Celltransmissions

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SIGMA RBI

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In this Issue...

Functions and Pharmacological Inhibitors of Cyclic Nucleotide Phosphodiesterases

Valeria Vasta and Joe Beavo

The discovery of the cyclic nucleotides **3',5'-cyclic monophosphate** (cAMP, Prod. No. **A 9501**, **A 4137**) and **guanosine 3',5'-cyclic monophosphate** (cGMP, Prod. No. **G 7504**, **G 6129**) led to the first formulation of the second messenger concept. These cyclic nucleotides are now known to be ubiquitous intracellular second messengers that mediate the response of cells to a variety of extracellular stimuli through the activation of cyclic nucleotide-dependent protein kinases, ion channels, GTP-exchange factors and their downstream effector systems [1]. The amplitude and duration of cAMP and cGMP signals are controlled by their rates of synthesis by adenylyl and guanylyl cyclases, respectively, and their degradation by 3',5'-cyclic nucleotide phosphodiesterases (PDEs) (Figure 1). PDEs, identified shortly after the discovery of cAMP and cGMP, are

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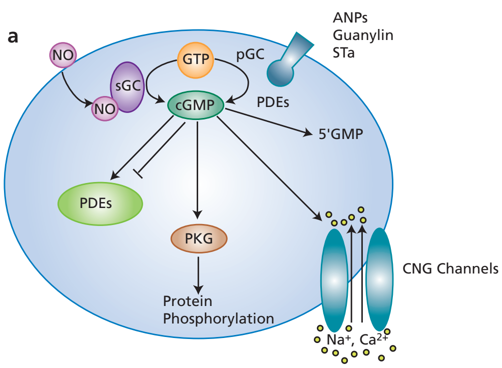


a large superfamily of enzymes that hydrolyze the 3' phosphodiester bond in cAMP or cGMP to form the corresponding 5'-nucleotide

continued on page 3

Application Note:

The New Panorama™ Antibody (Ab) Microarray Cell Signaling Kit: A Unique Tool for Protein Expression Analysis

Eliezer Kopf, Dalia Shnitzer and Dorit Zharhary

Introduction

There is a growing need for technologies that allow global molecular characterization of biological samples. The ability to identify multiple proteins simultaneously has many applications in basic biological research as well as in disease diagnosis and treatment. The use of DNA arrays for profiling mRNA expression in cells has provided valuable information in many biological areas. However, since there is not always a direct correlation between the mRNA level and the expression of the protein, a method that can directly assay proteins is required. Whereas DNA/RNA/oligo arrays give information on the genetic defects that may cause disease, protein microarrays provide

continued on page 20

sigma-aldrich.com/cellsignaling

New Products pp. 12-14

Anti-mTOR: marker for translation initiation activation
p. 9

XK469: selective topoisomerase II-β inhibitor
p. 10

Y-27632: selective inhibitor of Rho associated protein kinase p160ROCK
p. 11

SB-431542: potent, selective inhibitor of activin receptor-like kinase (ALK) receptors
p. 15

SCH-28080: potent inhibitor of gastric H^+/K^+-ATPase
p. 15

Peptidomimetic isoform selective nNOS inhibitors
p. 16

L-685,458: potent, selective γ-secretase inhibitor
p. 17

Anti-ILK: marker for integrin-mediated processes
p. 18

SB-228357: potent, selective $5\text{-}HT_{2C/2B}$ serotonin receptor antagonist
p. 18

Anti-Survivin: marker for blocking apoptosis
p. 19

Compound Libraries
pp. 22-23

SIGMA-ALDRICH

Panorama™ Antibody (Ab) Microarray Cell Signaling Kit: A Unique Tool for Protein Expression Analysis

