Panorama™ Human Protein Function Microarrays: A New Approach to Cancer Research

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Abstract

The recent use of DNA microarrays for parallel, multiplexed analysis of steady-state gene expression has become a powerful method for examining differences between normal and malignant cells and tissues. More recently, researchers have sought to apply similar technology to the study of protein expression and function. However, protein complexity and the ability to express large numbers of functionally active proteins have limited the use and applications of protein-based microarrays.

Sigma-Aldrich has partnered with Procognia, Ltd. to introduce the first Human Protein Functional Microarrays. The crucial core technology utilizes a proprietary tagging system that ensures the arrayed proteins are correctly folded and displayed on the microarray surface. This new technology enables functional characterization of proteins in a parallel, miniature, and multiplexed manner.

In order to test this new technology, protein microarrays were made of p53 mutant proteins, human cancer-related proteins, and human kinase proteins. The resulting arrays were used in binding studies of DNA and protein probes, and for analysis of kinase activities. In addition, the effects of small molecule inhibitors were studied. We found that specific protein-DNA and protein-protein interactions could be readily detected. Moreover, we were able to determine effects of a small molecule kinase inhibitor on over 100 human kinases simultaneously in one experiment. These protein function arrays should open new possibilities in the study of cancer biochemistry. Critical factors of functional protein immobilization and specific applications for these microarrays will be discussed.

Introduction

Goal

To test the utility of novel human protein function microarrays for applications in cancer research.

Approach

- Demonstrate functionality with p53 protein arrays.
- Test known and novel protein interactions on human cancer-related protein arrays.
- Perform multiplexed kinase assays using human protein kinase arrays.
- Test human kinase arrays for kinase inhibitor characterization.

Panorama Protein Function Arrays

Challenge	Solution
Clone libraries	Verified, full-length clones: NIH-MGC and rare clones
Non-denaturing surface	Glass slide, non-charged, hydrophilic coating, streptavidin linker
Stable immobilization	Streptavidin bound to biotinylated BCCP tag (K_D=10^{-15} M)
Functional protein conformation	BCCP tag biotinlyated during expression only when properly folded
Uniform orientation	BCCP tag at C-terminus of fusion proteins
Sterically available	BCCP tag provides a 50 Å spacer from slide
Normalization	c-Myc tag C-terminal to BCCP tag
Diverse assays	Biomolecule interactions, activity analysis, small molecule/drug effects

Fusion Protein Constructs

- Insect cell expression (polh promoter)
- Recombination sites for inserting protein coding (ORF) sequences
- BCCP tag at C-terminus of ORF
- c-Myc tag at C-terminus of fusion protein

Figure 1: Schematic representation of BCCP-tagged fusion protein constructs for expression in insect cells.

The BCCP Tag

- C-terminal BCCP tag serves as a reporter of protein conformation
- Derived from biotin carboxyl carrier protein (amino acids 74-156 encoded by AccB gene of *E. coli*)
- Biotinylated *in vivo* by biotin ligase (BirA) only when properly folded
- Fusion proteins immobilized on array only if BCCP tag biotinylated
- Consistent protein orientation away from slide surface, projecting into aqueous environment

Spotted Fusion Protein Architecture

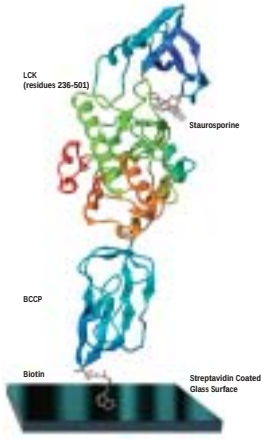

Figure 2: Schematic representation of a BCCP-tagged fusion protein immobilized on the streptavidin-coated slide surface via the biotinylated BCCP tag.

Protein Conformation

Wild-type p53 protein was expressed as a fusion protein with the C-terminal BCCP tag and was immobilized on streptavidin-coated slides.

Conformation of the p53 core domain was tested with a conformation-specific anti-p53 antibody (Pab1620) versus a conformation-independent antibody (anti-tag).

Pab1620 recognized the wild-type fusion protein on the slide, indicating proper folding of the core domain **(Figure 3)**.

Progressive heating of the slides caused loss of reactivity with this antibody, but not with a conformation independent antibody, anti-tag **(Figure 3)**.

