Methods in Molecular Biology

Protein Microarrays

Chapter 21: Inkjet Printing for the Production of Protein Microarrays

Iain McWilliam*, Marisa Chong Kwan, Duncan Hall

Arrayjet Ltd., MIC, Roslin, EH25 9RE, UK

* Corresponding Author

Keywords

i. Inkjet
ii. Microarrayer
iii. Protein microarray
iv. Printing buffer
v. Diagnostic microarray
vi. Non-contact
vii. JetSpyder
viii. JetGuard
ix. Microarray production
x. Antibody microarray

Introduction

Since their inception in 1995, microarrays have been the icon of the 'omics revolution. Whereas DNA can be printed onto a wide range of surface chemistries with a wide range of buffers, proteins must be printed in buffers which protect them and onto substrates which maintain their structural integrity, binding sites and activity. Common buffers for protein storage contain cryoprotectorants (e.g. glycerol (*1*) or ethylene glycol) which add to the often viscous nature of protein solutions, so the ability of a microarrayer to print

such solutions without extensive modification is a distinct advantage when producing protein microarrays. The most popular substrates for protein microarrays, designed specifically with structural protection in mind, are made with thin nitrocellulose or hydrogel coatings and are fragile themselves, which encourages the use of a non—contact method of printing. When addressing production throughput requirements, the ability to handle multiple samples simultaneously and to print those samples quickly on—the—fly is important to minimise production timescales. The technology should also be scalable and robust enough to accommodate a shift from R+D level production to full—scale, manufacturing levels of production. Arrayjet has used the key factors of being able to print potentially viscous protein solutions with a non—contact printing method to develop a range of microarrayers, all centred around an industrially proven inkjet printhead, which are highly suited to printing high quality protein microarrays at all levels of production.

This chapter contains a detailed explanation of Arrayjet technology followed by a historical look at the development of inkjet technologies for protein microarray production. The method described subsequently is a simple example of an antibody array printed onto nitrocellulose coated slides and subsequent detection with fluorescently labelled IgG. The method is linked to a notes section offering advice on best practice and further sources of useful information for protein microarray production.

Inkjet Printing

Inkjet printing is the ejection, from a nozzle, of liquid droplets (e.g., protein solution) which travel a short distance (1 – 5 mm) through the air to land on a substrate in a predetermined pattern. The different methods of inkjet droplet ejection are well reviewed (*2, 3*), there are three modes of action as follows;

1. Piezo actuation uses a volumetric change to induce the pressure required for droplet ejection.
2. Valve-jet uses a continuous pressure stream in conjunction with a valve which opens and closes to eject a stream of droplets.

3. Thermal inkjet, also known as bubble-jet, uses the rapid heating of samples to create a pocket of gas to induce the required pressure for droplet ejection (*4, 5*).

Arrayjet printheads use piezo actuation; the interior walls of the printhead channels are made from ceramic, piezoelectric material called lead zirconate titanate (PZT). When PZT is subjected to an electrical charge it changes shape causing a volumetric change and a subsequent acoustic wave which ejects a droplet of sample from the nozzle (Figure 21.1). Unlike thermal inkjet, with which Arrayjet was incorrectly associated (*6*), there is no heating of the sample with piezo actuated printing. Piezo actuation and acoustic wave drop formation impart almost no mechanical stress on the printhead; nozzles consistently print even after actuations exceeding 10^{13} per nozzle (*7*) making this technology ideal for printing reliable microarrays.

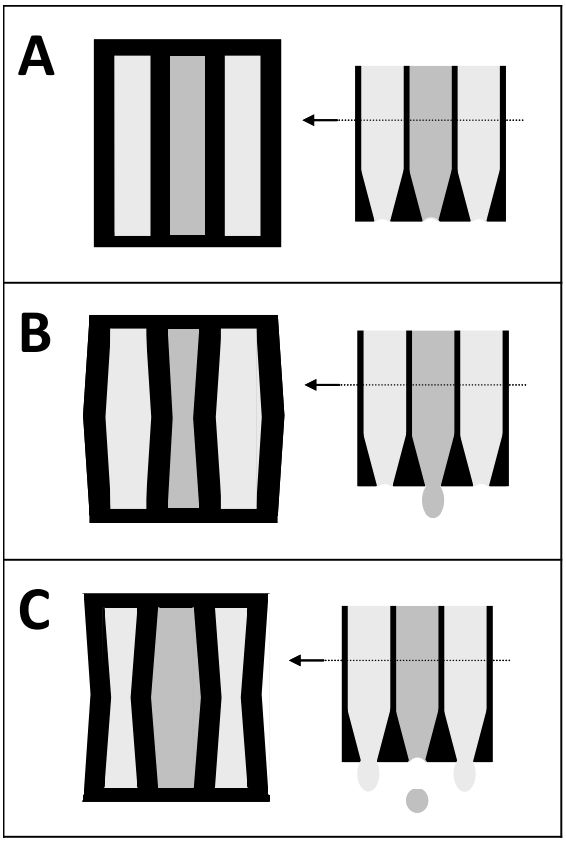

Figure 21.1. Simplified diagram of the mechanism of sample droplet ejection from a small section of a shared wall printhead shown in two cross—sectional planes. A: Three channels of the printhead, each terminating in a nozzle, in the non—actuated state. The dashed line indicates a cross section part way up the channels. B: Actuation of the piezoelectric walls of the central channel; the walls change form momentarily to create a pressure wave to eject a droplet of sample from the nozzle. Capillary action replenishes the sample to make up for the volume ejected so ensure the channel is ready for the next ejection. C: Neighbouring channels undergo the same process whilst the droplet from the central nozzle travels through the air to the substrate.

Arrayjet technology is comparatively new and there has been some confusion as to the nature and speed of the technology (*6, 8*), particularly with reference to printing speeds of inkjet microarraying in the literature published before 2004(*9, 10*). Use of Arrayjet technology is gaining momentum and whilst many users print different varieties of nucleic acid arrays (*11-15*), the very same microarrayers are being used increasingly for protein microarray production (*16*), including label—free protein techniques (*17-19*) and nanotechnology applications (*20*). There is, however, limited accurate technical information in the scientific literature on how Arrayjet technology works, best practice and troubleshooting tips; the present article addresses this need.