Eliezer Kopf, Dalia Shnitzer and Dorit Zharhary *(continued from cover)*

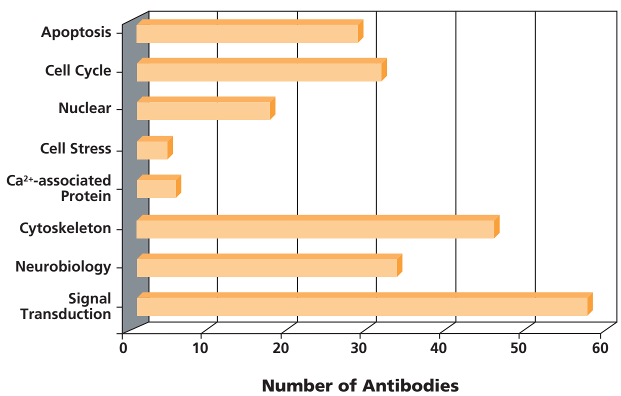
information about the corresponding functional state. Antibody arrays provide such a solution and can be used to profile expression of proteins in cell and tissue samples.

Antibody Content

Sigma's new **Panorama™ Ab Microarray Cell Signaling Kit** (Prod. No. **CSAA1**) is the first in a line of antibody-based microarrays. The array contains over 224 antibodies that have been carefully selected from the range of over 3,000 antibodies currently available from Sigma-Aldrich. Please refer to the Antibody Explorer at www.sigma-aldrich.com/antibody or www.sigma-aldrich.com/antibodyarray for further information about our antibodies and related products. Each spotted antibody in the array has been validated for its ability to bind proteins in the array assay using samples from human, mouse and rat. Some antibodies are specific for the phosphorylated protein of interest (FAK, MAPK, Raf, p38, Pyk2, PAK1, and DAPK).

The antibodies target proteins that are expressed in a variety of biological pathways such as apoptosis, cell cycle, nuclear proteins, neurobiology and phosphorylation (see distribution in Figure 1). In addition, cytoskeleton antibodies to actins, myosins, and tubulins are included for normalization purposes, as the expression of these housekeeping proteins does not change with different treatments of sample and control.

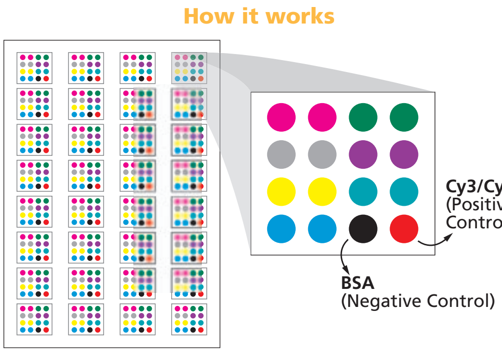
Figure 1. Biological Areas of Antibody Specificities.



Microarray Format

The antibodies are spotted onto nitrocellulose-coated glass slides. The three-dimensional (3D) structure of the nitrocellulose layer maintains a high protein-binding capacity, dynamic range and reproducibility. The slides contain 224 antibodies printed in duplicate onto a 32 grid array. Each grid contains 7 antibody duplicates plus a Cy3 and Cy5-conjugated BSA positive control and a non-labelled BSA negative control, resulting in 512 spots per slide (Figure 2).

Figure 2. Spotting Format.



Labeling and Detection Chemistry

Using the robust method of direct sample labeling with fluorophores, multiple proteins can be simultaneously measured in complex mixtures without the potential cross-specificity of antibody pairs that occurs in classic sandwich assays. Proteins from test and control samples are labeled with the Cy3™ or Cy5™ monofunctional reactive dyes, respectively. The non-conjugated free dye is removed from the labeled sample by applying the sample to **SigmaSpin™ Post-Reaction Clean-Up Columns** (Prod. No. **S 5059**). Both the test labeled and control-labeled protein samples are mixed and incubated on the microarray. After specific proteins have bound to antibodies on the array, the slides are read for differential analysis using a standard microarray scanning device. The total assay takes 5 to 6 hours.

For protocol details, please refer to the technical bulletin at www.sigma-aldrich.com/antibodyarray.

