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

Probing EGFR, HER2, and c-Met Protein-Protein Interactions Using an Antibody Array

Abstract

The Receptor Tyrosine Kinase (RTK) family includes important disease drivers and validated drug targets. Cooperation between RTKs is often achieved through physical interactions and is thought be a contributing factor for some cases of tumor resistance to targeted therapies. Epidermal Growth-Factor Receptor (EGFR), HER2/ErbB2, and c-Met are prominent examples of disease driver RTKs that interact to form hetero-dimers.

Current methods to detect RTK protein-protein interactions lack proper throughput, are not quantitative, or rely on highly engineered systems and cumbersome workflows. We have developed a planar surface antibody array-based sandwich assay that enables the detection and relative quantification of interactions between EGFR, HER2, and c-Met.

The antibody array-based assay revealed co-existing combinatorial receptor interactions that were unique to each cell type tested. It also alluded to the existence of distinct topological configurations of EGFR-c-Met and EGFR-HER2 hetero-dimers, unveiling previously undescribed nuances of these interactions. Lastly, the array-based assay allowed the disruption of receptor complexes using small-molecule inhibitors to be monitored and revealed differential sensitivity of EGFR-HER2, and EGFR-c-Met complexes to the small-molecule EGFR kinase inhibitor gefitinib.

The antibody array-based sandwich assay described here allows a large panel of affinity reagents to be used and enables parallel detection of RTK protein-protein interactions, allowing a more systematic monitoring of the response to activators or inhibitors.

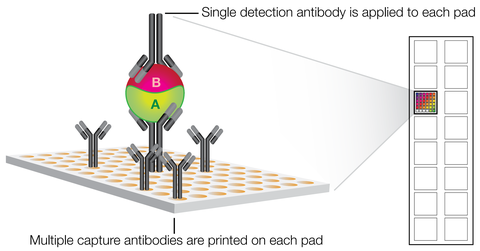
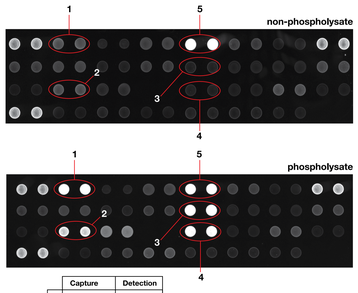


Figure 1. Schematic representation of the assay set-up. Protein-protein interactions are detected by capturing a target protein, **A**, with a specific antibody and detecting the interacting partner, **B**, with another specific antibody. In this assay configuration, multiple capture antibodies can be printed on each pad, and a single detection antibody is applied per pad. This format is amenable to multi data-point analysis. Both direct and indirect protein-protein interactions can be detected using this assay format.



	Capture	Detection
1	EGFR Y^{996}	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 cells, which express mutated and constitutively active EGFR and overexpress c-Met, were used. The specificity of the relevant capture antibodies printed on the pad is indicated (**1**-**5**). The pad was probed with a 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 at the same time. Non-phospholysate and phospholysate indicates the use of lysis buffer that was either devoid of or contains phosphatase inhibitors respectively. Slides were scanned using the Odyssey® CLx array scanner (LI-COR®) 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.

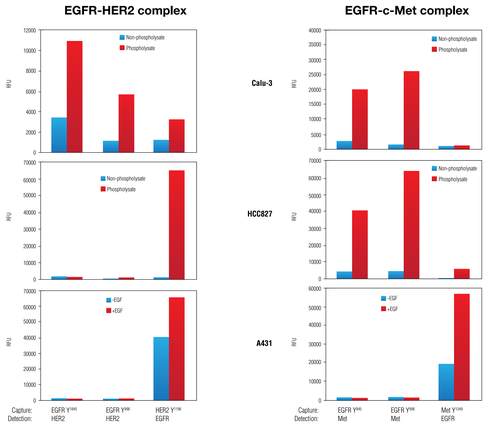


Figure 3. Detection of complex formation between EGFR, c-Met, and HER2 in tumor derived cell extracts using the antibody array-based assay. The interaction between the three RTKs in HCC827 and Calu-3 cells is constitutive and the cells were not treated prior to lysis. A431 cells were serum starved overnight then treated with 100 ng/ml EGF for 5 minutes, 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 the Odyssey® CLx array scanner (LI-COR®). Image studio 2.0 software was used to quantify the spot fluorescence intensity.

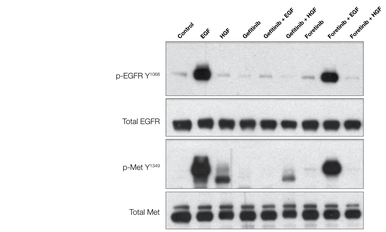


Figure 4. Western blot analysis to demonstrate activation of EGFR and c-Met by EGF and HGF in A431 cells. A431 cells were serum starved overnight then treated with various combinations of growth-factors and/or inhibitors as indicated. Gefitinib (EGFR inhibitor) and foretinib (c-Met inhibitor) were used at 1μM and cells were preincubated with inhibitors for 3.5 hours prior to growth-factor treatment. Cells were treated with 100 ng/ml EGF or HGF (where indicated) for 5 minutes. Western blot analysis shows EGFR and c-Met phosphorylation with the various growth factor/inhibitor combinations.

