Functional Nanoarrays for Protein Biomarker Profiling

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ABSTRACT

The success of microarrays for analyzing nucleic acid and, more recently, protein-protein interactions illustrates the advantages of bioassay miniaturization. However, the current level of miniaturization of useful bioanalytical tools still creates limits in terms of breadth of application. For example, analyses of picoliter sample volumes containing protein analytes that cannot be amplified or replaced (e.g., cancer biopsy material) require unprecedented levels of miniaturization and novel analytical approaches. Novel platform technologies comprised of instrumentation, methods and software must be developed to achieve the goals of ultraminiaturized biodiagnostics. We will describe one such system and its real-world application to biomarker profiling.

Keywords: nanoarray, microarray, protein array, cytokine, AFM

1 INTRODUCTION

One of the greatest benefits that protein array [1] technologies can offer to the public is the potential for biomarker profiling of disease states [2,3]. This information may lead to earlier diagnosis and treatment for ailments that exhibit distinct patterns of protein expression. Monitoring the molecular basis of disease progression will ultimately lead to new therapeutic targets. The predominant protein profiling technology is based on printing microarrays of antibodies generated against specific known proteins. Lysates from the cell population of interest are typically labeled by conjugation with a fluorophore and incubated with the antibody array to reveal relative expression levels. One of the main drawbacks of this protocol is the differential labeling efficiency of each protein. Alternatively, the lysates can be incubated on the array without any chemical labeling step and then detected with a cocktail of antibodies, which adds an additional degree of specificity to each interaction.

Either antibody microarray based protein profiling method will suffer from a requirement for relatively large sample volumes. Ten years ago 50 µl would not have been considered a large volume, however advances in high-throughput screening and microfluidics have redefined our perceptions. Our solution to the problem is the miniaturization of the entire assay. A new contact-printing instrument, the NanoArrayer™ [4] (Figure 1) was devised to print micron and sub-micron spots of each antibody. These nanoarrays can be created in virtually any pattern, as shown in Figure 2. The 1 µm diameter spots are resolved in the optical image because their spacing (pitch) is greater than 2 µm, the approximate resolving limit of far field optical systems. In this example a protein nanoarray was patterned to encode the nature of the array content, anti-interferon-gamma (IFN-γ). One of the applications we are developing with this technology is a multiplexed, nanoscale cytokine assay. Figure 5 illustrates the sensitivity and dynamic range of an early version. The array is resolvable by optical methods or by more novel approaches such as atomic force microscopy (AFM).

The applicability of nanoarrays to optical detection methods makes them immediately available to a large number of researchers. However, AFM analysis of biological nanoarrays offers unique advantages including the ability to operate in fluids, label-free detection, single molecule detection capability, and nanometer spatial resolution.

Our focus has been on the practical application of the emerging tools and concepts of nanotechnology to the development of ultraminiaturized bioanalytical methods and devices. A key question that remains is “how small is small enough?”. Numerous reports have shown that with relatively simple readout methods such as AFM topography and/or force measurements it is possible to explore molecular interactions at the single molecule level.

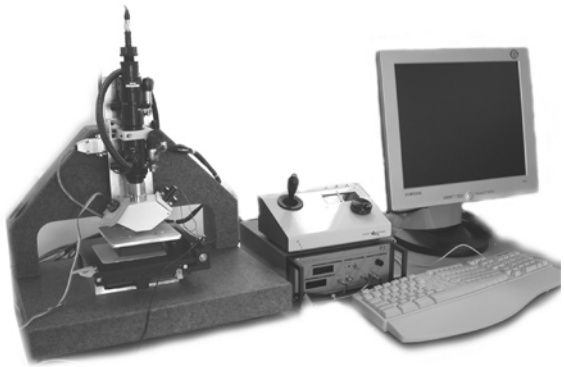

Figure 1. NanoArrayer III incorporates several novel features that create the most optimal NanoArraying platform available. Environmental chamber not shown.

However, the issue of data integrity must be addressed in these cases. As the assay size approaches the single-molecule level, the influence of molecular dynamics and statistics becomes more pronounced. Thus, in our current view, there is a "sweet spot" for ultraminiaturized bioanalysis that exploits the power of numbers (e.g., thousands of molecules per assay) while still reaping the benefits of ultraminiaturization.

Figure 2. Brightfield image of 1 μm diameter mouse anti-human IFN-γ antibody spots arrayed on a gold surface.

