

The FAST Guide to Protein Microarrays

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1. Introduction.....	2
2. Array Processing	5
2.1. Array Printing	5
General Considerations	5
Contact Printing.....	7
Non-Contact Printing	8
Methods.....	8
2.2. The Binding Experiment (Assay).....	9
Blocking.....	9
General Considerations	9
Methods.....	10
Incubation with Sample/Target	12
General Considerations	12
Methods.....	13
Washing	14
2.3. Detection	14
Imaging.....	15
A Comment on Donuts.....	19
The 4 Pillars of Successful Protein Array Processing using FAST Slides	19
3. Troubleshooting.....	21
4. Further Information	25
5. References	26

The FAST Guide to Protein Microarrays

1. Introduction

High-throughput DNA microarrays have provided a breakthrough in measuring expression levels of genes. However, it is difficult, if not impossible, to predict the highly dynamic cellular environment from transcription levels only. In fact, there is no reliable correlation between gene activity and protein concentration (Spector et al. 1994, Anderson and Seilhamer 1997). Post-translational modifications, which are of paramount importance for protein activity, cannot be deduced directly from DNA studies. Therefore, protein arrays are increasingly recognized as valuable tools in proteomics.

FAST Slides are the preferred surface for designing and building protein microarrays. The FAST Slide surface is a proprietary nitrocellulose-based polymer that offers excellent reproducibility and sensitivity for microarray assays. Unlike other microarray surfaces, FAST Slides have a 3-dimensional polymer coating, that has been used for immobilization of biomolecules for decades. The properties of the material, in combination with the very high surface area of its unique 3-dimensional structure (Fig. 1), enable higher binding capacities than planar and other 3-dimensional surfaces. Furthermore, the 3-dimensional nature of the matrix allows for retention of arrayed protein in near-quantitative fashion. This leads to linear increases in signal intensities in response to increased concentrations of printed proteins over a very wide range of concentrations (Fig. 2). This is especially important for the development of quantitative protein microarray assays for research and diagnostic applications. The high binding capacity leads to an unparalleled sensitivity of detection in the zeptomol range (10^{-21} mol, i.e. 3000-5000 molecules/spot, Pawletz et al. 2001, Nishizuka et al. 2003). Nitrocellulose formulations from Whatman have proven their reliability in millions of Western Blots and for the manufacturing of lateral flow immunoassays (also referred to as immunochromatographic assays), such as pregnancy tests, in the diagnostic industry.

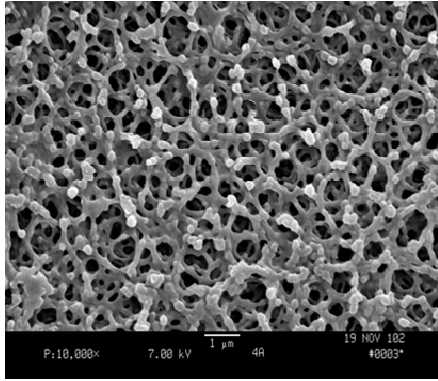


Fig. 1: 3D Structure of Whatman nitrocellulose, scanning electron microscopy, magnification 10000x

Nitrocellulose (NC) is an established platform for immobilizing and characterizing proteins under denaturing and non-denaturing conditions. It has been used for the detection of protein-protein (including antigen-antibody), protein-DNA, protein-RNA and protein-ligand interactions. FAST Slides are compatible with all of these reactions. Often protein arrays on FAST Slides are carried out in non-denaturing conditions. Unlike blots, proteins are directly arrayed and do not undergo SDS-PAGE and electro-transfer, procedures which can result in loss of protein activity. Contrary to conventional wisdom, proteins arrayed and dried on the nitrocellulose matrix of FAST Slides usually maintain native molecular recognition activity. This has been impressively demonstrated by publications investigating protein-protein interactions (Espejo et al. 2002, Schweitzer et al. 2003, Espejo and Bedford 2004).

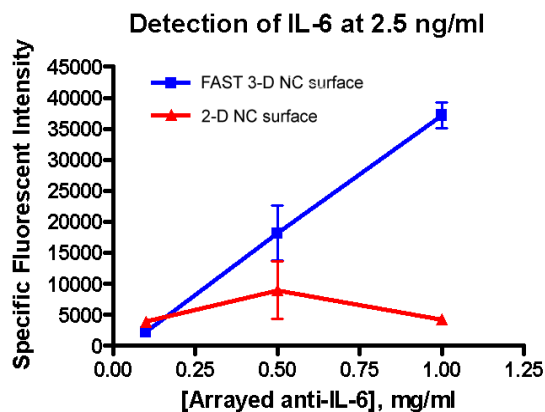


Fig. 2: Anti-IL-6 antibodies were printed from different source concentrations on FAST Slides and on slides coated with a 2-dimensional NC film, respectively. The arrays were incubated with solutions of recombinant IL-6 at 2.5 ng/ml. Detection was performed using a biotinylated anti-IL-6 antibody and streptavidine-Cy5. In contrast to the 2-D NC film, FAST Slides show a linear signal increase reflecting increased amounts of immobilized antibody.

Protein arrays comprise a wide variety of experimental designs. Antibodies may be arrayed as capture molecules to perform microspot ELISA-type experiments for quantitative profiling of protein expression (Fig. 3A) or for detecting the presence of their antigens in complex lysates after direct or hapten labelling (Fig. 3B). Recombinant or purified proteins can be immobilized to study protein-protein interaction (Fig. 3C) or to probe sera for the presence of specific antibodies (Fig. 3D). The latter was shown to be a suitable approach to study allergy (Kim et al. 2002) or autoimmune diseases (Gutjahr et al. 2005, Lueking et al. 2005). In so called reverse arrays, complex tissue or cell lysates (or fractions thereof) are immobilized and probed with a number of antibodies to profile the presence of antigens in many samples under identical conditions (Fig. 3E). It is obvious that a general protocol for array fabrication and processing will hardly be applicable for all experiments that can be realized on FAST Slides. The scope of this guide is to provide a foundation for the researcher to develop a protocol which suits his specific needs. All recommendations may require individual adaptation for optimization. However, we hope that the information provided will save a researcher time, and give guidelines for an optimum start to successful protein microarray assays and experiments.

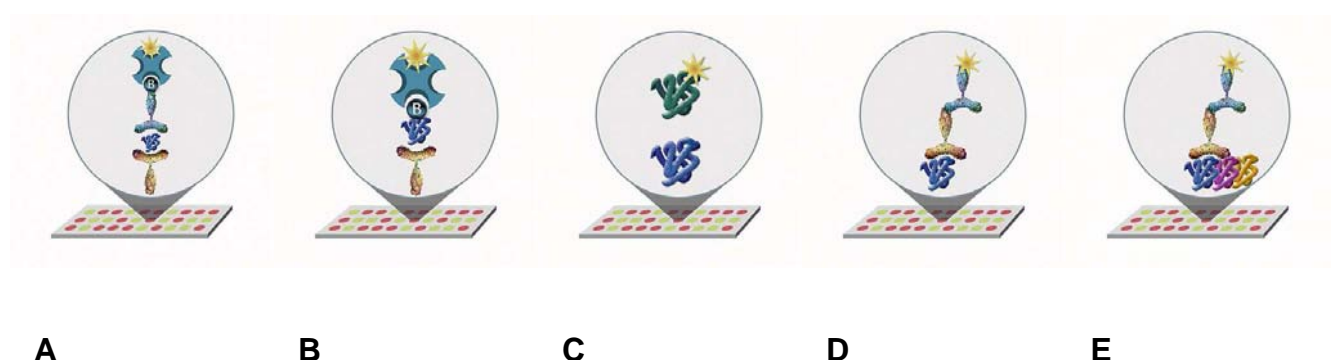


Fig. 3: Protein microarrays can be used for many different applications. (A) Antibody arrays can be used in sandwich ELISA-like assays or (B) to capture specific antigens which are directly labelled with a hapten. (C) Purified or recombinant proteins can be arrayed to study protein-protein interaction or (D) to probe serum samples for antibodies (e.g. to study allergy or autoimmune diseases). (E) Reverse-phase arrays are used to profile dozens or hundreds of arrayed samples (e.g. cell lysates) for the presence of a small number of antigens.