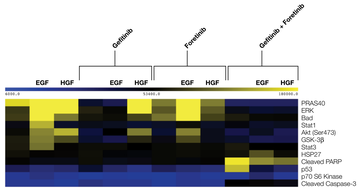


Figure 5. Downstream signaling nodes were activated by EGF and HGF, and this activation was inhibited by gefitinib and foretinib. The various cell treatments are indicated. The relative phosphorylation levels of some major signaling molecules as well as cleavage of pro-apoptotic proteins were assessed using the PathScan® Intracellular Signaling Antibody Array Kit (Fluorescent Readout, #7744). Raw fluorescence values, after subtraction of background fluorescence, were used for analysis and the heatmap was generated using Multi Experiment Viewer software.

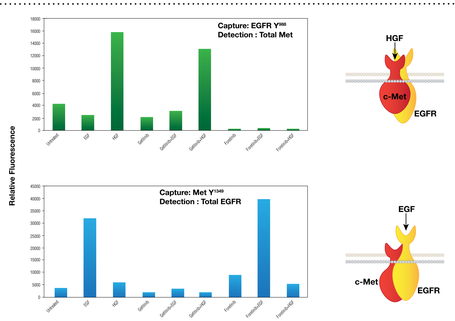


Figure 6. EGF and HGF induced complexes were detected in A431 cells: different conformations of EGFR-Met heterodimers were recognized using the antibody array-based assay. A431 cells were serum starved overnight then subjected to the various treatments indicated. Cells were treated with 100 ng/ml EGF or HGF for 5 minutes. Where indicated, cells were pretreated with 1 μM gefitinib or foretinib for 3.5 hours. Complexes between EGFR and c-Met were detected using two sets of antibody pairs. EGFR Y^{996} and total c-Met (upper), and Met Y^{1349} and total EGFR detection antibody (lower).

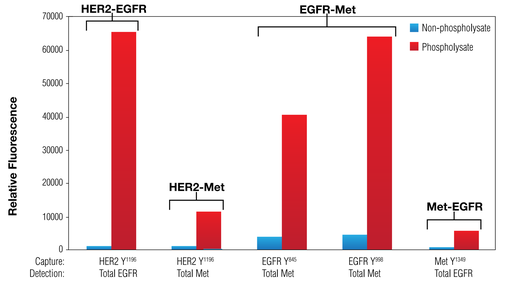


Figure 7. Various constitutive EGFR-HER2 and EGFR-c-Met complexes were detected in HCC827 cells. The specificity of the various capture antibodies is indicated below the graph. Antibodies to total EGFR and c-Met were used for detection. RTK complexes were detected using the array-based assay. Fluorescence intensity after background subtraction was used for the relative quantification.

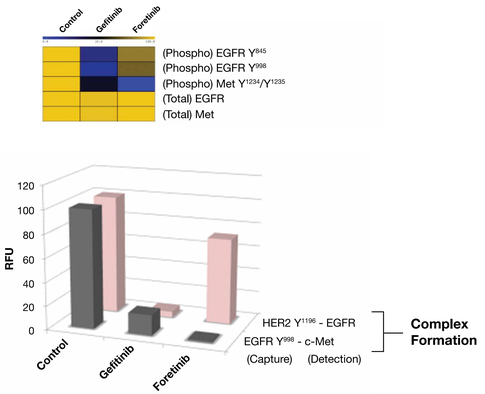


Figure 8. EGFR-c-Met dimerization was inhibited by gefitinib and foretinib. HCC827 cells were either untreated or treated with 1 μM gefitinib or foretinib for 16 hours in complete medium. The heatmap shows tyrosine phosphorylation of RTKs on indicated sites as well as levels of the total EGFR and c-Met protein. The bar graph shows the relative levels of EGFR-c-Met and EGFR-HER2 complexes under these conditions.

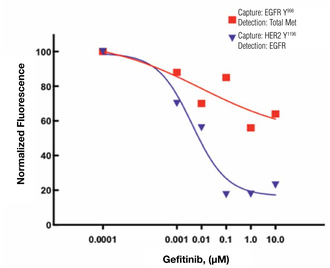


Figure 9. EGFR-HER2 and EGFR-c-Met complexes show differential sensitivity to gefitinib. HCC827 cells were treated for 2.5 hours with various concentrations of gefitinib in serum-free medium. Capture-detection antibody pairs used to detect the complexes are indicated in the graph. Normalized fluorescence intensity was used to create the plot.

Summary

- A planar surface antibody array was configured to detect and quantify Receptor Tyrosine Kinase (RTK) protein-protein interactions.
- Two sets of antibody pairs were identified that are able to distinguish between two combinations of EGFR-c-Met heterodimers, which differ in their joint conformation.
- The antibody array sandwich immunoassay allowed monitoring of the disruption of receptor heterodimers using small-molecule inhibitors.

Contact Information

Zev Gechtman

Cell Signaling Technology, Inc.
3 Trask Lane, Danvers, MA 01915

Email: zgechtman@cellsignal.com

Cell Signaling TECHNOLOGY®