Traditional commercially available piezo-electric microarrayers employ glass capillary dispenser(s) with a ceramic piezo-collar (*21*) whilst Arrayjet utilises the Xaar XJ126, an industrially proven, multi-nozzle, shared wall printhead which has 126 linearly arranged nozzles. Whereas glass capillary spotters use a low number (1-16) of dispensers, this does not apply, as was suggested (*6*), to Arrayjet printers which have 126 nozzles. The Arrayjet printhead has a unique internal surge control mechanism, ensuring consistent drop size and placement accuracy from each nozzle. The systems handle 12 or 32 samples simultaneously, and printing is performed on—the—fly, with a head speed of 0.2 metres per second. Arrayjet systems currently print at a rate of approximately 475 features per second which is understood to be significantly faster than competitors (*22, 23*). This translates into the ability to print an entire 384-well plate onto all 100 slides in under 25 minutes; tests performed in conjunction with DKFZ, Heidelberg indicated that the same task would take peer instruments between six and twelve hours to complete. This represents a paradigm shift in throughput when compared to both pin spotters and competitor non-contact microarrayers. As a real—life comparison with a pin—printer, one Arrayjet user prints a 576 feature array onto 100 Schott Nexterion 16 pad slides in fewer than 6 hours; a run which takes their MicroGrid II (BioRobotics), utilising four pins, 58 hours to print. Suggestions that printing rates of commercially available piezo-electric printers are less than (*9*) or typically similar to single-pin contact printing due to limitations of the number of tips on the spotter (*21*) are not applicable to Arrayjet technology.

Arrayjet's Contribution to Inkjet Microarrayers

Arrayjet offer four microarrayers to address the different levels of throughput required by users. All four instruments are built around the same core components, discussed below, which allow users to increase throughput as their requirements for production grow. The Sprint is an entry level benchtop research and development system which prints two microplates (4 with reloading) onto 20 slides. The other three systems are the Marathon, Super Marathon and Ultra Marathon which are built around a modular, upgradeable design. The Marathon prints 6 microplates (96 with reloading) onto 100 slides. With the addition of a microplate stacker module the Marathon becomes the Super Marathon; enabling 48 microplates to be loaded at once with a further 156 by reloading. The Ultra Marathon, which can also

accommodate the microplate stacker, critically has a slide stacking module which transforms it into a 1000 slide production platform for truly industrial-scale microarray printing; this level of throughput remains unique in the industry.

The JetSpyder™ Concept for Automated Sample Loading

The key components of Arrayjet systems are a 126 nozzle inkjet printhead, a liquid handling device called a JetSpyder™ and a stepper motor driven syringe. The printhead is located on a robotic gantry and is connected to a hydraulic system which incorporates the syringe. The JetSpyder™ is used during the automated loading of samples into the printhead and the syringe drives bulk liquid movements such as system purging and aspiration of samples.

Samples are aspirated into the printhead via the patented liquid handling adapter, the JetSpyder™ (Figure 21.2). The JetSpyder™ has a rubber seal on its upper surface comprising 12 or 32 diamond shaped enclosures arranged linearly. These enclosures correspond to the line of nozzles on the underside of the printhead that lead to the channels which hold samples during printing. The printhead can be docked to the JetSpyder™ which causes the upper surface of the rubber seal to compress around groups of nozzles. The walls of each enclosure isolate a group of nozzles which will ultimately share a common sample. The JetSpyder™ has 12 or 32 stainless steel capillaries which extend from the underside of the seal enclosures and are manipulated into a 4 x 8 or 3 x 4 Society for Biomolecular Screening (SBS) footprint. When the JetSpyder™ is lowered into a 96 or 384 microplate, 12 or 32 samples are simultaneously aspirated into the capillaries. Each sample travels through a separate capillary to the printhead where it enters an enclosure and continues into up to six adjacent nozzles. Air is then aspirated into the capillaries to displace the samples still in the JetSpyder™ to ensure dead volumes are minimised (Figure 21.2.D). The printhead is now loaded with samples and the JetSpyder™ is replaced into its cleaning station. Printing is performed on—the—fly to the substrates by the printhead alone and up to 500 drops can be printed per nozzle, with each droplet having a volume of 100 pL. This arrangement of separate sampling and printing components make Arrayjet systems uniquely compatible with JetGuard™, a disposable rubber cover for microplates with a valved septum for each well which

protects samples held in 96 or 384 microplates from evaporation and contamination at all times during microarraying (Figure 21.3).