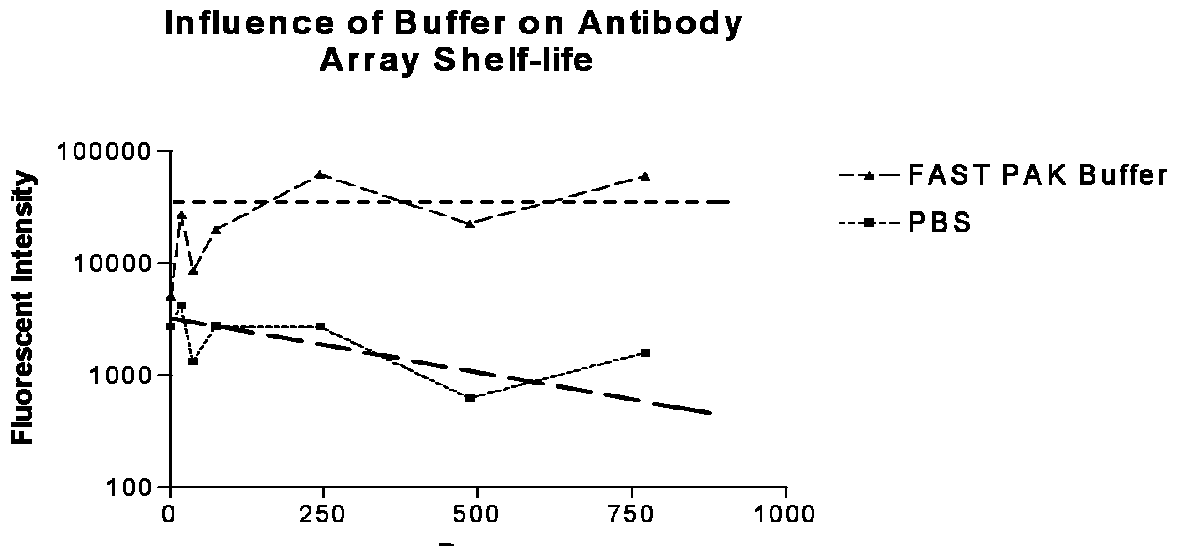


Fig. 4: Antibody against creatine kinase was printed on FAST Slides with Whatman Arraying Buffer or PBS. Detection of Cy5 labelled creatine kinase at different times after printing is shown. A marked decrease of the amount of creatine kinase bound was observed when PBS was used for printing of antibodies while no change could be detected with Whatman Arraying Buffer over a time period of 771 days.

2. Array Processing

2.1. Array Printing

General Considerations

Buffers

Protein arrays may utilize different kinds of capture molecules. Depending on applications, antibodies, antigens (recombinant or purified proteins or complex cell lysates) or other binding molecules may be arrayed. A suitable arraying solvent must keep the protein in a state to maintain its molecular recognition properties. Hence, it must consist of a buffer with suitable pH and ionic strength, and it may contain other stabilizing agents like protease inhibitors, chelators, etc.. In many cases PBS is a suitable basis, however, we recommend Whatman Arraying Buffer (cat. no. 10 485 331) which promotes long term stability of printed proteins (Fig. 4).

Buffer Additives

Some applications may require the presence of detergents and/or chaotropes (e.g. urea) for cell disruption and/or solubilization of proteins. Generally, substances like these are compatible with FAST Slides. Even solubilization buffers originally designed for 2D electrophoresis (containing 6 M urea and 2% CHAPS) have been successfully applied for array printing (Nishizuka et al. 2003). In this case the resulting protein solutions may be very viscous, causing further demands on the printing robot and pins. The use of DMSO is generally not recommended because high concentrations of DMSO can negatively affect the NC coating of FAST Slides and even destroy it. If DMSO is added to the printing buffer, final concentrations should not exceed 5%.

Protein Concentration

Source plate concentrations may vary from protein to protein. For capture antibodies, a concentration between 250 and 1000 µg/ml was found to be optimal for most applications. However, some formats may require vastly different concentrations. Testing the effect of increasing protein concentrations in an assay system is a typical step in assay development.

Drying Time

It has been found that stability of protein binding to the NC matrix increases with drying time. Hence, it is strongly recommended to store the slides overnight desiccated at room temperature after arraying, to maximize binding of the printed proteins before use (overnight curing).

Spot Size

Generally, the diameter of spots should be as small as possible because this allows for a higher analyte density and in consequence a better signal-to-noise ratio (Ekins and Chu 2003). However, there are technical constraints and theoretical limitations. For lysate arrays, the number of molecules of low abundant proteins may be too low for detection when the total amount of spotted lysate is very small. Technical constraints can come from viscosity of the sample that may dictate the choice of printing system, or from the resolution of the detection system. For quantitative analysis of array images, the pixel size should be no more than 1/10 of the spot diameter (i.e. at

least 10 pixels across the spot's diameter). Isotopic detection (autoradiography on x-ray film or image phosphor screens) and chemiluminescence have generally much lower spatial resolution than colorimetric or fluorescence detectors (CCD camera or scanner systems). If arrays are produced for quantitative measurement using these detection methods, spot size should be large enough to allow for the "10 pixel across the spot" rule. The spot pitch should be large enough to avoid spot overlap during detection, taking into consideration the possibility of lateral "bleeding" of signal onto substrates such as X-ray film.

Contact Printing

Contact printing utilizes pin-type arrayers that transfer a defined volume of sample by directly touching the surface of the slide. Despite the relatively soft NC surface of FAST Slides, this can be done without physically damaging the coating if the arrayer settings are appropriately adjusted. It is recommended to use contact arraying systems that feature free-floating pins in their print-heads, as opposed to spring-loaded pin mechanisms. Contact printers are usually simpler in design, less expensive, and faster than non-contact printers. They may be the arrayer of choice when large numbers of samples are to be spotted. Split pins and quill-type pins have been successfully used for printing proteins on FAST Slides; however, cleaning may cause problems because of higher viscosity and adhesiveness of proteins compared to nucleic acids. Addition of a surfactant such as Bioterge AS-40 in a very low concentration (e.g. 0.025 %) was found to be advantageous when using quill pins.

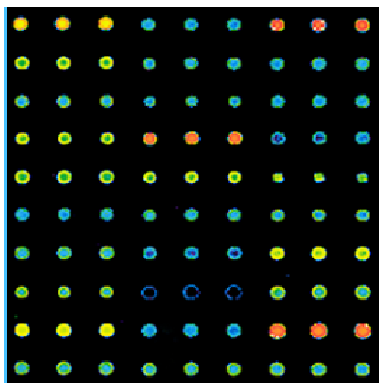


Fig. 5: Single-capture antibody array printed with a contact printer: Monoclonal antibodies against human biomarker proteins were printed in triplicate on a FAST Slide using Whatman Arraying Buffer. Detection of multiple proteins in human serum by direct labelling of the serum sample with DY647 fluorophore; spot diameter 110 μm .

