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Nanohole Arrays of Mixed Designs and Microwriting for Simultaneous and Multiple Protein Binding Studies

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Abstract

We demonstrate using nanohole arrays of mixed designs and a microwriting process based on dip-pen nanolithography to monitor multiple, different protein binding events simultaneously in real time based on the intensity of Extraordinary Optical Transmission of nanohole arrays. The microwriting process and small footprint of the individual nanohole arrays enabled us to observe *different* binding events located only 16µm apart, achieving high spatial resolution. We also present a novel concept that incorporates nanohole arrays of different designs to improve confidence and accuracy of binding studies. For proof of concept, two types of nanohole arrays, designed to exhibit opposite responses to protein bindings, were fabricated on one transducer. Initial studies indicate that the mixed designs could help to screen out artifacts such as protein intrinsic signals, providing improved accuracy of binding interpretation.

Keywords

nanohole array; microwriting; dip-pen lithography; protein binding

1. Introduction

An approach enabling multiple, different protein bindings in real time and a high-throughput fashion has long been desirable for researchers in proteomics, drug discovery, and system biology. Such approach not only provides dynamic characteristics of individual bindings through real-time monitoring, but also inter-dependence relations between different binding interactions through its multiplicity capability.

Commonly used methods towards this direction include label-free techniques such as Surface Plasmon Resonance (SPR), microcantilevers, and semiconductor nanowires. (Patolsky, Zheng et al. 2006; Saefsten, Klakamp et al. 2006; Campbell and Kim 2007; Kwon, Eom et al. 2007) Such techniques first immobilize one component of the binding pair on a signal transducer, then introduce the counter part of the immobilized component into the system. The binding triggers changes of the transducer signal. The binding characteristics can then be deciphered

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by analysis of the signal changes. These techniques have achieved various levels of multiplicity and throughput. For instance, SPR coupled with microfluidics has been shown to achieve up to 400 different binding events in near real time (Saefsten, Klakamp et al. 2006). SPR imaging (SPRI) and SPR microscopy developed by a number of research groups have demonstrated their capability of monitoring various binding events with time resolution of 1 s. (Li, Lee et al. 2006; Campbell and Kim 2007) Studies by Corn et al. demonstrated that spatial resolution of SPR methods is limited by propagation length of surface plasmons, which is 14 μm when choosing incident light of 676.4nm (Brockman, Nelson et al. 2000), though such resolution have not been demonstrated in multiple protein bindings. Recent work on scalable assembly of nanowires demonstrated thousands of nanowires packed on a platform with spatial resolution on micrometer scale (Jin, Whang et al. 2004), although the adaptation of these nanowires in multiple, different protein binding studies has not been demonstrated. Signals of these nanowires are also sensitive to solution ionic strength, posing a challenge for their use in biological samples (Patolsky, Zheng et al. 2006; De Leebeeck, Kumar et al. 2007).

Alternatively, biosensing based on nanohole arrays is an attractive technique that applies Extraordinary Optical Transmission (EOT) to probe protein interactions occurring on nanohole arrays. (De Leebeeck, Kumar et al. 2007; Ji, O'Connell et al. 2008) EOT is a unique optical phenomenon discovered in 1998 in which an array of subwavelength holes in an optically thick metallic film transmits significantly more light than what classical theory predicted. (Bethe and Von der Lage 1944; Ebbesen, Lezec et al. 1998; Ghaemi, Thio et al. 1998) Since the discovery, many studies have been done to understand its mechanism and explore its use in nano-optical devices. (Van der Molen, Segerink et al. 2004; Gao, Henzie et al. 2006; Liu and Lalanne 2008) Although not fully understood, the phenomenon is commonly explained as a result of resonant coupling between incident light and surface plasmon waves at the metal-dielectric interface through periodic nanoholes. The intensity of the EOT depends on several parameters including the refractive index of the medium on the metal surface, the wavelength and angle of the incident light, the hole geometry and periodicity. This gives rise to concepts that use those nanoholes as nanotransducers to probe binding interactions. Since EOT is affected by the refractive index of the medium on the metal surface, changes of EOT indicate the changes of surface refractive index, which is directly related to the physical and chemical activities of the material on the metal surface such as absorption and binding. Several advantages set these nanohole arrays apart from other transducers: (a) Extremely small footprint of individual array enables high density of the arrays, thus the high throughput (Stark, Halleck et al. 2005); (b) Easy fabrication enables the mass production of these nanohole arrays at fast rate and good reproducibility (Ji, O'Connell et al. 2008); (c) Using a light intensity analyzer such as charge-coupled detector (CCD), one can monitor events on multiple nanohole arrays simultaneously in real time (Ji, O'Connell et al. 2008); (d) Demonstrated in a recent work (Eftekhar, Gordon et al. 2008) as well as below, nanohole arrays with various designs can be packed on one sensing platform to provide different aspects of binding events.

