High-throughput multiplex protein analysis for limited sample size on a single slide

Sergey Rozhok, Yong Jie Ouyang, Doug Dixon, and Mike Nelson

NanoInk, Inc., Skokie, IL, USA, srozhok@nanoink.net

ABSTRACT

To meet the challenges of new genomic and proteomic applications, rapid and sensitive analysis of large numbers of interactions in parallel will be required. High-density microarrays are ideally suited for parallel multiplex screening of thousands of interactions with minimum use of materials. In this study, we report on high throughput screening of 10 different cytokines using both 48 and 96 well formats on a single slide. Multiple arrays of cytokine antibodies were printed on a single microarray slide utilizing Dip Pen Nanolithography® (DPN®). DPN allows parallel printing of hundreds of molecules over an area that is 100 times smaller than that of conventional microarray formats. Smaller feature sizes require significantly smaller amounts of synthesized and labeled materials, which is especially pertinent to drug targets that are expressed in vanishingly small quantities. The unique design of this high throughput format does not require gaskets, allowing reaction volumes of only a few microliters. To validate this new approach both conventional and high throughput formats were run in parallel and the results were compared. The performance of these high throughput formats meets or exceeds the performance of conventional assays, while requiring significantly smaller volumes of sample and reagents. Additionally, the gasket-less approach offers up to 384 individual reaction wells and sample sizes as low as 1 microliter affording the capability for parallel quantitative measurements on a scale not previously available.

Keywords: microarray, cytokine, nanolithography, glass slide

1 INTRODUCTION

Microarrays have demonstrated utility for numerous applications in genomic and proteomic studies and continue to be an increasingly powerful tool for immunological studies, protein profiling, biomarker identification and drug discovery. Most (if not all) microarray platforms that allow detection and screening of biological analytes *in vitro* use solid phase substrates like glass slides, membranes, microtiter wells, mass spectrometer plates, beads, or other particles in order to build arrays that capture target molecules from a solution. Current developments in the microarray field focus on achieving ever higher sensitivity to detect minute amounts of analytes along with multiplexing to allow the analysis of a significantly larger number of interactions that can be processed simultaneously. The microarray glass slide platform provides some specific technological advances that enable essential miniaturization of arrays and high-throughput analyte detection while offering the additional benefit of using very small sample volumes. Additionally, the glass surface offers a large variety of surface chemistries that allow multiple analyte immobilization methods. Finally the microarray slide format opens the door to significant opportunities for assay automation. However, the ability to run assays on a glass substrate has required a number of technical advances that allow the application of very small sample volumes on a planar surface while virtually eliminating sample cross-contamination. In this work we describe a new apparatus that enables an improved high-throughput (48-96-384 array) assay on a single microarray glass slide.

2 EXPERIMENTAL

The NanoBioDiscovery division of NanoInk Inc. has developed methods and techniques that employ the company's patterned Dip Pen Nanolithography (DPN) printing process [1] for proteomic studies. The DPN method allows simultaneous parallel printing of molecules at the micron and submicron scale that makes possible the measurement of a large number of molecular interactions in a single experiment. To achieve all of the benefits of high-throughput printing and performing assays on a micron scale it is crucial to prevent sample cross-contamination. A typical format for running assays on a glass slide utilizes specialized gaskets that localize each array to a single reaction well. However gaskets can not conform to very small wells due to the strong surface tension of liquid reagents caused by the gasket material that prevents liquid from contacting the glass surface. To overcome this obstacle we have developed a platform that does not require gaskets but still keeps liquid contained to a specific area on a plain glass slide surface without requiring any structural modifications to the glass.

2.1 Dip-Pen Nanolithography

In our studies we used commercially available Schott Nexterion slides designed for protein immobilization. To produce arrays of antibody molecules that will be used as capture molecules on the surface we used DPN technology, currently the only technology that enables direct printing at the micron to nanometer scale. Due to the intrinsic properties and technical advantages of the DPN method it is

