蛋白芯片制作与应用（3）－操作流程

一个经典的蛋白芯片操作流程：

Experimental Procedures for Protein Microarrays

Chemically Derivatized Glass Slides.

Aldehyde slides were purchased from TeleChem International (Cupertino, CA). BSA-NHS slides, displaying activated amino and carboxyl groups on the surface of an immobilized layer of bovine serum albumin (BSA), were fabricated as follows. 10.24 g N,N'-disuccinimidyl carbonate (100 mM) and 6.96 ml N,N-diisopropylethylamine (100 mM) were dissolved in 400 ml anhydrous N,N-dimethylformamide (DMF). 30 CMT-GAP slides (Corning Incorporated, Corning, NY), displaying amino groups on their surface, were immersed in this solution for 3 hr at room temperature. The slides were rinsed twice with 95% ethanol and then immersed in 400 ml of phosphate buffered saline (PBS), pH 7.5 containing 1% BSA (w/v) for 12 hr at room temperature. The slides were rinsed twice with ddH2O, twice with 95% ethanol, and centrifuged at 200 g for 1 min to remove excess solvent. The slides were then immersed in 400 ml DMF containing 100 mM N,N'-disuccinimidyl carbonate and 100 mM N,N-diisopropylethylamine for 3 hr at room temperature. The slides were rinsed four times with 95% ethanol and centrifuged as above to yield BSA-NHS slides. The slides were stored in a desiccator under vacuum at room temperature for up to two months without noticeable loss of activity.

Arraying Proteins on Glass Slides.

Proteins were dissolved in 40% glycerol, 60% PBS, pH 7.5 at a concentration of 100 μg/ml unless indicated otherwise. For Figs. 1, 5, 6, and 7, the proteins were spotted on

aldehyde slides using a GMS 417 Arrayer (Affymetrix, Santa Clara, CA). Following a 3 hr incubation in a humid chamber at room temperature, the slides were inverted and dropped onto a solution of PBS, pH 7.5 containing 1% BSA (w/v). After 1 min, the slides were turned right side up and immersed in the BSA solution for 1 hr at room temperature with gentle agitation. Following a brief rinse in PBS, the slides were ready for further processing (see below).

For Fig. 3, the proteins were spotted on BSA-NHS slides using a GMS 417 Arrayer. Following a 3 hr incubation in a humid chamber at room temperature, the slides were inverted and dropped onto a solution of PBS, pH 8.0 containing 500 mM glycine. After 1 min, the slides were turned right side up and immersed in the glycine solution for 1 hr at room temperature with gentle agitation. The slides were then ready for further processing (see below).

For Fig. 2, the proteins were spotted on a single aldehyde slide using a split pin arrayer constructed following directions on P. Brown's web page (http://cmgm.stanford.edu/pbrown/). Following a 3 hr incubation at room temperature, the slide was processed using the procedure employed for the aldehyde slides described above.

Screening for Protein-Protein Interactions.

Protein G was from Pierce (Rockford, IL) and BODIPY-FL-Goat-anti-Mouse IgG was from Molecular Probes (Eugene, OR). IKBalpha and p50 were kindly provided by T. Maniatis (Harvard University, Cambridge, MA) and GST-FRB and (His)6-FKBP12 were produced recombinantly in Escherichia coli. IKBalpha and (His)6-FKBP12 were labeled with Cy3 and Cy5, respectively, using monofunctional reactive dye from Amersham Pharmacia Biotech (Newark, NJ) and following the recommended protocol.

For Fig. 1, protein G, p50, and FRB were spotted in quadruplicate on aldehyde slides and processed as described above. To probe the slides, the labeled proteins were diluted into PBS, pH 7.5 supplemented with 0.1% Tween-20 (v/v) and 1% BSA (w/v). BODIPY-FL-IgG was used at a concentration of 0.5 μg/ml, Cy3-IKBalpha was used at a concentration of 0.1 μg/ml, and Cy5-FKBP12 was used at a concentration of 0.5 μg/ml. 0.55 ml of protein solution was applied to the slide using a PC500 CoverWell incubation chamber from Grace Biolabs (Bend, OR). Following a 1 hr incubation at room temperature, the slides were rinsed with PBS and then washed 3 times for 3 min each with PBST (PBS supplemented with 0.1% Tween-20). The slides were rinsed twice with PBS and centrifuged at 200 g for 1 min to remove excess buffer.

To visualize fluorescence, the slides were scanned using an ArrayWoRx fluorescence slide scanner (Applied Precision, Issaquah, WA). The scanner works by imaging successive 2.5 x 2.5 mm sections of a slide using excitation and emission filters coupled with a magnifying lens and CCD camera. The resulting panels are then stitched together to form one large image. The slides were visualized at 5 μm resolution, using CCD camera integration times ranging from 1 to 5 sec depending on the fluorophore. The emitted light was false-colored blue, green, and red to correspond to BODIPY-FL, Cy3, and Cy5, respectively. For all images, the intensity of the color was scaled linearly, with black corresponding to the background fluorescence of the slide and pure color corresponding to the brightest pixels in the image.

For Fig. 2, protein G and FRB were spotted on an aldehyde slide, probed with BODIPY-FL-IgG + Cy5-FKBP12 + 100 nM rapamycin, and visualized with an ArrayWoRx fluorescence slide scanner, all as described above. For Fig. 6, FRB (1 mg/ml) was spotted in triplicate on 12 separate areas of two aldehyde slides. The areas were then separated by drawing lines between them with a hydrophobic pen (PAP PEN from Newcomer Supply, Middleton, WI). The slides were processed as

described above. To probe the slides, Cy5-FKBP12 was serially diluted 2-fold into PBST containing 1 % BSA (w/v) and 1 μM rapamycin. 30 μl of each dilution were applied to separate sections of the slides. Following a 1 hr incubation at room temperature, the slides were washed as described above and scanned with a GenePix 4000A microarray scanner (Axon Instruments, Foster City, CA). The fluorescence intensity of each spot was taken as the median intensity of the spot minus the median intensity of the local background. In order to span the full range of intensities observed at different concentrations of Cy5-FKBP12, the two slides were scanned at different sensitivity settings (PMT voltage) and the data scaled to adjust for this difference.

