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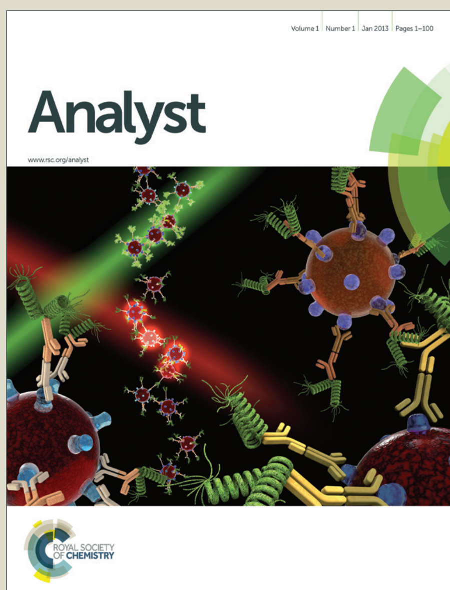

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A Critical Comparison of Protein Microarray Fabrication Technologies

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Abstract. Of the diverse analytical tools used in proteomics, protein microarrays possess the greatest potential for providing fundamental information on protein, ligand, analyte, receptor, and antibody affinity-based interactions, binding partners and high-throughput analysis. Microarrays have been used to develop tools for drug screening, disease diagnosis, biochemical pathway mapping, protein-protein interaction analysis, vaccine development, enzyme–substrate profiling, and immuno-profiling. While the promise of the technology is intriguing, it is yet to be realized. Many challenges remain to be addressed to allow these methods to meet technical and research expectations, provide reliable assay answers, and to reliably diversify their capabilities. Critical issues include: (1) inconsistent printed microspot morphologies and uniformities, (2) low signal-to-noise ratios due to factors such as complex surface capture protocols, contamination, and static or no-flow mass transport conditions, (3) inconsistent quantification of captured signal due to spot uniformity issues, (4) non-optimal protocol conditions such as pH, temperature, drying that promote variability in assay kinetics, and lastly (5) poor protein (e.g., antibody) printing, storage, or shelf-life compatibility with common microarray assay fabrication methods, directly related to microarray protocols. Conventional printing approaches, including contact (e.g., quill and solid pin), non-contact (e.g., piezo and inkjet), microfluidics-based, microstamping, lithography, and cell-free protein expression microarrays, have all been used with varying degrees of success with figures of merit often defined arbitrarily without comparisons to standards, or analytical or fiduciary controls. Many microarray performance reports use bench top analyte preparations lacking real-world relevance, akin to "fishing in a barrel", for proof of concept and determinations of figures of merit. This review critiques current protein-based microarray preparation techniques commonly used for analytical and function-based proteomics and their effects on array-based assay performance.

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Introduction

Proteomics has emerged over the past decade as a critical approach to dissecting biological pathways and mechanisms and to identifying key players in these mechanisms. This knowledge of pathways has led to efforts to identify bioactive agents that affect them, in native function, biological toxicity and importantly in new therapeutics discovery. Compelling advancements in analytical and clinical protein application developments have resulted[1]. Protein microarrays initially were advocated as a promising tool to enable these advances in proteomics[1–4]. Yet, as microarrays remain an important approach, challenges with the technology remain, limiting the contributions and impact that this technology provides to several fields[5,6]. Protein microarrays have been used to identify biomarkers for disease diagnosis and therapies, protein-protein interaction analysis, vaccine development, biochemical pathway mapping, enzyme–substrate profiling, and immunoprofiling[1–4,7]. The primary advantages of protein microarrays over traditional protein separation/identification techniques such as ELISAs and Western Blots include the: (1) ability to more detect low-abundance proteins in complex milieus with improved sensitivity and specificity), (2) ability to multiplex protein detection on a single platform that can be fabricated rationally by design, and (3) minimal sample consumption[8,9]. Since the original concept of the protein microarray was forwarded many different iterations on this idea and diverse resulting technologies to fabricate protein microarrays and microchips have been reported for analytical, clinical, and function-based proteomics. Few reviews are available that compare these fabrication technologies and critically evaluate their strengths and weaknesses to allow insight into key technological advances required for protein microarray capabilities [2–4,10] . Figure 1 encapsulates some of the more prominent technologies discussed and applied as described in current literature.

This review article discusses these technologies and their challenges, while also exploring and presenting more recent advances. Three general protein microarray designs are reported: (1) functional, (2) analytical, and (3) reverse phase[11]. Figure 2 illustrates these basic designs. Functional microarrays assay the functional properties of isolated, captured proteins and are used to screen protein interactions to identify post-translational modifications (PTMs), analyze enzymatic activities, and study protein pathways[12–14]. Analytical microarrays, also called capture or antigen microarrays, use antigens or affinity proteins for characterizing levels of protein expression in samples, antibody quantification, biomarker discovery, protein activity state determinations in signaling pathways, and for profiling antibody repertoires in autoimmunity, cancer, infection or following vaccination[15,16]. Moreover, antigen arrays are tools for determining the specificity of antibodies and related affinity reagents[17]. Reverse-phase arrays use cell lysates[18,19] or serum samples[20] for biomarker discovery by studying changes in the expression of specific proteins and PTMs during disease progression[11]. As a result of these broad applications and in response to multiple challenges in customizing the approach to different research and technology missions, multiple different protein array design and printing technologies have been developed to address different requirements desired for each assay and enable each type of array as a discovery tool.

The attraction of assay miniaturization using printed microspots plus the versatility of depositing thousands of such spots as individual assay "units" on a common substrate have enabled microarrays to achieve theoretically lower limits of detection, broad applications, less protein reagents per assay, faster assay speeds, and greater throughput than traditional immunoassays[21,22]. While widely adopted in research use currently, arrays have problems that hinder broader adoption of the technology, including the important expansion into clinical diagnostics[23]. These challenges include (1) poor antibody compatibility with the surface chemistries, with resulting shelf-life and quality control issues in the assay format[24], (2) inconsistent printed reagent spot morphologies, (3) inconsistent assay signal quantification (i.e., poor coefficient of variations for spot-spot, assay-assay, substrate-substrate and lab-lab comparisons), (4) unfavorable protocol conditions such as pH, temperature, or desiccation, (5) variability

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in assay kinetics and/or endpoints, (6) low signal-to-noise ratios due to assay sample complexity, difficult protocols, contamination, and static assay conditions[21,22], and (7) substantial primary-secondary antibody cross-reactivity leading to high false positive signals[25,26]. A survey of the number of different array printing solutions applied to various different slide surface chemistries provides evidence that (1) no single microarray printing format, reagent set or technology meets the assay needs of all microarray users, (2) no protocols to date provide sample-to-answer formats direct from complex biological media without either analyte amplification (e.g., PCR) or enrichment (e.g., immunoprecipitation or chromatography), or extensive sample purification and analyte isolation prior to array processing, and (3) challenges remain with microarray manufacturing technologies to yield consistent assay platforms capable of reliability, reproducibility and the figures of merit expected of this technology base[27,28]. While the Microarray Quality Control (MAQC) projects have focused on standardization issues for DNA microarrays, much of the fundamental concerns and principles of focus within the MAQC quality control initiative also apply to protein microarrays, including experience of the user, internal and external performance controls, and modeling selection and validation[29,30]. While protein microarray performance is not at the level of use and experience of nucleic acid microarrays, quality control studies for protein microarrays analogous to those applied by MAQC are warranted.

The theoretical limit of detection (LOD) for a microarray spot is reported to be 1 fg/mL[29,30]. Some researchers claim to reach this LOD with specific technologies and proteins[29]. Unfortunately, these claims seem to be lab- or condition- specific, and fail to apply broadly to most current arrays or real-world samples of interest. Often, the results are achieved by using a simple salt buffer rather than a more clinically relevant sample or milieu like serum, tissue lysate, CNS, urine, or other complex biological fluid. While these results are an important first step, they fail to validate array use for diagnostics and decision-making in clinical settings. The time has now come to move beyond buffer-based, laboratory-concocted simple assays to assert and validate array sensitivity in relevant conditions. Unfortunately, this problem remains a red herring for the microarray community in clinical applications as many diseases would benefit from more sensitive diagnostic biomarker assays - cancer biomarkers, acute cardiovascular events, infections, and bio-threat agents as prominent examples[31]. Over 90% of potential biomarkers for cancer require an LOD below 1 pg/mL for early detection. However most low abundance proteins are not reliably detected with the current-art immunoassays[31]. The compelling need for lower LODs in clinical use allows microarrays a unique opportunity to improve current biomarker assays.

The fabrication techniques applied to protein microarrays remain central to their performance and reliability[11]. Conventional printing technologies including contact (quill and solid pin), non-contact (piezo and inkjet), microfluidics, lithography, and cell-free protein expression printing have well-developed histories at this point, allowing for critical comparisons of each approach to anticipated bioanalytical, pharmaceutical and clinical chemistry goals. Understanding the strengths and weaknesses of these arraying systems should facilitate development of improved performance of these methods for diagnostic and drug discovery applications.

Pin Printing of Microarrays.

The most widely adopted technology for printing microarrays remains contact pin printing, primarily because of the pin printer's early and wide adoption in DNA microarray printing and direct transfer of lessons learned to later protein printing strategies[32]. Pin printing hardware uses solid pins, split or quill pins, tweezers, or other liquid transfer/deposition pin types to load and then deliver protein-containing aqueous solution droplets in printing buffers onto solid microarray surfaces[33–35]. Figure 3 shows the basic concept for pin-based printing. A robotically controlled print head contains from one to dozens of

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fluid-dispensing pins that are repeatedly dipped into wells of a protein-source microtiter plate containing wells of purified proteins in print buffer. The robot captures a known, controlled volume of protein solution from each well into each pin and then lightly touches each pin onto the surface of the functionalized glass or other microarray surface to dispense nanoliters of its liquid payload as a droplet[36,37]. This technology relies on surface energetics (i.e., liquid wetting on array, and controlled interfacial tensions) between the solution, the substrate and the pin surface to deposit controlled amounts of protein solution onto the substrate and release it reliably from the pin[33]. This deposition, the resulting droplet wetting and subsequent evaporation process that provide the final array microspot footprint and morphology, are pin-, printing solution-, and substrate chemistry- dependent. These considerations for array substrate chemistry have been reviewed elsewhere [25,26,37].

Pin printing technology has advanced considerably since solid pin printing was introduced by Brown et al.[38]. Early contact printing technology revolved around the use of a solid pin for dipping into reagent pools and subsequently carrying the sample over to the surface for printing [39]. This system evolved to include better fluid sample handling and hence reduce cross-contamination through the introduction of split pins. Further improvements included increased reservoir size and modified pin designs that have come to be known as quill pins[40]. The sample volume picked up by the pin then depends on the diameter of the pin itself and the pin-solution interfacial energy (capillarity), essentially the combination of surface tension and adhesive forces to overcome the effects of gravity[33]. A primary challenge lies in creating pins with a precise diameter where pin-head tolerances must be micron or sub-micron in size. These pins are usually designed from stainless steel, titanium or tungsten[41]. These pin materials, their respective surface finish/treatments, cleaning under simple rinse conditions, and chemical composition of the print solution all play major roles in their capillarity and therefore the final microarray print quality[33]. The liquid to be deposited is drawn into the pin slits on the order of 10 to 100 µm in diameter[33]. The pin then deposits each liquid droplet by slight surface contact with the array surface, exchanging one set of designed wetting energetics for another set on the substrate to control deposited volumes. However the added benefit of the reservoir or the split is that it can produce several hundred pico-liter sized prints before having to return to the source plate reservoir to rinse and re-fill [33,42]. These pins are relatively easy to use, maintain, and, if well-controlled, can generate reproducible microspot results[43].

