Immunostaining with dissociable antibody microarrays

The availability of a large number of biological materials such as cDNA, antibodies, recombinant proteins, and tissues has promoted the development of microarray technologies that make use of these materials in high-throughput screening assays. However, because microarray technologies have been less successful in examining proteins than DNA and mRNA, there is a need for improved protein microarray systems. To address this need, we developed an antibody microarray-based immunostaining method that can analyze the properties of a large number of proteins simultaneously.

In this method, antibodies are arrayed and immobilized on a solid support and cells bearing antigens of interest are attached to a second support. Apposition of the two supports allows the antibodies to dissociate from the array support and bind to the cellular antigens. After separation of the supports, antigen-bound antibodies can be detected by standard secondary antibody techniques. These “dissociable” antibody arrays were used to detect both the expression and subcellular localization of a large number of specific proteins in various cultured cell types.

**Keywords:** Dissociable antibody microarrays / Immunostaining / Protein expression / Protein microarrays / Protein subcellular localization

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**1 Introduction**

A large number of proteins from human and other organisms have been identified, largely through recent DNA sequencing projects. The human genome was found to encode about 30,000 proteins [1, 2]. In a given cell type, only a subpopulation of the proteome is expressed and a specific protein may be present in multiple forms due to post-translational modifications. Because proteins are such important components of cells, a major task of biological research in the post-genome era is to reveal the protein expression profiles and the functions of those expressed proteins. Assays based on protein microarrays have been used previously to examine multiple proteins. For example, Chang [3] employed antibody microarray-based assays to capture antigen-bearing cells to the areas of a support where antibodies against each of the specific antigens were immobilized. In the last few years, partly encouraged by the success of DNA microarrays [4], various protein microarrays and related technologies were developed for the global studies of proteins [5–10].

They have been used in studying protein expressions, protein post-translational modifications, protein-protein interactions, and enzymatic activities [11–16], all of which are critical in determining the functions of proteins.

Immunostaining is a versatile method for determining both the expression and cellular localization of proteins [17]. Typically, however, cells are stained with only one or at most a few antibodies at a time. This limitation prevents the use of immunostaining in many applications that require the detection of a large number of proteins. To take advantage of the numerous antibodies available and the power of protein microarrays, we have developed a novel method to detect and localize a large number of cellular antigens in a single experiment.

**2 Materials and methods**

**2.1 Antibodies and cell cultures**

Primary antibodies were from several sources (Labvision, Santa Cruz Biotechnology, Transduction Laboratory, and Upstate Biotechnology), and secondary antibodies were from Pierce and Jackson Immunoresearch.

Madin-Darby canine kidney (MDCK), A431, and ME180 cells were obtained from American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM supple-
ментed with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were maintained under standard cell culture conditions at 37°C and 5% CO₂ in a humid environment. Cells were seeded either in culture dishes or on cover glasses and cultured for 2 days until fresh confluence. Then the cells were fixed and permeabilized in methanol/acetone (1:1) for 15 min at -20°C followed by rinsing with phosphate-buffered saline (PBS).

2.2 Production of dissociable antibody microarrays

Antibodies in their original solutions (0.5 µg/µL) were arrayed on Nylon membranes (Amersham Biosciences, Piscataway, NJ, USA) by a custom-made robotic arrayer. For arrays used in fluorescent staining, antibodies (about 5 ng/10 nL) were deposited at each spot using a 0.3 mm solid pin, spaced 300 µm apart. For arrays to be used with an alkaline phosphatase-mediated color reaction, antibodies were spotted at higher volume (about 40 ng/80 nL) with 0.6 mm solid pins, 600 µm apart. Antibody arrays were either used immediately or stored at 4°C and used within 48 h.