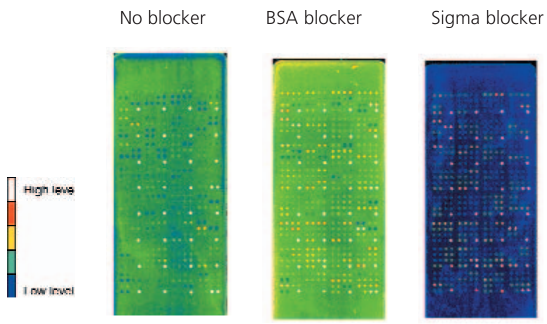
Panorama™ Antibody (Ab) Microarray

Panorama ™ Antibody (Ab) Microarray Cell Signaling Kit...*(continued)*

Background Reduction

Obtaining high signal to noise ratio is an important requirement for meaningful analysis. In the antibody array assay, two factors can contribute to the background signal: the nitrocellulose, which is known to have some fluorescent background, and the non-specific binding of proteins in the labelled sample. We have developed a proprietary blocking buffer that reduces most of the non-specific background in the assay (Figure 3) and extends the shelf life of the kit to 12 months. This background reduction enables the direct fluorescent labeling method to be used to its full capabilities.

Figure 3. Cy3 labelled NIH-3T3 cell extracts were applied to slides treated with different blocking solutions after spotting.

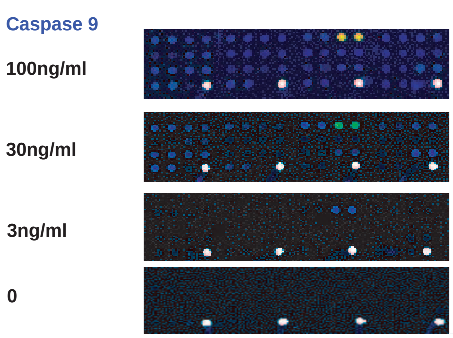


Specificity, Sensitivity and Reproducibility

To determine specificity and sensitivity, purified caspase 9 was labelled and incubated on the microarray at different concentrations from 100 ng/ml down to 3 ng/ml (Figure 4). Specific binding of caspase 9 to its spotted antibody was observed. The sensitivity was at least 3 ng/ml. It is important to note that for other antibodies, sensitivity levels will depend on the binding and dissociation constants of the antibody-antigen complexes. Therefore, a lower or higher sensitivity may be observed for other antigens.

The variance of signal detected from duplicate spots within the same slide is less than 8%, whereas the variance of those signals detected from spots between slides is approximately 10% (data not shown).

Figure 4. Cy3 labelled recombinant caspase 9 was applied on array slides at different concentrations. The position of anti-Caspase 9 antibody on the array is circled.

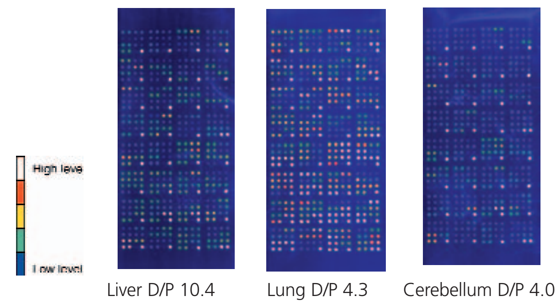


Protein Expression Profiles obtained using the Panorama Ab Microarray

Differential expression of proteins in tissues.

The Panorama Ab Microarray Cell Signaling Kit was used to profile the expression of proteins in several mouse tissues. Proteins were extracted from mouse liver, lung and cerebellum, labelled with Cy3, and applied to the array. As expected, each tissue showed a different pattern of protein expression (Figure 5).

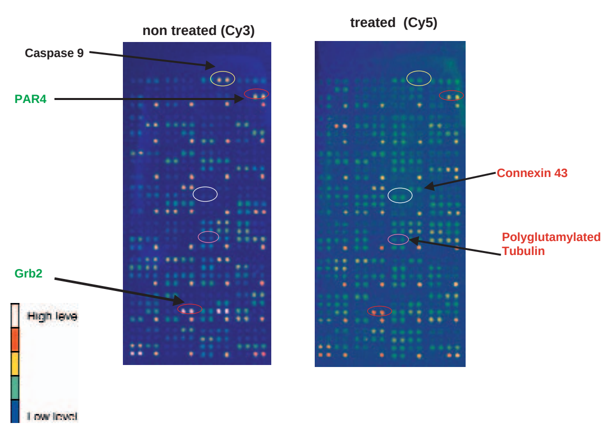
Figure 5. Differential Protein Expression in Mouse Tissues



Protein expression profiling of the differentiation of mouse F9 stem cells by stimulation with all-trans-retinoic acid.

