JACOB KNOWLES, STEPHEN CAPODILUPO, RICHARD NORING, ROBERTO CAMPOS-GONZÁLEZ, JULIO E. HERRERA, AND **ZEV GECHTMAN**. CELL SIGNALING TECHNOLOGY, INC. 3 TRASK LANE, DANVERS, MA 01923

Highly Parallel Planar Surface Antibody Array-based Assay to Monitor Protein-protein Interactions

Abstract

Protein-protein interactions have been receiving increasing attention as targets for pharmacological intervention. However, current methods used to detect protein-protein interactions either lack proper throughput, are not quantitative, or rely on highly engineered systems and cumbersome workflows. To address this, we developed a planar surface antibody array-based sandwich assay that enables simultaneous detection and relative quantification of protein-protein interactions from complex solutions. We validated our approach by applying it to receptor tyrosine kinases (RTKs), which are well characterized and are known to form disease relevant, dynamic interactions. The antibody array-based assay revealed co-existing combinatorial RTK interactions that were unique to each cell type tested. The assay allowed the monitoring of ligand-mediated formation of RTK complexes as well as their disruption by small molecule inhibitors. The antibody array sandwich assay described can be applied to a broad range of cases with various topological configurations, thus offering an advantage over technologies that have strict proximity requirement. This protein interaction array-based assay can be used to screen for inhibitors or inducers of protein-protein interactions, thereby aiding in the identification of new chemical modulators with unique properties and novel mechanisms of action.

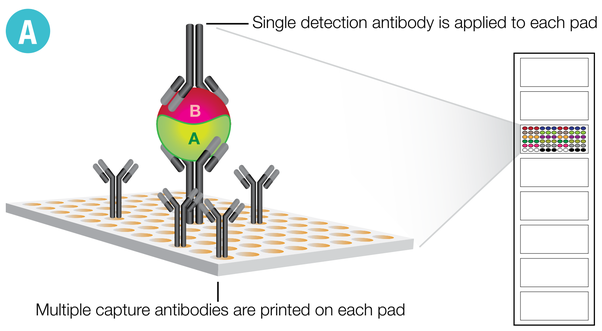
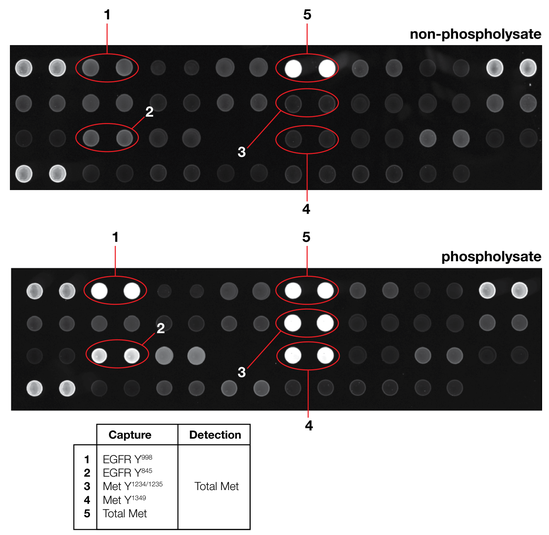


Figure 1. A) Schematic representation of the antibody array based assay set-up. Protein-protein interactions are detected by capturing target protein, **A**, with one specific antibody and detecting the interacting partner, **B**, with another specific antibody. In this assay configuration multiple capture antibodies are printed on each pad and a single detection antibody is applied per pad. This format is amenable to a highly parallel assay and both direct and indirect protein-protein interactions can be detected using this assay. **B**) A photo of an eight-pad nitrocellulose-coated glass slide on which the capture antibodies are printed.



	Capture	Detection
1	EGFR Y^{998}	Total Met
2	EGFR Y^{845}	
3	Met $Y^{1234/1235}$	
4	Met Y^{1349}	
5	Total Met	

Figure 2. Representative fluorescent slide images. HCC827 Non-Small Cell Lung Cancer cells, which express constitutively activated EGFR as well as overexpress c-Met, were used. The specificity of the relevant capture antibodies printed is indicated (1-5). The pad was probed with a single total c-Met detection antibody so the EGFR-c-Met interaction (1 and 2), c-Met phosphorylation (3 and 4), and total c-Met levels (5) were visualized all at once. Non-phospholysate and phospholysate indicate the use of lysis buffer that was either devoid of or contained phosphatase inhibitors respectively. Slides were scanned using an Odyssey® CLx array scanner (LiCOR®) at a wavelength of 700 nm (compatible with DyLight™ 680-conjugated streptavidin). Image studio 2.0 software was used to quantify the spot fluorescence intensity.

