events in multiple pathways. In this study, a four-color panel was used in a cell-based high content assay to examine the effect of specific kinase inhibitors on cellular signaling. A human breast cancer cell line, MCF7, was stimulated with free and with kinase inhibitors. Cells were cultured, treated, and fixed in multwell plates, then indirectly labeled with a cocktail of three primary antibodies targeting phospho-Akt (Ser473), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), and phospho-S6 ribosomal protein (Ser235/236), detected with fluorescent secondary reagents, and labeled with Hoechst 33342. Fluorescent signal was quantified using an Acumen eX3 that is capable of simultaneously quantifying fluorescence from up to four different antibodies. Immunofluorescent signals from the three antibodies were individually quantified per cell basis in cells treated with compounds (alone or in combination). Neuregulin treatment alone increased signaling in the MAPK and Akt pathways in the breast cancer cell line MCF7. Effective monitoring of this signaling is important for the discovery and development of drugs to combat, treat, and cure diseases driven by mutations in these proteins. Using a combination of specific antibodies and RTK signaling in general – phospho-Akt (Ser473), phospho-p44/42 MAPK (Erk1/2), and a downstream target of both, phospho-S6 ribosomal protein (Ser235/236). The cells were then labeled with a detect a cocktail of Alexa Fluor® conjugated secondary antibodies (488, PE, 447) for 10 minutes at 22°C. Nuclei were labeled with Hoechst 33342 for at least 30 minutes at 22°C. Fluorescence quantification was performed using a TTP LabTech Acumen® eX3 high content screening platform, and images were acquired using a CellSens® Acumen® v3.6. The fluorescent intensities per well were normalized to 100% of the vehicle control, and inhibition induction curves were generated using Tophic®.

Methods

MCF7 cells were grown under normal culture conditions in 96-well plates and treated for 2 hours at 37°C with vehicle (dimethyl sulfoxide) or increasing concentrations (0 – 1000 ng/mL) of LY294002 (PI3 kinase inhibitor), U0126 (MEK1/2 inhibitor), or (0.1 – 100 nM) Rapamycin (mTORC1/2 inhibitor), either alone or in combination. The cells were then stimulated with 100 ng/mL human neuregulin-1 (NRG-1) (HER/ErbB family ligand) for 20 minutes. In a separate experiment, MCF7 cells were exposed to increasing concentrations (0 – 1000 ng/mL) of EGF for 20 minutes without the presence of kinase inhibitors. After treatment, all cells were fixed with 4% formaldehyde for 15 minutes and subsequently labeled with a cocktail of three primary antibodies targeting phospho-Akt (Ser473), phospho-p44/42 MAPK (Erk1/2), and phospho-S6 ribosomal protein (Ser235/236), detected with fluorescent secondary reagents, and labeled with Hoechst 33342. Fluorescent signal was quantified using an Acumen eX3 that is capable of simultaneously quantifying fluorescence from up to four different antibodies. Immunofluorescent signals from the three antibodies were individually quantified per cell basis in cells treated with compounds (alone or in combination). Neuregulin treatment alone increased signaling in the MAPK and Akt pathways in the breast cancer cell line MCF7.

Results

Conclusions

• Phosphorylation-specific antibodies can be used to effectively monitor receptor tyrosine kinase signaling as a simple on/off readout.
• Multiplexing allows the simultaneous analysis of different cellular signaling endpoints within a single sample.
• High throughput laser-scanning cytometers (e.g., Acumen® eX3) combined with activation-state-specific antibodies allow for the rapid quantification of cellular signaling, and avoid the need for complex, time-consuming image analysis.