As mentioned earlier, while the greatest advantage of this hollow pin system is its ability to print hundreds of times before recharging, each time the pin strikes the surface a slight change in spot size and volume can occur[27]. Microspot surface areas are often reduced with subsequent prints (i.e., steady reductions from 325 um to 100 um diameters in a single run[44]). Quills pins are better suited to low viscosity liquids[45] liquids as the effectiveness of capillary action depends on the consistency of the printing solution drawn up into the pin with each source plate loading. This requirement was appropriate to their introductory use in genomic arraying. However, with frequent application in both antibody and protein printing[46], viscous print additives common to protein print buffers (i.e., glycerol, concentrated sugars, or high molecular weight polymers[47]) challenge the reliability of these pins (i.e., clogging and carry-over issues) but are necessary to maintain the proteins in an aqueous environment. Limited pin clogging[48]and the ability to print different viscosity print buffers are benefits unique to solid pin designs[49]. By contrast, limitation of quill printing is that the tiny slit or reservoir can clog due to solution composition or environmental conditions (e.g., drying)[45]. This clogging translates into a greater risk of cross-contamination between prints[50]. As with solid pins, quill pins are produced using the same materials; however, silicon has also been used[51]. The fact that the print liquid is drawn into a slit means that pin-based evaporation will not be as pronounced compared to solid pins. Viscous solution additives further reduce environmental effects on printing. Carry-over and cross-contamination may be more of

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an issue with pins containing reservoirs. While standard pin washing protocols may clean the outside of the pin, an ultrasonic bath is necessary to clean the deeper portions of the pin reservoir and lumen in order to dislodge any evaporative build-up and pin-adsorbed reagent proteins[28]. This build-up means that a cleaning step between successive sourcing is required to evacuate any cleaning fluids lodged in the reservoir. As such, it is imperative that the pin system is set up in a dust-free environment[52]. Reagent and plate handling, fluidics, pin loading substrate transfer, drying and well-plates must use protocols to ensure that ambient contamination and air-borne particles are not introduced into printed reagents[53,54].

Another major disadvantage of the pin printing system in array fabrication is the basic operational design. The robotic head and pins must travel back and forth between the substrate deposition site, the source plate and the cleaning reservoir after only a few prints of each print reagent (limited by pin number and fluidics controls in the head), which unfortunately increases the printing time[28]. The time required may be reduced by increasing the number of pins and associated fluidics channels interfaced on the print head; however, this expansion will significantly increase printer complexity and costs. Secondly, the contact nature of this print method also means that during every deposition there is a chance that the array surface may be damaged[39,55]. The associated issue is the possible change in pin wetting and capillarity properties associated with substrate contact and transfer of surface chemistry to the pin lumen that could alter arrayed print volumes and spot sizes, and potentially block the reservoir[56,57].

Other considerations are important in the use of pin printing for protein microarray fabrication. For example, the number of arrayed spots required per printing area is a concern as substrates are expensive, some requiring complex assay protocols that increase handling time and material costs[58]. Furthermore, large numbers of pins in the robotic heads are usually accommodated in oversized pin holders that allow for smooth insertion and convenient pin removal. However, one problem stems from the natural pin wobble within these holders on contact deposition, resulting in variation in actual printing positions[45]. In addition, the actual contact of these wobbly pins with the surface[59] introduces imprecision: for two or more pins not aligned to contact the surface at the same height or time as all others in the head, some spots will not print correctly (or at all) in that particular row or column, leading to array printing errors[60].

In a typical pin printing setup, environmental conditions are usually controlled by enclosing the entire arraying system in a humidity-controlled environmental chamber, also acting to limit ambient dust and atmospheric contamination on the array substrate. Careful control of ambient humidity is crucial to spot desiccation, final print morphology and subsequent image analysis[59,61]. The deposited microspots may exhibit variable dried morphologies based on environmental conditions, and a certain risk of reagent evaporation is present even before the pin contacts the surface[62]. A typical solution load on a solid pin may be on the order of pico-liters; hence, pin evaporation can quickly become a significant issue in both deposited final volume as well as concentration known to influence droplet drying[62]. Drying of the protein can result in destabilization of secondary and tertiary structures, resulting in a change or loss of protein functionality. As pointed out by Papp et al., controlling environmental conditions goes beyond the geometric features of printed spots[63]. Solutions of varying print compositions may require specific temperature and humidity controls in order to preserve capture reagent structure and its analyte binding competence post-print[63]. While newer printing systems have been developed that claim to improve on some of the aforementioned issues, most of the marketing claims are made with model, stable proteins and under artificial, often irrelevant, assay

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conditions. While the pin printer is suitable in pin printed microarrays for many proteins, many other proteins have trouble with structure and function due to hydrophobic and drying condition of a pin array. This problem occurs more prominently in functional and antibody microarrays [47,59,61,64–66].

Current publication trends suggest that solid pin printing is still used albeit not as frequently as current array-based research branches out into many new, different areas where solid pins expose their vulnerabilities. Some of these areas include printing microparticles for optimization in immune response once exposed to dendritic cells[67] and label-free microorganism detection on SERS chips[68].

Microstamping of microarrays.

A popular alternative to pin printing is array transfer by microstamping[69–71]also referred to as microcontact printing or soft lithography[72]. Introduced by Whiteside et al.[73], soft lithography became the prime technique for developing small-scale elastomer-based features pre-designated based on lithographed master patterns. The term "soft lithography" now encapsulates a number of different, related pattern transfer technologies using patterned polymer monolithic substrates, the most prominent being microstamping[74]. Soft lithography is the fabrication method by which microstamps are created[75,76]. Both pin printing and microstamping are essentially different versions of serial deposition techniques where pins and stamps are each iteratively loaded with reagents for surface transfer, then cleaned and re-loaded after each substrate deposition (see Figure 3). While pins are fluidically individually addressable with unique print solutions, microstamping is generally a more parallel deposition method where many stamp features are inked and printed with the identical reagent solution. Elastomeric stamps, usually made of crosslinked polydimethylsiloxane (PDMS, silicone elastomer), are fabricated with specific micro-features using routine positive-negative lithography transfer. These features are then inked using soak, spray-on, or robotic feature-feature ink transfer of printing reagent solutions. Stamps then can be dried or used wet to transfer the inked printing solution to the array substrate by physical contact, very similar to using a rubber stamp to transfer ink[77,78]. A diverse array of different protein arrays are reported from both dry and wet stamp transfer, using many different protocols and reagent sets. Feature sizes for stamped array span the micron to sub-micron range, with line resolutions for final protein features in the less than ones of microns with low CVs on spot morphologies [77,79].

Further, Figure 4 shows one type of lithographic-based printing using pattern transfer to soft polymer stamps that can then be inked with reagents for array printing and stamping. Micro-molding of the stamps is a relatively straight-forward[33] adapting well-known photolithography methods using patterned photomasks. Uncured elastomer such as PDMS is spread or injected onto the patterned negative mask substrate and cured (heat or light). The cured PDMS device is then removed, cleaned and trimmed. Multiple stamps can be generated simultaneously at low cost and can be discarded after printing is completed[33]. Contamination between prints is no longer an issue if PDMS curing by-products and uncured additives are fully removed prior to printing[80], minimizing printing time and cleaning costs[33]. Clear advantages of microstamping are its ease of use and low cost, making it readily available for general lab use[33]. The main benefit of this technique is that it permits high throughput microarray

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fabrication[81]. The stamping process varies depending on the type of application, stamped reagents, buffers, array substrates, and environmental controls[72].

Microstamping has been used to produce many different arrays, including proteins of many types[73,79,82], quantum dots[83–85], nanoparticles [84,86], microbes [87,88], studies of cell interactions[78,89,90], and DNA[91]82. Stamp surfaces can be coated with different solvents and depending on the elastomer used, can be made more hydrophobic or hydrophilic[33,92]. Such versatility in surface modification not only results in improved reagent transfer from stamp to substrate, but also in preventing reagent adsorption and aggregation[83]. Further, masks can be made with nm-sized features, allowing for greater flexibility in creating complex geometries and sub-micron features and size and shape not accessible using pin printing methods[90,92]. With such enhanced geometrical controls, arrays printed with microstamps tend to be highly reproducible[93]. In addition, such control over stamping features means precise mechanically placed deposition can be achieved, resulting in increased printing resolution. While current literature refers mainly to microstamping, analogous nanostamping is slowly gaining traction through advanced fabrication methods[76,94]. The clear advantage is then reduced printing area and reduced sample consumption, balanced against the challenges of feature resolution, and capillarity/wetting issues as topographic dimensions are reduced. A number of different printing solutions can be inked onto different regions of the same stamp face for transfer of multiplexed features for high throughput analysis[33,95].

Microstamping has other general challenges. While fabricating the stamps may be an inexpensive process, the initial lithographic and clean-room equipment or equipment usage fees may be quite high[33]. Stamps are usually designed using relatively elastic materials such as PDMS that facilitates surface transfer of aqueous analyte while also establishing a tight fluid-transfer seal against a substrate to prevent cross-feature reagent bleed[33]. However, hydrophobic methacrylate (e.g., PMMA resin) has also been utilized for patterning and microprinting because of its rigidity, low interfacial tension, and low glass transition temperature, facilitating efficient stamp manipulation[91]. PMMA's added benefit as a more rigid stamp is in allowing much smaller feature topographies and resolutions on the stamp. Using PDMS, extremely fine features risk mechanical deformation due to fluid wetting deformations and compression under transfer[33]. Stamp materials should consider the nature of the reagents, solvents, and features for printing. Swelling of the elastomer during inking with certain solvents can be a severe problem[96]; however, it can be mitigated through surface modification and is generally not an issue for aqueous media with PDMS or PMMA.

Careful cleaning of the virgin stamp surface is essential since many hydrophobic stamp resins have surface-active components (e.g., liquid hydrophobic oligomers, monomers, additives, initiators) bleeding to the stamp surface under ambient conditions. These stamp "bleeds" can occur throughout the stamp lifetime as well, meaning that successive careful cleaning is required to prevent stamp contamination transfer[97]. If several different reagents are printed, cleaning is required between prints. Rinsing of fine surface structures is complicated by capillarity and surface tension where solvent vapor pressures are different from bulk due to fine geometry. Cleaning in-between geometries on the stamp requires dedicated equipment such as ultrasonication and even ultrasonic jet technology[33,95]. Furthermore, not

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all inked content or cleaning reagents will rinse readily from fine topographies, a potential source of inking contamination[97,98]. Two other major considerations in microstamping included deposition efficiency and protein integrity. Small and commonly inconsistent amount of the proteins are transferred to the array surface[72]. Also, the transfer occurs in "semidry" conditions which just as is the case with pin printing, potentially and commonly affect protein structure and function, especially with sensitive proteins[72].

Lithographically prepared microarrays.

Photolithography is a type of projection lithography where a surface pattern is created on a substrate using a mask exposed to high energy radiation, usually ultraviolet light (UV)[33,99]. A spin-coated layer of photoresist (e.g., a radiation-sensitive organic polymer) is selectively exposed to UV or e-beam irradiation through a patterned mask[100]. The polymer chains of the resist either cross-linked or break in irradiated areas. Positive resists are soluble to development solutions whereas negative resists are insoluble (see Figure 5). The exposed substrate then is immersed in development solution and a pattern is generated on the substrate by removal of resist, governed by the pattern. A positive resist produces an identical replica of the mask template, while a negative resist is a complimentary geometrical image to the mask. The process is completed by developing the surface to form micrometer-sized regions where adhesion-promoting molecules will bind[33]. Often PDMS-molded or cast replicas of the resulting patterned template can be used as a patterned substrate or as stamps for soft- lithographic processes. These PDMS molds are often produced in a parallel, single-step fabrication mode. Photolithography often requires expensive equipment, clean rooms, and high maintenance costs that limit its complete wide-scale implementation. Photolithographic patterning for protein microarray applications is highly accurate and as such is a major technique for producing micron to submicron scale patterns on solid surfaces[100]. Photolithography benefits from parallel microarray fabrication where, instead of individual spot fabrication, the entire substrate can be coated and patterned at once[101]. Further, it is a highly reproducible technique for fabricating highly complex geometries[101]. However, while photolithography can be used for creating extremely dense microarrays, they are limited to the evaluation of a single protein at any one time[102]. Furthermore, to ensure consistent layering, the substrate must be properly cleaned of contaminants[102].

Interference lithography is a closely another type of photolithography where the photoresist is selectively exposed to the exposed light radiation based on projected light interference patterns[100]. The interference pattern is created by joining two or more light beams originally split, usually through a prism and diffraction gratings, and then recombined, creating the optimal interference fringes. The interference fringes are nanometer-sized to produce patterns on photoresist surfaces of this dimension. However, these generated patterns are limited based on the configuration of the fringes generated. Using this approach, chemical patterns with a resolution of tens of nm have been fabricated[100].