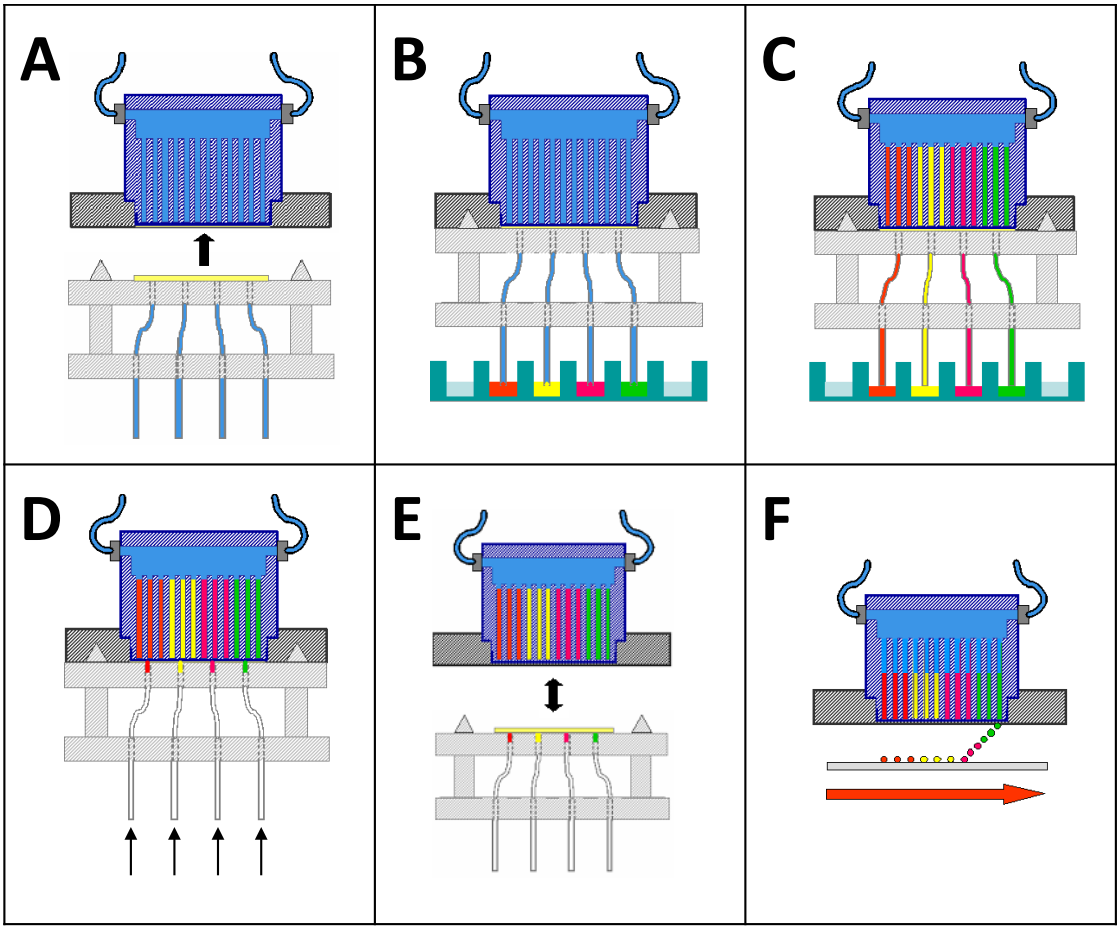

Figure 21.2. A: The JetSpyder™ is docked to the printhead and held in place by vacuum. System buffer is pushed through the printhead and JetSpyder™ to prime them before sample aspiration. B: The docked JetSpyder™ is positioned over a 96 or 384 microplate and the capillaries are lowered into the samples. C: A servo-driven syringe (not shown) is used to aspirate 12 or 32 separate samples simultaneously into the printhead. D: The JetSpyder™ is removed from the samples and air is aspirated (arrows) forcing the sample deeper into the printhead and reducing the dead volume. E: The printhead is undocked, depositing the JetSpyder™ in its cleaning station. F: The printhead performs on-the-fly, non-contact printing onto the substrates.

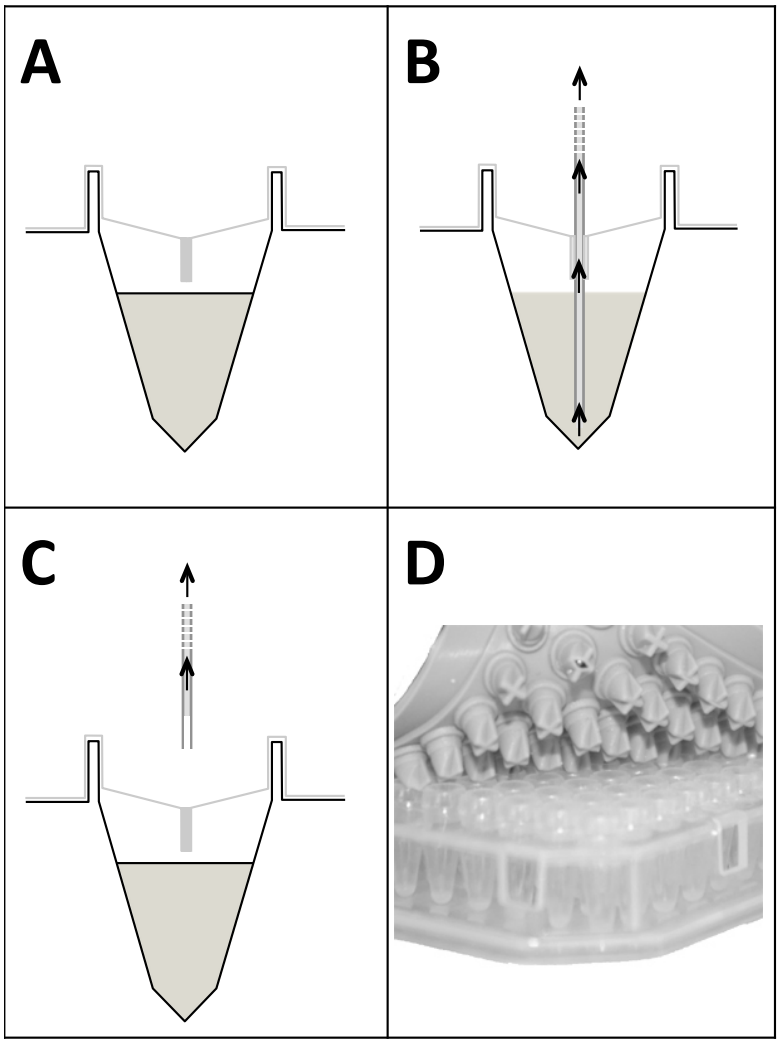

Figure 21.3. A: The JetGuard™ seal creates a microclimate with 100 % relative humidity around the sample, thus minimising sample evaporation. The sample is also protected from foreign body contamination. B: A JetSpyder™ capillary has entered the well through the duckbill valve and sample is aspirated; the arrows indicate the direction of flow towards the printhead and the dashes through top of the capillary indicate its extension towards the printhead. C: The duckbill valve closes as the JetSpyder™ capillary is removed from the well. Air is aspirated into the capillary to displace the sample further into the printhead. D: A photograph of a JetGuard™ seal being pulled peeled away from a 384 well microplate.

A brief history of non-contact printing technology in microarray production

Early microarray fabrication technologies employed photolithography, as used by Affymetrix, or pin technology championed by their academic inventors and proponents, both described in now famous articles published in Science by Steven Fodor *et al* (*24*) and Mark Schena *et al* (*25*), respectively. Non-contact technology had previously been demonstrated to have potential in this area by Schober *et al* (*26*), who showed the precise deposition of bacterial colonies onto agar plates in frame-like layouts that resembled printed microarrays. These publications were swiftly followed by arguably the first publication

involving the use of inkjet technology for microarray production, adapted by Leroy Hood at the California Institute of Technology for microarray production via *in situ* synthesis of nucleotides on a solid substrate (*27*). Later publications also followed, including a chapter in the widely selling DNA Microarrays – A Practical Approach, which described a non-contact spotting instrument called the GeneJet (*28*). Hood, meanwhile, together with Leland Hartwell and Steven Friend founded Rosetta Inpharmatics in 1996. A strategic partnership agreement with Agilent Technologies to commercialise Rosetta's DNA microarrays followed in 1999 and the Gene Expression Solution, which used inkjet printed nucleotides for *in situ* oligo synthesis.