2 MATERIALS AND METHODS

2.1 Instrumentation

The results reported here were all obtained using the third generation of an instrument we have constructed and termed the NanoArrayer™. The underlying principle of the NanoArrayer™ is mechanically mediated direct transfer of materials on surfaces with high spatial precision, surface sensing capabilities and environmental control. Initial development of the first hand crafted NanoArrayer (NanoArrayer™ I) was promising and led to development of a second generation NanoArrayer (NanoArrayer™ II), which incorporated closed loop motion control, environmental controls, and surface sensing. A third version (NanoArrayer™ III) has since been developed and the full details of the NanoArrayer™ instrumentation development will be reported elsewhere [5]. Briefly, a microfabricated deposition tool is loaded by immersing the distal end in a drop of the protein solution. The surface to be patterned is positioned on a piezoelectric inchworm-driven XY stage with 20 nm resolution over 25 mm of travel. Manipulating the local humidity at the interface between the deposition tool and surface with gentle bursts of wet or dry air allows precise control of the molecular transfer rate. All stage movement and patterning parameters are controlled from within a custom software package called NanoWare™.

In the proof-of-principle experiments shown here the deposition tools used to physically place proteins on the substrates were 0.58 N/m DNP AFM probes from Digital Instruments. AFM probes were pretreated with UV and ozone for 30 min in a UV-TipCleaner (BioForce Nanosciences) to remove silicone oils and organic debris. While adequate for these demonstrations, commercially available AFM probes have proven to be less than ideal for macromolecular deposition. Therefore, non-AFM probe deposition tools for the NanoArrayer™ are currently under development [6]. These custom microfabricated silicon dioxide surface patterning tools (SPTs) provide a significant increase in reproducibility and writing duration.

2.2 Surfaces

The nanoarrays presented here were created on 4 mm squares of diced silicon wafer (Montco Silicon Technologies, Inc.) that were first coated with 5 nm of chromium and 10 nm of gold by ion beam sputtering through an alphanumeric indexed electron microscopy specimen grid (Electron Microscopy Sciences). Gold-coated silicon wafers or "chips" were removed from the sputterer and used for arraying (Fig. 2, Fig. 3) or immediately immersed in 0.5 mM solutions of dithiobis-succinimidyl undecanoate (DSU) [7] in 1,4-dioxane. Following successive washes in dioxane and dry 100% ethanol, the chips were blown dry with argon and used for deposition (Fig. 4). The nanoarrays from which the plot in Figure 5 is derived were constructed on glass slides with a 3-D polymer matrix coating (Full Moon Biosystems).

2.3 Proteins

The antibody deposited in the nanoarrays in Figures 2-5 was a monoclonal mouse anti-human interferon-γ at 0.9 mg/ml in 10 mM Tris-HCl pH 8.0, 10 mM NaCl (Pierce Endogen). The Cy3-goat anti-mouse $F(ab')_2$ used in Figure 3 was obtained from Jackson ImmunoResearch Laboratories. The cytokine sandwich assays in Figure 4 and Figure 5 were performed using matched mouse monoclonal antibody pairs and recombinant proteins generously provided by Pierce Endogen. Our ELISA data from these pairs of antibodies (not shown) indicates high specificity and minimal cross-reactivity with other cytokine matched pairs.

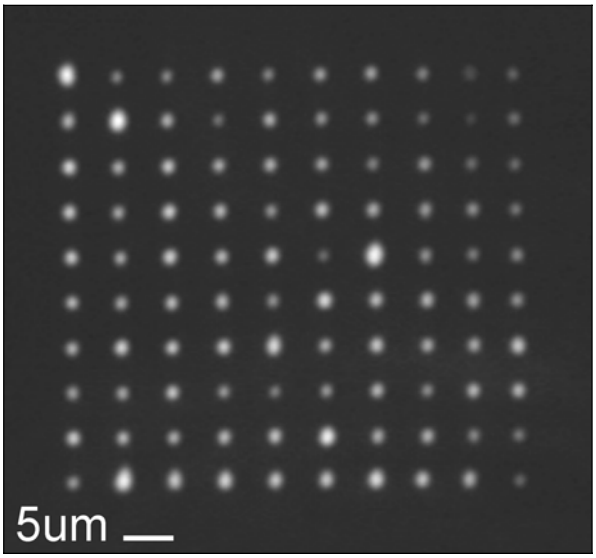

Figure 3. Image of mouse anti-human IFN-γ antibody on gold and labeled with Cy3-goat anti-mouse $F(ab')_2$.

