Hypromatrix
The Proteomics Company

AntibodyArray™ Staining Instruction Manual

www.hypromatrix.com

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I. INTRODUCTION

Immunochemical staining is a versatile technique for determining both the presence and localization of an antigen (Harlow and Lane, 1988). This information is of immense value to biomedical research and clinical medicine. Most of the current methods, all of which involve incubating cells with an antibody solution, only allow cell staining with one or a few antibodies at a time. These methods are not suitable for applications in which the expressions and sub-cellular localizations of a large number of different proteins need to be examined.

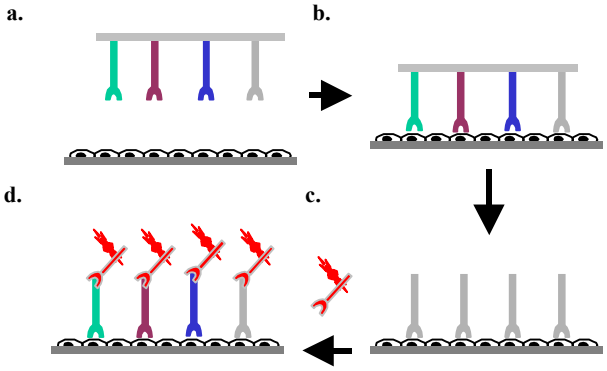

Fig. 1: Overview of AntibodyArray™ staining technology. **a**, make contact between staining antibody array and adherent cells; **b**, incubate arrays and cells to allow antibodies to bind to their antigens; **c**, remove array support; **d**, detect bound antibodies.

The AntibodyArray™ staining method takes advantage of "Dissociable Protein Array" technology, which allows the delivery of a large number of proteins to their targets in a position-addressable manner. In staining AntibodyArray™s, the antibodies are immobilized on a membrane in such a manner that when they make contact with cells fixed on another support, the antibodies can bind to their respective antigens. When the array support is separated from the cells, the antibodies will be dissociated from the support and remain bound to the antigens. Therefore, the method enables the staining of multiple antibodies simultaneously, each at a predetermined position.

AntibodyArray™ staining technology offers a high-throughput method for examining *in vivo* protein activities. It has many applications, including:

1. Examining protein expressions
2. Revealing protein sub-cellular localizations

Measuring protein expressions has applications in a variety of fields, including biomedical research, disease diagnosis, and drug discovery. Antibody array-staining provides an unique approach to reveal protein expression patterns. It is useful in comparing the expressions of a large number of proteins between different biological samples (see Fig. 2).

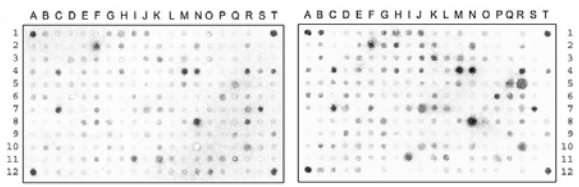

Fig. 2: Comparison of protein expressions between ME180 cells (left) and A431cells (right) using antibody arrays with 240 antibodies. Alkaline phosphatase-labeled secondary antibodies were used and the staining was visualized by color reaction with BCIP/NBT as substrates

Knowledge of a protein's sub-cellular localization can provide important information about the protein's functional state. For example, change of a transcriptional factor's location from cytoplasm to nucleus often suggests its activation. When observed under a fluorescence microscope, AntibodyArray™ staining can reveal sub-cellular localizations of many proteins simultaneously.