Ring-and-pin printers are a variant of contact printers. Samples are taken up from the source plate with rings mounted in front of the spotting pins. The pin passes through

the ring to deposit sample on the slide surface. This technology has been successfully used with very viscous tissue lysates containing high concentrations of urea and detergents (Nishizuka et al. 2003). Ring-and-pin printers were produced by Affymetrix (Genetic Micro Systems GMS 417) but were discontinued recently.

Examples of contact printers that have been successfully used for array production on FAST Slides include Q-array (Genetix), Flexys, Microsys, OmniGrid and MicroGrid (Genomics Solutions).

Non-Contact Printing

Non-contact printers can be syringe-based (solenoid) or piezo type. With these technologies, sample droplets are dispensed onto the slide, avoiding contact of the printer pins with the surface. Sample volume can be varied in steps by firing multiple times on the same spot. This technique enables very high reproducibility. Examples of systems which were successfully used with FAST Slides are Biochip Arrayer (Perkin-Elmer), GeSiM NanoPlotter, and Scienion sciFLEXARRAYER.

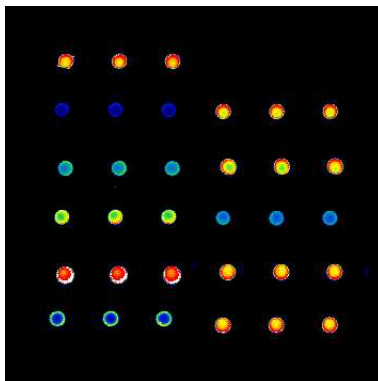


Fig. 6: Detection of multiple cytokines utilizing a sandwich assay and fluorescence detection. Monoclonal antibodies to cytokines were printed in triplicate in Whatman Arraying Buffer with a non-contact printer (triplicates from upper left to bottom right: Donkey anti-goat positive control, GM-CSF, IL-10, IL-1 β , IL-12p70, IL-2, MCP-1, IL-4, RANTES, IL-6, Donkey anti-goat positive control).

Methods

FAST Slides are ready for arraying straight from the box. Due to the nature of FAST Slides, no activation steps are necessary to immobilize proteins. Do not pre-wet FAST Slides. Arraying onto a wet slide will cause the spots to diffuse.

Attention to environmental conditions is critical for accurate spotting. Temperature and humidity should be controlled to avoid evaporation of source plate solution dur-

ing the arraying process, and, for contact printing, to avoid evaporation of sample within quill pins during pin travel. On the other hand, too high humidity may cause problems with water condensation. Refer to the manual of the arraying system.

Pure proteins should be arrayed using a source plate concentration of 0.05–1 mg/ml. A concentration between 250 and 1000 µg/ml is optimal for most applications. For antibodies, the upper end of this range is recommended. If using a fluorescent scanner for subsequent detection, the spot diameter should be no more than 250 micrometers (Telechem Chipmaker2 and Chipmaker3 pins have yielded excellent results on FAST Slides), and the array pitch (distance of neighbouring spots from center to center) can be as low as 300 micrometers. For chemiluminescent or isotopic detection, spot diameter can be significantly larger than for fluorescent detection. However, a pitch of 1000 micrometers or greater will be needed to allow for sufficient resolution. FAST Slides arrayed with antibodies should be stored desiccated overnight at room temperature, in order to maximize the binding of the immobilized protein. For some antibodies, drying overnight at 37 °C may be advantageous. Drying conditions should be determined empirically for individual proteins.

If the arrayed FAST Slide will not be used immediately, store in a cool, low humidity environment (10–30% RH) protected from light.

2.2. *The Binding Experiment (Assay)*

Blocking

General Considerations

The blocking step is carried out after arraying and before addition of the sample solution. Many researchers utilize blocking protocols identical to those used with Western Blots on NC membranes. The type of blocker used will depend on the nature of the experiment. A physiological buffer (1x PBS or 1x TBS) containing 1–5% non-fat milk is compatible with isotopic and chemiluminescent detection. For fluorescent detection, 1x TBS containing 0.1% Tween 20 (1x TBS-T) may be sufficient. The percentage of Tween 20 may be increased to 2% if needed. Preliminary experiments should

be conducted to determine the optimal blocker and concentration for fluorescent systems. Some blockers can add to the fluorescent background. For this reason, blocking buffers containing protein, such as BSA, casein or non-fat dry milk, should be examined on an empirical basis. 1% BSA (e.g. Sigma cat. no. A-7638, "cold alcohol precipitation fractionation, prepared from Fraction V bovine albumin") + 0.05% Tween 20, in PBS (pH 7.2-7.5) is a good starting point. Casein-based solutions are highly efficient blockers due to a statistical distribution of molecules of different sizes. However, because of poor solubility, casein-based blocking bears the risk of speckles on the arrays due to precipitates.

Whatman Protein Array Blocking Buffer (cat. no. 10 485 356) is compatible with all detection methods and is recommended for its superior blocking power and the resulting low non-specific binding and background (Fig. 7).

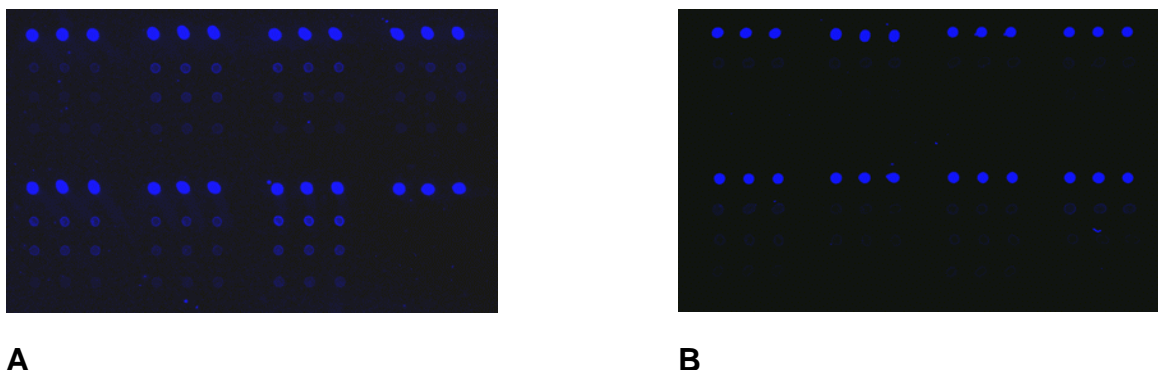


Fig. 7: Monoclonal capture antibodies were arrayed on FAST Slides and incubated with an antibody cocktail consisting of 16 biotinylated polyclonal antibodies followed by Streptavidin-Cy5 detection. Both images were taken at identical laser/PMT settings. The first row of each field (3 columns x 4 rows) is a positive detection control. Unspecific binding of biotinylated antibodies can be detected when blocking is performed with 0.1% Tween 20 in TBS (A). This unspecific interaction is reduced by the Protein Array Blocking Buffer (cat. no. 10 485 356, image B).

Methods

For blocking, as for all incubation steps, FAST protein array incubation chambers are recommended. Protein array incubation chambers are available in 3 different formats: single-well, double-well and 16-wells (16-well chambers fit 8-pad and 16-pad slides). To use the incubation chambers, place the appropriate chamber onto a FAST

Slide and insert it into a FAST Frame (cat. no. 10486001, Fig. 8) or ChipClip (cat. no. 10486081). Add the appropriate volume of blocking solution, depending on the slide and chamber type (see Table 1). For blocking and washing steps, the max. volumes are recommended. Incubate at appropriate temperature with gentle agitation, ensuring that mixing occurs. Fast motion should be avoided. A speed of ≤ 40 rpm is recommended with most shaking devices. Depending on the sample, blocking times can range from 15 min to overnight and need to be determined empirically. For long incubation times (i.e. hours) care must be taken that the slides do not dry out. The use of a humid chamber (e.g. a zip-lock bag with wetted paper towel) is highly advisable.