2.4 Cytokine Profiling Nanoarrays

Overnight hydration of the mouse antibody arrayed chips at 25°C and 60% RH facilitated complete binding of the mouse IgG to the surface. The remaining reactive sites were blocked by incubation in ViriBlock™ (BioForce Nanosciences) for 30 min at 25°C, followed by a 5 min wash in PBST. Chips were incubated with recombinant human interferon-γ (Pierce Endogen) at the indicated concentrations in PBS + 4% IgG-free, protease-free BSA (Jackson ImmunoResearch Laboratories) with agitation for one hour at 25°C. Biotinylated mouse anti-IFN-γ detection antibody (Pierce Endogen) was added to the reaction to 250 ng/ml and incubated with agitation for another hour at 25°C. The chips were then transferred into 500 µl of Cy3-streptavidin (Jackson ImmunoResearch Laboratories) at 1.8 µg/ml in PBST and incubated with agitation for 30 min at 25°C. Three successive washes were carried out for 1 min each in 500 µl of 50 mM Tris pH 8.0 + 0.2% Tween-20 prior to image acquisition.

2.5 Detection

Nanoarrays that utilized fluorescent reporting molecules were visualized on a Nikon TE-2000U inverted microscope equipped with a HYQ Cy3 filter #41007a from Chroma Technology. Images were captured using a Cohu cooled CCD camera. Immediately prior to visualization, chips were briefly rinsed with a few drops of ddH_2O and inverted onto a drop of n-propyl gallate/glycerol anti-fade solution (5% n-propyl gallate, 70% glycerol, 25% 0.5M Tris-HCl pH 9.0, then adjusted to a final pH of 7.4). Fluorescent images were analyzed for spot size, intensity, and coefficient of variance with the Array Pro Analyzer software package (Media Cybernetics).

3 RESULTS

Antibody spots with diameters of 1 µm and estimated volumes of 30 attoliters were deposited on a gold surface with a high degree of precision using NanoArrayer™ III, as illustrated by the brightfield image in Figure 2. The NanoWare™ software interface allowed for creative array design in the form of text. The presence of mouse antibody in the transferred material was confirmed by labeling a 10 x 10 x 5 µm pitch array with Cy3-goat anti-mouse $F(ab')_2$ as shown in Figure 3 (negative controls not shown here). Incubation of a 5 x 5 x 5 µm antibody array with a biologically relevant concentration of 5 ng/ml IFN-γ in a sandwich style assay demonstrated the functionality of the antibodies (Figure 4). Specificity controls confirmed the ELISA cross reactivity data for the matched antibody pairs (not shown). An eight point standard curve was processed for IFN-γ nanoarrays to determine the sensitivity and dynamic range. The results plotted in Figure 5 suggest that current labeling strategies have sub-ng/ml detection limits and approximately 2.5 logs of dynamic range. The "hook effect" displayed at higher concentrations of IFN-γ was caused by adding the detection antibody to the chip without first removing the excess IFN-γ.

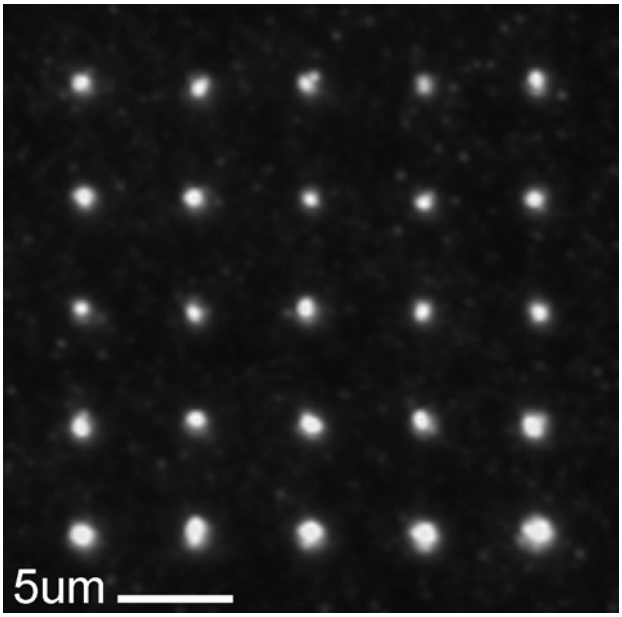

Figure 4. Fluorescent image of an antibody array exposed to 5 ng/ml IFN-γ. Detection provided by a biotinylated detection antibody and Cy3-streptavidin.

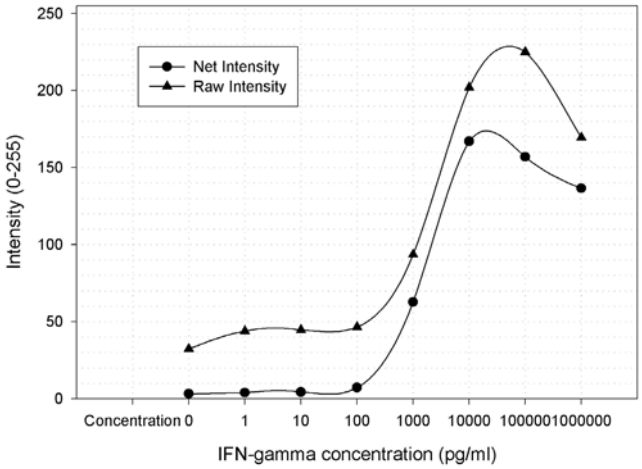

Figure 5. A preliminary concentration series indicates at least 2.5 logs of dynamic range and sub-ng/ml sensitivity.