2.3 Antibody microarray staining

Dissociable antibody microarrays and fixed cells were first blocked in PBS solution containing 5% BSA for 1 h. Then the arrays were placed on top of the cells with the antibodies facing the cells. Close contact between antibodies and cells was maintained by a pressure generated by a weight of 250 g placed on top of the array. After 1–2 h incubation, the arrays were removed from the coverslips or culture dishes on which cells are seeded. Then the cells were rinsed with PBS and either alkaline phosphatase- or fluorescent-labeled secondary antibodies were added to the cells for 1 h. After washing, the staining was either visualized by color reaction with substrates for alkaline phosphatase (3-bromo-4-chloro-5-indolyl phosphate/nitroblue tetrazolium; BCIP/NBT) or the fluorescent label was directly observed by fluorescence microscopy. For comparing protein expressions, all samples were processed in parallel and the enzymatic reactions were stopped by washing off substrates for alkaline phosphatase or fluorescent-labeled secondary antibodies and cells was maintained by a pressure generated by a weight of 250 g placed on top of the array. After 1–2 h incubation, the arrays were removed from the coverslips or culture dishes on which cells are seeded. Then the cells were rinsed with PBS and either alkaline phosphatase- or fluorescent-labeled secondary antibodies were added to the cells for 1 h. After washing, the staining was either visualized by color reaction with substrates for alkaline phosphatase (3-bromo-4-chloro-5-indolyl phosphate/nitroblue tetrazolium; BCIP/NBT) or the fluorescent label was directly observed by fluorescence microscopy. For comparing protein expressions, all samples were processed in parallel and the enzymatic reactions were stopped by washing off substrates with PBS.

2.4 Fluorescence microscopy

To obtain images at subcellular resolution, fluorescent staining was observed under an inverted microscope (Olympus IX-71) and photographed with a digital camera controlled by a PC running the Metamorph image acquisition and analysis software package (Universal Imaging). All images were pseudocolored with Photoshop (Adobe System).

2.5 Data acquisition and analysis

To analyze staining results, the images of array staining visualized via color reaction were scanned on a standard flatbed scanner and the digitized images were analyzed by custom-written software. The numerical intensity of each spot in array staining was defined as the total pixel intensity within a defined area. For comparing spots between two array images, the two images were first normalized by equalizing the intensities of the corresponding reference spots whose expression was identical under the two conditions. For experiments described in Fig. 3a, Ets-1 staining at 1A, 1T, 12A, and 12T, was used as reference. Then the numerical intensities of the corresponding spots with the same diameters were calculated. The ratio of the numerical intensities of two spots was calculated. If the ratio is larger than 10, a value of 10 was arbitrarily used in plotting. The analysis results were displayed in a spreadsheet format and the graph was generated in Excel (Microsoft). Similar procedures were also used to analyze Western blotting data.

3 Results

The key to the method is the production of “dissociable” antibody arrays that can deliver multiple antibodies to antigens on cells in a position-addressable manner. In the current example, dissociable antibody arrays were produced by arraying and immobilizing antibodies on Nylon membranes. Nonspecific interaction between antibodies and the Nylon membrane immobilizes the antibodies to their spotted positions but allows them to dissociate from the membrane and bind to antigens on cells that are immobilized on another support.

The diagram in Fig. 1a shows the basic steps of the method. Adherent cells are first seeded on a cell support, which can be the bottom of a tissue culture dish or a glass coverslip in a tissue culture dish. After growth to desired states, the adherent cells are fixed and permeabilized with standard methods. A dissociable array of antibodies is then placed on top of the cell support with the antibodies facing the cells and incubated for 1 h. Pressure is applied to maintain close contact between antibody arrays and cells. During the incubation, antibodies dissociate from the array support and bind to their respective antigens in the cells without significant lateral diffusion. Afterwards, the array support is removed, leaving the bound antibodies attached to the cellular antigens in situ.
Figure 1. Immunostaining of adherent cells with antibody arrays. (a) Basic steps in antibody array staining method: 1, prepare staining antibody array and adherent cells; 2, make contact between the array and cells; 3, remove array support; 4, detect bound antibodies. Array support, immobilized antibodies, fixed cells, and cell support are labeled. Cell supports are either tissue culture dishes or glass coverslips; array supports are nylon membranes. (b) Staining of MDCK cells with an array of 200 antibodies. Cells were seeded in a tissue culture dish and fixed and permeabilized before being stained. Center-to-center distance of the array is 600 μm. The staining was observed via alkaline phosphatase-mediated color reaction with BCIP/NBT as substrates.