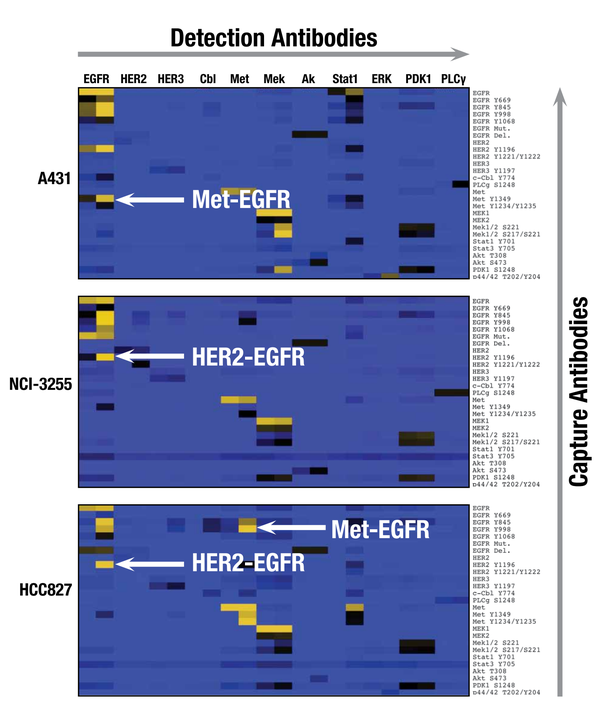


Figure 3. Heatmaps displaying the full data set including protein-protein interactions. Each capture antibody was screened against every detection antibody, and the entire set of capture and detection antibodies was tested using three different cell types. Raw fluorescence values were used after subtraction of background fluorescence, and the heatmaps were generated using Multi Experiment Viewer software. Low fluorescence signal is blue, intermediate signal is black, and a strong signal is yellow. This assay format (printing multiple capture antibodies on a planar surface and then probing with a single detection antibody) allows the following observations to be made simultaneously: 1, detection of a target protein through a traditional sandwich assay; 2, assessment of the cross-reactivity of a given detection antibody with multiple printed capture antibodies; 3, detection of protein-protein interactions (indicated with arrows above and see **figure 1**).

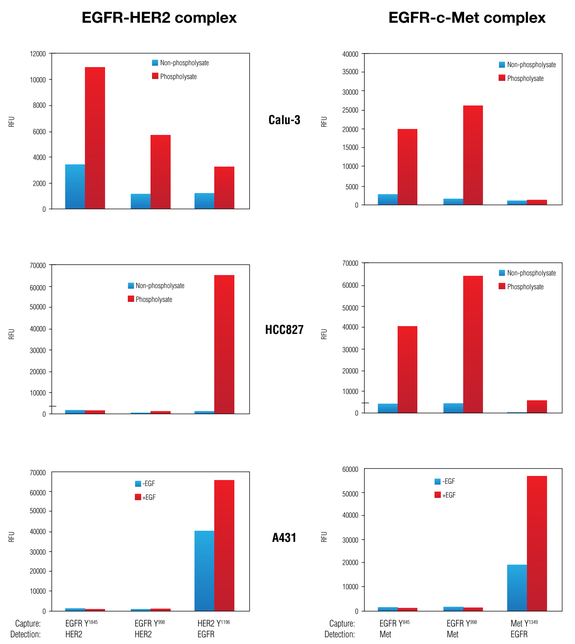


Figure 4. Detection of complex formation between EGFR, c-Met and HER2 in tumor-derived cell extracts. The interaction between the three RTKs in HCC827 and Calu-3 cells is constitutive and the cells were not treated prior to lysis. A431 cellswere serum starved overnight then treated with 100 ng/ml EGF for 5 min, where indicated, to induce RTK interaction. Receptor complexes were detected using the specified RTK antibodies and quantified using the array-based assay. Slides were scanned using an Odyssey® CLx array scanner (LiCOR®). Image studio 2.0 software was used to quantify the spot fluorescence intensity.