Lithographed "Direct Write" Array Patterns. In addition to aiding micropatterned elastomer stamp fabrication and soft lithography, conventional photolithography as shown in Figure 5 has been utilized directly in a number of approaches for developing high density biomolecule microarrays[101–105]. methods share the common approach of using high-energy focused electromagnetic radiation applied to coated surfaces to produce desired surface patterns. However, instead of using pre-fabricated patterns from photomasks, direct-write computer-controlled beam rastering can be used across surfaces pre-coated with capture reagents (not resist) to ablate some molecules off only in certain regions, while

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leaving them unadulterated in others as surface-immobilized patterns. Direct write lithography utilizes collimated, highly focused light (typically x-ray and deep UV), focused electrons, and focused ion beams capable of controlled surface rastering to initialize localized chemical changes in surface layers including ablation of immobilized biomolecules to create desired patterns[101–105].

Laser Writing. Several techniques exist for direct laser writing of microarrays., In one, a pulsed laser is scanned across a quartz disk coated with an array of protein solutions [51,106–108]. The laser causes localized evaporation in the disk coating. As the sample evaporates, liquid droplets accumulate on the substrate, resulting in uniform spots from 10 to 50 µm diameters. Very little sample is used in this method. In laser ablation fabrication, UV lasers directly ablate deposited protein-blocking layers according to the design of the mask. Ablated protein is washed away, leaving proteins only in the desired masked areas. Proteins can also then also be re-applied to the ablated areas. The main disadvantage in laser writing that limits adoption is the relatively low throughput of the technology[33,72].

Electron-beam (e-beam) lithography (see Figure 6)[109] is used to perform two surface functions relevant to arraying: increase crosslinking within the targeted area to retain and stabilize it, or degrade the surface chemistry to remove it, creating areas of negative or positive resist or topological features, respectively[110]. E-beam's main advantage is production of geometrically complex shapes at 10^1–10^6 nm resolution, although the creation of complex nanopatterned surfaces is time-consuming[110]. Further, it can be utilized to immobilize molecules on a substrate both in 2D and 3D[110]. The ability to pattern and print onto different substrates is derived from the ability to repetitively or cyclically deposit molecules of varying chemistries onto surfaces[110]. For example, e-beam has been used to locally alter the hydrophobicity and/or functionality of conventional lithographic polymer resists after which proteins are immobilized in patterns by hydrophobic interaction or coupling chemistries. Other applications include using it to create gold surface features as sites for formation of self-assembled monolayers (SAMs), SAMs ablation including silane, thiol-on-gold, and phosphonic acid-on-aluminum SAMs, and negative resists such as poly(ethylene glycol) (PEG)[110].

Techniques that exploit e-beams to create chemical or protein patterns can be classified into resist-based, resist-less and e-beam-initiated chemical reactions. Resist-based patterns are formed using e-beam lithography on PMMA-deposited surfaces[111]. Selective removal of PMMA polymer by resist lithographic methods (vida infra) creates high-resolution surface patterns. Direct ablation, on the other hand, utilizes an e-beam to locally remove a perfluorosilane SAM pre-adsorbed on silicon oxide surfaces[112]. This process functionalizes the exposed areas with animosilanes that can then be chemically derivatized with a biotin linker, resulting in 100-nm wide protein patterns of avidin and biotinylated GFP[112]. Alternatively, e-beam can be used to selectively remove a thin layer of SiO2 deposited on TiO2. In a second step, molecules, such as dodecyl phosphate, can be adsorbed directly and selectively on the exposed TiO2 and then the exposed SiO2 regions can be coated with PLL-g-PEG protein-repellent polymer[113]. Finally, e-beam lithography can be used to initiate chemical reactions directly on a surface. Chemically well-defined surface patterns on the 100-nm scale have been formed by using an electron beam to locally reduce nitro functionalities in SAMs of 3-(4-nitrophenoxy)-propyltrimethoxysilane to amino functionalities[114].

E-beam's disadvantages are its intrinsic expense, requiring clean room conditions, computer-generated beam rastering, and mask-generating technologies[115]. Additionally, low throughput limits the use of e-beam lithography because of long exposure times. Furthermore, this technique utilizes serial printing techniques, creating individual areas of interest, hence suffering from large printing times compared to photolithography [110]. However, e-beam lithography primary applications usually

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require fewer substrates, higher resolutions, and/or small inter-feature spacings not readily produced by other methods.

Focused ion beam direct-write[116–119] and UV/soft x-ray lithography[117,120] are analogous technologies to e-beam writing methods. These technologies, especially focused ion beam (FIB) milling, have been used to fabricate nanostructures on surfaces smaller than 5 nm. These open nanochannels can be used for single-molecule DNA and protein microarray studies[118].

Molecular Assembly Patterning) **by Lift-off** (MAPL) is also a type of photolithography. Nanoimprint lithography (NIL) and molecular assembly patterning by lift-off have been used to produce streptavidin patterns with feature sizes in the order of 100 nm[121]. The nanolithographed stamp is embossed into a heated PMMA film. The resulting PMMA stamp is then dry etched to create the pattern topography in a PMMA/Nb_2O_5 contrast. After stamp manufacturing, a biotin-functionalized copolymer is selectively adsorbed onto the metal oxide surface[100]. After stamp manufacturing, a biotin functionalized copolymer is adsorbed onto the oxide surface[122]. The PMMA lifted-off and protein-resistant PLL-*g*-PEG fills the background[113,114]. The patterns of the printed proteins have nanoscale resolution[91]. MAPL allows for parallel patterning and is fast, reproducible, and economic. The surface chemistry can be functionalized with a variety of bioactive groups[121].

The MAPL technique is versatile allowing for patterning multiple biomolecules with 2D and 3D spatial control. Surfaces can have a patterned combination of motifs each with specific properties. MAPL is inexpensive and can be performed with little cost and with little experience. MAPL, however, is a low throughput method currently. MAPL allows for control over the pattern geometry and the type and density of bioligands on the surface. MAPL generates arrays with low background, layers of different types of biomolecules, and as variety of form, size, and geometry of the templates[100].

Dip-Pen This technique utilizes a scanning surface stylus-like probe adapted from atomic force microscopy, where the probe tip is immersed in biomolecule ink (protein solution) and this tip-inked solution is then transferred to the array surface in 'write' mode and in specific patterns based on rastering the probe in a desired pattern (see Figure 7)[123,124]. This technique has been utilized to produce a number of different protein, antibody or lipid arrays and creation of miniaturized immunoassays[125–128]. Surface printing involves the slow deposition of the probe-tip-resident biological ink via capillary action off the probe tip onto the dry surface[129]. Molecular interactions on the surface vary depending on the substrate type and reagent "ink" used. Surface interactions with the deposited ink can be covalent through use of complementary functional groups between the molecule and surface, electro-chemisorption or by physisorption[130]. This process was first described using the scanning probe as the nib, the substrate as the paper and ink molecules with the correct affinity as the ink[115]. Hence, the major advantage of the method is that one delivers a variety of different molecules onto a single substrate by inking the probe tip sequentially with different inks[131] or by using multiplexed probe tips scanning simultaneously with different inks, each controlled by independent rastering processes[130]. Further, as the DPN name suggests, this system is able to retain line resolution with prints to 15 nm[130]. However, when creating microarrays a number of factors must considered, including the type of substrate, the geometry of the tip, tip-surface contact time, write time and the environment[130]. Further, if ink-surface attachment occurs by physisorption with a pre-adsorption phase then large or high-throughput array generation limited[112]. DPN enjoys broad academic use with over 200 labs utilizing the technology[87]; however clinical applications remain limited due to the high cost of AFM and the limited throughput of the technology. A listing of applications of DPN is extensive [123,124,131].

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Scanning probe lithography. In contrast to DPN use of the rastering probe to directly print ink, scanning probe microscopes can also fabricate arrays using a tunneling microscope (STM) or atomic force microscope (AFM) to produce nanoscale structures by scribing patterns into a substrate[122]. Further scanning probe lithography can simultaneously be used to probe material properties with the scanning tip[122]. To overcome limitations in speed, investigators have developed parallel devices such as the Millipede[132] and Snomipede[133]. The main advantages of scanning probe lithography include reproducibility, medium to high throughput, alignment accuracy, high spatial resolution, and flexibility in adapting to a variety of study conditions[100,134]. While AFM instruments and tips are commercially available, the cost of such equipment as well as the problems associated with cross-contamination and surface damage limit wide scale adoption[122]. Additionally, these finely fabricated features require subsequent inking to produce arrays, a task not readily accomplished at the scale of surface structures produced.

Noncontact printing methods in array fabrication.

Noncontact Printing. Non-contact printing's distinctions from contact printing are intuitive from its name: no contact exists between the printing tool and microarray surface for molecular deposition to occur. Print solution is instead ejected as a droplet or stream from a reservoir through an orifice at a precise distance directly onto the microarray surface[135]. A common non-contact technique uses commercial inkjet printing technology adapted to biomolecules. Protein solution is contained in the printer's ink cartridges and ejected from the print head nozzle at a distance of 1-5 mm[136] mm[129] when prompted by the interfaced computer controller. Three types of ink jet ejection mechanisms include: (1) piezo actuation[136–138], (2) valve-jet [139], and (3) thermal inkjet[33,136,140]. Figure 8 schematically depicts this ejection process through the inkjet reservoir orifice[136,141]. In piezo actuation, a volumetric change in the ink reservoir induces an abrupt pressure change in the reservoir, causing ejection of the droplet[136–138]. In valve-jet ejection, a reservoir valve is opened and closed under computer control while under continuous high pressure, resulting in ejection of a stream of drops. In thermal ejection, rapid heating of gas in the ink chamber induces an abrupt pressure change that ejects the droplet[136]. All three noncontact microarray printing technologies attempt to deposit uniformly dense arrays of small droplets of probe molecules while seeking to prevent solution contamination and loss of function due to structural changes to the ejected and deposited protein[33,43,140].

The primary advantage of noncontact printing is a lack of direct contact and surface damage, especially detrimental to nitrocellulose and hydrogel array substrates[55,142]. In addition, proteins in inking solutions often adsorb onto metal surfaces within contact printing pin lumens, resulting in fouling and cross-contamination between print runs[143]. The large non-contact ink reservoir is also an additional advantage over contact printers, resulting in larger print runs before ink depletion. This greatly improves spot reproducibility[142]. Some non-contact printing systems are also very fast, printing approximately 475 features per second, and from multiple different ink sources[142]. This speed and multiplexing is unrivaled compared to any other printing technology and "represents a paradigm shift in throughput"[142].

A main challenge associated with noncontact printing methods regards print artifacts associated with accumulation of ink proteins at the print-head nozzle[142]. This problem (i.e., protein accumulation on a surface) is not unique to noncontact printing, but requires overall system optimization including buffer surface tension and rheology, surface wetting, humidity, blocking, and other printing conditions[136,144].

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Artifacts common to noncontact printing are failed spots, satellite spots, misplaced spots, and sample carryover commonly caused by low solubility protein inks or high salt content[136]. Highly viscous liquids with low surface tension and high ionic strength (sometimes common to protein array printing[47]) are particularly challenging as are inks that rapidly change properties to affect nozzle performance[136]. Common approaches to address these problems include use of fluorocarbons in print media, high salt buffers, organic co-solvents, or protein stabilizers such as serum albumin[142]. Fluorocarbons minimize humidity-related variabilities[142]. Protein stabilizers such as serum albumin and high salt buffers allow the protein ink solution to behave more consistently[142]. . Lastly, some water-miscible organic solvents such as DMSO help optimize spot wetting and humidity conditions[142].

Microfluidics-interfaced "Flow-Based" or "Wet" Print Arraying.

While contact and noncontact print methods remain the most widely adopted for array generation, persistent, difficult problems with resulting microspot quality and morphology remain concerns for the assay metrics and signal, especially for difficult or complex proteins. Most of these printing problems stem from microspot drying during and/or after protein adsorption or covalent attachment to the array surface[59,61,145]. These problems have been observed for nucleic acid arrays, but are even more of a concern for proteins arrays where microspot drying more profoundly affects resulting protein bioactivity on surfaces compared to nucleic acids [37,47].