Enzyme-labeled or fluorophore-conjugated secondary antibodies are then used to detect and localize the bound primary antibodies on the cells. In this way, both the presence and subcellular localization of multiple antigens can be determined.

To illustrate the antibody array staining method, a dissociable antibody array was produced by immobilizing 200 different rabbit polyclonal antibodies on a Nylon membrane. About 40 ng antibodies were immobilized at each spot on the membrane, with a distance of 600 μm between spots. The array was used to stain MDCK cells. MDCK cells were seeded on a tissue dish and, after growth to fresh confluence, they were fixed, permeabilized, washed, blocked, and exposed to the array as described above. After incubation and separation, alkaline phosphatase-conjugated goat-anti-rabbit secondary antibodies were used to visualize the bound primary antibodies on the MDCK cells. Phosphatase-mediated reaction produced a brown color signal in a regular pattern that matched the pattern in which the antibodies were arrayed (Fig. 1b). There was little diffusion of the antibodies in the procedure, as judged by the lack of staining between spots. As in other immunoelectrochemical methods, the signal intensity at each position reflects both the expression level of the antigen and the binding properties of the antibody.

To confirm that the antibodies did bind to their respective antigens and the signals are due to specific antibody binding, array staining of MDCK cells was performed with antibody arrays, in which 5 ng of antibodies were immobilized at each spot, 300 μm apart. After apposition and separation, the cell cultures were stained with Cy2-labeled secondary antibodies and examined by fluorescence microscopy. At low magnifications, each stained spot was about 300 μm in diameter and consisted of a cluster of 400 cells (Fig. 2a, c, e, and g). There were clear boundaries between the stained areas and nonstained areas indicating low diffusion of antibodies. At high magnifications, the subcellular localization of the antigens was apparent. For example, the transcriptional factor IRF1 was expressed in the nucleus (Fig. 2b), signaling molecule 14–3-3 β and transcriptional factor Ets-1 were in the cytoplasm (Fig. 2d and 2h), and cell adhesion protein β-catenin was evident at cell-cell contacts (Fig. 2f). The staining patterns were indistinguishable from those obtained with standard immunostaining procedures (data not shown), indicating that dissociable antibody array technology can reliably detect cellular antigens in situ.

Simultaneous staining of two proteins (double staining) is a unique tool for studying two functionally related proteins. For example, evidence of protein interactions often includes the demonstration that the proteins colocalize in the same subcellular structure. Array staining is unique in that it allows the examination of multiple proteins individually as well as simultaneously in the same cell preparation. To illustrate this, we stained human epidermoid carcinoma A431 cells with an antibody array, in which rabbit anti YY1, mouse anti-p130cas, and a mixture of the two antibodies were immobilized at neighboring positions. Cy2-labeled goat anti-rabbit secondary antibodies (green fluorescence) and Cy3-labeled goat anti-mouse secondary antibodies (red fluorescence) were used to visualize the antigens. The nuclear localization of YY1 and membrane localization of p130cas were clearly seen separately (Fig. 2i, left and middle) and combined (Fig. 2i, right; Fig. 2j).
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**Microarray staining**

Figure 2. Fluorescent staining with antibody arrays. An array of 200 rabbit polyclonal antibodies were used and the staining at four positions are shown here as representatives (a–h). (a), (b) Transcriptional factor IRF1; (c), (d) Signaling molecule 14-3-3β; (e), (f) Cell adhesion protein β-catenin; (g), (h) Transcriptional factor Ets-1. The low magnification (a, c, e, and g) shows the stained cells and surrounding nonstained areas; the high magnification shows (b) the detailed nuclear localization of IRF1, (d) cytoplasmic staining of 14-3-3β; (f) membrane staining of β-catenin at cell-cell contacts, and (h) cytoplasmic staining of Ets-1. Scale bar in (a, 300 μm, in (b) 30 μm. (i) Low magnification of A431 cells staining with an array containing rabbit anti YY1 antibodies (left; green), mouse anti-p130cas antibodies (middle; red), and both YY1 (right; green) and p130cas (right; red) antibodies at neighboring positions. Goat anti-rabbit Cy2-labeled secondary antibodies and goat anti-mouse Cy3-labeled secondary antibodies were used. (j) Enlarged view of the double staining of YY1 (green) and p130cas (red) from (i).