Evidence for Correct Protein Folding

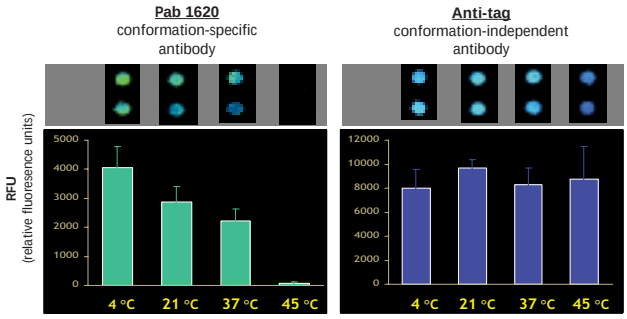

Figure 3: An antibody specific to the properly folded core domain of p53 recognizes properly folded p53-BCCP fusion protein printed on a streptavidin-coated slide. Prototype p53 arrays were preheated at different temperatures and probed with an antibody (Pab1620) that reacts only with the properly folded core domain of p53 (left panel) or with an antibody (anti-tag) that is not p53 structure dependent (right panel).

Cancer Array

- 128 human proteins involved in cancer (kinases, DNA binding, transcription, Reference 1)
- Genetic mutations implicated in cancer development
- Encode crucial regulatory proteins (cell proliferation, differentiation, cell death, DNA-repair)
- 71 tumor types (including epithelial, mesenchymal and lymphoma tissue types)

Binding Assays

Calmodulin interacts with specific proteins, including calmodulin-dependent kinases, in a calcium-dependent manner.

The specific interaction of calmodulin in the presence of calcium with calmodulin-dependent kinase IV (CAMK4) was detected on a human cancer protein microarray **(Figure 4)**.

Cancer Arrays Binding Assays

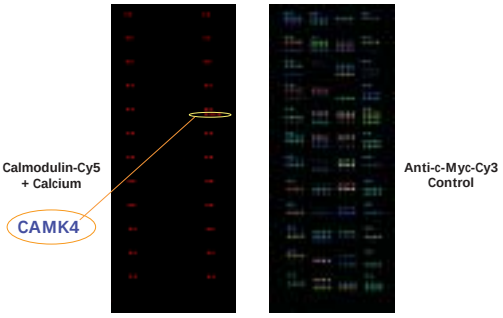

Figure 4: Calmodulin, in the presence of calcium, specifically binds to only one protein, calmodulin-dependent kinase IV (CAMK4), on the Panorama Human Protein Function Microarray, Cancer v1. Cy5-labeled calmodulin (30 nM) was incubated with a cancer microarray. After washing and drying, the slide was scanned for Cy5. Only pairs of labeled control spots and the set of four CAMK4 spots (yellow oval) were seen, showing that only the expected, specific protein-protein interaction was detected (left slide). All spotted proteins could be visualized with an anti-c-Myc-Cy3 antibody (right slide).

Binding Curves

The human cancer protein microarrays were used to study the binding kinetics of the interaction between calmodulin and CAMK4 **(Figure 5)**.

Calmodulin Binding Kinetics on Cancer Array

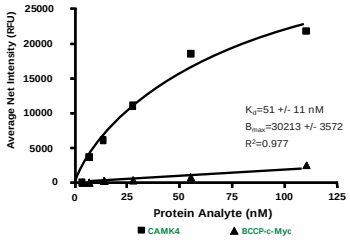

Figure 5: A series of cancer array slides were incubated with different concentrations of Cy5-labeled calmodulin. The results display saturation kinetics of calmodulin binding to CAMK4. The K_d and B_{max} were similar to those previously reported.

Discovering Interactions

The human cancer protein arrays were employed for discovery of new protein-protein interactions.

Human importin 2α binds to nuclear localization sequences of many regulatory proteins which must enter the nucleus.

Importin 2α was found to interact with a number of proteins on the human cancer protein arrays **(Figure 6)**.

Importin 2α Interactions on Cancer Array

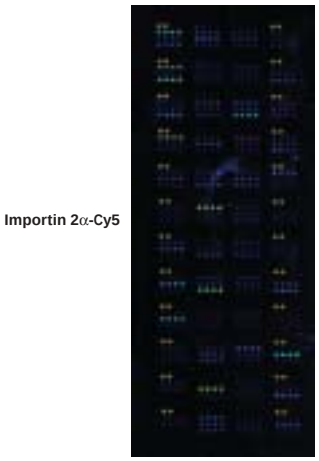

Figure 6: Importin 2α binds proteins on the Panorama Human Protein Function Microarray, Cancer v1. Cy5-labeled importin 2α was incubated with a human cancer protein microarray slide. After washing and drying, the slide was scanned for Cy5. The importin protein appeared to bind specifically to a number of spotted cancer array proteins (bright quadruplicate sets of spots).

Human Kinase Array

- 152 human kinase proteins
- Detection of phosphorylation by radioactivity (e.g., [γ^{33}P]-dATP)
- Detection of phosphorylation by phosphorylation site-specific antibodies (e.g., anti-phosphotyrosine-Cy5 conjugate)

Kinase Inhibitor Specificity Assay

An autophosphorylation assay was performed on the array in the absence and presence of a broad-spectrum kinase inhibitor, staurosporine.