Commercial non-contact microarrayers – in the beginning

As the microarray boom continued in the latter part of the 1990's and on into the early part of this century, technology companies were attracted to the possibilities it opened up. There has been a general trend towards systems which are increasingly robust and easy to use, and that offer printing that is faster and more consistent, resulting in smaller spots with better morphology and higher density. Home-spotting technologies were initially thought to be reserved to contact spotters, though non-contact technology to produce microarrays, such as BioDot's Microdoser was available. In 1997, BioDot spun out Cartesian Technologies to focus on the life sciences market and microarrays in particular. The company was very successful, reporting an installed base of over 170 microarrayers worldwide at the time of its acquisition in 2001 by Genomic Solutions. A close rival to Cartesian in this regard was Packard Biosciences who developed the BioChip Arrayer 1 (BCA1) based on technology owned and developed by Microdrop, and which launched in 1999 after 5 years of development. Packard Biosciences was acquired by Perkin Elmer Life Sciences in 2001 prompting a significant revision of the BCA1 (with improvements to the linear motions and operating system) before its re—launch as the Piezoarray in 2003. The revisions made the Piezoarray more user—friendly but it still required high levels of maintenance and frequent recalibration. Ninety Piezoarray units were shipped during the product's life time before being withdrawn from the market. Concurrently, a number of start-up companies launched new products– specifically two German

companies, GeSiM (Gesellschaft für Silizium-Mikrosysteme mbH, translated as Silicon Microsystems Company mbh) and Scienion AG. GeSiM's Nanoplotter range of instruments was launched at the end of the 1990s. The first SciFLEXARRAYER of Scienion, which followed shortly after, was an instrument of an unusual design in that the substrate tray and motions were based on a turntable.

Therefore by 2003 a number of commercial possibilities existed for scientists interested in using non-contact technology for microarray production. All of these microarrayers were, however, based largely on the same technology: the use of micro-dosing piezoelectric tip dispensers. The tips are dipped into the wells holding the probe sample material to be arrayed, in order to aspirate the samples into the dispensing device. They are then located over the substrates to allow the probe samples to be dispensed in droplets during microarray production.

The need for speed – accelerating non-contact microarray production

Whilst Agilent Technologies was able to produce very large numbers of high quality DNA microarrays with its proprietary inkjet technology, many scientists were frustrated that they could not acquire the technology themselves for use in their own laboratories. The Agilent array writers, as they were often described, were complex machines which required intensive maintenance and engineering support to obtain maximum performance. This level of support is not available to most scientists and the prospect of owning and running such a device was beyond their means. In its early years, non-contact technology was not especially reliable or robust and it was certainly not fast. Only Packard Biosciences had been able to develop and bring to market a high throughput version of its microarray technology. The Spot Array Enterprise appeared in 2001 and was based on a new dispensing head which held 8 tips, as opposed to the usual 4 on the BCA1 and Piezoarray. Whilst the Spot Array product was never officially launched, 15 units were shipped between 2001 and 2003, when the product was discontinued.

In 2005, following successful trials at three locations beginning in 2003, Arrayjet officially launched its first product: the Aj100- an inkjet microarrayer which uniquely employed modern piezoelectric print head

technology from Xaar as described above. The launch of the Aj100 was followed by the Aj120 (now rebranded the Super Marathon) which had a microplate stacker to increase its walk-away printing capacity from 6 to 48 microtitre plates, making it a true core facility scale microarrayer. Shortly afterward, Arrayjet announced that it had developed a new liquid handling device which increased sample handling from 12 to 32 samples simultaneously, thereby reducing the time required to print 384 samples to 100 slides from 48 to only 25 minutes. In 2007, Arrayjet launched the Sprint Inkjet Microarrayer, an R&D-scale instrument capable of printing microarrays on up to 20 slides from a maximum of 4 microtitre plates, and simultaneously re-branded the entire product line. Then, in 2008, the Ultra-Marathon Inkjet Microarrayer was unveiled at the Advances in Microarray Technology conference in Barcelona. The Ultra-Marathon represented a paradigm shift in microarray production, taking it to a new level of throughput. As Arrayjet launched its new products, the world of microarray was being reinvigorated. Whilst many scientists had ceased making their own DNA microarrays, the technology was constantly maturing, and many had turned their attention to protein microarray technology as a biomarker discovery and validation platform.

Protein Microarrays -- A Renaissance for Microarrayers

Since the beginning of proteomics, the number of protein array applications has increased greatly. Protein arrays are being used as a crucial tool in major research projects which have the ability to change the world of diagnostics and, therefore, therapeutics. Areas such as biomarker discovery, drug interactions and expression profiling require high-throughput technology compatible with proteins. Unlike DNA applications, where commercially available chips are widely used, the protein field often requires a customised spotting for both research and manufacturing for diagnostic purposes. Many researchers have found themselves forced to manufacture their own protein microarrays and have found their old DNA microarrayer unsuited to the task. Diagnostic companies, with greater demands for speed and quality, find themselves in a position where the chosen technology needs to fulfill more specific challenges including high throughput. Antibody arrays are especially powerful as a proteomic technology. Multiplexed and ultra-sensitive assays, specifically targeting several analytes in a single experiment, can be performed, while consuming only minute amounts of the sample (*29*). Many antibody applications

mimic the Enzyme—Linked Immunosorbent Assay (ELISA) technique in that they either employ a capture reagent and a detector reagent, often both IgG molecules, as per a sandwich ELISA. Alternatively a target is adsorbed to a suitable substrate prior to detection with either a primary, or a primary and a secondary antibody, as in a direct ELISA. Colorimetric and fluorescent labelling approaches are both widely used. Many routine diagnostic tests are ELISA-based, and the prospect of migrating these tests to protein microarray is attractive not only for the savings in reagents, sample and time, but also due to the potential advantages in sensitivity and parallel-ability known as multiplexing. For these reasons, a method to effectively print antibodies has been the chosen subject for this manuscript.