Screening for Substrates of Protein Kinases.

All proteins used for these studies were purchased from New England Biolabs (Beverly, MA). EasyTides gamma-33P-adenosine 5'-triphosphate (gamma-33P -ATP) was from NEN Life Science Products (Boston, MA). NTB-2 autoradiography emulsion, Dektol developer, and Fixer were from Eastman Kodak Company (Rochester, NY).

Kemptide, I-2, and Elk1 were spotted in quadruplicate on BSA-NHS slides and processed as described above. The slides were then washed 3 times for 10 min each with Wash Buffer (WB; 20 mM Tris, 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, pH 7.5). The slides were subsequently washed once for 10 min with Kinase Buffer (KB; 50 mM Tris, 10 mM MgCl2, 1 mM DTT, pH 7.5), incubated for 10 min with KB supplemented with 100 μM ATP, and washed for an additional 10 min with KB. The slides were then incubated for 1 hr at room temperature with 200 μl of kinase solution, applied to the slides under a PC200 CoverWell incubation chamber (Grace Biolabs). The kinase solution was composed of the recommended buffer for each kinase supplemented with the recommended amount of ATP, 2 μl of

gamma-33P-ATP (20 μCi), and 2 μl of purified enzyme (10 units of cAMP-dependent protein kinase (catalytic subunit), 1000 units of casein kinase II, or 100 units of Erk2). Following the 1 hr incubation, the slides were washed 6 times for 5 min each with WB, twice for 5 min each with WB lacking Triton X-100, and 3 times for 3 min each with ddH2O. The slides were then centrifuged at 200 g for 1 min to remove excess water.

To visualize the radioactive decay, NTB-2 autoradiography emulsion was melted at 45 oC for 45 min in a dark room. The slides were dipped in the emulsion for 3 sec and allowed to dry vertically at room temperature for 4 hr. The slides were then sealed in a ?-radiation box with desiccant and incubated in the dark at 4 oC for 4 to 10 days. The slides were subsequently developed by immersing them successively in Dektol developer for 2 min, ddH2O for 10 sec, Fixer for 5 min, and ddH2O for 5 min. To visualize the slides, successive images were taken using a DeltaVision automated microscope (Applied Precision) in DIC mode and the individual panels stitched together to form a single larger image. The same settings were used for all three slides.

Screening for Targets of Small Molecules.

Mouse anti-digoxigenin IgG clone 1.71.256 (Anti-DIG) was from Boehringer Mannheim (Indianapolis, IN), streptavidin was from Pierce, and bovine serum albumin (BSA) was from Sigma (St. Louis, MO). Alexa488-BSA was prepared using an Alexa Fluor 488 protein labeling kit from Molecular Probes. Alexa488-BSA-Dig was prepared by labeling Alexa488-BSA with 3-amino-3-deoxydigoxigenin hemisuccinamide, succinimidyl ester (Molecular Probes). Cy3-BSA and Cy5-BSA were prepared by labeling BSA with Cy3 and Cy5 monofunctional reactive dyes (Amersham Pharmacia Biotech). Cy5-BSA-biotin was prepared by labeling Cy5-BSA with Sulfo-NHS-LC-Biotin (Pierce). Cy3-BSA-maleimide was prepared by labeling Cy3-BSA with Sulfo-GMBS (Pierce). All labeling reactions were performed

according to the recommended protocols.

Cy3-BSA-AP1497, Cy3-BSA-AP1767, and Cy3-BSA-AP1780 were prepared as follows. AP1497, AP1767, and AP1780 were kindly provided by D. Holt (Ariad Pharmaceuticals, Cambridge, MA). Each compound was coupled to polystyrene beads via a 6-carbon linker and 4-methoxytrityl-protected cysteine according to our previously published protocol (http://www.cgr.harvard.edu/macbeath/protocols/smmicroarrays.html). For each compound, about 15 beads were incubated in 100 µl of a 17:2:1 mixture of chloroform, trifluoroacetic acid, and triethylsilane for 2 hr at room temperature. The cleavage solution was then removed in vacuo, leaving about 750 nmol of each compound. About 165 equivalents of thiol-labeled small molecule was incubated with Cy3-BSA-maleimide in PBS for 6 hr at room temperature. Following a 1 hr incubation with 200 mM 2-mercaptoethanol, the conjugates were dialyzed extensively against PBS, yielding Cy3-BSA-AP1497, Cy3-BSA-AP1767, and Cy3-BSA-AP1780.

Anti-DIG, streptavidin, and FKBP12 were spotted in quadruplicate on aldehyde slides and processed as described above. To probe the slides, the doubly labeled BSA conjugates were diluted into PBST supplemented with 1% BSA (w/v) at a concentration of 10 µg/ml. 0.55 ml of protein solution was applied to the slide, using a PC500 CoverWell incubation chamber. Following a 1 hr incubation at room temperature, the slides were rinsed with PBS and then washed 3 times for 3 min each with PBST. The slides were rinsed twice with PBS, centrifuged at 200 g for 1 min to remove excess buffer, and imaged on an ArrayWoRx fluorescence slide scanner as above.