# How to Print Protein Microarrays With the BioOdyssey™ Calligrapher™ MiniArrayer

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## Introduction

While microarrays have become a common tool in molecular biology and gene expression analysis, only recently has this valuable technique gained attention in the field of proteomics. This paper, divided into three sections, will help the novice protein arrayer decide what and how to print. The first section presents initial considerations and includes a table with pertinent topics reviewed in recent papers. Next is a detailed synopsis of the protocols currently being used by investigators, with specific conditions that researchers at Bio-Rad use in the laboratory. The third section is a glossary of terms commonly used in the protein array field. Using this information, printing protein arrays with the BioOdyssey Calligrapher miniarrayer for the first time should be a less daunting task.

## Initial Considerations

Before starting to print, refer to Table 1. Then consider the following points:

- Method of detection
- Size of spot
- Method of sample preparation

The choice of detection method and spot size are interrelated. The most common method of detection is based on Cy dye fluorescence, which is detected using a laser-based scanner or other imager. Small spot diameters (120 µm) require a high-resolution (5–10 µm) laser scanner for imaging, but allow printing of higher density arrays. Larger spot diameters (~400 µm or greater) allow use of instruments such as the Molecular Imager® PharosFX™ system for imaging, but reduce the number of spots that can be printed.

Sample preparation is application dependent. Many laboratories prepare tissue or cell lysates in buffers containing sodium dodecyl sulfate (SDS), Tween 20, or guanidine. It might also be useful to prepare your lysates in a non-detergent-based buffer and to disrupt the cells by sonication. The choice will, in part, be determined by the detection method selected.

If the proteins are not denatured, they should be maintained at low temperature to avoid denaturation. The work surface of the Calligrapher miniarrayer, including both the source plate area and the slide section, can be cooled to ~12°C. Even with denatured samples, the work surface can be cooled to 20°C to minimize evaporation, thereby maintaining the sample at the proper concentration.

## Methods

### Protein Printing Protocol

**Step 1. Pin selection.** Solid or quill pins may be used for arraying. Solid pins are often used to avoid contamination between different samples, but quill pins can be used if stringent washing is performed. It is important to remember that during spotting of the array, washing the pins takes the most time. If quill pins are selected, identical spots can be printed repeatedly, eliminating the need for long washing steps.

**Tip:** When designing a source plate, arrange the dilution series such that the most dilute sample is spotted first and the most concentrated last. If spotting with solid pins, wash only between unique samples. Calligrapher™ BackTracker™ software assists in designing the plate.

**Step 2. Substrate selection.** The most commonly used slide for protein arrays is a nitrocellulose-coated glass slide (FAST slide; Whatman, Grace BioLabs). Other formats include three-dimensional surfaces (Full Moon Biosystems), silylated slides (CEL Associates), poly-L-lysine slides, and superaldehyde slides (TeleChem International).

Many companies (for example, Whatman and Grenier) produce slides with multiple pad formats. These are convenient for establishing assay conditions and for assessing different serum samples. To program the Calligrapher miniarrayer to print on these slides, enter the parameters as shown in Figure 1.

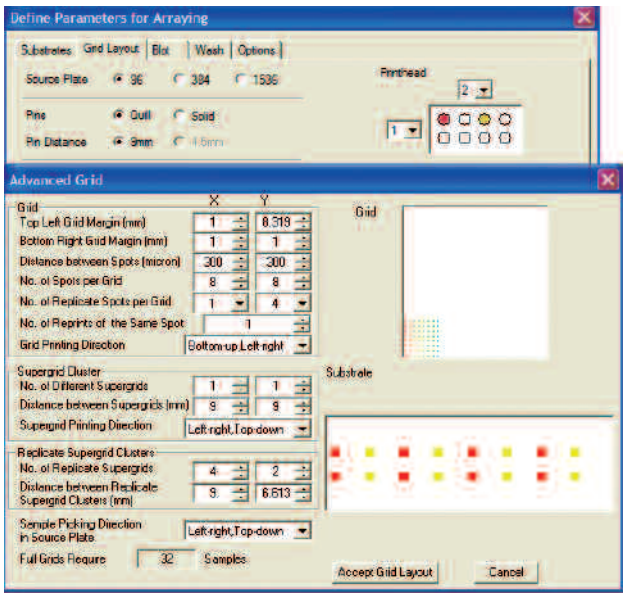


**Fig. 1. Parameters for printing onto pad-type slides.** Note the margin and supergrid distances.

**Step 3. Test print.** If this is the first print run, it is important to optimize the Calligrapher miniarrayer for printing. Prepare the detection molecule in a source plate at an appropriate concentration to be visualized. Place pins, properly conditioned, into the printhead and set up a printing program to test.