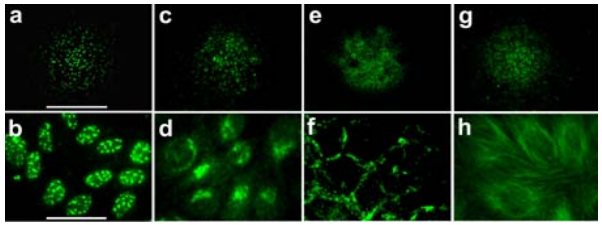

Fig. 3: Fluorescent staining with staining AntibodyArray™. An array of 200 rabbit polyclonal antibodies were used and the staining at four positions are shown here as representatives. **a, b**, transcriptional factor IRF1. **c, d**, signaling molecule 14-3-3 β. **e, f**, cell adhesion protein β-catenin. **g, h**, transcriptional factor Ets-1. Low magnification (**a**, **c**, **e**, **g**, and **i**) shows the stained cells and surrounding non-stained area; and high magnification shows the detailed nuclei localization of IRF1 (**b**), cytoplasmic staining of 14-3-3 β (**d**) and Ets-1 (**h**), and membrane staining of β-catenin at cell-cell contacts (**d**). Scale bar in **a**, 300 μm; Scale bar in **b**, 30 μm.

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 co-localize in the same cellular structure. Double staining is also useful when the protein of interest is only expressed in small number of target cells among a heterogeneous cell population, and the protein of interest needs to be observed together with a protein marker which is used to denote the target cells. Array staining is unique in that it allows the examination of multiple proteins individually as well as simultaneously in the same cell preparation.

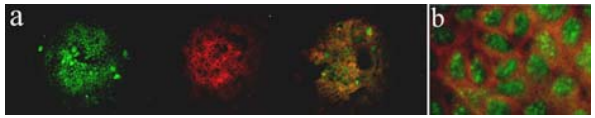

Fig. 4: Fluorescent double staining with AntibodyArray™. **a**, Low magnification of A431 cells staining with an array containing rabbit anti-YY1 antibodies (left in green), mouse anti-p130^{cas} antibodies (middle in red), and both YY1 (right in green) and p130^{cas} (right in red) antibodies at neighboring positions. Goat anti-rabbit Cy2-labeled secondary antibodies and goat anti-mouse Cy3-labeled secondary antibodies were used. **b**, Enlarged view of the double staining of YY1 (green) and p130^{cas} (red) from **a**.

Hypromatrix's Staining Antibody Microrrays are designed for the following applications:

1. Revealing a protein's novel functions;
2. Understanding molecular mechanisms of a protein's function.
3. Dissecting signaling pathways activated by specific stimulations;
4. Screening cellular effects of drug candidates;
5. Discovering novel diagnostic markers.

For additional information regarding other applications and custom-tailored staining arrays, please contact Hypromatrix, Inc.

II. LIST OF COMPONENTS

Staining AntibodyArray™
Store at 4 °C. Stable for at least 4 weeks.

III. ADDITIONAL MATERIALS REQUIRED

The following materials are needed but not supplied:

A. Cells or tissue sections

B. Reagents required for cell fixation permeabilization
Methanol, Acetone, Formaldehyde, Glutaldehyde

C. Materials required for performing AntibodyArray™ staining
AntibodyArray™ staining apparatus
Filter papers
Filter pads

D. Solutions
1. Cell fixation/permeabilization:
   50% Aceton/50% Methanol.

2. Blocking solution
   5% BSA (bovine serum albumin) in PBS

3. Washing buffer:
   PBS (phosphate buffered saline)

E. Reagents required for detection
1. Secondary antibodies:
   fluorophore-conjugated goat anti-mouse and anti-rabbit antibodies.

3. Anti-bleaching reagent
4. Mounting medium

F. Equipment required for observing fluorescent immunostaining
1. Fluorescence microscope
2. Motorized stages: Hypromatrix Cat. # HM8020 & HM8021
3. Stage control Software: Hypromatrix Cat. #HM8010

IV. METHODS

A. General considerations

1. Before using staining AntibodyArray™, tests should be done to get information on the suitability of the cells for AntibodyArray™ staining. Some cells may fall off the support during the staining procedure. Cell culture conditions and fixation conditions should be carefully examined before experiments. Testing Staining AntibodyArray™ can be used for this purpose.

2. One cell fixation condition may be optimal only for the staining of a subset of the proteins. Therefore, several conditions may be needed to obtain proper staining for all the antigens.

3. The following protocols are for reference only. Researchers should carefully study their systems and select proper experimental conditions.