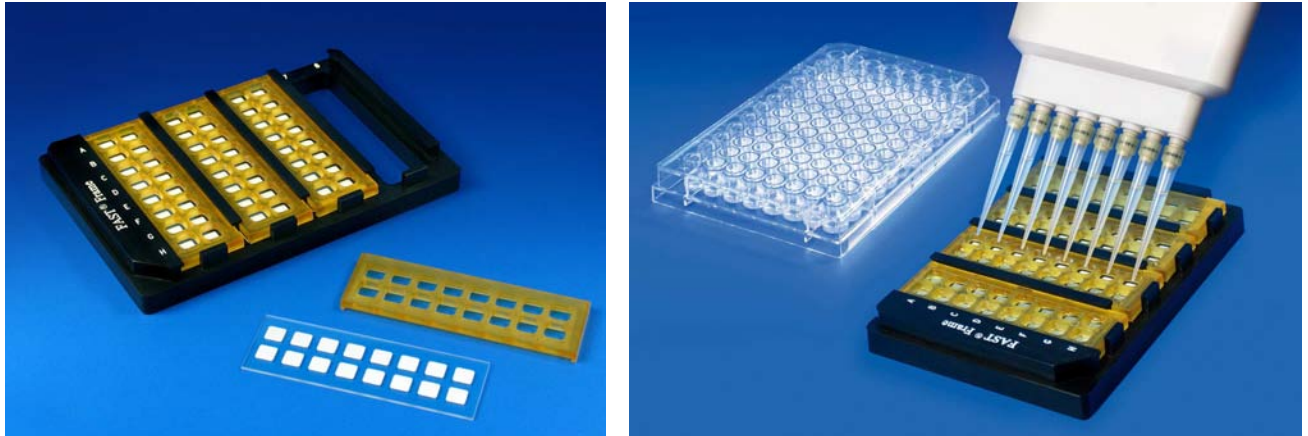


Fig. 8: The FAST Frame (cat. no. 10486001) is a reusable holder for up to four slides. Having the footprint of a microtiter plate it makes handling of FAST Slides like that of a standard ELISA.

Table 1: Suitable liquid volumes for FAST incubation chambers

Format	Volume (min-max)
1 Pad	700-2000 μ l
2 Pad	250-1000 μ l
16 Pad (=8 Pad)	70-90 μ l

Some researchers perform incubation without using incubation chambers by simply covering the slide with liquid kept in place by encircling the array with a hydrophobic marker pen (e.g. PAP pen, Kukar et al. 2002). With this method evaporation may occur even for short incubations. The arrays can be protected by addition of a cover slip (Kersten et al. 2003). In this case, it is necessary to remove the cover after incubation for further processing. This must be done very carefully to avoid damaging the surface of the array. Additionally, it is recommended to prolong incubation times because these methods rely only on diffusion of reagents.

Single-pad FAST Slides have been processed automatically using systems designed for processing of immunohistochemistry slides (Paweletz et al. 2001) or automated hybridization chambers (e.g. GeneTac Hybridization Station) for processing of DNA microarrays (Madoz-Gúrpide et al. 2001).

Incubation with Sample/Target

General Considerations

In many applications, pure capture molecules (antibodies, recombinant proteins) are immobilized on the slide surface. Generally, this array is incubated with a complex solution to be analyzed for the presence (and concentration) of specific binding partners. In the following, the defined capture molecules will be referred to as *probes*. The complex solution containing target molecules for these probes will be referred to as *sample*. In lysate arrays, complex samples are immobilized on the solid phase and analyzed with solution-phase probes. Based on this nomenclature lysate arrays are often referred to as reverse-phase arrays.

Buffers

In order to define a suitable solvent for the sample molecules, the same general considerations apply as for the printing buffer: it must keep the proteins in a state to maintain their molecular recognition properties. In addition to stabilizing agents, it is recommended to include a blocking agent. An appropriate buffer for sample incubation can be PBS (pH 7.2-7.5) containing 0.05% Tween 20, 0.1% BSA, and including

other additives such as protease inhibitors. This may also serve as a generic dilution buffer if samples are to be diluted. A 1:2 dilution (i.e. mix of equal volumes) of serum samples using this buffer was found to reduce matrix effects. Hence, this dilution does not result in a 50% loss of sensitivity as would be expected.

Sample Concentration

Protein concentrations of the sample will vary based on the nature of the experiment. In microspot ELISA-type assays, they will be governed both by concentration of the molecules to be measured and affinity of the arrayed antibodies for them. Hence, individual assay conditions must be determined empirically. Different sample dilutions (e.g. 1:2, 1:4,...) should be tested.

Incubation Times

The time needed to establish equilibrium binding will vary with the type of experiment. If probing with an antibody in an experiment analogous to a Western Blot, 1–2 hours at room temperature are usually sufficient. Other types of samples may need to be incubated overnight in order to maximize intensity of interaction. Appropriate conditions should be determined empirically. Depending on assay configuration, there may be 2 or more separate incubation steps, each separated by washing steps.

Methods

For general recommendations on incubation methods, please refer to paragraph *Blocking->Methods*. Incubation times for the samples being tested are generally the longest step in the protocol (from one hour to overnight). Hence, care must be taken to prevent the arrays from drying out. A microtiter plate lid can be used to cover the FAST Frame. The use of a humid chamber is highly advisable (zip-lock bag or similar). In some shaking devices evaporation of samples and condensation of water under the lid can occur due to heat production from the instrument's motor. This will in turn increase concentrations of analytes in the sample leading to errors in quantitative assays. In this case, a mat of polystyrene foam should be placed under the FAST Frame for insulation. If the sample is directly labelled with a fluorochrome, protection from light during incubation is also recommended.

Washing

A physiological buffer should be used that preserves the protein-protein or protein-nucleic acid interaction, yet washes away unbound sample. This may consist of the blocking solution used minus the blocking agent. Whatman Protein Array Wash Buffer (cat. no. 10 485 330) is an ideal wash reagent for protein microarrays. For most applications (including Western-based formats), 3-4 washes at room temperature with an incubation time of 1-20 min is sufficient. An alternative wash strategy is to use a plastic or glass container, instead of washing within the chambers. Devices designed for histological staining of microscope slides are well suited. This method is especially useful for the final wash in water before drying and scanning the slides as is recommended for fluorescent detection.

2.3. Detection

FAST Slides are compatible with essentially all detection methods (Fig. 9). Detection using fluorescent dyes is very convenient as it is simple, has high spatial resolution as well as very high sensitivity. Commonly used fluorophores include Cy3, Cy5, corresponding Alexa- and DY-fluorophores, Phycoerythrin and others. Infra-red fluorophores such as IR800 have also been used with excellent results (Calvert et al. 2004, Yeretssian et al. 2005). In general it has been observed that longer wavelength fluorophores such as Cy5 (and analogs) or IR800 are often advantageous. Many biomolecules present in blocking reagents and samples have an inherent autofluorescence and will bind to the surface thus contributing to background. This phenomenon is less pronounced when using red and far-red wavelengths for detection.

For some applications signal amplification will be necessary. FAST Slide have been used with rolling circle amplification followed by fluorescent detection (Zhou et al. 2004). Frequently, systems employing horseradish peroxidase (HRP) are used. With either HRP or alkaline phosphatase (AP), both chemiluminescent or chromogenic substrates can be used. If using AP-based chemiluminescent substrates, enhancers for nitrocellulose may be required, depending on the reagent system employed. Interestingly, the highest sensitivities on protein arrays reported so far were achieved by an enzyme amplified colorimetric method (Paweletz et al. 2001, Nishizuka et al

2003). The method of choice used for protein detection will depend on the application. For analysis of phosphorylation, labelling with γ - ^{32}P -ATP and detection by autoradiography is still considered the most reliable method.