**General Recommendations —** Use PBS or TBS, pH 7.5, with 0.1% Tween 20 to prepare antibodies for spotting. Design arrays so that each pin prints a sample in triplicate and then prints buffer only in triplicate (Figure 2, upper screen). In the options window, reduce the z-offset by –.015 (Figure 2, lower screen). Using this robot file, prepare a BackTracker file (Figure 3) that will allow a source plate design containing only Cy5-labeled anti-goat IgG (1 mg/ml, Sigma, diluted 1:5,000 in PBS containing 0.1% Tween 20) and buffer. Load and run the arraying file. After the print run, dry the slide for 15 min at room temperature before imaging. If the spots have the appearance of a pig snout (Figure 4), adjust the z-offset by another –.015. Quantitate the spots to ensure that they have equivalent spot intensity from pin to pin and spot to spot, with CV values of 15% or less. In addition, quantitate buffer-only spots to detect carryover contamination; signal intensities >5% warrant addition of 0.1% Tween 20 to the static water bath. When the quality of the print run is satisfactory, it is time to begin the experiment.

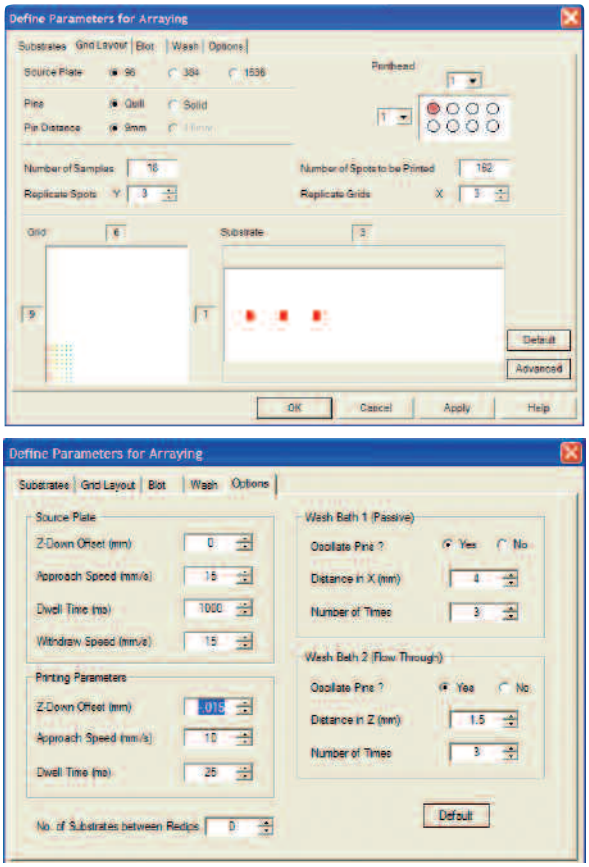


**Fig. 2. Arraying program.** Upper screen, grid layout window; lower screen, options window.

**Step 4. Sample preparation.** Samples can be prepared in most biological buffers (Table 1). The most common are PBS or TBS, pH 7.5. Addition of 0.1% Tween 20 or other nonionic detergent can help to solubilize the proteins. To denature proteins, detergents such as SDS or guanidine can be used. To denature cell samples and prepare extracts, use 2% SDS in 50 mM Bis-Tris, pH 6.8, containing β-mercaptoethanol, ProteaseArrest (G Biosciences), and phosphatase inhibitor cocktail set II (Calbiochem).