Non-contact microarrayers offer benefits for the production of protein microarrays. This has stimulated the replacement market in microarrayers, and further validates recent product offerings not only from Arrayjet, but from its non-contact competitors GeSiM and Scienion. The latter has recently updated its existing product offering and added new products in the form of an entry level system, an updated mid-scale system, the SciFLEXARRAYER SX, and a high throughput version, the S100. Furthermore, during 2009 two new arrivals in non-contact microarrayer space have emerged in the shape of M2 Automation and Olivetti. M2's microarrayer - iONE– is without doubt the fastest moving microarrayer on the market, though its limited input and output capacity, and number of dispensers makes it suitable only for low throughput, low density microarrays. Moreover, the dispenser type employed by M2 is very similar to those known to have problems of reproducibility and robustness – only time will tell if M2 has been able to address these issues with its new products. Olivetti's thermal BioJet technology is clearly aimed at the high throughput market though to date it has only been seen at the proof of concept stage.

Materials

1. Arrayjet Sprint, Marathon, Super-Marathon or Ultra-Marathon Inkjet Microarrayer.
2. JetMosphere environmental control system.
3. 2x JetStar™ Optimum Protein Printing Buffer (Arrayjet, UK).

4. Arrayjet System buffer: 47 % (v/v) glycerol (G6279, Sigma Aldrich, UK), 0.06 % (v/v) Triton X100 (T8787, Sigma Aldrich, UK)

5. Command Centre™ GUI microarray printing software provided with the microarrayer.

6. Mouse monoclonal IgG_1 antibody P-Tyr (PY20), 200 $\mu g.ml^{-1}$ (sc-508, Santa Cruz Biotechnology, USA).

7. Mouse monoclonal IgG_{2b} antibody p-Tyr (PY99), 200 $\mu g.ml^{-1}$ (sc-7020, Santa Cruz Biotechnology, USA).

8. Mouse monoclonal IgG_{2a} antibody EGFR (528), 200 $\mu g.ml^{-1}$ (sc-120, Santa Cruz Biotechnology, USA).

9. Mouse monoclonal IgG_1 antibody β-Actin (C4), 200 $\mu g.ml^{-1}$ (sc-47778, Santa Cruz Biotechnology, USA).

10. Blocking solution: 2 % Bovine Serum Albumin (A9647, Sigma Aldrich, UK) in PBS-T (P3563, Sigma Aldrich, UK).

11. Incubation solution: Atto 550 goat anti-Mouse IgG (43394, Lot No. 1267477, Sigma Aldrich) diluted to 2.8 $\mu g.ml^{-1}$ in PBS-T (as before).

12. Wash buffers: 1 x PBS-T and 1 x PBS (both as before).

13. Whatman® FAST® nitrocellulose coated slides (10484182, Sigma Aldrich, UK) (See note 1).

14. Lifterslips (22x25l—2—4816, Erie Scientific Company, USA).

15. JetGuard™ Probe Protector as shown in Figure 21.3 (AJPP20, Arrayjet, UK).

16. JetGuard™ compatible 384 well microplate (PCR-384-55-C, Axygen, UK).

17. Innoscan 700 microarray scanner (Innopsys, France).

18. Mapix image analysis software v2.9.5 (Innopsys, France).

Methods

1. Create a serial dilution of each antibody with 2x JetStar™ Optimum Protein Printing Buffer (100, 20, 8, 4 and 2 $\mu g.ml^{-1}$) (See note 2).

2. Overlay JetGuard™ probe protector onto the 384 well microplate.

3. Populate a 384 well microplate with > 5 μl of sample per well.

4. Centrifuge the microplate at 3000 x g for 5 minutes to remove any bubbles from the sample.

5. Program a printrun in Command Centre™ graphical user interface software to print 6 slides, each with 3 miniarrays per slide, three replicate spots per miniarray (9 replicate spots per slide), and one drop per spot (100 pL) spot volume (see Note 3).

6. Mount the slides into the spring—loaded holders.

7. Set the JetMosphere™ environmental control system to 50 % relative humidity and 5 ºC below ambient (See Note 4).

8. Initialise the microarrayer; this will take about 1 minute.

9. Print a test slide, an automated procedure taking about 20 seconds, and verify that all nozzles are functioning correctly by visually inspecting the slide.

10. If test slide is not satisfactory, request automatic printhead purging and reprint a test slide.

11. If any nozzles are performing sub-optimally they can be switched off in the software. There is inbuilt redundancy in the printhead allowing automatic reallocation of nozzles without affecting the printrun.

12. Start the printrun (the above array takes under 10 minutes to print in its entirety).

13. Once printing is complete, incubate the slides 37 °C and 50 % relative humidity for 3 hours (see Note 5).

14. Immerse the printed slides in the blocking solution for 1 hour at room temperature with constant, gentle agitation.

15. Wash the slides twice in PBS-T for 5 minutes.

16. Overlay the array with sufficient incubation solution, cover with a lifter—slip and incubate at room temperature for 1 hour. Protect the slides from the light during the incubation and for the remainder of the process until they have been scanned.

17. Wash the slides in PBS-T for 10 minutes with gentle agitation to remove unbound detection antibody.

18. Wash the slides twice in PBS for 5 minutes with gentle agitation.

19. Centrifuge the slides for 30 seconds or until dry.

20. Scan the slides using the 532 nm channel (see Note 6). A scanned image is shown in figure 21.4. The pmt was set at 1 and laser power was low.

21.Extract the signal intensity data from the images with Mapix analysis software using local background correction. The data extracted from this experiment is shown in figure 21.5.