possible to create a nearly unlimited number of arrays on the glass substrate. At present, because of limitations in the detection methods available to image submicron particles, the size of individual dots is restricted to ≥ 3 um. The latest DPN MEMS accessories [2], such as multiple pen arrays and specialized inkwells, provide users with multiplex capabilities and open a route to fabricating arrays with up to a hundred different antibodies printed simultaneously. NanoInk has developed proprietary techniques and protocols that allow the generation of tens of thousands of features in the form of multiple subarrays on a single glass slide. Parallel printing capabilities reduces array printing time to 10-20 minutes depending on the printing format. While current microarray printing technologies suffer from poor spot-to-spot reproducibility, nanoarrays exhibit excellent uniformity and repeatability of features within and between arrays. Each nanoscale feature covers only a fraction of the surface area occupied by a single conventional microarray spot. Figure 1 shows an array of protein spots printed by multipen array on a glass slide in multiplicative format. The size of the array is 300x140 um^2 while the individual dots are less than 5 um in diameter. Due to the small size of the dots the number of arrays that can be printed on the glass slide can be extremely high. NanoInk's standards for protein subarrays are 18, 48 and 96 and are defined by the number of individual reaction volumes that can be created over each subarray. To the best of our knowledge these are the largest numbers of subarrays on a single glass slide currently available commercially.

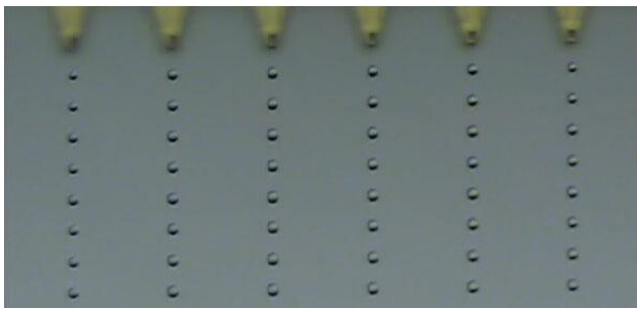

Figure 1. Deposition of femtoliter droplets of capture antybody with multiple pen array

2.1 Slide Apparatus

While printing submicron and nanoscale arrays has been demonstrated in the past, processing high density arrays on a single slide is still a challenge. A typical format for running assay on a glass slide involves applying gaskets that creates individual reagent pools around the arrays. However, processing high density arrays on a slide using gaskets creates issues with sealing between the wells, as well as dispensing and aspiration of solution from the wells. To overcome these problems we have developed an approach that does not require a gasket but can contain small reaction volumes over indivitual printed areas. The design concept is shown on Figure 2 and is based on creating a sandwich between two slides: a sample slide with printed arrays and a slide with small reaction volumes placed within small microwells created by teflon coating (Figure 2a). When two slides are put together at a distance smaller than the height of the droplets the solution flows from one slide to another creating a meniscus bridge between the two surfaces (Figure 2b). In this manner two slides form an incubation chamber with individual reactions volumes encircling each subarray. The amount of solution put in each reaction volume can vary from nanoliters to a few microliters depending on format. In the assembled position the volume between the slides is sealed thus protecting the solutions from evaporation and outside contamination. Within the chamber water vapor comes to equilibrium with the liquid phase thus guaranteeing a constant humidity environment during long incubation times (if required). With the gasket-less approach, the number of individual reaction wells can be as many as 384, offering the capability for parallel quantitative measurements at a scale previously unavailable. The gasket-less platform can be engineered to adapt various experimental settings for different applications. The described setup has been validated and compared to conventional microarray platforms and ELISA in dose-response studies for up to 10 multiplexed cytokines.

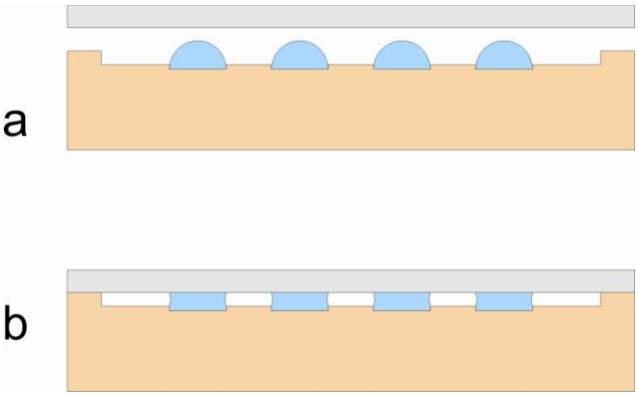

Figure 2. Schematic presentation of high-throughput assay setup for microarray slide

2.2 Cytokine Assay

To validate this miniaturized protein assay concept both conventional and high-throughput microarray formats were run in parallel and the results were carefully compared. It has been demonstrated that the performance of this high-throughput format meets or exceeds the performance of conventional assay while saving significant amounts of costly or valuable reagents. We have developed protocols

for screening cytokines in a range of matrices including biological buffer, plasma or serum samples, and eluted dried blood samples (DBS). Some results of multiplex protein screening are shown on Figure 3 and Figure 4.