**Step 5. Arraying.** Array proteins according to their concentration. Use BackTracker software to generate a map of how to fill the source plate to ensure easy visualization of the array. Print each concentration in triplicate to ensure statistical reliability. The concentration of the protein should be as high as possible. Include positive and negative controls. A good positive control is an IgG that recognizes the secondary antibody.

**Table 1. Who's printing what?** Protocols from published papers showing the variety of ways protein arrays are being used, from immunoassays to biomarker discovery.

| Material Arrayed | Concentration of Material Arrayed | Sample Buffer | Substrate | Detection Protein |
|---|---|---|---|---|
| Different cloned and purified epitopes | 400, 100, 25.5, 0.05 mg/L; 40 pL/pin | PBS, pH 7.5 with 400 ml/L glycerol | Silylated slides (CEL Associates) | Cy5-conjugated goat anti-huma a Cy5 antibody labeling kit (GE |
| GST-fused, expressed coronovirus proteins | Dependent upon expressed level; measured with anti-GST antibody after spotting | — | FAST slides (Schleicher and Schuell) | Cy3- or Cy5-conjugated anti-h |
| Antibodies, antigens | 1.6–50 pg (antibody); 5–500 ng/ml (antigen); ~1 nl spotted | PBS | Poly-L-lysine or superaldehyde slides (Telechem, International) | Cy3 or Cy5 |
| 149 *Y. pestis* recombinant proteins | 100–200 µg/ml | PBS | Silylated glass slides (CEL Associates) | Cy5-conjugated goat antirabbi (1:1,000 dilution) generated us antibody labeling kit (GE Health |
| 35 antibodies | 1–5 mg protein per ml, arrayed with SMP8 spotting pins | Micro printing buffer (TeleChem International) | SuperEpoxy microarray slides (TeleChem International) | Eosin Y and Cy3 monofunction (GE Healthcare) were tested fo cells by directly incubating the |
| Protein antigens | Serial dilutions starting at 15 fmol and ending at 1.5 fmol of protein/spot; arrayed with 16 or 24 blunt-ended stainless-steel print tips with a tip diameter of 150 µm | 2% (w/v) BSA, TBST | FAST slides (Schleicher and Schuell) | 1:100 dilution of serum in 2% ( followed by tertiary antibody (C anti-mouse IgG) in 2% (w/v) BS terminal RGS-$His_6$ tagged anti |
| Recombinant histidine-tagged proteins | 1 mg/ml | 125 mM Tris-HCl (pH 6.8), 0.4% SDS, and 2% Tris-HCl (pH 6.8), 0.4% SDS, and 2% β-mercaptoethanol (Sigma-Aldrich) in a final volume of 25 µl | FAST slides (Schleicher and Schuell) | Cy5-conjugated streptavidin (1:1 Jackson ImmunoResearch Labo against total human serum (seria to 1:800 and 1:1,000; Jackson I Laboratories) included as contro |
| Reverse-phase protein lysate microarrays that included 60 cell lines plus controls | 10 serial 2-fold dilutions | — | FAST slides | Catalyzed signal amplification ( (DAKO), based on horseradish diaminobenzidine |
| NCI-60 cell lines lysed in buffer containing 9 M urea (Sigma-Aldrich), 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS; Calbiochem), 2%, pH 8.0–10.5 Pharmalyte (GE Healthcare), and 65 mM DTT (GE Healthcare) | Functional sensitivity (defined as the lowest concentration measured with a coefficient of variation of 20% within an array) is ~5,000 molecules/spot | Tris-HCl buffer | FAST slides (Schleicher and Schuell) | CSA system (DAKO); arrays st Ruby protein blot stain (Molecu detect total protein |

**Fig. 4. Pig snout formed by improper z-height of the solid pins.**

**Step 6. Processing the arrays.** After printing, air-dry the slides. If the slides are not used immediately, store under appropriate conditions (see Storage Conditions in Table 1). To process arrays, first block unreacted sites, then incubate with the partner protein, which may also serve as the detector molecule. In some cases, a third and occasionally a fourth incubation are required. The arrays can be rinsed 3 times with excess TBST or PBST between incubation steps.

Total protein printed can be assessed by staining the array with SYPRO Ruby protein blot stain followed by imaging.