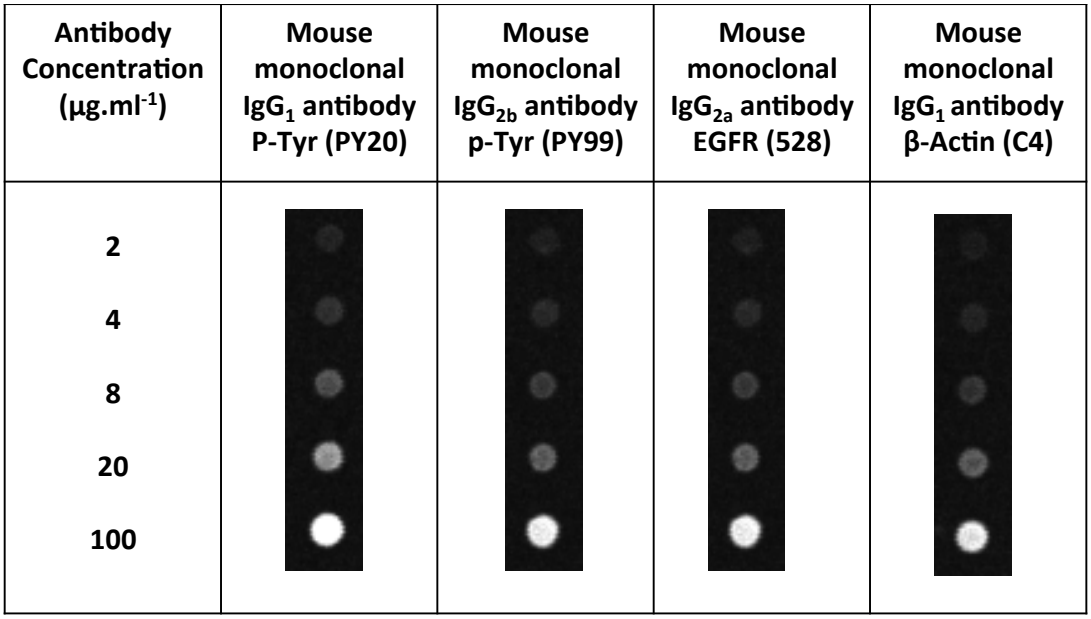

Figure 21.4. Scanned microarray images of four mouse monoclonal antibodies printed in a serial dilution after incubation with goat cy3-anti mouse IgG as described. Spot morphology, intra-spot signal distribution and spot placement accuracy are uniformly good.

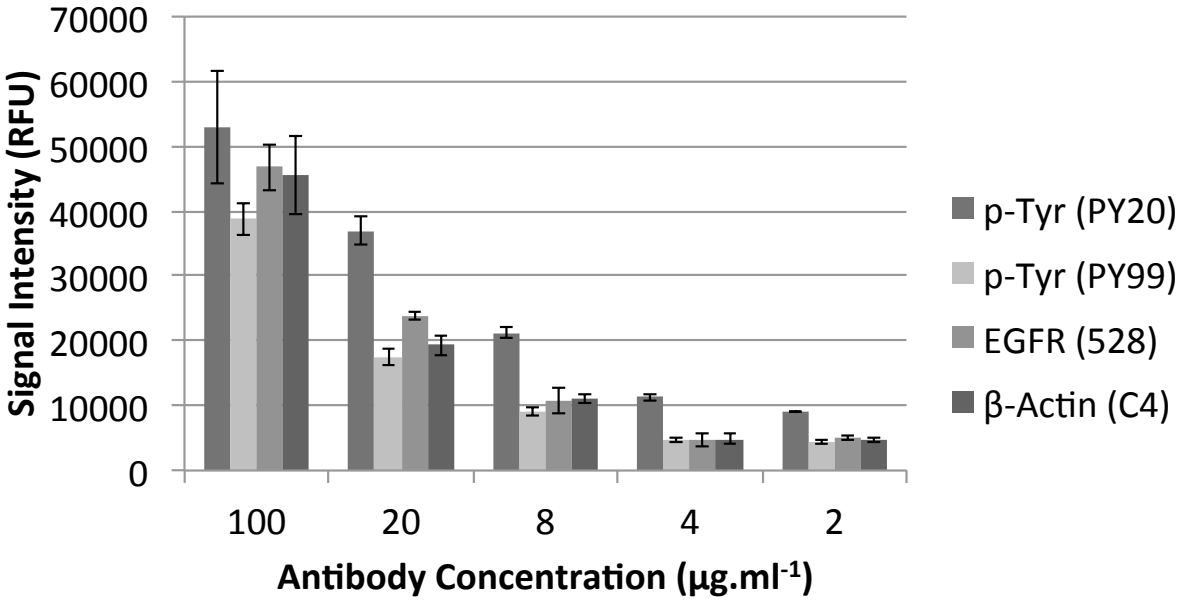

Figure 21.5. Signal intensities of four mouse monoclonal antibodies printed in a serial dilution after incubation with goat cy3-anti mouse IgG as described. Error bars are +/- standard deviation.

Notes

1. In our experience, protein microarrays are most commonly printed onto nitrocellulose membranes bonded to glass slides (Whatman FAST, Gentel PATH, JetStar™ Nitrocellulose, Schott Nexterion NC), nitrocellulose or nylon membrane sheets; they are polysorbant and hold the proteins in a fibrous matrix. Other coated slides employ aldehyde, epoxy or NHS ester chemistry either as a dry coating or hydrogel matrix to chemically bind proteins to the substrate randomly via functional groups on the protein surface. Oriented protein binding can be achieved by introducing affinity tags such as His-6 (*30*) or biotin (*31*) groups at specific sites in the protein and then arraying them onto nickel—NTA or streptavidin coated substrates respectively. Protein microarrays are also finding their way onto novel surfaces for label free signal detection on slides compatible with surface plasmon resonance spectroscopy (SPR) or pre-patterned substrates for electrical detection of analytes, both covered in a review by Yu *et al (32)*. Each surface type has advantages but consideration must be given to aspects such as membrane pore size, which will affect spot size and adsorption efficiency, or whether chemical binding of the protein may disrupt the protein conformation and function detrimentally. The reader is directed to detailed reviews of available slide substrates (*33-35*).