A microfluidic-interfaced printer called the continuous flow microspotter (CFM) is shown in Figure 9 that addresses this issue directly using a three-dimensional microfluidic array-based mask that prints proteins onto a surface directly under microfluidic flow. Spatial control of patterns on the surface are controlled under flow using a microfluidic channel network interfaced with a microfluidic pumping manifold that links the protein reagents in a source plate to a PDMS micropatterned flow-through mask on the substrate[66]. The PDMS template seals on the array substrate, confines the solution, and subsequently deposits protein only to the area of each patterned micro-flowcell. The PDMS printhead interfaces directly on the substrate during the printing process, and the individual fluidic flowcells of the printhead circulate the protein sample into each defined spotted area confined by the flowcell. Here the protein in solution is exposed to the substrate under flow and can adsorb to or react with the substrate directly, or can be captured by a recognition molecule already residing there from previous deposition. Maintaining the protein in a liquid environment helps stabilize the secondary and tertiary structures that are essential in preserving protein folding and function[66]. The PDMS printhead produces individual continuous flow channels (inlet/outlet) between each well of the source-plate, the fluidics pumping manifold, and each flowcell outlet (i.e., to waste or to a collection plate). This micro-flowcell channel allows for bidirectional, stopped or continuous flow of solution across the substrate under fluidics control of shear rate and residence time. The surrounding, bulk PDMS of the printhead seals onto the surface and creates the array of individual spots[66]. During operation, the CFM is mounted on a platform with a machined polycarbonate manifold placed over it. An integrated circuit board is used to control the direction, flow, and shear rate of the solution reagents as they are pumped through the CFM by applying either positive or negative pressures[66].

A primary advantage of microfluidic-interfaced printing in this design is the resulting templated spot quality, achieved through bidirectional flow in each flow cell[139] without exposure to air or desiccation[66]. Bidirectional flow across each spot is achieved through the printhead's spot interface. Each spot interface is linked to an inlet that allows inking fluid to circulate across the spotted area as it passes through the microchannels over the array surface. The ink solution can be oscillated or recycled back and forth (bi-directionally) between the inlet and outlet wells, or stopped and equilibrated, until the desired mixing or substrate deposition level is achieved. Resulting prints spots are significantly more

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uniform and with tighter spot morphology than those printed by other methods[66]. The method also produces reliable spots at 100-fold lower protein concentrations. Arrays produced using the CFM can capture analyte from crude sample solutions in which the background protein milieu is 10,000 times more concentrated than the target protein[66]. Another unique advantage of the strategy is that sequential assays and affinity capture manipulations can be conducted sequentially without spot drying or even breaking the flow cell. This provides important alternatives to problems associated with desiccated microspots and possible adverse effects on printed proteins[66].

Because of the capability to print high quality microarrays without drying or opening the flow cell, the main application of microfluidic microarray technology involves surface plasmon resonance (SPR) detection where printing occurs directly on thin gold SPR films [4,66,146–148]. Current SPR systems have limitations in throughput [149] but the flowcell array printing method enables increased SPR throughput while maintaining assay data quality and ease of use[66,146–148]. In high-throughput screening mode, the 96-channel integrated printing/arraying/sensing platform is capable of collecting data for >30,000 samples in less than 24 hours. The combination of high-throughput and enhanced sensitivity of the integrated format allows small molecule drug screening for drug discovery efforts.

The integrated format also has a niche in membrane-based protein microarrays[150]. Planar supported lipid bilayers (PSLBs) serve as good models of biological membranes[74] and also possess intrinsic resistance to nonspecific biomolecule adsorption and nonfouling appropriate for use in arraying and molecular capture.[150,151]. High-density PSLB arrays can be printed using the microfluidic-based flow system onto substrates[151] by known vesicle-surface fusion methods[152]. These high density micropatterned lipid bilayer arrays (MLBAs) in microarray form have potential applications in many fields such as biosensing, drug discovery, proteomics, and clinical diagnostics[151].

While microfluidics-interfaced arraying yields significantly improved spot morphology, several factors limit wide adoption of this technology. A primary challenge for this microfluidic printing remains throughput, especially when compared to traditional microarray fabrication technologies. To print a complete glass slide encompassing an area of 25 mm x 75 mm at 10 minutes per print would take approximately six hours, with 1920 printed spots. A comparable print with other technologies could be completed in one to five minutes.

Cell-free protein microarray fabrication.

Protein arrays are conventionally produced using pre-purified recombinant proteins each placed first into individual wells and then deposited onto functionalized glass surfaces using several different methods. Approaches to allow proteins to self-select surface sites from mixed solutions are in their infancy and currently complex and limited in their design[153]. Alternatively, flow-cell methods with multi-port, reagent-controlled and site addressable capabilities could enable in situ generation of multiple recombinant proteins, with a unique protein synthesis occurring in situ in real time on each array site using microfluidics reagent feed. High-throughput production of a large number of proteins using conventional expression systems, based on *E. coli* or other eukaryotic cell-based expression systems, is generally a tedious, time-consuming process requiring substantial reagents and resources [154,155]. Purified recombinant protein production in real-time thus has never been a realistic consideration for innovating protein microarrays. Further compounding the challenge for improving sourcing of recombinant proteins are associated problems with aggregation, expense, poor expression, and/or protein degradation[154]. In this technique drying effects on protein structure and function are only dependent on the host system used to produce the protein, which is a consideration in all microarrays[72]. Cell-free

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systems thus have reduced concerns for problems with protein structure and function because of lower drying effects.

Exploitation of commercial kits for cell-free expression systems in tandem with flow-cell microarray technology has been proposed as a potential solution to address limitations with recombinant protein production, purification and printing stability[154]. These cell-free expression systems have the potential to provide simultaneous production and immobilization of protein products as they are produced by converting printed, more stable DNA precursor arrays into protein arrays on demand by expressing the immobilized DNA in situ on array [156,157]. The result is the 'protein in situ array' (PISA), described in Figure 10. PISA was developed where protein microarrays are rapidly generated by in situ immobilization of the DNA gene for a protein on a surface and subsequent cell-free protein expression of this gene based on this immobilized DNA template. The in situ produced proteins at that site then attach to the surface. Introduction of reagents from a cell-free protein synthesis kit initiates synthesis of the protein with a double (His)6-tag for capture by affinity nickel ion-affinity NTA ligands present on the array surface, allowing the protein to couple in real time as it is produced [154,158]. The slide is then ready for array assay without leaving the flow cell.

Cell-free expression systems make use of cell extracts that contain all of the key molecular components necessary for carrying outperforming transcription and translation *in vitro* [158]. Typically, these extracts can be purified from cell lysates of different types of cells. The most commonly used are obtained from E. coli, rabbit reticulocytes and wheat germ, although more specialized cell extracts from hyperthermophiles, hybridomas, insect, and human cell lines can also be employed [156]. This large variety of available cell-free expression systems ensures that recombinant proteins can be expressed under different cell-free conditions[159]. Cell-free systems have also been used to introduce different biophysical probes during translation for protein detection and/or immobilization[160–162] These arrayed products can be divided into three approaches: 1) protein in situ arrays[159], 2) nucleic acid programmable protein arrays[163], and 3) in situ puromycin-capture from mRNA arrays[164].

Chief advantages of this method are that proteins generated by cell-free synthesis are usually soluble and functional[165]. The template for protein expression is the specific DNA that encodes an individual protein or its domains, produced by PCR using primers designed from information in DNA databases. Coupled mRNA transcription and protein translation is performed with this milieu on an affinity surface to which the desired individual tagged protein product adheres as soon as it is synthesized without purification or separation required[154,156]. The template for protein expression is the specific DNA that encodes an individual protein or its domains, produced by PCR using primers designed from information in DNA databases. Coupled mRNA transcription and protein translation is performed with this milieu on an affinity surface to which the desired individual tagged protein product adheres as soon as it is synthesized without purification or separation required [159]. . An affinity tag sequence is also frequently encoded into the N- or C-terminus of the protein to enable immobilization on the surface following the translation step (Figure 10) [159]. Once the protein is translated and specifically immobilized onto the surface, any unbound material can be washed away [22,159]. Because proteins generated by cell-free synthesis are usually soluble and functional, this method can overcome problems of insolubility or degradation associated with bacterial expression of recombinant proteins [165]. Moreover, the use of PCR-generated DNA enables rapid production of proteins or domains based on genome information alone and will be particularly useful where cloned material is not available[165]. Using this approach, many different proteins can be expressed in parallel using the appropriate, PCR-based, protein-specific *in vitro* transcription/translation systems [159] .

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The PISA method was originally demonstrated using a small set of proteins, which included several antibody fragments and the enzyme, luciferase[159]. These proteins were immobilized onto microliter wells and magnetic beads[159]. PISA was used in a macro format in which ~25 µL of cell-free expression reaction was used for the immobilization of individual proteins. More recently, PISA has also been miniaturized (using ≈40 nL of reagents per well) and adapted for direct production of microarrays on glass slides[21]. In this method, the transcription/translation reaction is performed for two hours at 30°C before array spotting of each product[156].

Hoheisel and co-workers have further developed the miniaturization of PISA using an on-chip system based on a multiple spotting technique (MIST) [166]. In this approach, the DNA template is first spotted (≈350 pL) on the surface followed by addition of in vitro transcription/translation mixture onto the same spot. His-tagged GFP was used as a model protein, immobilized onto Ni ion-NTA-coated glass slides [159]. Estimated suggests that with as little as 35 fg (≈22,500 molecules) of DNA was sufficient for detection of GFP expression in sub-nL volumes[166]. The same authors also adapted the system for high-throughput expression of libraries by designing a single specific primer pair for the introduction of the required T7 promoter and terminator, and demonstrated *in situ* expression using 384 randomly chosen clones from a human fetal brain library[166]. In principle, the optimized and miniaturized version of PISA should be able to produce high-density protein microarrays containing as many as 13,000 spots per slide using a variety of different genomic sources in a relatively uncomplicated fashion [22].

Nucleic acid programmable protein array (NAPPA) is another approach that allows the on-chip surface, cell-free transformation of DNA arrays into protein arrays. This is depicted in Figure 11. NAPPA was initially developed by LaBaer et al.[163] using transcription and translation from an immobilized DNA template [166,167], as opposed to PISA, where the DNA template is kept in solution. In NAPPA, the expression plasmids encoding the proteins, as glutathione s-transferase (GST) fusions are biotinylated and immobilized onto a glass slide that has been coated with avidin and an anti-GST antibody which acts as the protein capture reagent. This plasmid array is then used for in situ expression of the proteins using rabbit reticulocytea cell lysate or a similar cell-free expression system[163]. Once the proteins are translated, they are immediately captured by the immobilized antibody within each spot. This process generates a protein array where every protein is co-localized with its analogous expression plasmid. In general, NAPPA creates good quality protein spots with limited horizontal spreading, although some variation can be observed in the quality of the arrays[163]. Unfortunately, this method produces a mixed protein array in which the different GST fusion proteins are co-localized with their corresponding expression plasmids, avidin, and capture antibody[163].

The first demonstration of the NAPPA approach was carried out by immobilization of 8 different cell cycle proteins at a density of 512 spots per slide[166]. Approximately ≈10 fmol of free protein were captured on average per spot, ranging from 4 to 29 fmol for the different proteins, sufficient for functional studies. The authors used this protein array to map and identify new interactions between 29 human proteins involved in initiation of DNA replication. This data was used to establish the regulation of Cdt1 binding to select replication proteins and map its geminin-binding domain[166].

As with PISA, NAPPA allows the protein array to be generated *in situ*, thus removing any concerns about protein stability during production, separation and storage[22]. However, this requires the cloning of the genes of interest and biotinylation of the resulting expression plasmids to facilitate their immobilization onto the chip. Furthermore, the technology does not generate a pure protein microarray, but rather a mixed array in which the different GST fusion proteins are co- localized with their corresponding expression plasmids, avidin and the capture antibody[22,167].