**Step 7. Imaging the arrays.** In general, to obtain an accurate measurement of spot intensity, the resolution of the arraying device should be 10-fold less than the spot diameter. If the spots are 100 µm, a 10 µm scanner should be adequate. For larger spot sizes, 400 µm or more, less sensitive but more flexible imagers, such as the Molecular Imager PharosFX or VersaDoc™ MP system, can be used (for more information, refer to bulletin 5436).

#### Bio-Rad Laboratories Technique

After arrays are printed onto FAST slides, the print date and slide number are recorded with a diamond pen on the bottom of each slide. If the entire slide is processed, it is rinsed briefly with PBST to wet the membrane and then transferred to a

| | Imager | Storage Conditions | Authors | Title | Journal Citation |
|---|---|---|---|---|---|
| an IgG generated using Healthcare) | Laser scanner | 4°C; used within 2 weeks | Chen Z, Pei D, Jiang L, Song Y,Wang J, Wang H, Zhou D, Zhai J, Du Z, Li B, Qiu M, Han Y, Guo Z, Yang R | Antigenicity analysis of different regions of the severe acute respiratory syndrome coronavirus nucleocapsid protein | Clin Chem 50, 988–995 (2004) |
| uman IgG | Laser scanner | –20°C | Zhu H, Hu S, Jona G, Zhu X, Kreiswirth N, Willey BM, Mazzulli T, Liu G, Song Q, Chen P, Cameron M, Tyler A, Wang J, Wen J, Chen W, Compton S, Snyder M | Severe acute respiratory syndrome diagnostics using a coronavirus protein microarray | Proc Natl Acad Sci USA 103, 4011–4016 (2006) |
| | Laser scanner | — | Sreekumar A, Nyati MK, Varambally S, Barrette TR, Ghosh D, Lawrence TS, Chinnaiyan AM | Profiling of cancer cells using protein microarrays: discovery of novel radiation-regulated protein | Cancer Res 61, 7585–7593 (2001) |
| t IgG ing a Cy5 hcare) | Laser scanner | 4°C | Li B, Jiang L, Song Q, Yang J, Chen Z, Guo Z, Zhou D, Du Z, Song Y, Wang J, Wang H, Yu S, Wang J, Yang R | Protein microarray for profiling antibody responses to *Yersinia pestis* live vaccine | Infect Immun 73, 3734–3739 (2005) |
| al reactive dye r labeling *Salmonella* cells with the dyes | Laser scanner | Printed slides stored at room temperature in a slide box up to a week before use | Cai HY, Lu L, Muckle CA, Prescott JF, Chen S | Development of a novel protein microarray method for serotyping *Salmonella enterica* strains | J Clin Microbiol 43, 3427–3430 (2005) |
| w/v) BSA, TBST Cy3-conjugated rabbit SA, TBST: amino- body (Qiagen) | Laser scanner | — | Lueking A, Huber O, Wirths C, Schulte K, Stieler KM, Blume-Peytavi U, Kowald A, Hensel-Wiegel K, Tauber R, Lehrach H, Meyer HE, Cahill DJ | Profiling of *Alopecia areata* autoantigens based on protein microarray technology | Mol Cell Proteomics 4, 1382–1390 (2005) |
| 100 and 1:1,000, oratories) and antibodies al dilutions from 1:100 mmuno Research l proteins | Laser scanner | 4°C | Sreekumar A, Laxman B, Rhodes DR, Bhagavathula S, Harwood J, Giacherio D, Ghosh D, Sanda MG, Rubin MA, Chinnaiyan AM | Humoral immune response to α-methylacyl-CoA racemase and prostate cancer | J Natl Cancer Inst 96, 834–843 (2004) |
| CSA) system peroxidase and | — | — | Nishizuka S, Chen ST, Gwadry FG, Alexander J, Major SM, Scherf U, Reinhold WC, Waltham M, Charboneau L, Young L, Bussey KJ, Kim S, Lababidi S, Lee JK, Pittaluga S, Scudiero DA, Sausville EA, Munson PJ, Petricoin EF 3rd, Liotta LA, Hewitt SM, Raffeld M, Weinstein JN | Diagnostic markers that distinguish colon and ovarian adenocarcinomas: identification tissue array profiling | Cancer Res 63, 5243–5250 (2003) |
| ained with SYPRO ular Probes) to | FluoroImager SI system (GE Healthcare) at 100 µm resolution | | Nishizuka S, Charboneau L, Young L, Major S, Reinhold WC, Waltham M, Kouros-Mehr H, Bussey KJ, Lee JK, Espina V, Munson PJ, Petricoin E 3rd, Liotta LA, Weinstein JN | Proteomic profiling of the NCI-60 cancer cell lines using new high-density reverse-phase lysate microarrays | Proc Natl Acad Sci USA 100, 14229–14234 (2003) |