2. For optimal printing and best spot morphology, the printed samples should have a fluid viscosity between 4 and 20 centipoises (cP, a measure of fluid viscosity). As a guide, water has a viscosity of 1 cP and a 67.5 % (v/v) glycerol solution has a viscosity of 20 cP. The correct viscosity ensures that each droplet of sample remains intact during its flight between printhead and substrate. Protein solutions are often inherently viscous but where modulation is required, a viscosifier can be added to increase the Newtonian viscosity of the samples. Recommendations include glycerol or ethylene glycol at concentrations up to 50 %; both of which confer the added benefit of protein stabilization and cryoprotection. Sets of samples aspirated together should have a viscosity range within +/- 40 % of the mean viscosity to ensure print consistency. Other hydroxylated printing buffer additives (e.g., polyvinyl alcohol 9000, 0.05 – 0.5 % (*36*)) or carrier protein additives (e.g., BSA, 0.1 % (w/v) (*37*)) can be used to further enhance spot morphology and signal intensity. There is a detailed review of the printability of

proteins by Joseph Delaney *et al (38)*. The JetStar™ range of printing buffers has been developed and optimised by Arrayjet for a wide range of sample types, including proteins and peptides.

3. The assay performed here gave sufficient signal from features of ~100 μm in diameter but users may wish to amplify the signal or achieve larger spots. This can be achieved by printing multiple drops of the same sample to each spot location. Every time the printhead passes over a slide, up to 6 drops (600 pL) per spot can be printed and repeat passes allow the spot volume to be increased to a maximum of 10 nL. Spot size will increase with spot volume; 100 pL spots are ~100 μm in diameter, 600 pL spots will be ~ 300 μm; but the precise ratio between spot volume and diameter will depend on sample surface tension and the substrate in use; a comprehensive explanation is given by Frits Dijksman and Anke Pierik (*39*).

4. Consistent environmental conditions are critical in protein microarray production to ensure that spots on every batch of slides have optimal morphology, and also to maintain samples in their optimal state to maximize stability and limit evaporation from microplates. Ideally the printing environment should be HEPA filtered to prevent foreign bodies from contaminating source microplates and printed substrates. Different assays and substrates will have different optimal operating conditions. The environmental operating range of Arrayjet systems is 6 – 30 ºC, and a relative humidity between 40 and 60%. An ideal printing environment for protein array printing can be created with JetMosphere™ environmental control system, which can be installed on all Arrayjet systems. It allows the user to define, create and maintain a stable printing environment as low as 6 ºC with a relative humidity between 40 - 60 %. JetMosphere™ comprises a humidifier, dehumidifier, refrigerating element and an air pump with built-in HEPA filter which circulates the air within the arrayer. The refrigerating element can be mounted directly onto the arrayer to cool up to 5 ºC below ambient, or the arrayer can be placed within an insulated unit and cooled as low as 6 ºC. There are no consumable elements in JetMosphere™ and the only user intervention is to empty or fill water tanks before the start of a printrun.

5. Post-printing environmental conditions can be as important as printing conditions for the highest quality arrays as they affect the evaporation rates of spots and hence their morphology and distribution of immobilised protein. An alternative post-printing incubation would be overnight at room temperature and 50 % relative humidity. Spots should be dry before proceeding to the blocking step to avoid “comet tail”

artifacts. JetMosphere™ can also be used to maintain appropriate post-printing conditions for consistent protein immobilisation between different production batches.

6. Slides should be scanned with PMT and laser power set to get maximum signal intensity without pixel saturation to maximise the dynamic range of the assay.

References

1. Olle, E. W., Messamore, J., Deogracias, M. P., McClintock, S. D., Anderson, T. D., and Johnson, K. J. (2005) Comparison of antibody array substrates and the use of glycerol to normalize spot morphology, *Exp Mol Pathol* **79**, 206-209.
2. Derby, B. (2008) Bioprinting: inkjet printing proteins and hybrid cell-containing materials and structures, *Journal of Materials Chemistry* **18**, 5717-5721.
3. Cooley, P., Wallace, D, Antohe, B. (2001) Applications of ink-jet printing technology to BioMEMS and microfluidic systems. *J Assoc Lab Autom* **7**, 33-39.
4. Okamoto, T., Suzuki, T., and Yamamoto, N. (2000) Microarray fabrication with covalent attachment of DNA using Bubble Jet technology, *Nat Biotech* **18**, 438-441.
5. Roda, A., Guardigli, M., Russo, C., Pasini, P., and Baraldini, M. (2000) Protein Microdeposition Using a Conventional Ink-Jet Printer *Biotechniques* **28**, 492-496.
6. Lausted, C. G., Warren, C. B., Hood, L. E., and Lasky, S. R. (2006) Printing your own inkjet microarrays, In Kimmel, A. and Oliver B. (eds.) *DNA Microarrays,* Academic Press, California.
7. Alexander, M. (2008) The Xaar Guide to Single Pass Printing, http://www.xaar.com/regdownload.aspx?file=uploads/xaar_guide_to_single_pass_printing_white_paper.pdf. Accessed 15 August 2010.
8. Dufva, M. (2009) Fabrication of DNA Microarray, in Dufva M. (ed) *DNA Microarrays for Biomedical Research: Methods and Protocols*, Humana Press Inc, New York.
9. Martinsky, T. (2003) Printing technologies and microarray manufacturing techniques: making the perfect microarray, in Blalock, E. (ed) *A beginner's guide to microarrays*, Kluwer Academic Publishers, Norwell.
10. Heller, M. J. (2002) DNA microarray technology: devices, systems, and applications, *Annu Rev Biomed Eng* **4**, 129-153.
11. Sun, A., Devi-Rao, G. V., Rice, M. K., Gary, L. W., Bloom, D. C., Sandri-Goldin, R. M., Wagner, P., and Wager, E. K. (2004) The TATGARAT box of the HSV-1 ICP27 gene is essential for immediate early expression but not critical for efficient replication in vitro or in vivo, *Virus Genes* **29**, 335-343.
12. Evans, H., Mello, L.V., Yongxiang, F., Wit, E., Thompson, F.J., Viney, M.E., Paterson, S. (2008) Microarray analysis of gender-and parasite-specific gene transcription in Strongyloides ratti, *Int J Parasitol* **38**, Elsevier, Oxford, UK.
13. Grinfeld, E., Ross, A., Forster, T., Ghazal, P., Kennedy, P. (2009) Genome-wide reduction in transcriptomal profiles of varicella-zoster virus vaccine strains compared with Parental Oka strain using long oligonucleotide microarrays, *Virus Genes* **38**, 19-29.
14. Williams, D. R., Li, W., Hughes, M. A., Gonzalez, S. F., Vernon, C., Vidal, M. C., Jeney, Z., Jeney, G., Dixon, P., McAndrew, B., Bartfai, R., Orban, L., Trudeau, V., Rogers, J., Matthews, L., Fraser, E. J., Gracey, A. Y., and Cossins, A. R. (2008) Genomic resources and microarrays for the common carp *Cyprinus carpio* L. *Journal of Fish Biology* **72**, 2095-2117.