B. Protocol for AntibodyArray™ staining

1. Fix and permeabilize cells to exposure antigens (for detail see Protocols D to I).
2. Block staining AntibodyArray™ and fixed cells in PBS solution containing 5% BSA for 0.5—1 hour.
3. Place the staining AntibodyArray™ on top of the cells and assemble the components as shown in Fig. 4.

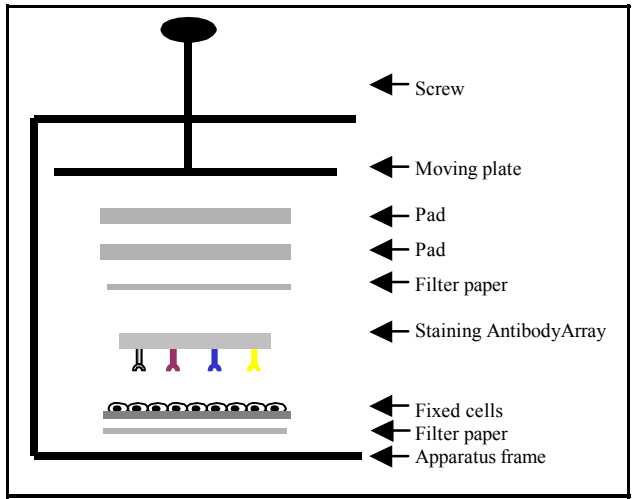

Fig. 4: Assembly of various staining components

4. Apply pressure and secure the assembly.
5. Stain for 1- to 2-hour.
6. Remove the array from the cell support and put the cells in a container.
7. Rinse the cells with PBS.

8. Apply fluorescence-labeled secondary antibodies (a mixture of anti-rabbit and anti-mouse antibodies); and incubate for an hour.
9. Wash with PBS.
10. Visualize the staining under fluorescent microscope.

Note:

1. For comparing protein expressions, all samples were processed in parallel and the enzymatic reactions can be stopped by washing off substrates with PBS.
2. Care should be taken to avoid trapping air between cells and arrays.
3. Arrays should be carefully aligned with cells and the alignment should be recorded to avoid any confusion at later steps.
4. After arrays contact cells, any movement between them should be avoided. The interaction between antibodies and antigens begins in minutes

C. Some considerations for observing and analyzing staining results

For semi-quantitative analysis of protein expressions, array staining can be detected with enzyme-conjugated secondary antibodies and visualized via color reaction. For example, one may use alkaline phosphatase conjugated secondary antibodies in the following manner:

1. After array staining, place the coverslip in a washing container. Remember the orientation of the coverslip in the container.
2. Wash with PBS three times.
3. Apply alkaline phosphotase-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies to the cells.
4. Incubate for 1 hour.
5. Wash with PBS three times.
6. Add phosphotase substrate 3-bromo-4-chloro-5-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT).
7. Incubate until a brown color develops. When proper intensity is reached, stop the reaction by washing the substrate with PBS.

The image of the StainingArray can be scanned on a standard flatbed scanner. The digitized images can then be analyzed.

When comparing two stainings, the overall intensities must be normalized first. This may be accomplished by equalizing the intensities of the corresponding reference spots whose expression is identical in control and experimental conditions. Then the numerical intensities of the corresponding spots may be calculated and compared.

Because an antibody will bind both its intended characterized antigen(s) and some unintended proteins which have similar structure to the immunogen (non-specific binding), there *may be* some false positive signals.

To obtain a more quantitative result, fluorescence conjugated secondary antibody should be used and array staining should be observed under a fluorescence microscope. The problem of non-specific binding can be minimized since both the fluorescence intensity and subcellular localization of the antigens can be detected and analyzed.

A general procedure for fluorescent AntibodyArray staining is:

1. After array staining, place the coverslip in a washing container. Remember the orientation of the coverslip.
2. Wash with PBS three times.
3. Apply Alexa 488-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies to the cells.
4. Wash with PBS three times.
5. Mount the coverslip on a glass slide and observe under a fluorescence microscope.