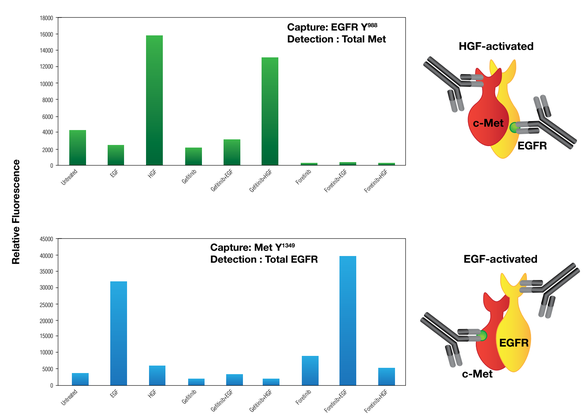


Figure 5. EGF and HGF induced complexes were detected in A431 cells: two conformations of EGFR-c-Met heterodimers were recognized using the antibody array-based assay. A431 cells were serum starved overnight and then subjected to the indicated treatments. Cells were treated with 100 ng/ml EGF or HGF for 5 min. Where indicated, cells were pretreated with 1 μM Gefitinib or Foretinib for 3.5 hr. Complexes between EGFR and c-Met were detected using two sets of antibody pairs: EGFR Y^{998} (capture antibody) and total c-Met (detection antibody, upper panel) and Met Y^{1349} (capture antibody) and EGFR (detection antibody, lower panel).

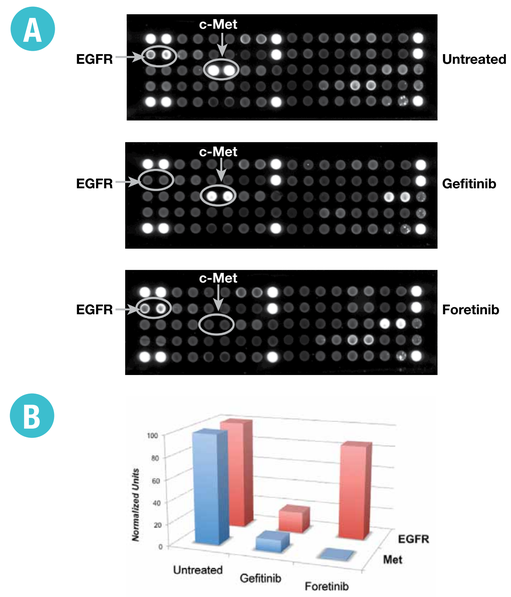


Figure 6. Inhibition of EGFR and c-Met tyrosine phosphorylation by Gefitinib and Foretinib. HCC827 cells were either untreated or treated with 1 μM Gefitinib or Foretinib for 16 hr in a complete medium. The relative levels of tyrosine phosphorylated EGFR and c-Met were assessed using the PathScan® RTK Signaling Antibody Array Kit (Fluorescent readout) #7949. **A**) Fluorescent images of the slides were acquired with an Odyssey® CLx array scanner. **B**) Bar graph showing relative levels of tyrosine phosphorylation associated with EGFR and c-Met expressed as normalized units.

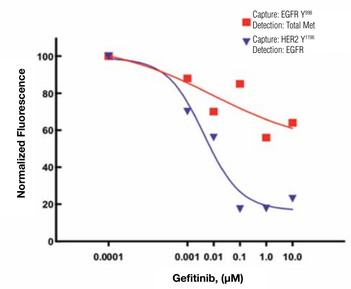


Figure 7. Disruption of EGFR-HER2 and EGFR-c-Met complexes by the small molecule EGFR inhibitor Gefitinib. HCC827 cells were treated for 2.5 hr with various concentrations of Gefitinib in serum-free medium. Capture-detection antibody pairs used to detect the complexes are indicated. Normalized fluorescence intensity was used to create the plot.

Summary

:: A planar surface antibody array was configured to detect and quantify RTK protein-protein interactions.

:: Two sets of antibody pairs were used to distinguish between two conformations of EGFR-c-Met heterodimers.

:: The antibody array-based sandwich immunoassay was used to monitor the disruption of receptor heterodimers by small molecule inhibitors.

Contact Information

Zev Gechtman
Cell Signaling Technology, Inc.
3 Trask Lane, Danvers, MA 01923
Email: zgechtman@cellsignal.com

Cell Signaling TECHNOLOGY®