The Panorama Ab Microarray-Cell Signaling Kit was used to monitor the changes in the protein expression pattern of mouse F9 stem cells induced by all-trans-retinoic acid. F9 cells were treated for 96 hours with 100 nM **all-trans-retinoic acid** (Prod. No. **R 2625**). Extracts were prepared from untreated and treated cells using the extraction/labeling buffer provided in the kit, and labelled with Cy3 and Cy5, respectively. A mixture containing equal amounts of each labelled extract (5 µg/ml) was incubated on the array as described in the kit protocol. The results obtained are illustrated in Figure 6, which shows the same slide at the two fluorescence emission wavelengths for Cy3 and Cy5. (Note that the blue background for Cy5 represents the normal and unavoidable interference of the nitro-

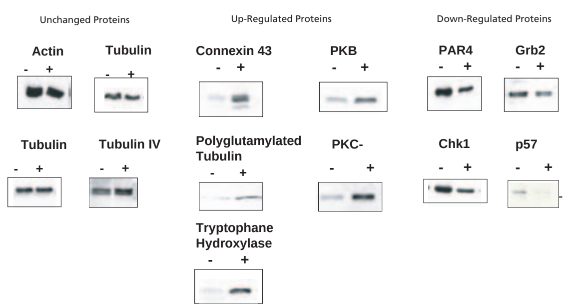
Figure 6. Expression of Proteins in F9 mouse stem cells induced to differentiate by Retinoic acid.



Panorama™ Antibody (Ab) Microarray

cellulose membrane). Proteins that showed changes in protein expression were further analyzed by immunoblotting to confirm the results. These results are summarized in Figure 7. Equal amounts of protein extract (20 µg per lane) from treated and untreated cells were separated by SDS-PAGE and blotted onto nitrocellulose membranes. The proteins were probed with the monoclonal or polyclonal antibodies corresponding to the array and visualized using chemiluminescence.

Figure 7



Summary

We have described a novel antibody array system called Panorama™ Ab Microarray Cell Signaling kit that contains 224 antibodies spotted on FAST™ nitrocellulose-coated slides that can detect protein levels as low as a few nanograms per ml. The antibodies are specific for proteins important in various areas of cell signaling such as phosphorylation, cell cycle, apoptosis, nuclear signaling and cytoskeleton proteins. Furthermore, the Panorama ™ Ab Microarray can detect phosphorylated and non phosphorylated forms of selected target proteins. We found that treatment of the slides post spotting is important for array performance (ratio of signal to background) and stability. The Panorama™ Ab Microarray was used to analyze changes in protein expression in F9 embryonic carcinoma (EC) cells induced by all-trans retinoic acid. We found that the level of several proteins, among them cell cycle regulators and kinases, was either up or down regulated. For more than ten protein targets, the results obtained by the Panorama™ Ab Microarray were confirmed by immunoblotting.

About the Authors

Dalia Shnitzer received her M.Sc. in Immunology from BarIlan University, Ramat-Gan, Israel, where she studied under the supervision of Dr. Abraham Klein. Dalia joined Sigma-Aldrich Israel in 1983 as a Research Scientist in the R&D Cell Biology department where she has been responsible for the development of monoclonal antibodies.

Eliezer Kopf received his Ph.D. in Biochemistry from the Weizmann Institute of Science, Rehovot, Israel following which he undertook his post-doctoral studies at University Pasteur in Strasbourg, France with Prof. Pierre Chambon. Dr. Kopf joined Sigma-Aldrich Israel in 2000 as a Research Scientist in the R&D Cell Biology department and since 2001 has served as the Head of the department.

Dorit Zharhary received her Ph.D. in Immunology from the Technion – Israel Institute of Technology, Haifa, Israel. After post-doctoral studies at Scripps Clinic, LaJolla, California with Dr. Norman Klinman, she joined the Weizmann Institute of Science in Rehovot, Israel as a faculty member. Dr. Zharhary joined Sigma-Aldrich Israel in 1992 where she now manages all Israel R&D.