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In situ puromycin-capture from mRNA arrays were generated by ingeniously adapting mRNA display technology for the production of protein microarrays, capturing the nascent polypeptides using puromycin as depicted in Figure 12[164]. In this approach, the PCR-amplified DNA construct is transcribed into mRNA *in vitro*, and the 3'-end of the mRNA is hybridized with a single-stranded DNA oligonucleotide that has been modified with biotin and puromycin[164]. These modified RNA/DNAs are then arrayed by affinity binding on a streptavidin-coated glass slide and allowed to react with a cell-free lysate for *in vitro* translation[164]. During the cell-free in situ translation step, added ribosomes stall when reaching the RNA/DNA hybrid section of the molecule, and the DNA is then cross-linked to the nascent polypeptide through the puromycin moiety. Once the translation reaction is finished, mRNA is digested with added RNase, leaving a protein array immobilized through the C-termini to the DNA linker, which is in turn immobilized through a biotin/streptavidin interaction to the surface[164]. This technology was first demonstrated by the immobilization of GST, two kinases, and two transcription factors[164]. The transcription factors retained the ability to specifically bind DNA on-chip. This approach provides well-defined non-diffused protein spots as a result of the precise co-localization of the mRNA with puromycin and the 1:1 stoichiometry of mRNA versus protein. However, this method requires extra manipulations involving the RNA modifications to enable reverse transcription and modification of the RNA before transcription prior to spotting process, which may limit its practical use for the creation of large protein microarrays[164]. Furthermore, the amount of protein produced is proportional to the amount of mRNA spotted, since there is no enzymatic amplification involved, unlike the previously mentioned techniques [164].

An important aspect to consider when preparing in situ protein arrays is the level of protein expression required for efficient, reliable assay. While many proteins can be readily expressed, others may require modifications in the expression protocol or to the protein construct, for example by fusing them to a well-expressed fusion protein[168]. He and co-workers have shown that using fusion protein constructs containing the constant domain of immunoglobulin κ light chain can significantly improve the expression levels of many proteins in *E. coli*-based cell-free expression system[168]. This provides a new opportunity to improve general expression in cell-free systems to levels desired for array use.

Furthermore, the presence of disulfide bonds, special requirements required for folding and post-translational modifications in some proteins, especially those of human origin, may require more specialized and expensive expression systems such as mammalian cells or baculovirus[22,168]. Another potential issue of adapting this cell-free in situ expression approach to protein microarrays is the resulting stability of immobilized, folded proteins in an immobilized state over long periods of storage, and the heterogeneity and variability in different such cell-free expression protein products with regard to stability and shelf-life in multiplexed array formats.

A recent advance affecting cell-free protein arrays is the commercialization of the Protein synthesis Using Recombinant Elements (PURE) expression system[169,170]. The PURE approach is based on modular reconstitution of the translational machinery of the cell from purified protein[171,172]. These recombinant protein components are combined with ribosomes and tRNAs, and other necessary cell machinery to complete transcription and translation to create a self-contained reaction system. This system can be programmed for protein synthesis using a variety of DNA templates. The PURE cell-free protein synthesis system has been commercialized through PURESYSTEM in Tokyo, Japan and PURExpress through New England Biolabs in the USA, making it readily available for a variety of laboratory research

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applications [171,172]. Extension of the technology to in situ cell-free protein synthesis and capture on array should prove interesting.

The marriage of cell-free protein expression and microfluidic printing offers an intriguing technological advance for microarray fabrication technologies. While throughput would remain an issue, merging the two technologies could deliver a fully automated, high-quality, multiplexed array while bypassing the tedious efforts of protein purification. In addition, the application of these technologies for SPR applications could facilitate an optimized determination of functional protein folding conditions.

Other Microarray Fabrications Technologies.

Electrospray Deposition (ESD). Thin films of protein are deposited onto a surface using an electrical potential to pull the protein in solution out of a nebulizing nozzle (analogous to electrospray mass spectrometry), through a mask, and onto a surface to generate an array using ESD[173,174]. A capillary tube with the protein solution to be printed is placed between a dielectric mask. Next, the solution is driven out of the capillary nozzle using a high voltage electrostatic field created between the capillary and the substrate[33,174]. The protein solution is pulled to the surface through the mask. A microarray is created by moving between capillaries containing different protein solutions, applying each solution to a different area in the mask. ESD allows for fast and parallel fabrication of protein microarrays. As with inkjet printing, electrospray deposition (ESD) is an application of existing technology applied to microarray fabrication goal. A variety of studies conducted in recent years using ESD in academic environments[175–180]. ESD has shown great potential for miniaturization as arrays of 1 mm^2 can contain as many as 1×10^5 spots. Moreover, research labs could readily fabricate printed spots greater 50 µm. ESD also allows functional proteins, including enzymes and antibodies, to be printed while maintaining their specific activity or kinetics with proper printing conditions[62].

A^2 hybrid reagent multiplexed microarrays. Quantiscientific (USA) has developed quantitative multiplexed microarray products called A^2. The A^2 technology addresses a major concern in protein microarrays: capture antibody/ligand adherence to the array surface is not specific. In the A^2 multiplexed microarray format, an array of capture oligonucleotides is first covalently patterned on the surface. After oligoDNA probe surface tethering, complimentary DNA oligos pre-conjugated to capture antibodies are exposed to the array and each antibody then spontaneously immobilizes to each array address by complimentary DNA hybridization. The immunoassay is then developed like other immunoassays. The A^2 technology enables fabrication of reproducible, sensitive and robust multiplexed immuno-capture arrays. Tethering of the DNA-antibody hybrid away from the surface may also permit more efficient capture of analytes. This technology also permits optimal storage of pre-printed oligo-DNA slides for subsequent on-demand DNA-antibody capture, and also more rapid kinetics for immunoassay[181]. Hypothetically, one would presume that the target antibody structure would be different when compared to either non specific adsorption or the direct covalent binding to the surface through.

Conclusions.

Protein microarrays in their varied forms represent powerful screening and identification tools for: discovering and validating new interaction partners, biomarker and drug target detection, post-translational modifications, and relative protein quantification in complex samples. Microarrays have and will continue to contribute greatly to the investigation and understanding of the proteome, diagnostics, vaccine development, and drug discovery. However, despite substantial advances in proteomics and protein microarray technologies, practical adoption and clinical applications of protein

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microarrays are hindered by technical challenges and associated unreliable or insensitive assay metrics[6,64,182]. A listing of the different types of microarray fabrication techniques as well as advantages and disadvantages are shown in Table 1. Long-standing biomedical applications for the technology beyond medical research[14] include clinical diagnostics[183], drug testing[64,182,184], disease monitoring[7,185], drug discovery[186–188], medical, expression profiling[189–191], protein function characterization[6,64,182], and characterization of protein molecular interactions[6,64,182]. The greatest promise for the assay format is multiplexed and ultra-sensitive assays that reliably target several specific analytes in a biological complex milieu without amplification or purification[192].

Nucleic acid microarray technologies enjoy greater appeal and commercial success than their protein counterparts. Nonetheless, protein and DNA microarrays share similar array printing technologies. Both contact and non-contact printing techniques transfer reagent solutions onto arraying substrates that are usually coated, functionalized glass slides or adsorbent membranes[1]. Importantly, however, proteins have capture functions highly dependent on retaining their complex 3-D quaternary structure. Some techniques such as the microfluidics printing minimize problems with changes to protein structure/function; nevertheless, all microarray fabrication technquiqes suffer in varying degrees from a loss of protein structure and function. Table 1 outlines to what degree in the Spot Quality column. This structure dependence is distinct from nucleic acid-based arrays where duplexing (capture) activity is adapted in real-time from random coil nucleic acids by complementarity; higher order DNA structure that produces array specificity need not be present after array printing or storage. Thus, protein microarrays have unique printing and post-printing stability challenges in both solution, desiccated and stored array formats that limit their utility as quantitative analytical tools. As a result, protein arrays have failed to live up to the success of DNA arrays in a variety of applications[23]Another issue involves the confounding levels of non-specific background and adsorbed protein noise that is a more significant barrier to protein assays versus DNA arrays[23].

The vast number of surfaces designed for microarrays is a testament to the struggle to overcome these problems with the protein microarray platform[37]. Array surfaces play a critical role in the success of microarrays but there is no single surface solution to the diversity of protein assay issues. Criteria for an effective surface include: (1) high binding capacity for the capture ligand (~50-150 μg/cm^2)[64], (2) resistance to nonspecific adsorption by other proteins[23], (3) sufficient stability and three-dimensional structure of the printed capture ligand[23], (4) control of proper capture ligand orientation[23], (5) low background in the assay, resulting in a high signal-to-noise ratio[64]and (6) high specificity of the capture ligand to facilitate minimal to no pre-purification of sample media[23]. While highly specific surfaces are frequently reported to address these needs, a "generic surface" successful in these design criteria does not exist. Additionally, relatively easy surface modification procedures that increase the reproducibility of the array performance and reduce the requirement of cumbersome and sophisticated sample pre-treatment and instrumentation are not available[193].

Maintenance of post-printing protein reagent stability is often cited as both a critical concern as well as a technical performance mandate for arraying surfaces and shelf-life. However, little data is published to document or validate these claims nor general stability requirements. Certainly, the many general thermodynamic components for protein stability at interfaces are long-recognized (REF: T.A. HORBETT & W. Norde below), particularly during solution-phase deposition on substrates and subsequent dessication. Loss of protein structure and consequent function at interfaces, particularly for larger, globular proteins, has a long, documented history, often compromising the performance of technologies

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that employ these adsorbed protein layers[194]. Protein array spots resulting from solution-phase deposition and drying have therefore included various soluble stabilizers and lyoprotectants to facilitate protein transitions from hydrated to dehydrated states without structural compromise on surfaces[47]. However, the effectiveness of this approach is very case-dependent, as each protein printed onto each surface has distinct interfacial stability requirements that may or may not benefit from such added stabilizers[195]. Currently little consensus exists about how to select or choose such stabilizers for a given arraying system. As excess protein is actually deposited compared to required array spot area protein density, the excess protein in the droplet provides a reservoir of "filler" in creating these spots during drying on surfaces, and might also help limit protein denaturation on surfaces both by sterically crowding proteins as they dry, and acting as an endogenous sacrificial lyoprotectant. However, few studies have documented the percent of bound proteins per spot versus the fraction of active protein per spot for arraying systems, so the actual fractional bioactivities and recipes for rationally fabricating such systems are not yet available. Additionally, array spot morphologies will change as a result of these added spotting excipients[195], altering the spot optical integration features used for array assay "answers" and metrology - a significant concern. Microarray spot quality concerns and resulting changes and artifacts in spot optical features upon deposition and spot drying for nucleic acid array models recently reported[59,145] are also generally relevant and translatable to many protein arraying methods.

Nonetheless, despite the critical connections between protein-surface interactions, immobilized functional preservation and array quality, reliability and assay performance, few systematic studies that connect arraying deposition conditions, solution phase additives, protein reagent selection, surface chemistry, drying procedures, an reagent shelf-life to array assay performance are known. For those published, few general protocols or rules applicable to general protein arraying procedures can be translated across diverse protein printing methods. Hence, direct connections between array spot quality, protein stability, protein printed structure and its important functional preservation are not yet controlled and understood.

As much as improved surfaces and protein stability are critical to microarray advances, two major technical hurdles related to printing must also be overcome before microarrays can attain their promise. These are the development of 1) high-throughput methods for producing high affinity, high stability, high specificity proteins engineered for use as microarray capture reagents, and 2) a robust, consistent, high- throughput printing technology that survives storage and multiple assay conditions and lab-lab variations[5]. While secondary challenges are likely to remain in protein printing, overcoming these primary technical challenges will remain a significant driving force for innovative solutions as none of the current technologies address these two hurdles satisfactorily[5].

A chorus has erupted for the standardization of protein microarray production and read-out including preparing and analyzing the arrays[22]. Given the diversity of printing methods, requirements, and projects, this standardization, while noble, seems a logistical nightmare. Nevertheless, the theory that "the adoption of stringent standards by the scientific community for the production and analysis of these valuable reagents should, in principle allow the generation of data that could be compared and exchanged across different studies and different research groups"[22] deserves resources and effort necessary to accomplish the goal. The value of this effort is summed up by Berrade when stating, "At this point, we strongly believe that the protein microarray technology is on the brink of becoming a standard technique in research in the same way as DNA microarray technology is used today."[22] This brings us back to where we began this review: these technologies have broad application in three

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types of microarrays (1) functional, (2) analytical, and (3) reverse phase[7] (see Figure 1). Nonetheless, validation of this assay value remains a scientific curiosity without regulatory approval and requisite accuracy and precision; extension of the technology to clinical and biomedical domains lacks credibility to date.