Without a motorized stage:
Because of the large number of antigens needed to be examined for each array staining, the staining is best observed under a fluorescence microscope with a motorized stage, which allows the movement to each spot automatically. However, the staining can also be observed without a motorized stage.

When a motorized stage is not available, the AntibodyArray should be aligned with the cells on a coverslip as good as possible. The orientation of the staining (e.g., flips, rotations) during the staining and washing processes should also be recorded. This is important for later steps when the staining is observed under a microscope.

A general procedure to follow when observing a StainingArray without a motorized stage is:

After staining
1. Mount the coverslip with cells on a slide such that the cells are facing up.
2. Align the coverslip with the slide correctly;
3. Apply mount medium and overlay another coverslip to cover the cells. Try not to move the cells. If you did, you can correct the movement by inserting a piece of paper or a razor blade between the slide and the overlaying coverslip to move the coverslip with the cells. Be careful to avoid introducing air bubble.
4. Place the slide on the stage of a microscope, with the coverslip facing the objective lens. Record the orientation.
5. Examine the slide under low magnification (e.g., 10X objective) first. When you move around the field, you should see many bright areas, about 1mm apart, separated by dark non-stained areas. Exam all of the spots quickly first and check the orientation of the staining image relative to the staining AntibodyArray.
6. Find the reference spots: go to the approximate position of a reference spot, locate the reference spot by its unique staining pattern (e.g., nuclear staining of Histone H1). Then find additional reference spots.
7. Go to the spot of interest by moving the microscope stage. You can use the ruler in the microscope eyepiece to measure the moving distance. In most cases you should be able to identify the next spot by the fluorescence which is lacking outside the stained areas.

With a motorized stage:
Array staining is best observed under a fluorescence microscope equipped with a motorized ArrayStage from Hypromatrix, which also comes with software specifically written for this purpose. ArrayStage from Hypromatrix is compatible with the following manufacturers' fluorescent microscopes:

Manufacturer		Model
Nikon	Upright	E400, E600, E800, E1000, Optiphot I and II
	Inverted	TE200, 300, TE2000, TS100
Olympus	Upright	BH, BX40,50,60, BX41,51,61, BX45
	Inverted	IX50, 70, IX51, 71, 81, GX51, 71
Leica,	Upright	DMLB, DMR, DMLM, Dialux, Laborlux, Diaplan
	Inverted	DMIR, DMIL
Zeiss	Upright	Universal, Axioskop 1 and 2, Axioplan 1 and 2, Axiophot 1 and 2
	Inverted	Axiovert 1 and 2

For compatibility with other microscopes, please ask.

With a motorized stage and its control software, you can observe one array staining at a time or several array stainings (e.g., staining of different samples with the same staining microarrays) together.

For detailed information on ArrayStage and its control software, please contact Hypromatrix, Inc.

There are generic motorized stages from several manufactures, which can also be used for observing array staining. Please contact individual manufacturer for specifications and control software.

D. Protocol I for fixation and permeabilization of cultured cells
1. Grow cells on cover slips until desired state.
2. Aspire the medium.
3. Fix the cells by 50% Methanol and 50% Acetone (cold -20 degree).
4. Wash for three times and then put into freezer for 10 to 20 minutes.
5. Rinse with PBS for 3 times.
6. Blocking in 5% BSA (in PBS) at 4 degree for 1 hour.

E. Protocol II for fixation and permeabilization of cultured cells
1. Grow cells on a coverslip in a 6-well.
2. Wash cells 1x with media without serum and 2x PBS.
3. Block the cells 15' in PBS-BSA.
4. Wash 2x with PBS.
5. Fix the cells 20' in 4% paraformaldehyde at room temperature.
6. Wash 3x with PBS.
7. Permeabilize the cells 10' with 0.2 % Triton-X-100.
8. Wash 3x with PBS.
9. Block the cells 15' in PBS-BSA.
10. Wash 2x with PBS.

