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

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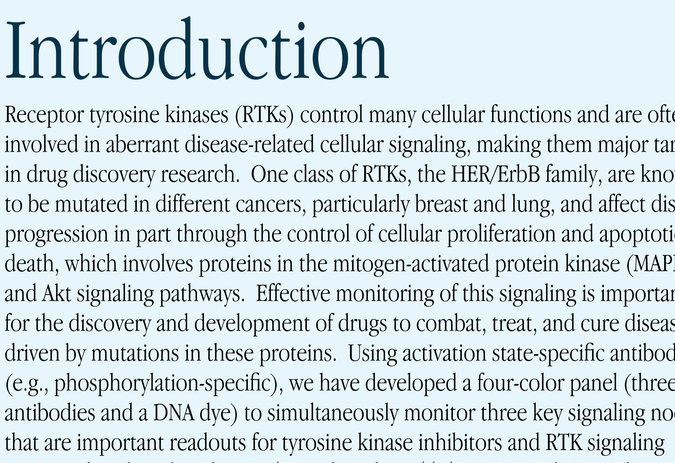
Abstract

Traditional biochemical and lysate-based assays (e.g., western blot, immunoprecipitation, ELISA) 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 neuregulin (HER/ErbB family ligand) and treated with well-characterized PI3 kinase, MEK1/2, and FRAP/mTOR inhibitors. Cells were cultured, treated, and fixed in multiwell 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 analytes. Immunofluorescent signals from the three antibodies were individually quantified on a per cell basis in cells treated with compounds (alone or in combination). Neuregulin treatment alone increased signaling in the MAPK and Akt pathways in a dose-dependent manner. Kinase-specific inhibitors directly affected signaling in the expected pathways; the PI3 kinase inhibitor decreased signal from the phospho-Akt antibody, the MEK inhibitor decreased signal from the phospho-Erk antibody, and combinations of the three inhibitors dramatically diminished signal from all endpoints – phospho-Akt, phospho-Erk, and phospho-S6. This study demonstrates that multiple endpoints can be combined in a single well to simultaneously monitor the activity of signaling pathways in cells treated with different kinase inhibitors. Further, the on/off signal from phospho-specific antibodies enables the rapid quantification of signaling using a multiplex laser-scanning platform like the Acumen eX3.

Introduction

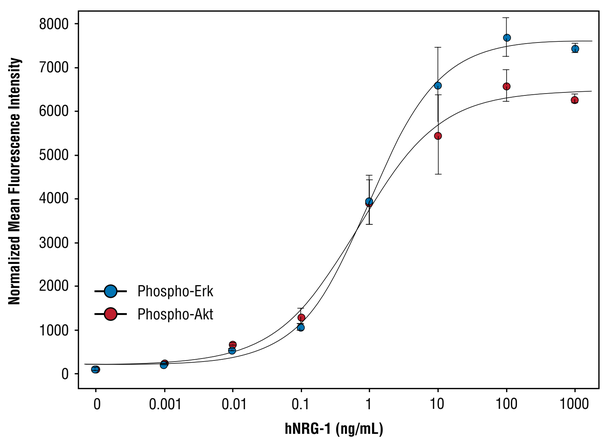
Receptor tyrosine kinases (RTKs) control many cellular functions and are often involved in aberrant disease-related cellular signaling, making them major targets in drug discovery research. One class of RTKs, the HER/ErbB family, are known to be mutated in different cancers, particularly breast and lung, and affect disease progression in part through the control of cellular proliferation and apoptotic cell death, which involves proteins in the mitogen-activated protein kinase (MAPK) and Akt signaling pathways. 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 activation state-specific antibodies (e.g., phosphorylation-specific), we have developed a four-color panel (three antibodies and a DNA dye) to simultaneously monitor three key signaling nodes that are important readouts for tyrosine kinase inhibitors and RTK signaling in general – phospho-Akt (Ser473), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), and a downstream target of both, phospho-S6 ribosomal protein (Ser235/236). Here, we demonstrate the use of this panel and the power of multiplexing using a high content laser-scanning platform to yield fast, reliable results in an efficient and cost-effective manner.