Microarrays generally promise greater sensitivity, multiplexing, reduced reagent use, higher throughput and greater robustness compared to current technologies[8,9]. While multiplexing and higher throughput are important advantages, reaching lower LODs (i.e., with greater sensitivity in biological samples) perhaps holds the greatest opportunity[196]. In many diseases, the development of a more sensitive diagnostic assay is monumentally beneficial if it can be conducted reliably by labs worldwide under reasonable (non-exotic) conditions. Cancer diagnosis provides an important example of this where hundreds if not thousands of biomarkers have been identified as potential cancer biomarkers, but which a large majority (over 90%) require assays with a detection level of < 1 pg/mL[196]. The problem is compounded by the fact that while these low abundance prospect proteins often are better biomarkers than current biomarkers, they are not reliably detected with the current immunoassays[196]. Microarray development, especially in the area of microarray printing, is crucial in contributing to the next-generation assays capable of impacting these and other biomedically important analytical scenarios[196]. Customized, complex printing and assay protocols typical of protein microarrays currently limit both the reliability and appeal of the format in both clinical and research settings[136]. Researchers are still forced to manufacture custom protein microarrays often using ill-suited adapted printing conditions co-opted from a more successful yet more lenient DNA array history[136]. This approach is a first mistake: equating nucleic acid array printing lessons to protein arraying. Diagnostic companies forced to use high throughput technologies for protein reagents find a lack of quality or even rational printing options, hindering progress in the protein space[136]. Protein and antibody array applications will not reach their pre-technology hype for some years, yet little doubt existsthat this technology remains a wave of future in many fields, especially in clinical diagnostics[197]. One possibility is that should demonstrable improvements in protein array technology not occur in a timely manner, the field could be overtaken by next-generation technology, the same way that next-generation DNA sequencing technology has supplanted much of the former DNA array value recently. Thus, efforts to improve protein microarray fabrication must continue rapidly or the technology risks becoming a mere niche application.

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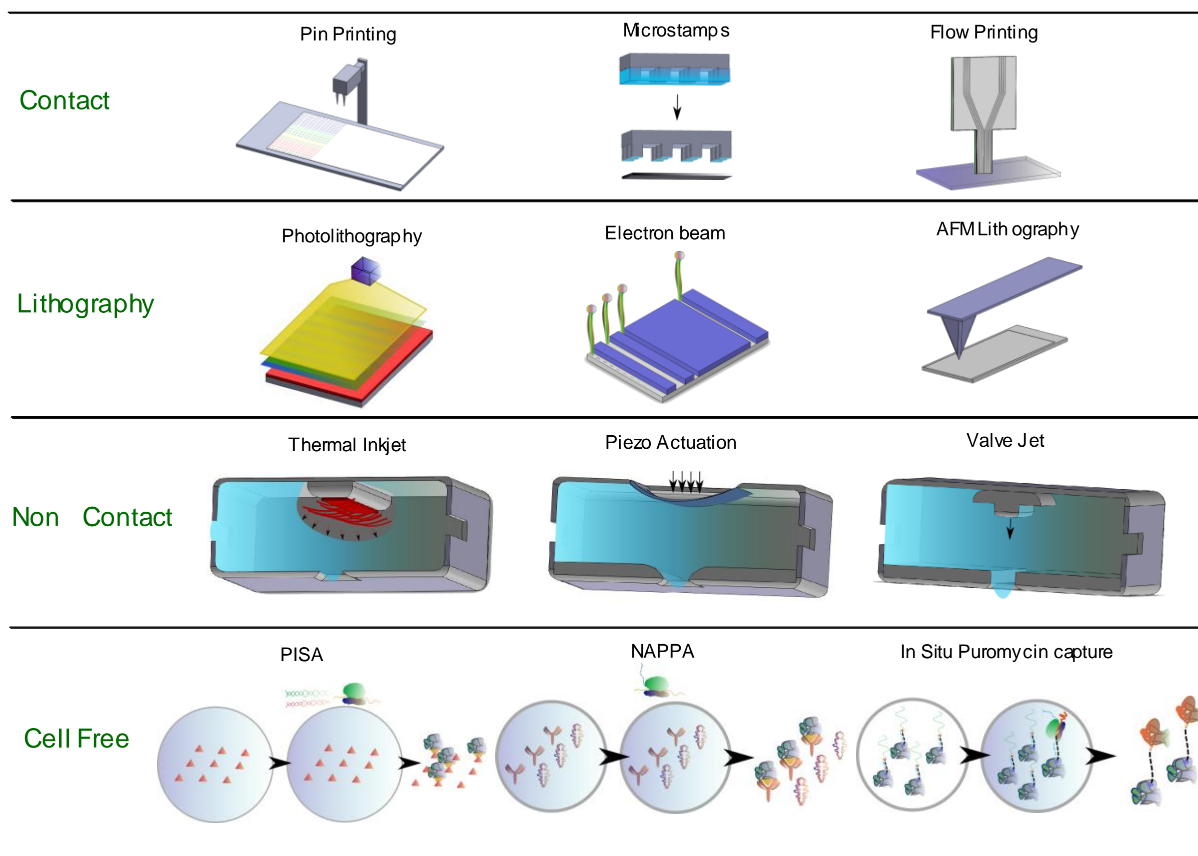

Figure 1. A summary of the more prominent array fabrication technologies addressed in this review.

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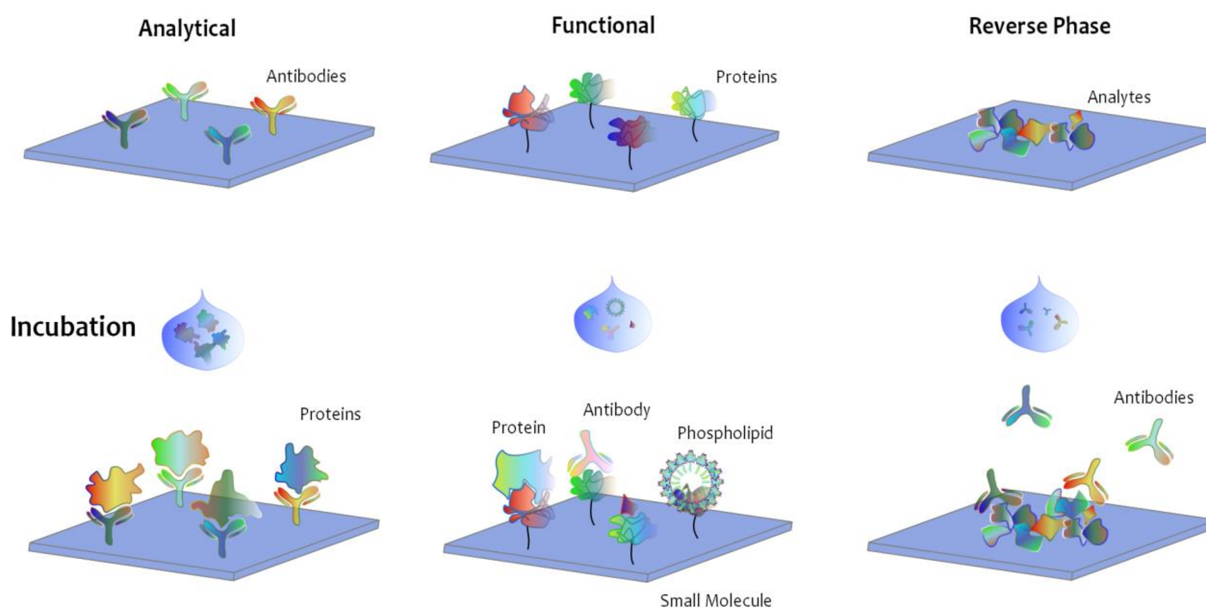

Figure 2. Three general protein microarray types: (left) Analytical. Antibodies may be probed with cell lysate for determining protein expression levels as well as the specificity of the resultant interaction. (middle) Functional. Can be used to study the biochemical properties and activities of target proteins. (right) Reverse phase. Can be used for investigating post-translational modifications and biomarker identification.

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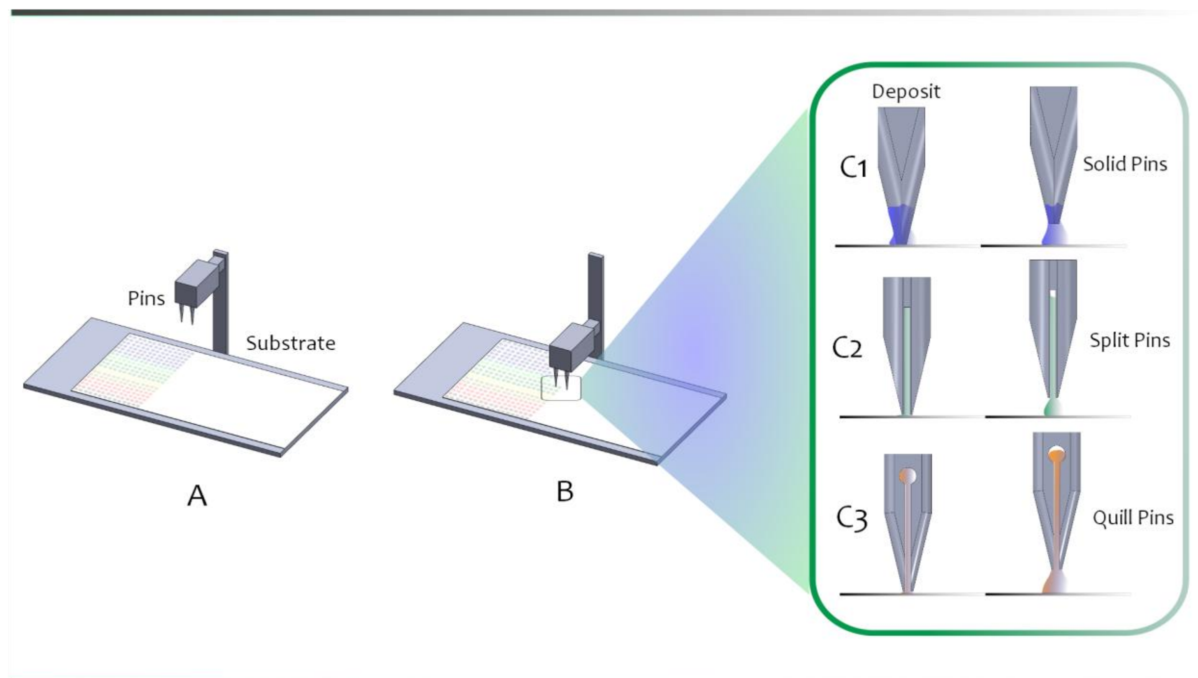

Figure 3. Schematic of pin printing. (A) A robotic print head with multiple printing pins is loaded with print solutions from a source plate and then contacts the substrate surface to deposit protein solution in (B). Various types of pins: C1 is a solid pin. C2 is a slotted pin. C3 is a quill pin, distinguished from the split pin by the inclusion of a reservoir.

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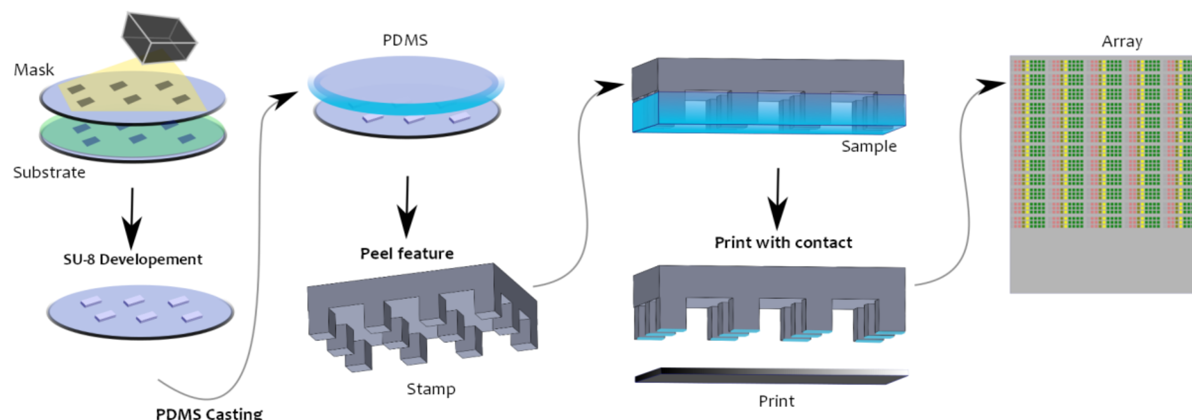

Figure 4. Schematic of microstamping fabrication and printing method. After the creation of a mask and subsequent exposure onto the substrate, features are developed with the aid of SU-8. Conformal sealing with PDMS creates chambers in the final product where SU-8 created raised indentations. Once the stamp is created, sample may be transferred onto its surface for subsequent printing.