Comparison of protein expression patterns between different protein samples is an important application of antibody array staining. To demonstrate this application, a dissociable antibody array containing 237 antibodies (including both mouse monoclonal and rabbit polyclonal) against proteins involved in various signaling pathways were used to compare protein expression between A431 and ME180 cells, two human cancer cell lines widely used in research. Because A431 and ME180 cells express the same level of transcriptional factor Ets-1 (see Fig. 3b), antibodies against Ets-1 were spotted at four corners and used as reference spots. After apposition, incubation, and separation, the cells were stained with alkaline phosphatase-labeled secondary antibodies and reacted with BCIP/NBT substrates to produce color signals. Among the 237 target proteins, many are expressed differently in A431 and ME180 cells (Fig. 3a). For example, A431 cells express more Cbl, cortactin, Neu, HSP 70, JNK1, p53, Raf-1, and Stat1, but less GSK-3β, Skp2 p45, Plk3, and Stat5α than ME180 cells do. The expressions of these proteins were also examined by Western blotting (Fig. 3b). The results obtained with the two methods were very well correlated (Fig. 3c), indicating that antibody array staining is a valid method for profiling protein expression.

### 4 Discussion

The protein microarrays used in earlier studies [3, 5] are capture arrays: they capture ligands (proteins, cells, etc.) onto the array supports from protein samples. The captured ligands are then analyzed *in situ*, each at a predetermined position. In contrast, the antibody microarrays described here are dissociable arrays: the immobilized antibodies can dissociate from the array support and bind to antigens that are immobilized on another support. The application of these dissociable antibody arrays is to deliver multiple antibodies simultaneously to their respective antigens in a position-addressable manner. Covalent
Figure 3. Detection of protein expression. (a) Protein expression in ME180 cells (left) and A431 cells (right) were examined with antibody arrays containing 237 antibodies. Cells were seeded on the bottom of tissue culture dishes. They were fixed and permeabilized as described in Section 2 before being stained. The staining was observed via alkaline phosphatase-mediated color reaction with BCIP/NBT as substrates. Center-to-center distance of the array is 600 μm. (b) Expressions of 19 proteins in ME180 cells (left lanes) and in A431 cells (right lanes) as detected by Western blotting. Proteins examined are: A, Cb1 (120 kDa); B, Cdc2 (34 kDa); C, cortactin (80 kDa); D, Neu (185 kDa); E, ERK1 (44 kDa); F, Ets-1 (51 kDa); G, GSK-3β (51 kDa); H, HSP 70 (70 kDa); I, JNK1 (46 kDa); J, Lyn (56 kDa); K, NFκB p50 (50 kDa); L, Skp2 p45 (45 kDa); M, p53 (53 kDa); N, Plk3 (70 kDa); O, SH-PTP2 (60 kDa); P, Raf-1 (74 kDa); Q, Rb p107 (107 kDa); R, Stat1 (84 kDa); S, Stat5a (95 kDa). The corresponding position for each of the antibodies in (a) is indicated on the top. Protein lysates of A431 and ME180 cells occupying equal culture areas were loaded in each lane. (c) Comparison of protein expressions. The ratios of the expressions of the 19 proteins between A431 and ME180 cells as determined by array staining in (a) and by Western blotting in (b) are plotted in logarithmic scale.
suggesting that array staining can reliably reveal the in situ properties of proteins. As demonstrated in this study, with carefully selected antibodies, array staining can be just as specific and quantitative as the standard methods of immunostaining. The ability of array staining to stain one cell preparation with hundreds of different antibodies should facilitate high-throughput screening applications. Although only adherent cells were stained by the current array staining method, nonadherent cells can also be stained after immobilization on a solid support. Immunostaining with dissociable antibody arrays as described here is best performed on homogeneous cell populations, rather than on tissue sections, which are inherently heterogeneous. This problem can be partially overcome if the heterogeneous cell population can be rendered homogeneous, e.g., via trypsinization and mixing. Some tissues, such as muscle, are relatively homogeneous: if each staining locus contained the same cell type and if high resolution analysis at the single cell level by fluorescence microscopy were done, this problem may be overcome.