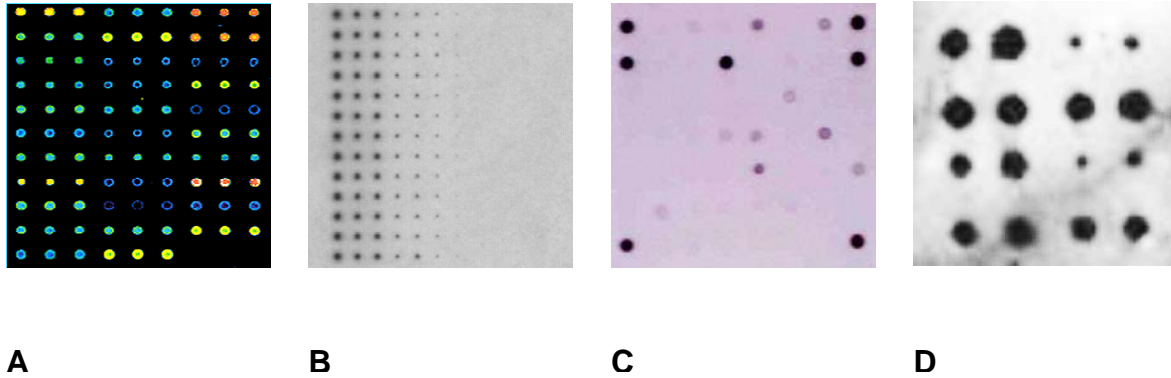


Fig. 9: (A) FAST Slides are compatible with fluorescent, (B) chemiluminescent, (C) colorimetric and (D) isotopic detection. A: Monoclonal antibodies against human bio-marker proteins were printed in triplicate on a FAST Slide using Whatman Arraying Buffer. Detection of multiple proteins in human serum by direct labelling of the serum sample with DY647 fluorophore; spot diameter 110 μm . B: Array of 1:2 dilutions of horseradish peroxidase enzyme on FAST Slide. Activity of arrayed enzyme measured via enhanced chemiluminescence reagents; spot size 150 μm . C: Array of peptide antigens on FAST Slide. Capture of antibodies from human serum; detection with anti-human-IgG alkaline phosphatase conjugate and chromogenic substrate; spot size 140 μm . D: Array of various protein kinases on FAST Slide. Detection of auto-phosphorylation (after incubation with ^{33}P -ATP + cold ATP at 30 $^{\circ}\text{C}$ for 80 minutes) by exposure to X-ray film; spot size 120 μm .

Imaging

Preparation of Slides

For chemiluminescent, colorimetric and isotopic applications, follow standard nitrocellulose membrane protocols for the specific detection reagents being used. In some instances (e.g. chemiluminescence and some staining protocols) the slide will remain wet and can be placed in plastic wrap or other material to prevent drying. For fluorescent detection using a microarray-based imager, the slide should be dried after the final wash. Excess water droplets on the edges can be removed gently with a lint-free tissue or a compressed N_2 stream. Be careful not to damage the surface as this may lead to background artifacts. An additional brief drying step at 80 $^{\circ}\text{C}$ for

5 min is optional. If heat is used, allow slides to cool for several minutes prior to scanning. An alternative drying method is to spin the slides briefly in a suitable centrifuge (2-3 minutes at 150 x g). Store slides in a dust-free dark place until imaging. If stored as recommended the signals remain stable over weeks and months after processing

Imaging Instruments and Image Resolution

FAST Slides can be read using a variety of laser scanners or CCD imaging systems. As a general rule of thumb, spot diameter should be at least 10x pixel size in order to sample sufficient data for a quantitative analysis. For arrays printed according to the recommended settings in 2.1., a resolution of 10 μm will be optimal. Imaging instruments such as gel-imagers usually work at considerably lower resolutions (pixel size 25 μm or larger). Thus, other imaging systems may require larger spot sizes if results are to be quantified. For optimum use of the dynamic range of the scanning system, it is recommended to save data using the maximum depth compatible with the evaluation software (e.g. 16-bit tiff-file).

Scanner Settings

When imaging FAST Slides, the default imager parameters for glass slides will not be suitable for detection. Due to the higher binding capacity of FAST Slides, as well as the unique light scattering properties of the polymeric surface, laser power and/or PMT settings (voltage, gain) will need to be set lower than for glass slides. If the scanner has confocal optics and focal depth adjustment, the focal depth should be optimized since the nitrocellulose coating is approximately 11 μm thick. On non-confocal systems this is not necessary as the fixed focal depth of field is usually larger than the thickness of the NC layer (e.g. the Axon GenePix 4100A has 40 μm depth of field). The laser and PMT settings will also depend on the type of experiment and blocking agent used. Typical starting parameters for some popular instruments are given in Table 2. For best results, these settings should be further optimized. In order to take full advantage of the dynamic range of the scanner, signal intensities should be as high as possible without reaching or exceeding the maximum of the system (i.e. pixel intensity = 65535 on a 16-bit system). Signals exceeding this limit (i.e. saturated spots, Fig. 11) cannot be analyzed quantitatively.

Table 2: Laser and amplifier settings

Scanner Model	Laser Power	PMT (Gain)
GenePix 4100A	not variable	400
GenePix 4200A	95	400
Tecan LS200	not variable	95
Perkin Elmer ScanArray 4000	80	40

An untreated slide will show some “autofluorescence” due to scattering and reflection of light by the NC coating. However, this light reflection is very homogeneous and can be easily subtracted by most analysis software programs (Fig. 10). Thus this baseline shift does not significantly reduce sensitivity and dynamic range of the experiment. It has been suggested that the reflective properties of the FAST Slides have a signal enhancing effect in fluorescent measurements (Madoz-Gúrpidé et al. 2001).

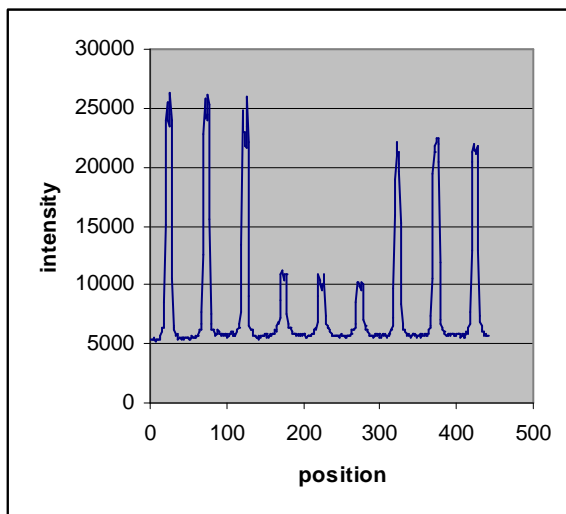
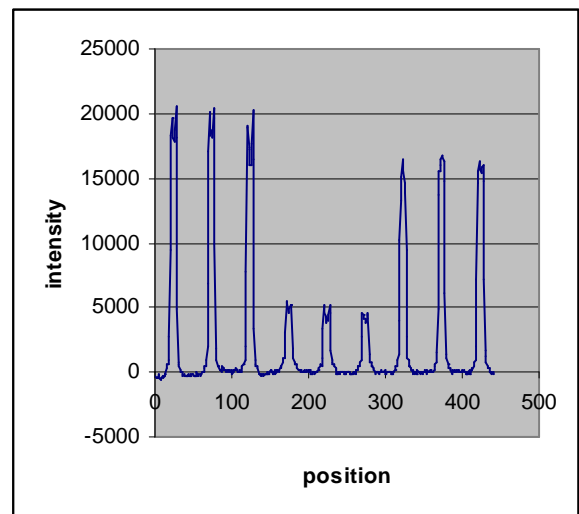
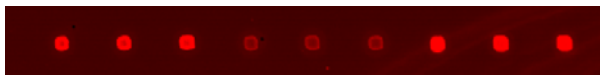
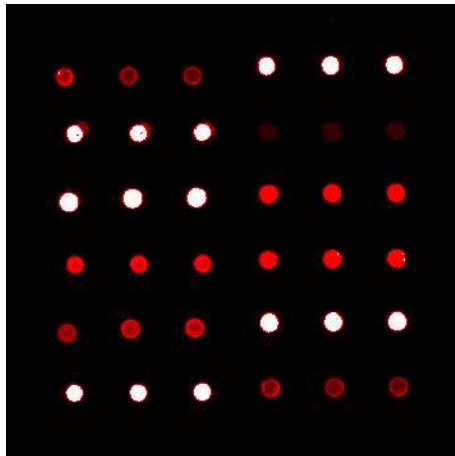
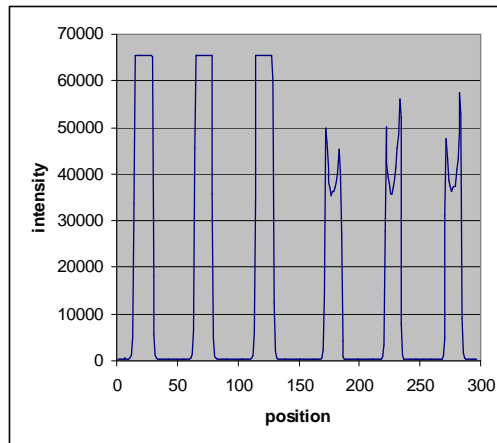
**A****B****C**