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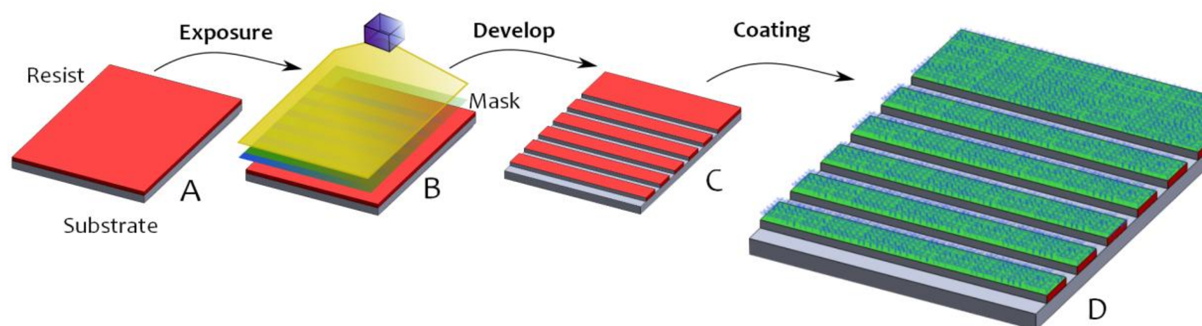

Figure 5. Schematic of Photolithography. (A) Undeveloped photoresist (red). (B) Photoresist is exposed to light (yellow) through the photomask. (C) Development removes the exposed, softened photoresist and a nanopatterned photoresist is generated. (D) Microarray is generated by attachment of proteins to patterned photoresist.

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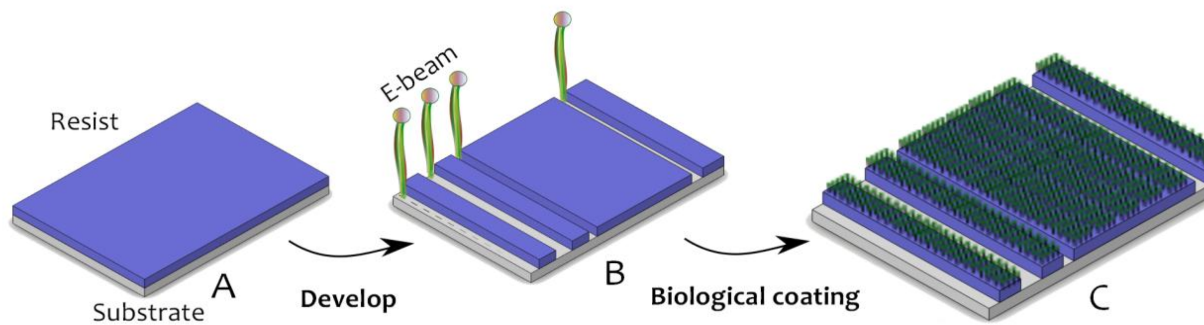

Figure 6. Schematic of E-Beam Lithography. (A) Undeveloped photoresist. (B) E-beam ablates photoresist. (C) Protein/antibodies attach to photoresist to generate array.

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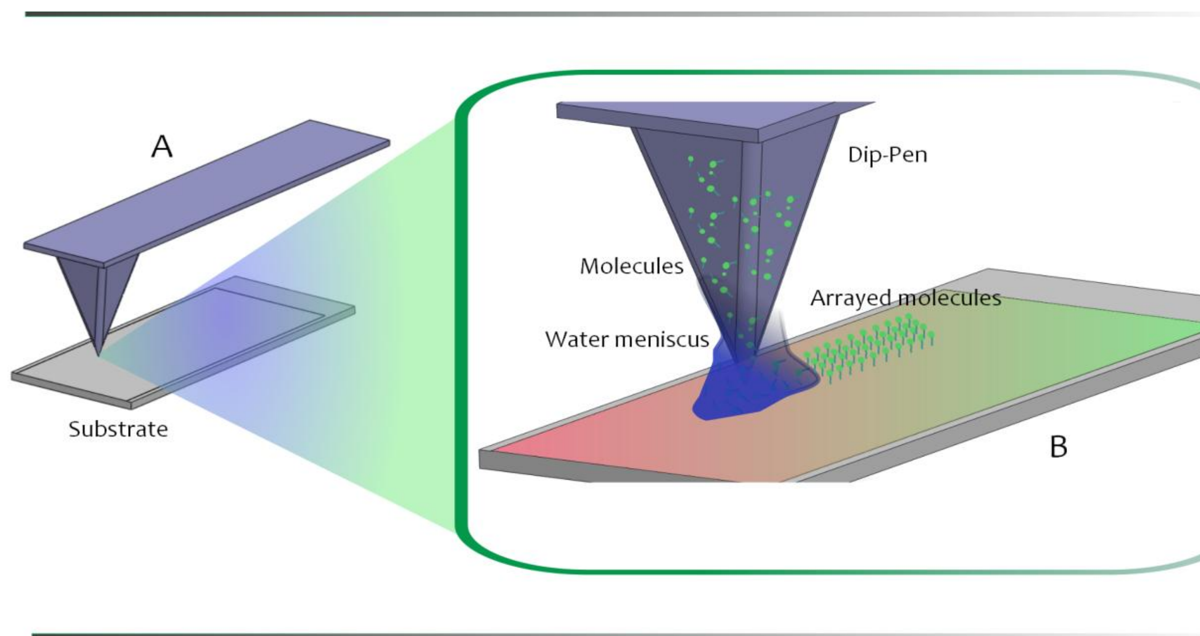

Figure 7. Schematic of Dip Pen Nanolithography. (A) Patterned microarray generated by AFM. (B) Previously dipped AFM tip transferring protein solution to surface. A meniscus of protein solution on the AFM tip transports molecules to the surface in the desired pattern.

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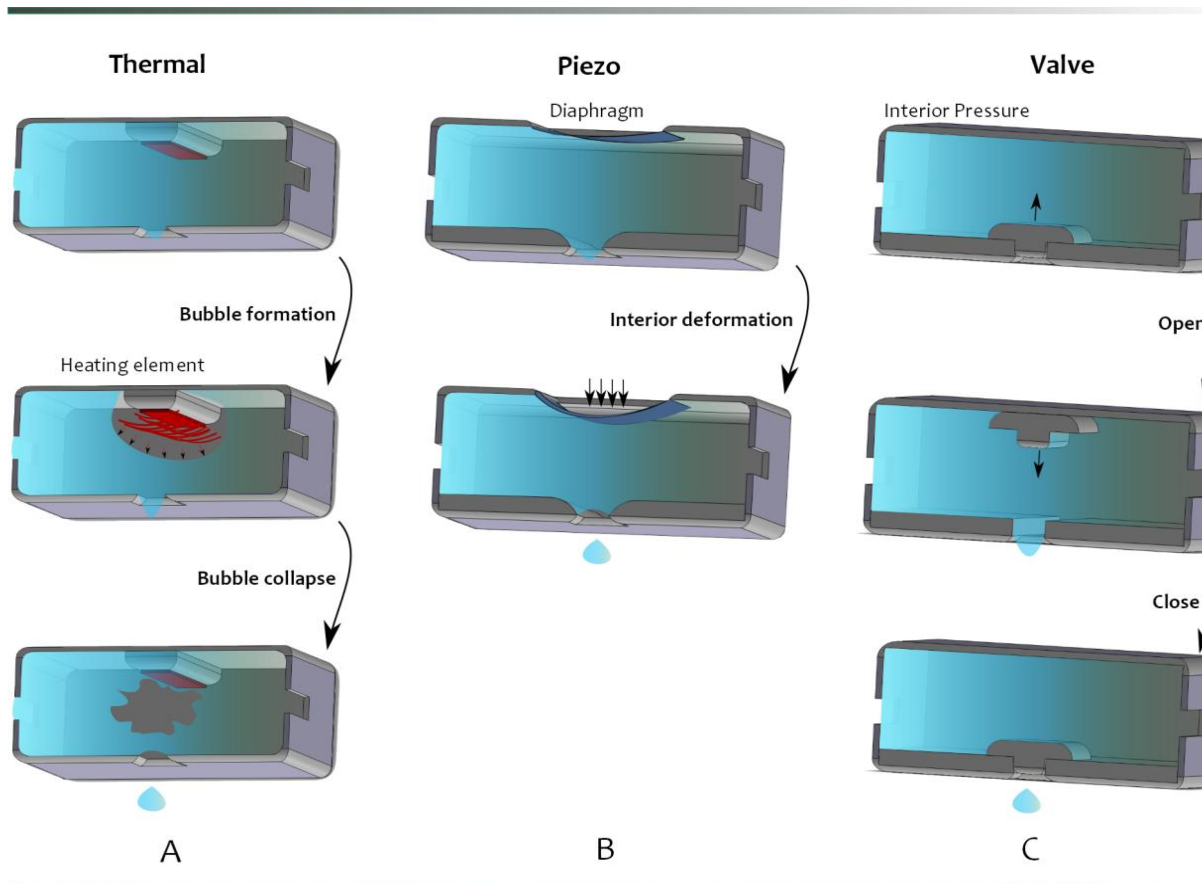

Figure 8. Schematic of noncontact printing for a single droplet ejection through a single inkjet reservoir orifice plate. (A) Thermal Inkjet. A heating element rapidly creates a bubble within the chamber. As the bubble propagates, liquid is further squeezed out of the orifice. Upon bubble collapse, sample is ejected, (B) Piezo actuation. A diaphragm is used to displace the sample within

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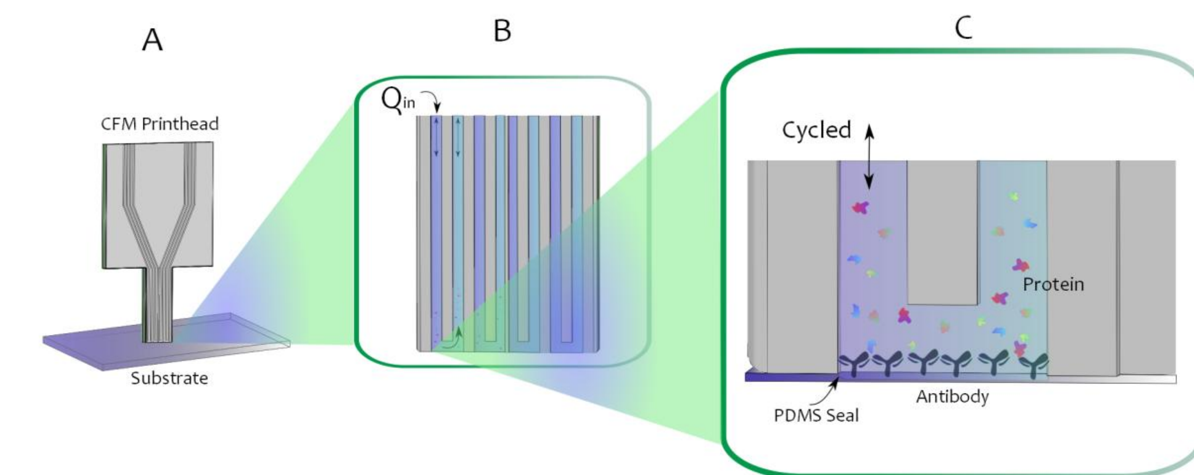

Figure 9. A microfluidic-interfaced printer. (A) CFM print head is docked against the surface. (B) Close-up of the flow cells within the print head. (C) Close-up of one channel. Solution can be cycled back and forth over the surface, ensuring total coverage of the surface.