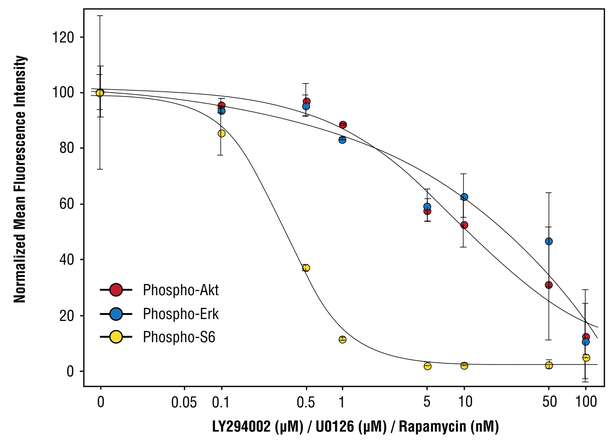
Methods

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 (0.1 – 100 μM) of LY294002 (PI3 kinase inhibitor), U0126 (MEK1/2 inhibitor), or (0.1 – 100 nM) Rapamycin (FRAP/mTOR inhibitor), either alone or in combination. The cells were then stimulated with 100 ng/mL human neuregulin-1 (hNRG-1) (HER/ErbB family ligand) for 20 minutes. In a separate experiment, MCF7 cells were exposed to increasing concentrations (0 – 1000 ng/mL) of hNRG-1 for 20 minutes without the presence of kinase inhibitors. After treatment, all cells were fixed with 4% formaldehyde for 15 minutes and subsequently labeled overnight at 4°C with the PathScan® Signaling Nodes Multiplex IF Kit primary antibody cocktail, which contains three antibodies targeting phospho-Akt (Ser473), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), and phospho-S6 ribosomal protein (Ser235/236). The cells were then labeled with a detection cocktail of Alexa Fluor® conjugated secondary antibodies (488, PE, 647) for 1 hour 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 Cellomics® ArrayScan® VTI. The fluorescence intensities per well were normalized to 100% of the vehicle control, and inhibition/induction curves were generated using Spotfire®.

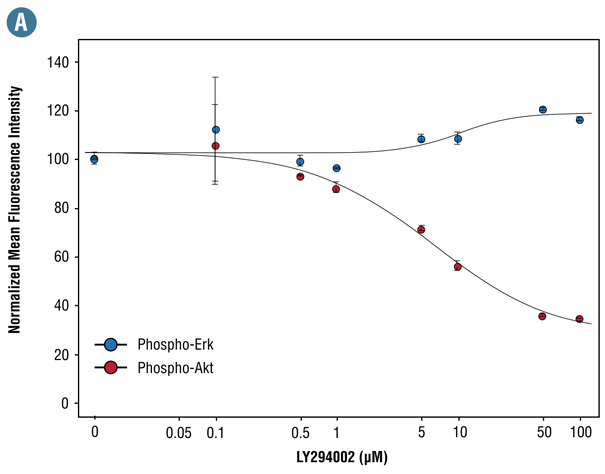
Results

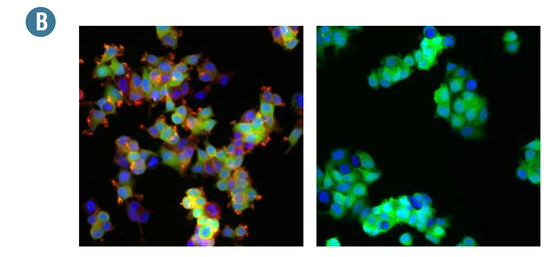


HER/ErbB ligand (hNRG-1) activates MAPK and Akt signaling pathways in the breast cancer cell line MCF7

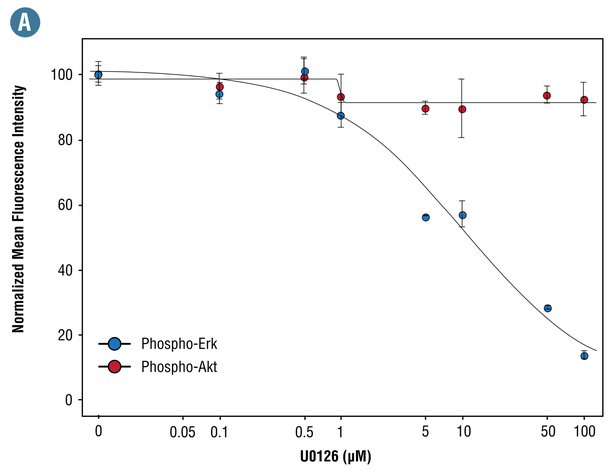


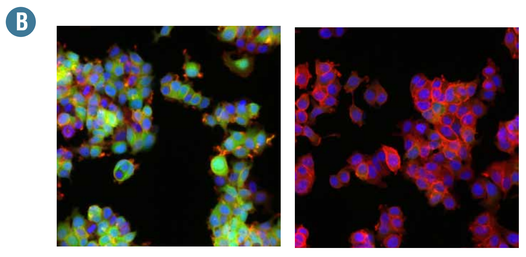
A combination of specific kinase inhibitors decreases phospho-Erk and phospho-Akt, along with a downstream signaling protein of both pathways, phospho-S6





A. PI3 kinase inhibitor (LY294002) decreases phospho-Akt but not phospho-Erk
B. Multiplex image; left 0μM, right 100μM; red = phospho-Akt (Ser473), green = phospho-Erk (Thr202/Tyr204), blue = nuclei





A. MEK1/2 inhibitor (U0126) decreases phospho-Erk but not phospho-Akt
B. Multiplex image; left 0μM, right 100μM; red = phospho-Akt (Ser473), green = phospho-Erk (Thr202/Tyr204), blue = nuclei

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.

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