Anti-phosphotyrosine-Cy5 antibody conjugate was used to detect tyrosine-phosphorylated kinases on the array.

Autophosphorylation of tyrosine residues was observed for a number of the spotted kinases in the absence of staurosporine **(Figure 7,** left panel**)**.

The autophosphorlyation of many of these kinases was inhibited in the presence of staurosporine **(Figure 7,** right panel**)**.

Autophosphorylation and Inhibition by Staurosporine

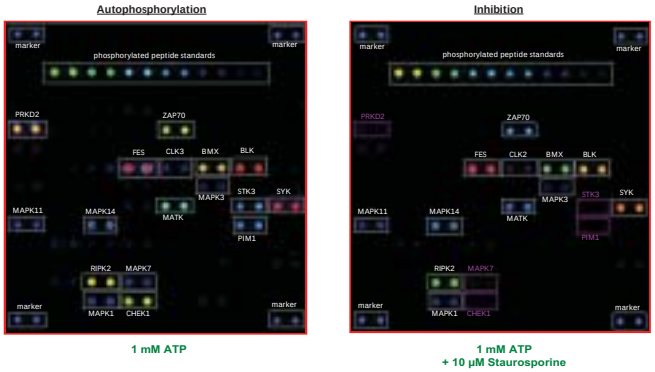

Figure 7: Autophosphorylation of many spotted human tyrosine kinases is inhibited by the broad-spectrum kinase inhibitor, staurosporine. Prototype human kinase arrays were incubated for 30 min at 30 °C with 1 mM ATP in the absence (left panel) or presence (right panel) of 10 µM staurosoprine. The phosphorylation state of the kinases spotted on the arrays was detected with an anti-phosphotyrosine-Cy5 antibody conjugate.

Autophosphorylation Inhibition Curves

Applying a series of concentrations of an inhibitor to kinase arrays allows the multiplexed, parallel determination of inhibition constants (IC_{50}) for multiple kinases in the same experiment.

Examples of staurosporine inhibition curves for four of the human kinases spotted on the arrays are depicted in **Figure 8**.

Multiplexed Inhibition Analysis

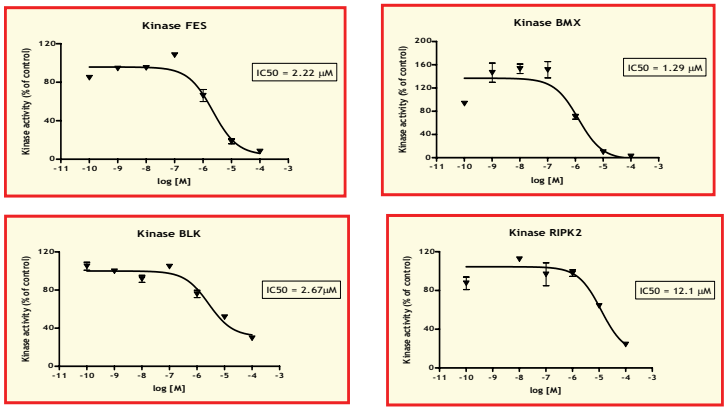

Figure 8: The IC50 concentrations for inhibition of multiple human kinases can be determined simultaneously in one experiment. A series of human kinase arrays was incubated with a dilution series of staurosporine. After scanning the array slides, inhibition concentration curves were generated for all the kinases spotted on the arrays. Examples of the inhibition curves for four representative kinases are shown above.

Discussion

1. The unique BCCP tagging method allows expressed fusion proteins with functional conformations to be printed onto streptavidin-coated slides.
2. Binding of the fusion proteins via biotin-streptavidin interaction ensures stable immobilization in an oriented, available manner.
3. Here we demonstrate functionality of these novel protein microarrays for the detection of protein-protein interactions, kinase phosphorylation reactions, and kinase inhibitor specificity assays.

Conclusion

The Panorama Human Protein Function Microarrays provide a unique platform for cancer research by providing the ability to do miniature, parallel, multiplexed assays for protein interactions with biomolecules, for kinase activities, and for small molecule inhibitor studies.

The ability to perform parallel, multiplexed kinase assays for testing specificity of kinase inhibitors provides a powerful tool for cancer drug discovery and development.

This functional protein array technology provides a powerful tool that should accelerate cancer research by providing a rapid, multiplexed assay platform for the study of human proteins related to cancer.

References

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Materials

Product Name	Cat. No.
Panorama™ Human Protein Function Microarray, Cancer v1	HPFM2
Panorama™ Human Protein Function Microarray, Kinase v1	HPFM3
Calmodulin	P2277
Importin 2α	I9656
Staurosporine	S4400
ATP	A6559