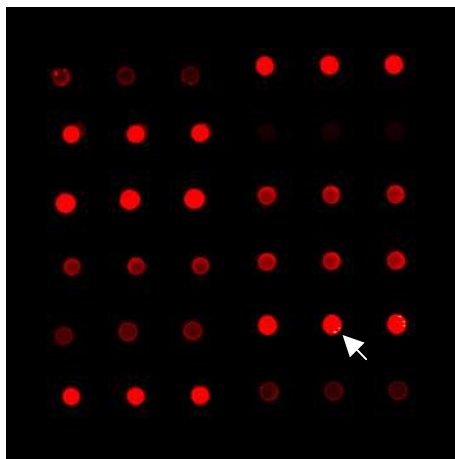
Fig. 10: A: Intensity profile through 9 spots shown in C. Baseline shift (i.e. mean of noise between peaks) = 5717, background (i.e. SD between spots) = 174; B: The same profile baseline-corrected.



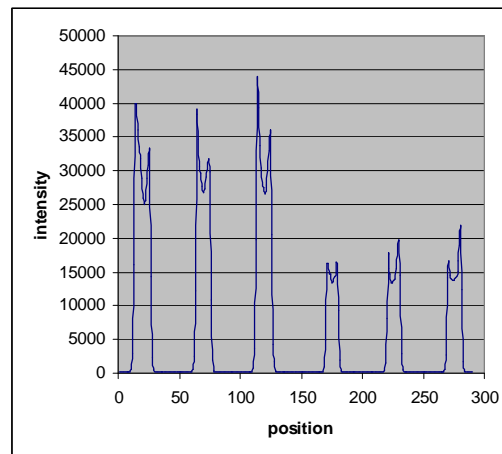
A



B



C



D

Fig. 11: An array of monoclonal antibodies against human cytokines was incubated with a cocktail of recombinant cytokines. Detection was performed with biotinylated secondary antibodies and Streptavidin-Cy5. The array was scanned with a GenePix 4100A scanner using the red channel at a pixel size of 10 μm . In the upper row (A and B) PMT voltage was set to 450. This value was too high. Many spots are saturated, i.e. the signal intensity exceeds the dynamic range of the scanner's detection system. This is indicated by white coloring of these spots in the image (A). The intensity profile (B) was measured through the 3rd row of spots. The 3 peaks on the left half are cut at 65535 units intensity. A quantitative evaluation of these spots is impossible. In the lower row (C and D), the same arrays were scanned at PMT voltage 390. This is the maximum amplification that can be used for this array as already some pixels are saturated (see arrow in C).

A Comment on Donuts

In fluorescent assays, so-called donuts may become apparent upon imaging the array: The signals on the outside of the spot are higher than on the inside. This may be due to an asymmetric deposition and/or drying of protein during printing and is influenced by buffer composition, type and concentration of protein, relative humidity, etc. For assays on FAST Slides, with their high binding capacity, this usually does not change results in quantitative analysis. The higher amount of protein present on the spot outside is translated to a proportionally higher signal. The integrated pixel intensity of the spot will reflect the total amount of analyte detected as long as all signal intensities are within the linear range of the scanner and the overall binding capacity of the FAST Slide surface is not exceeded.

The 4 Pillars of Successful Protein Array Processing using FAST Slides

1. Do not print on a wet slide! FAST Slides are ready for arraying out of the box. Do not pre-wet FAST Slides.
2. Maximize irreversible binding of protein to the NC matrix: leave FAST Slides after printing at room temperature and low humidity for several hours (e.g. overnight) before use.
3. Never let FAST Slides dry out during processing. Use humid chambers for long incubation steps.
4. Adjust scanner settings before reading FAST Slides. Default DNA microarray settings are usually not suitable (too high) for FAST Slides.

When creating and processing protein arrays on FAST Slides, the guidelines contained here can help yield results with clear signals, good spot morphology and low background (Fig. 12).