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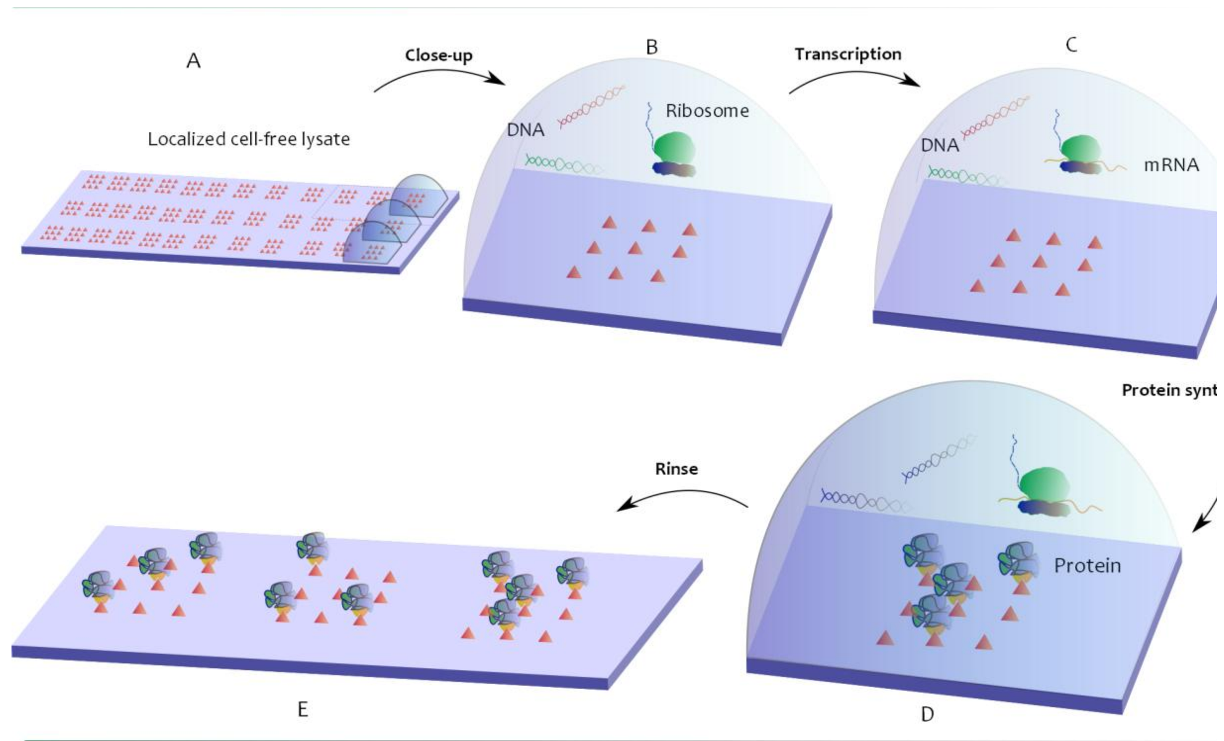

Figure 10. Schematic of PISA. 'Protein in situ array' (A) Protein capture tags are array on the surface (B) DNA and cell-free extract is added to the slide (C) mRNA is produced via the cDNA template (D) Newly synthesized protein is captured by the capture agent via a tag (E) Slide is washed to remove any non-specific binding and is ready for quantification.

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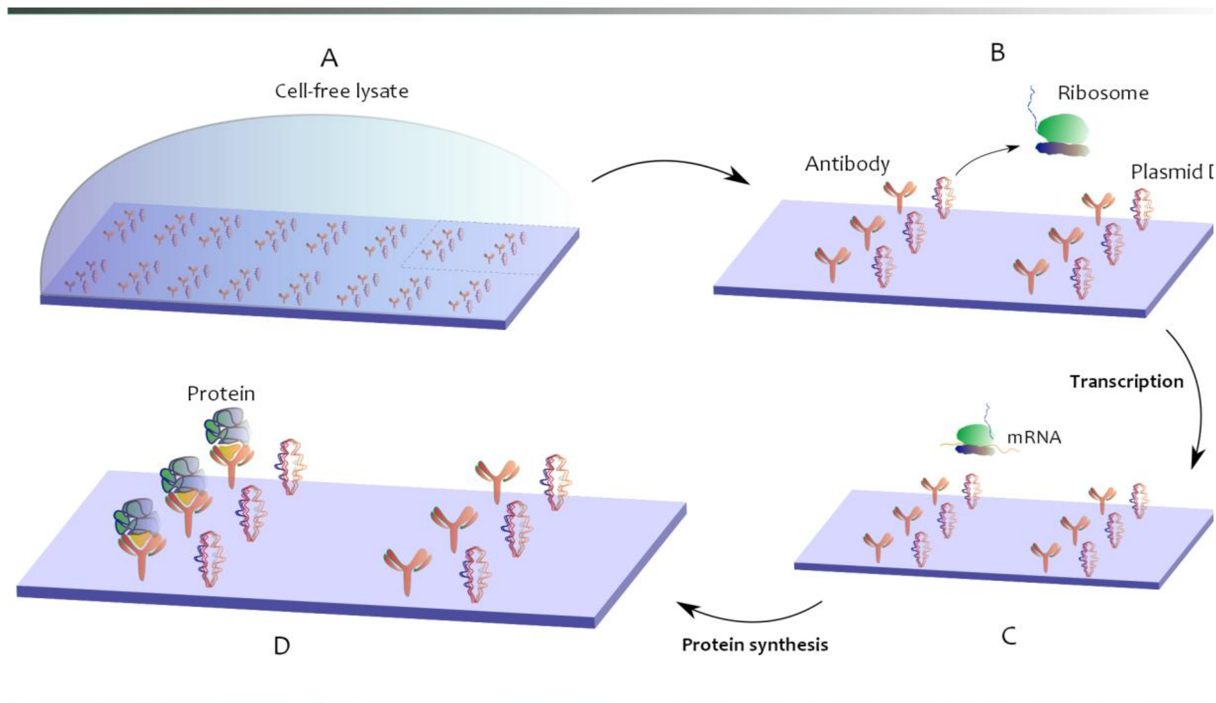

Figure 11. Schematic of NAPPA. (A) Expression plasmids encoding the proteins, as glutathione s-transferase (GST) fusions are biotinylated and immobilized onto a glass slide that has been coated with avidin and an anti-GST antibody which acts as the protein capture reagent. (B) Plasmid array is then used for *in situ* expression of the proteins using rabbit reticulocyte cell lysate or a similar cell-free expression system. (C) The protein is synthesized. (D) The protein is immediately captured by the immobilized antibody within each spot. This process generates a protein array where every protein is co-localized with its analogous expression plasmid.

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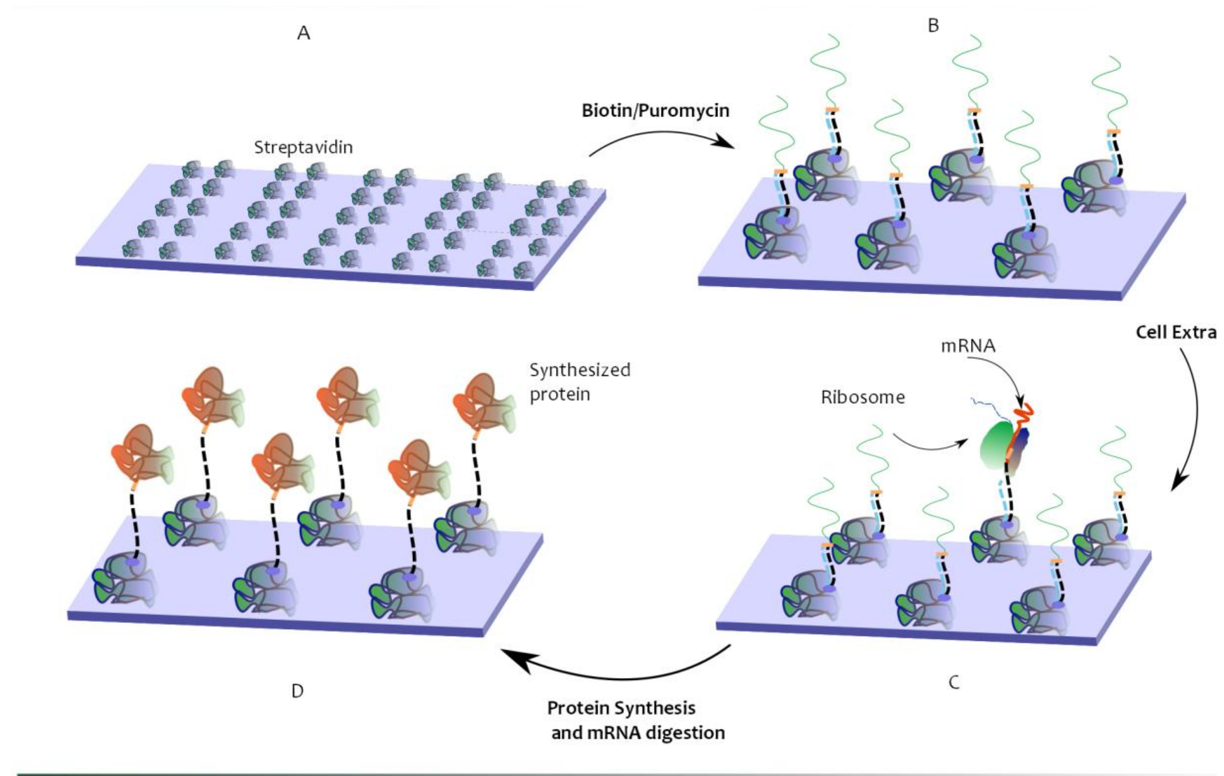

Figure 12. ***In situ*** **Puromycin capture.** (A) A streptavidin surface, (B) mRNA is hybridized with a single-stranded DNA oligonucleotide that has been modified with biotin and puromycin, (C) The ribosome interacts with the RNA/DNA section of the molecule, where DNA is cross-linked to the nascent polypeptide through the puromycin moiety, (D) mRNA is digested with added RNase, leaving a protein array immobilized through the C-termini to the DNA linker, which is in turn immobilized through a biotin/streptavidin interaction to the surface.

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	Throughput	Spot Quality	Array Fabrication Flexibility (ability to print different biomolecules: cells, antibodies, proteins, lipids etc)	Maintenance	Special Requirements
Pin Printing	++++	++	+++	+++	++[ε, η, Ϯ, δ]
Microstamping	+++	+++	+++	++++	+++[ς, ε]
Photolithography	+++	+++	+++	++++	+++[ς, ε]
DPN	++	+++	+++	+++	++[ε, η, Ϯ, δ]
E-Beam	++	+++	+++	+++	++[ς, ε]
Thermal InkJet	+++	+++	++++	++	++[ε, η, Ϯ, δ]
Piezo Actuation	+++	+++	++++	++	++[ε, η, Ϯ, δ]
Valve Jet	+++	+++	++++	++	++[ε, η, Ϯ, δ]
Microfluidics	+	++++	++++	++++	++++[υ]
PISA Cell Free	++	+++	++	N/A	+++
NAPPA Cell Free	++	+++	++	N/A	+++
Ratings Criteria					
++++	1,000's spots per second	Consistent Inter and Intra Spot Morphology	Can be used to generate arrays employing a very large and diverse number of biomolecules.	Very little maintenance, can be performed by the user	Very few external and internal factors need to be controlled. Samples can be printed straight out of the box or as directed.
+++	100's spots per second	Usually consistent requiring very little adjustment	Can be used to generate arrays using a number of different biomolecules.	Maintenance is required however user can be guided to fix most issues	A few variables with need to be controlled. Some modification of protocols is necessary to insure effective printing.
++	10's spots per second	Some inconsistency in morphology, requiring consistent user readjustment	Is limited to printing a small amount of biomolecules.	Maintenance requires outside expertise	A large number of external or internal factors will need to be accounted for.
+	< 1 spot per second	Inconsistent Spot Morphology	Can effectively array a certain type of biomolecule	Intricate maintenance, requires outside expertise, with potentially long machine downtimes.	Samples need to be modified in order to be used with the system. Most variables will need to be controlled.
Legend	υ = viscosity, ε = ongoing expenses, η = humidity, Ϯ = temperature, δ = buffers, ς = cleanroom conditions,				

Table 1. A direct comparison of the reviewed technologies.

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