Because a larger number of antibodies with different binding properties are used in antibody microarray staining assays, there are several intrinsic difficulties in such complex assays. Antibody cross-reactivity is a problem for all immunological methods. This problem is partially overcome in Western blotting by using molecular weight as a criterion. Antibody cross-reactivity is behind most problems of capture antibody microarray assays [5] because it is difficult if not impossible to use other independent criteria in distinguishing true signals from false signals in these assays. If only signal intensity is detected in array staining as in Figs. 1b and 3a, there is a tendency for false positive signals due to cross-reactivity. One way to minimize such false signals is through use of high-affinity, well-characterized antibodies. In addition, a key advantage of the dissociable antibody microarray method is that it can detect both the expression and subcellular localization of antigens (e.g., nuclear, cytoplasmic or membrane) by fluorescence microscopy. This unique reliability on multiple criteria tends to minimize (though it does not completely eliminate) the problem of antibody cross-reactivity. Another problem unique to antibody microarray is the cross-talk between different antibodies, a problem that limits the number of different antibodies that can be used in a multiplex assay to fewer than a hundred [18]. Antibody cross-talk is avoided in the microarray staining method because each antibody is restricted to its own predefined position without mixing with other antibodies.

Different antibodies may need different fixation and permeabilization conditions for use in immunostaining. Therefore, one technical difficulty in array staining is that of finding an optimal staining condition for all of the target proteins. Moreover, neither the mechanism of antibody dissociation from the support nor the process of antibody diffusion and binding to their antigens is fully understood, and the most efficient conditions for these processes may vary for different antibodies. Understanding these processes will be helpful in designing better dissociable antibody arrays. The complex nature of array staining assays therefore requires careful interpretation of the assay results. For example, the signal intensity is dependent not only on the expression of the antigen but also on the accessibility of the antigen and the antigen-antibody binding properties. It is possible to compare relative expression levels of the same set of proteins between cell samples but impossible to compare relative expression levels of two different proteins in the same cell sample.

As a proteomic tool, the dissociable antibody array technique has certain advantages over other antibody array systems. (i) It employs a simple procedure without the need to solubilize proteins or label them with detectable tags. (ii) It can reveal the cellular and subcellular localizations of proteins. Because a protein’s subcellular localization relates to its function, the information on changes in protein distribution may provide important insight to the activities and functions of proteins. (iii) It can easily be combined with other protein separation and detection techniques. Although the present report describes the staining of fixed cells, similar antibody arrays can be used to study proteins in other biological samples, such as protein lysates after separation according to molecular weight and/or isoelectric point. Such versatility will both expand the use of antibody arrays and increase assay specificities.

Current capture protein microarray technology has had only limited success in examining protein expression profiles, in contrast to the success of DNA microarrays in examining mRNA expression profiles. Many research groups are striving to develop technologies capable of revealing global protein expression profiles. We suggest that dissociable antibody array-staining provides an attractive means to achieve this goal. As an example, pro-
tein expressions between the two human cancer cell lines ME180 and A431 were compared by array staining in this study (Fig. 3), and the two cell lines were found to express different levels of many of the target proteins. The biological significance of these differences remains to be seen. But the confirmation of the differences by Western blotting validated the method. The accuracy of array staining in quantitating protein expressions can be further improved by using high-affinity, well-characterized antibodies and including additional identification criteria such as protein subcellular localizations. The quantitation of multiple proteins by antibody array-staining will have immediate uses in many important applications, such as simultaneous examination of multiple signal transduction pathways and the use of multiple biomarkers to facilitate clinical diagnosis.

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5 References