Coplin slide jar containing 5% nonfat dry milk (Bio-Rad catalog #170-6404) in PBST. The slide is incubated, with shaking, for 1 hr at room temperature or overnight at 4°C. When pad slides or arrays that have been designed for a FAST frame (Whatman) are used, the appropriate areas are blocked.

**Tip:** After preparing the blocking reagent, centrifuge the reagent to eliminate insoluble material. This reduces background fluorescence.

Primary antibodies are diluted in blocking buffer at the concentration recommended for immunofluorescence. When a whole slide is used, the slide and antibody solution are placed into an appropriate vessel and incubated with shaking for 1 hr at room temperature. The FAST frame requires 60–70 µl per well, and can be sealed with optical sealing tape to prevent evaporation.

After washing the slide 3 times for 5 min with PBST, the Cy5-labeled secondary antibody is applied, following the directions for primary antibodies. The slide is incubated in the dark (for example, wrapped in foil), with shaking, for 1 hr at room temperature. After washing with PBST 3 times for 5 min, the slides are placed in a slide minicentrifuge to spin off excess PBS, then air-dried and imaged.

## Glossary

Antibody (Ab) — A molecule produced by an organism's immune system as a defense against foreign molecules (i.e., molecules not recognized as produced by the organism). Antibody molecules are composed of a variable region and a constant region

Antibody constant region (Fc) — The portion of an antibody that is used by the host organism after the antibody binds to the antigen. It is specific for the species it is produced in

Antibody variable region (Fv) — The portion of an antibody that binds to a specific epitope. This region consists of a heavy chain and light chain that interact to bind the antigen

Antigen — A molecule or portion of a molecule that stimulates the formation of an antibody

BSA — A protein, bovine serum albumin

Continuous epitope — An epitope that is formed by adjacent amino acids; tertiary structure plays little or no role

Discontinuous epitope — An epitope that is formed by nonadjacent amino acids; proper tertiary structure is required for the antibody to bind the epitope

Epitope — The portion of an antigen that an antibody binds to

Forward-phase protein microarray (FPPM) — A technique in which an antibody specific to an analyte (protein, antigen) is spotted on a slide. The slide is incubated with a sample carrying the protein of interest. After washing, the slide is incubated with a second antibody that is specific to the captured protein. This antibody may be labeled with a detection molecule such as Cy3. If it is not labeled, the slide is incubated with another molecule, such as a species-specific immunoglobulin, that is labeled with a detection molecule. This technique is also known as a sandwich assay or indirect ELISA

Immunogen — An antigen

Immunoglobulin (Ig) — An antibody; several classes of Igs exist, including IgG, IgM, IgE and IgA. IgG is the most common for research purposes

PBS — A buffer, phosphate buffered saline

Primary antibody — In an immunoassay, this is an antibody that is generated against the protein of interest

Reverse-phase protein microarray (RPPM) — A technique in which a denatured analyte (protein, antigen) is spotted on the slide. The slide is incubated with an antibody specific for the protein. This antibody may be labeled with a detection molecule, such as Cy3

Secondary antibody — In an immunoassay, this is the antibody that recognizes the primary antibody and may be labeled with a detection molecule

Species-specific antibody — An antibody that recognizes the Fc portion of antibodies of a particular species; especially useful for detection molecules

TBST — a buffer, Tris-buffered saline with Tween 20 (0.01%)

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