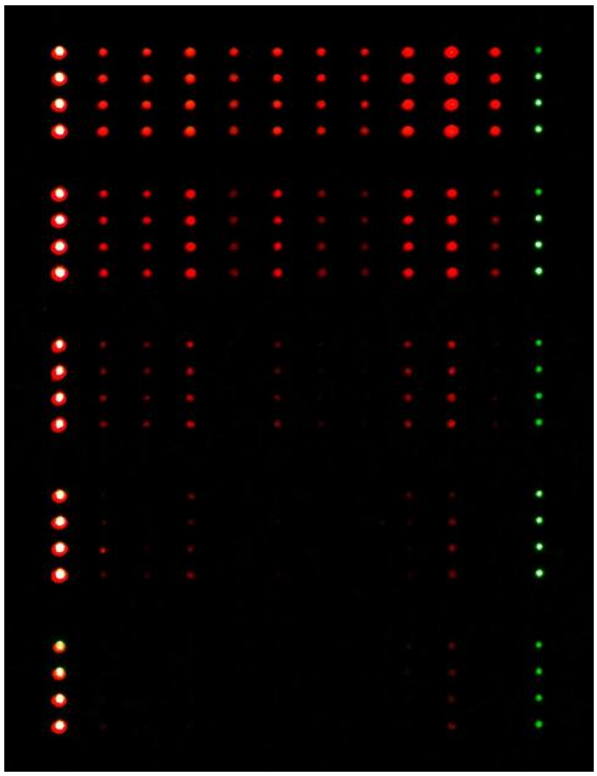

Figure 3. Fluoresence images of cytokine assay at different concentrations of antigens including positive and negative controls

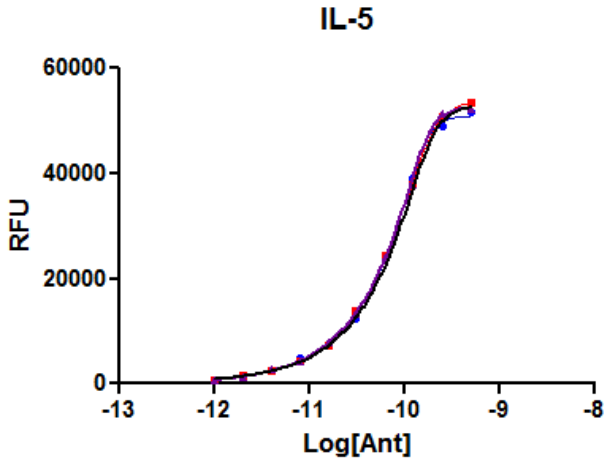

Figure 3. Dose-response curves for IL-5 from two individual subarrays

The study showed that this high-throughput format generates reliable quantitative results with CV's in the single digits and percent of recoveries in the +/- 15% range, consistent with the best assay formats presently commercially available but in a miniaturized format. The results of those studies were used to build the first commercially-available nanoscale cytokine assay kit. The kit is developed for the purpose of evaluating multiple biomarkers simultaneously in a single sub-array, allowing the measurement of a panel of 10 cytokines: IL-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IFNgamma and TNFalpha.

2.3 Scanning Microarray

Along with the advancement of microarray technologies, the development of sensitive and reliable detection systems has also been imperative. To obtain high resolution images of the micron size dots we used high-resolution fluorescent scanners built by Innopsys. The obtained fluorescence images (Figure 3) were analyzed to build standard curves a representative example of which (Figure 4) shows the sensitivity and repeatability of these data. The sensitivity of the assay was in the low femtogram/ml range for most cytokines.

3 CONCLUSION

NanoInk's miniaturized protein microarrays fabricated using the DPN process offer high-throughput, sensitivity, reproducibility, and low cost. Both the 48 and 96 array assay formats can be performed on a single slide and offer excellent adaptability and versatility allowing them to be used for multiple applications while offering better performance than conventional microassay methods. Our studies have validated an assay protocol that offers the possibility of using sub-microliter sample volumes in a high-throughput format to generate reliable and sensitive quantitative results. This high-throughput multiplexed assay format can generate a large data set while requiring significantly less labor, sample volume and reagents than conventional ELISA or large gasket assay formats. In addition the entire process can be adapted to function both manually on the bench and with automated robotics systems.

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