4 DISCUSSION

In this report we have demonstrated the construction of protein nanoarrays containing 1 µm spots of an antibody directed against the human cytokine IFN-γ. These protein spots can be arranged on the surface as text, in an array, or any desired pattern. A preliminary concentration series for IFN-γ revealed at least 2.5 logs of dynamic range through a

biologically relevant concentration range (Fig. 5). The next step in the progression of this technology is multiplexing the assay to allow evaluation of complex biomarker profiles from ≤1 μl of sample. Custom microfabricated surface patterning tools [6] have been designed for parallel deposition of large numbers of individual molecular species. These SPTs are currently being optimized for throughput and reproducibility.

The concept of a profiling panel for screening 100 or more protein biomarkers in the same area occupied by a single microarray spot offers nearly unlimited applications. It is anticipated that this technology will be first introduced for screening biomarkers from tiny protein samples harvested by Laser Capture Microdissection (LCM) of a subpopulation of cancerous cells [8]. Other potential applications include cytokine profiling for small animal models, as well as pre-natal biomarker testing.

The trend toward miniaturization in biotechnology has led to nothing short of a revolution in the way research is conducted. The practical limits of this trend must be carefully considered. Spot size is directly related to the number of available capture molecules and total binding capacity. Based on our best estimates, a 100 μm microarray spot could contain 10^7-10^8 randomly oriented IgG molecules. A similarly packed 1 μm nanoarray spot would contain only 10^3-10^4 IgG molecules. The number of antibody molecules present in a 100 nm spot such as those constructed via dip pen nanolithography (DPN) [9] is easily less than 100. It is reasonable to assume that a certain percentage of capture molecules in any size spot will be inactive or oriented improperly. While "single-molecule analysis" may sound seductive, it lacks statistical validity and value as a quantitative assay. Therefore, we have focused our efforts on the creation of protein nanoarrays with 1 μm spots which will contain enough antibodies to provide an adequate dynamic range. The preliminary standard curve for IFN-γ illustrated in Figure 5 demonstrates that a protein nanoarray on this scale can still offer at least 2.5 logs of dynamic range.

Another factor determining the optimal size of capture domains is the detection scheme. One benefit of protein nanoarrays is their ability to be analyzed by common optical methods [10]. The size scale of nanoarrays makes them more accessible than microarrays since the entire nanoarray field can be read in a single optical microscope image. Moreover, a typical 100 μm diameter microarray spot scanned in an expensive microarray scanner has a pixel density on the order of ~300 pixels/spot at 5 μm resolution or ~80 pixels/spot at 10 μm resolution. In comparison, an optical microscope with a 1.3 megapixel cooled CCD camera yields a pixel density for a 1 μm nanoarray spot of ~50 pixels/spot when viewed with a 60x objective or ~110 pixels when viewed with a 90x objective. This sampling density should be sufficient for statistically significant sampling of each nanoarray spot by optical microscope. Our work to date has utilized standard fluorescent labeling techniques, although greater sensitivity may be achieved through the use of quantum dots, dendrimers, resonance light scattering particles, or alternative methods such as total internal reflectance microscopy (TIRFM).

Atomic force microscopy (AFM) may also emerge as an attractive alternative to optical detection of nanoarrays. Microarrays are much too large to efficiently employ AFM, however an entire nanoarray can easily fit within the limits of a typical 100 μm XY piezo scanner. The principle driving force behind AFM-based detection is the potential for single molecule sensitivity. There are also additional benefits that cannon be realized with optical technologies such as the simultaneous acquisition of friction, adhesion, and viscoelastic data.

Advanced approaches to sample delivery must be integrated to take full advantage of the kinetics [11] and diminutive size scale of nanoarrays. We are presently exploring simple microfluidic technologies that will enable us to constrain small volumes of analyte to the active capture regions. More sophisticated components such as microvalves for sample circulation or oscillators for on-chip agitation have been demonstrated by others and will be incorporated as our design matures.

This work was supported in part by NIH/NIBIB SBIR Phase II grant R44-EB00316, NIH/NIDDK SBIR Phase I grant R43-DK064463, and Army BCRP grant DAMD17-00-1-0362.

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