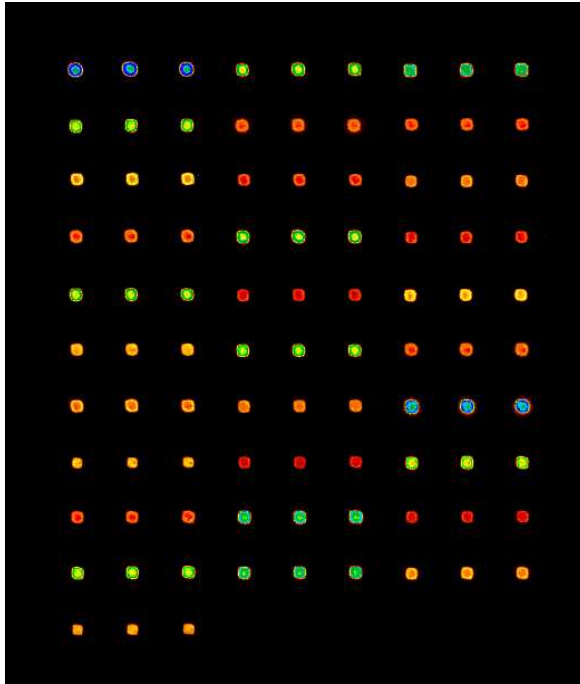
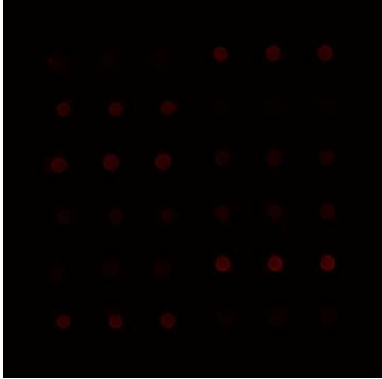
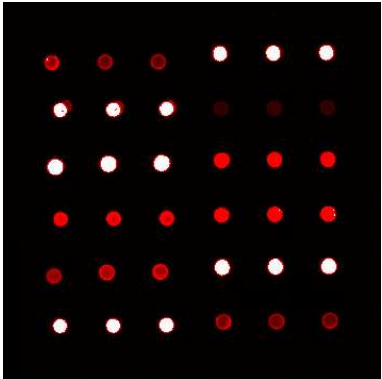
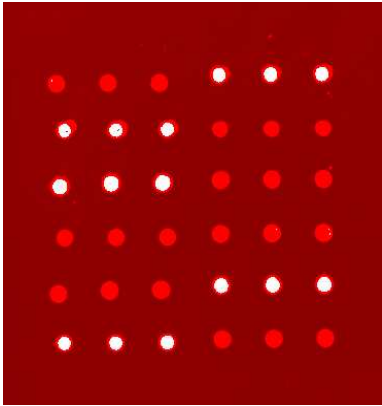
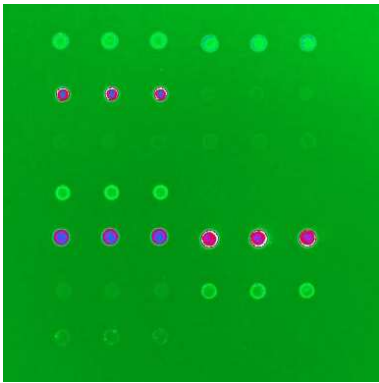
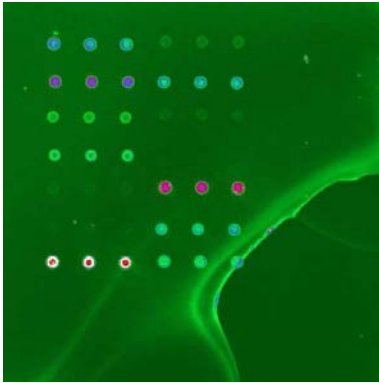
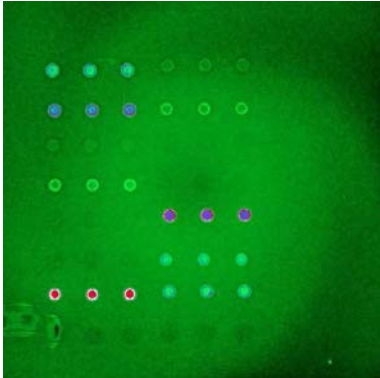
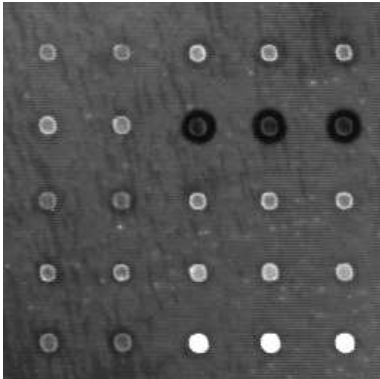
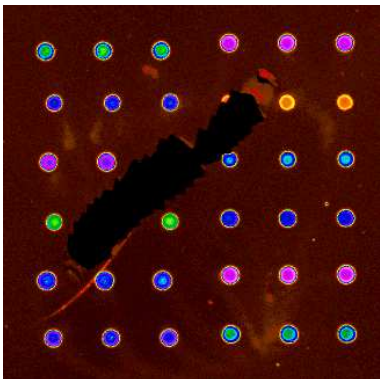


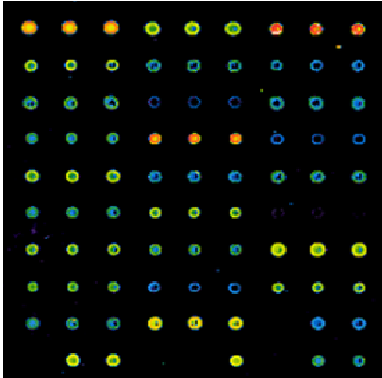
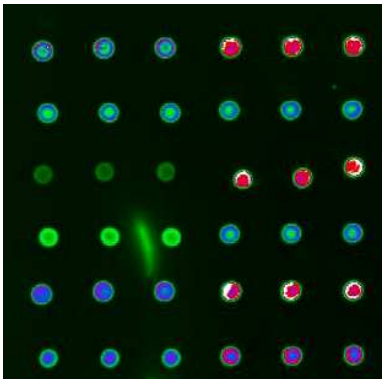
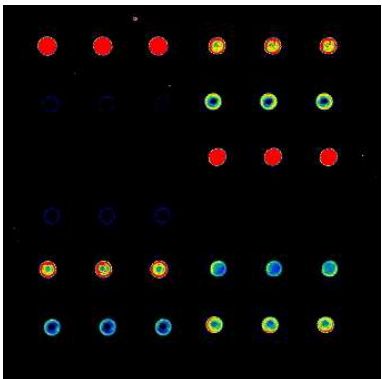
Fig. 12: Single capture antibody array with good spot shape and high signal/background ratio. Monoclonal antibodies against human cancer markers were arrayed on a FAST Slide using a contact printing robot. Spot diameter is 110 μm . The array was incubated with a human serum sample that had been labelled with biotin-ULS utilizing the Whatman Two Color Labelling and Detection Kit (cat. no. 10 486 085). The very slight donut effect which can be seen in some spots is normal and will not influence analysis.

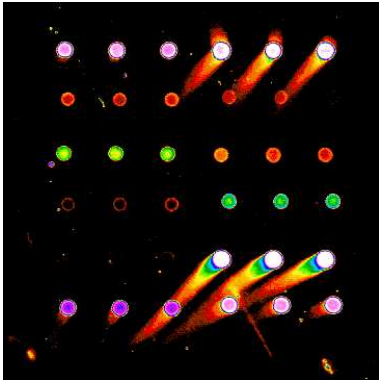
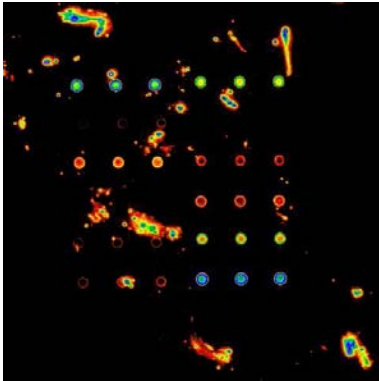
3. Troubleshooting

Symptom	Cause	Remedy
<p>1. Low signals</p> 	<ul style="list-style-type: none"> • scanning: PMT/laser power too low • sample: no binding, sample concentration too low • detection antibody does not bind • fluor conjugate concentration too low 	<ul style="list-style-type: none"> • increase PMT/laser power of scanner • use more concentrated sample • include suitable positive controls • increase fluor conjugate concentration
<p>2. Saturated spots</p> 	<ul style="list-style-type: none"> • white colouring indicates spot saturation: PMT/laser power too high 	<ul style="list-style-type: none"> • avoid saturated spots (cannot be quantified), reduce PMT/laser settings
<p>3. High background and saturated spots</p> 	<ul style="list-style-type: none"> • white colouring indicates spot saturation: PMT/laser power too high • brightness/contrast in imaging software not set appropriately 	<ul style="list-style-type: none"> • avoid saturated spots (cannot be quantified), reduce PMT/laser settings • reduce brightness, increase contrast • background cannot be judged visually: measure background with analysis software, if possible use "intensity