F. Protocol for preparing tissue sections

1. Fix the tissue in 10% formalin at 4°C overnight.
2. Paraffin-embed the fixed tissue.
3. Mount tissue sections on slides.
5. Clear the paraffin with xylene for ten minutes; move slides to a fresh dish of xylene for an additional ten minutes. (**NOTE:** Perform all xylene washes in a fume hood!)
6. Rinse the slides twice for 2 minutes in 100% alcohols (18:1:1 100% ethanol:100% methanol:100% isopropanol).
7. Rinse the slides twice for 2 minutes in a 95% solution of the 100% alcohols.
8. Place slides in an 80% solution of the 100% alcohols for 2 minutes, followed by de-ionized water for 5 minutes.
9. Rinse slides several times with fresh deionized water followed by another five-minute wash using fresh water.
10. SDS Antigen Retrieval: place slides face-up in incubation tray and cover each section with 1% SDS in TBS (100mM Tris pH 7.4, 138mM NaCl, 27mM KCl). Incubate for five minutes at room temperature, followed by three five minute washes with TBS.
11. Blocking: immerse slides in a dish containing blocking buffer (serum from host species of secondary antibody to be used, diluted 1:10 in TBS). Incubate at 37°C for one hour.

G. Protocol I for fixation of frozen sections and cell preps

1. Place section or cell prep on slide.
2. Air dry: 1 hour for cell preps and from 2 hrs. to overnight for sections.
3. Place in acetone for 5 - 10 minutes
4. Air dry for 10 minutes.
5. Rehydrate by placing 5 minutes in each of three separate solutions of PBS (with 2% BSA to reduce background).
6. IMPORTANT: Use of methanol or other alcohols at any stage of this process may prevent staining with certain antibodies.

H. Protocol II for fixation of frozen sections and cell preps

1. Obtain frozen sections, 4 or 8-well chamber slides or cover-slips containing cultured cells.
2. Fix in 4 % paraformaldehyde in PBS for 30 min at room temperature or min (chamber-slides or cover-slips) in PBS containing 0.05%-0. 1% Triton X-100.
3. For examination of cell surface components, methanol or Triton X-100 extraction should not be used.
4. After a 30-min incubation in PBS containing 0.05% Twen-20 (PBST) and 3%-BSA,

I. Steps to take if tissue keeps falling off slides

1. Cut no thicker than 4 microns. (3 is ideal)
2. Dry 2-4 hours at 58° C in oven (4 hours minimum for breast tissue or other fatty tissue). You can also dry overnight at 37° C. Finally, there are microwave methods of drying tissue, but the protocol depends on your microwave and the number of slides. Generally, a 1000W microwave can dry up to 100 slides in about 3 minutes. You should examine each slide to make sure the paraffin has melted uniformly across the tissue section.
3. Make sure you use positively charged slides. If the tissue adhesion problem coincides with the use of a newly received shipment of slides, you may have a bad lot of slides.

4. When lifting the tissue on to the slide out of the water-bath, at no point before or during this process should you touch the flat surface of the slide. Hold the slides by the edges or by the front end.

V. TROUBLESHOOTING

If the results you obtained are different from what you have expected, use the following guide for troubleshooting. For further help, please contact Hypromatrix.

Observations	Possible Causes	Solutions
No signal	Cells fell off the support.	Check cells under microscope to make sure that most cells are still there after the staining. Change cell fixation conditions or use an additional step to further immobilize cells on the support. Use properly treated support to grow cells.
	Improper 2nd antibodies.	Check the 2nd antibodies to use correct anti-mouse or anti-rabbit antibodies. Use correct enzyme- or fluorescent-conjugated antibodies.
	Improper use of Staining AntibodyArrays	Make sure that the correct side of the array is contacted with the cells. Follow the instructions.
Staining is weak	Secondary Antibody is not sensitive enough.	Use high-quality enzyme- or fluorescent-conjugated 2nd antibodies.
	Bleaching.	Use anti-bleaching mounting medium and use less bleaching flurophore.
	Insufficient incubation time between arrays and cells.	Increase the incubation time.
Too much background	Blocking is not complete	Block the cells overnight.
	Antibodies contain reactivities to some components in blocking reagents	Pre-incubate the antibodies with the blocking reagents. Add blocking reagents in wash solution.
Positive staining at spots which should be negative	Antibodies recognize proteins other than the supposed antigens in the cells.	Contact Hypromatrix and request some control.