15. Taggart, J. B., Bron, J. E., Martin, S. A., Seear, P. J., Hoyheim, B., Talbot, R., Carmichael, S. N., Villeneuve, L. A., Sweeney, G. E., Houlihan, D. F., Secombes, C. J., Tocher, D. R., and Teale, A. J. (2008) A description of the origins, design and performance of the TRAITS-SGP Atlantic salmon Salmo salar L. cDNA microarray, *J Fish Biol* **72**, 2071-2094.
16. Korf, U., Henjes, F., Schmidt, C., Tresch, A., Mannsperger, H., Löbke, C., Beissbarth, T., and Poustka, A. (2008) Antibody Microarrays as an Experimental Platform for the Analysis of Signal Transduction Networks, *Adv Biochem Eng Biotechnol* **110,** 153-175.
17. Olkhov, R. V., and Shaw, A. M. (2009) Quantitative label-free screening for antibodies using scattering biophotonic microarray imaging, *Anal Biochem* **396**, 30-35.
18. Olkhov, R. V., and Shaw, A. M. (2008) Whole serum BSA antibody screening using a label-free biophotonic nanoparticle array, *Anal Biochem* **385**, 234-241.
19. Olkhov, R. V. and Shaw, A. M. (2008) Label-free antibody-antigen binding detection by optical sensor array based on surface-synthesized gold nanoparticles, *Biosens Bioelectron* **23**, 1298-1302.
20. Tan, C. P., Cipriany, B. R., Lin, D. M., and Craighead, H. G. (2010) Nanoscale Resolution, Multicomponent Biomolecular Arrays Generated By Aligned Printing With Parylene Peel-Off, *Nano Lett* **10,** 719-725.
21. Clarke, S. (2005) Protein and Peptide Microarray- Based Assay Technology, in Walker J. M. (ed) *The Proteomics Protocols Handbook*, Humana Press Inc., Totowa, New Jersey.
22. Scienion AG, (2010) sciFLEXARRAYER S5 and S11 - versatile tool for ultra-low volume applications in array manufacturing. http://www.scienion.com/index.php?mid=42&vid=&lang=en. Accessed 15 August 2010.
23. Leung, F. L., Pang C. P. and Browne K. (2001) Guide to microarray hardware- a researcher perspective. http://www.images.technologynetworks.net/resources/comptab.asp. Accessed 15 August 2010.
24. Fodor, S. P., Read, J. L., Pirrung, M. C., Stryer, L., Lu, A. T., and Solas, D. (1991) Light-directed, spatially addressable parallel chemical synthesis, *Science* **251**, 767-773.
25. Schena, M., Shalon, D., Davis, R. W., and Brown, P. O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray, *Science* **270**, 467-470.
26. Schober, A., Gunther, R., Schwienhorst, A., Doring, M., and Lindemann, B. F. (1993) Accurate high-speed liquid handling of very small biological samples, *Biotechniques* **15**, 324-329.
27. Blanchard, A. P., Kaiser, R.J. and Hood, L.E. (1998) High-density oligonucleotide arrays *Biosens Bioelectron* **11**, 687-690
28. Theriault, T. P., Winder, S.C. and Gamble, R.C. (1999) Application of ink-jet printing technology to the manufacture of molecular arrays, in Schena, M (ed) *DNA Microarrays - A Practical Approach*, Oxford University Press, Oxford.
29. Wingren, C., and Borrebaeck, C. A. (2009) Antibody-based microarrays, in Bilitewski U. (ed) *Microchip Methods in Diagnostics*, Humana Press, New York.
30. Cornelia, S., Christer, W., Farid, K., Mingyue, H., Michael, J. T., and Carl, A. K. B. (2006) Improved affinity coupling for antibody microarrays: Engineering of double-(His)6-tagged single framework recombinant antibody fragments, *Proteomics* **6**, 4227-4234.
31. Peluso, P., Wilson, D. S., Do, D., Tran, H., Venkatasubbaiah, M., Quincy, D., Heidecker, B., Poindexter, K., Tolani, N., Phelan, M., Witte, K., Jung, L. S., Wagner, P., and Nock, S. (2003) Optimizing antibody immobilization strategies for the construction of protein microarrays, *Anal Biochem* **312**, 113-124.
32. Yu, X., Xu, D., and Cheng, Q. (2006) Label-free detection methods for protein microarrays, *Proteomics* **6**, 5493-5503.

33. Angenendt, P., Glokler, J., Sobek, J., Lehrach, H., and Cahill, D. J. (2003) Next generation of protein microarray support materials: evaluation for protein and antibody microarray applications, *J Chromatogr A* **1009**, 97-104.
34. Seurynck-Servoss, S. L., White, A. M., Baird, C. L., Rodland, K. D., and Zangar, R. C. (2007) Evaluation of surface chemistries for antibody microarrays, *Anal Biochem* **371**, 105-115.
35. Grainger, D. W., Greef, C. H., Gong, P., and Lochhead, M. J. (2007) Current microarray surface chemistries, in Rampal J.B. (ed) *Microarrays: Volume 1: Synthesis Methods*, Humana Press, New York.
36. Wu, P., and Grainger, D. W. (2006) Comparison of Hydroxylated Print Additives on Antibody Microarray Performance, *J Proteome Res* **5**, 2956-2965.
37. Delehanty, J. B. (2004) Printing Functional Protein Microarrays Using Piezoelectric Capillaries, in Fung E.T. (ed) *Protein Arrays: Methods and Protocols*, Humana Press, New York.
38. Delaney, J. T., Smith, P.J., Schubert, U.S. (2009) Inkjet printing of proteins, *Roy Soc Chem* **5**, 4866-4877.
39. Dijksman, J. F., and Pierik, A. (2008) Fluid dynamical analysis of the distribution of ink jet printed biomolecules in microarray substrates for genotyping applications, *Biomicrofluidics 2*, 044101-044122.