		<p>possible use “intensity profile tool”; if this corroborates high values, refer to “Symptom 4”</p>
<p>4. High background, but not very high signals</p> 	<ul style="list-style-type: none"> • insufficient blocking • matrix-effect of sample • direct sample labelling: unbound dye in solution, protein concentration too high • indirect labelling: fluor conjugate concentration too high 	<ul style="list-style-type: none"> • prolong blocking time (> 30 min, overnight) • optimize blocking buffer (see section 2.2) • use FAST blocking buffer • dilute sample • direct sample labelling: remove unbound dye (use spin column) • indirect labelling: reduce fluor conjugate concentration
<p>5. Cloudy background</p> 	<ul style="list-style-type: none"> • insufficient washing • dryout of slide during processing • final wash step (water rinse) left out or not long enough • post-processing slide drying protocol not followed correctly 	<ul style="list-style-type: none"> • use more wash steps (minimum 3 changes) • increase detergent concentration in wash buffer (Tween 20 up to 2%) • use FAST wash buffer • increase wash temperature (37 °C) • never let slide dry out during processing! Use humid chamber for all incubation steps longer than just a few minutes, work as quick as possible when changing solutions, use multichannel pipette

<p>6. Swirls or smeary stripes</p> 	<ul style="list-style-type: none"> • vortexes and wave pattern forming during shaking with sample • portion of pads dried out during incubations 	<ul style="list-style-type: none"> • reduce speed of shaker (≤ 40 rpm) • use humid chamber for long incubations • cover FAST Frame with lid; if condensation of water is observed under cover: place piece of polystyrene foam under frame for insulation from heat generated by shaking instrument
<p>7. Black holes (spot appears darker than surrounding background)</p> 	<ul style="list-style-type: none"> • arrayed probe does not bind sample, but shows blocking effect 	<ul style="list-style-type: none"> • normal effect • change buffer for the proteins arrayed (i.e. remove additives that lead to "blocking effects")
<p>8. Scratches</p> 	<ul style="list-style-type: none"> • surface of slide touched with pipette tip 	<ul style="list-style-type: none"> • always take care not to touch the surface of FAST Slides; always remove/aspirate liquid from corners of pads

<p>9. Missing spots</p> 	<ul style="list-style-type: none"> • bent or broken pin(s) • salt/other material on tip of pin(s) • clogged pin(s) • volume difference in source plate • wet slide surface 	<ul style="list-style-type: none"> • check integrity of pins (under microscope), adjust position, replace bent/broken pin(s) • clean pins thoroughly after each printing run, optimize washing protocol (see manual of printing instrument) • check source plate • do not use cold (out of the fridge) slides (risk of condensation water on surface)
<p>10. Misaligned spots</p> 	<ul style="list-style-type: none"> • if contact printing: bent or broken pin(s) • if non-contact arraying: arrayer tip is misfiring 	<ul style="list-style-type: none"> • contact printer: check integrity of pins (under microscope), adjust position, replace bent/broken pin(s) • non-contact printer: check tip status/settings
<p>11. Donut-shaped spots</p> 	<ul style="list-style-type: none"> • strike-force of pin on surface too high • viscosity of sample very high • humidity too low 	<ul style="list-style-type: none"> • reduce strike-force • add glycerol to source solution (5 to 10%) • increase humidity of printing chamber • donut effect can hardly be completely avoided; if spot finding in the data reduction software is

		performed properly it has little effect on the results
<p>12. Comets and Tadpoles</p> 	<ul style="list-style-type: none"> • loosely bound capture protein moves during sample incubation • wet slide surface during printing (see above) • protein arrayed using too high concentration 	<ul style="list-style-type: none"> • prolong drying time after printing (-> overnight) • do not use cold (out of the fridge) slides (risk of condensation water on surface) • reduce concentration of capture protein
<p>13. Speckled background</p> 	<ul style="list-style-type: none"> • particles in solution 	<ul style="list-style-type: none"> • replace with fresh, particle-free solvents • make sure that no precipitate is in sample • centrifuge sample before incubation • filter sample/solvents through 0.45 µm syringe filter

4. Further Information

The reference list below includes several articles that give a review of specific fields of application of protein microarrays. Espina et al. (2003) review the use of reverse-phase arrays in cancer research. Array platforms for analysis of autoimmune diseases are reviewed by Balboni et al. (2006). A review of microarrays for glycosylation research was written by Feizi and Chai (2004). Sakanyan (2005) and Schweitzer et al. (2003) review the use of protein arrays to study protein-protein interaction. A general review on proteomic studies using microarrays was written by Feilner et al. (2004).

Additional information can be found on the Whatman web pages www.whatman.com and www.arraying.com.

5. References

1. Anderson, L. and Seilhamer, J. (1997). A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis* 18(3-4):533-537
2. Balboni, I. et al. (2006). Multiplexed protein array platforms for analysis of autoimmune diseases. *Annu Rev Immunol* 24: 391-418
3. Calvert, V. S. et al. (2004). Development of multiplexed protein profiling and detection using near infrared detection of reverse-phase protein microarrays. *Clin Proteomics* 1: 81-89
4. Ekins, R. and Chu, F. (2003). Ultrasensitive microarray-based ligand assay technology. *In: Protein Arrays, Biochips, and Proteomics. The next Phase of Genomic Discovery.* Albala, J.S. and Humphrey-Smith, I. (Eds.), Marcel Dreyer Inc. New York, Basel. PP 81-125
5. Espejo, A. et al. (2002). A protein-domain microarray identifies novel protein-protein interactions. *Biochem J* 367: 697-702
6. Espejo, A., Bedford, M.T. (2004). Protein-domain microarrays. *Methods Mol Biol* 264: 173-184
7. Feilner, T. et al. (2004). Proteomic studies using microarrays. *Current Proteomics* 1(4): 293-295
8. Feizi, T. and Chai, W. (2004). Oligosaccharide microarrays to decipher the glyco-code. *Nature Reviews/Mol Cell Biol* 5: 582-588
9. Gutjahr, C. et al. (2005). Mouse protein arrays from a Th1 cell cDNA library for antibody screening and serum profiling. *Genomics* 85: 285-296
10. Kim, T.-E. et al. (2002). Quantitative measurement of serum allergen-specific IgE on protein chip. *Exp Mol Med* 34(2): 152-158

11. Kersten, B. et al. (2003). Generation of *Arabidopsis* protein chips for antibody serum screening. *Plant Mol Biol* 52: 999-1010
12. Kukar, T. et al. (2002). Protein microarrays to detect protein-protein interactions using Red and Green Fluorescent Proteins. *Anal Biochem* 306: 50-54
13. Lueking, A. et al. (2005). Profiling of *Alopecia areata* autoantigens based on protein microarray technology. *Mol Cell Proteomics* 4(9): 1382-1390
14. Madoz-Gúrpide, J. et al. (2001). Protein based microarrays: a tool for probing the proteome of cancer cells and tissues. *Proteomics* 1: 1279-1287
15. Nishizuka, S. et al. (2003). Proteomic profiling of the NCI-60 cancer cell lines using new high-density reverse-phase lysate microarrays. *PNAS* 100(24): 14229-14234
16. Paweletz, C.P. et al. (2001). Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. *Oncogene* 20: 1981-1989
17. Sakanyan, V. (2005). High-throughput and multiplexed protein array technology: protein-DNA and protein-protein interactions. *J Chromatography B* 815: 77-95
18. Schweitzer, B. et al. (2003). Microarrays to characterize protein interactions on a whole-proteome scale. *Proteomics* 3: 2190-2199
19. Spector, N. L. et al. (1994). Regulation of the 28 kDa heat shock protein by retinoic acid during differentiation of human leukemic HL-60 cells. *FEBS Lett* 337: 184-188
20. Yeretssian, G. et al. (2005). Competition on nitrocellulose-immobilized antibody arrays. From bacterial protein binding assay to protein profiling in breast cancer cells. *Mol Cell Proteomics* 4: 605-617
21. Zhou, H. et al. (2004). Two-color, rolling-circle amplification on antibody microarrays for sensitive, multiplexed serum-profile measurements. *Genome Biology* 5(4): R28