VI. REFERENCES

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APPENDIX I. ANTIBODY LIST BY POSITIONS

A. Staining AntibodyArray™ I

Catalog Number HM8100

	A	B	C	D	E	F	G	H	I	J	K	L
1	Histon H1	14-3-3	c-Abl	Annexin VI	Bak	BRCA1	gamma-catenin	c-Cbl	CBP	Cdc6	Cdk1/Cdc2	Histon H1
2	Cdk2	Histon H1	Cdk6	c-Fgr	CREB	Cyclin B	Cyclin E	Dynamin	E2F1	Egr-1	Egr-3	erb2 (Neu, Her 2)
3	ERK1	ERK2	Ets-1/2	Fas/CD95/ APO-1	FGFR4	Flt-3/2	GRK 2	GSK-3 alpha	HSP-70	Id1	IFN-g R a	Ikappa B-b
4	IKKb	IL 1 R	IL 2 R alpha	IL 2 R, beta	IL4R a	Integrin a V	Integrin b 1	IRAK	IRF1	ISGF3 gamma	Jak1	Jak3
5	JNK1,2,3	Lck	Mad-1	Max	MDM2	Histon H1	MEK1	MEKK2	Met	c-Myc	NF2	NF-kappa B 50
6	NF-kappa B p65	p130Cas	p38 MAPK	p45 skp2	p53	P63 (KET)	PCNA	PSD-95	PTP1 (SH)	PTP2 (SH)	Rab3	RACK1
7	c-Raf-1	RAIDD	Rap1	Rb p107	RbP130 (Rb2)	Rel B	Rho A	Rsk-1	Sam68	Smad1 (1/2/3)	Smad4	Sos1/2
8	Histon H1	sp1	Stat1	Stat3	Stat4	Stat5a	TRADD	TRAF2	Vav	YY1	ZAP70 Kinase	Histon H1

Note: the spots of Histone H1 shown in red are recommended reference spots.

D. Trial Staining AntibodyArray™

Catalog Number HM8900

	A	B	C	D
1	Histone H1	14-3-3 beta	r-catenin	Histone H1
2	ERK1	Histone H1	Ets 1	IRF-1
3	Histone H1	p130cas	YY1	Histone H1

Note: the spots of Histone H1 shown in red are recommended reference spots.

APPENDIX II. PRODUCTS FROM HYPROMATRIX, INC

A. Staining AntibodyArray™s

1. Staining AntibodyArray™ I
   Catalog Number HM8100

2. Trial Staining AntibodyArray™
   Catalog Number HM8900

B. AntibodyArray™s

1. Signal Transduction AntibodyArray™
   Catalog Number HM3000

2. Apoptosis AntibodyArray™
   Catalog Number HM4000

3. Cell Cycle AntibodyArray™
   Catalog Number HM5000

4. Custom AntibodyArray™
   Catalog Number HM6000

C. Antibodies

1. Primary antibodies
   Hypromatrix offers a variety of high quality antibodies. For a complete list of antibodies and their specificities, please visit our web site at www.hypromatrix.com.

2. Secondary antibodies
   Alexa 488 conjugated Goat anti-rabbit secondary antibody
   Catalog Number HM2101

   Alexa 488 conjugated Goat anti-mouse secondary antibody
   Catalog Number HM2105

D. Others

1. AntibodyArray™ staining apparatus
   Catalog Number HM8000

2. Motorized ArrayStage for upright microscopes
   Catalog Number HM8020

3. Motorized ArrayStage for inverted microscopes
   Catalog Number HM8021

4. ArrayStage Control program
   Catalog Number HM8010

Limit liability claims

All of the antibodies work under conditions tested by Hypromatrix. They may not work in other conditions. Hypromatrix may change antibodies without notification. Returns will be only accepted with Hypromatrix's authorization. Hypromatrix, Inc. shall have no liability for any damage as the result of improper using of its products

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