Our group recently demonstrated the use of 25 nanohole arrays of same designs on 100nm thick gold film and their EOT signal to monitor redundant binding events between Glutathione-S-transferase (GST) and anti-GST in real time. (Ji, O'Connell et al. 2008) Our studies also showed that the approach has the potential of extremely high throughput, accommodating up to 20,164 binding events with high temporal resolution on milliseconds scale that is decided only by the camera speed and exposure time. However, lacking a nanodeposition technique to modify individual nanohole arrays with separate reagents, we could only monitor 25 same, redundant binding events simultaneously. To make this technique truly significant and versatile, each nanohole array needs to be individually controlled and functionally varied from each other.

Developing methods allowing depositing reagents in resolution of micro-scale or nano-scale is an exciting challenge in nanosciences because it opens up gates for new possibilities in fields ranging from biomedical to nanolithography. The invention of the scanning tunneling microscope (STM) and its descendents such as Atomic Force Microscope (AFM) to manipulate nanostructures inspired researchers to create similar but more comprehensive techniques for nanofabrication and nanodeposition. In 1999, Dip-pen nanolithography (DPN) was introduced as a new tool for nanofabrication. (Piner, Zhu et al. 1999) Such technique uses an AFM tip to write chemicals directly on a gold thin film with 30-nanometer line width in a manner analogous of a dip pen. Molecules are delivered from the AFM tip to a solid substrate in submicrometer dimensions via capillary transport, making DPN a useful tool for nanolithography and nanodeposition. Microcontact printing, which uses an elastomer stamp, has also been demonstrated to deposit patterns of thiol-functionalized molecules directly onto Au substrates. (Kim, Xia et al. 1995; Xia, Kim et al. 1996) This method is a parallel method that allows one to deposit an entire pattern or a series of patterns with a same reagent. In this study, we developed a microwriting process that applied DPN principle to deposit various protein solutions on to individual nanohole arrays in a sequential manner. Using this process, we modified individual nanohole arrays with various proteins for simultaneous studies of their bindings to respective counterparts. Further, we varied the designs of nanohole arrays, compared their abilities of monitoring binding events. To demonstrate the use of various nanohole array designs in complex proteomic and drug discovery studies, we also present a sensing platform that contains two dramatically different types of nanohole arrays that provide opposite signals upon binding for improved confidence. For proof of concept, we show the studies done with three different binding pairs on 12 individual nanohole arrays, although the microwriting process and the population of nanohole arrays can be scaled up to study hundreds and thousands of protein binding pairs.

2. Materials and Methods

2.1. Materials

All proteins were received as purified proteins and used without further modification. Rat IgG, anti-Rat IgG were from Abcam. GST and anti-Human IgG were obtained from Sigma. Anti-GST was from GE health. Human IgG was from Pierce. Anti-Human IgG with Cy5 label was obtained from Invitrogen.

2.2. Nanohole Array Fabrication and System Setup

All nanohole arrays in this study were fabricated in 100nm thick gold films with FEI DB235 focused ion beam system with 30KV acceleration voltage and 10 pA ion current. Transmission spectra and real-time EOT signal monitoring were collected using an inverted microscope with bright-field accessories (Nikon eclipse TE 300). To achieve multiplexed spectral acquisition and real-time monitoring, a white light source and an intensity imaging CCD were used. Non-polarized white light from a 75W Xenon lamp was passed through a double monochromator (Jobin-Yvon SPEX 1680B) and a condenser lens. The light transmitted through nanohole arrays was collected with a 20x objective (NA = 0.5) and then focused on an uncooled CCD (QImage, Intensified Retiga). The spectra were corrected by the lamp transmission profile through a 190μm thick glass, which was used as substrate for the gold films.

3. Results and Discussions

3.1. Nanohole Array Designs and Their Use in Protein Binding Studies

Previous reports and our studies indicated that the design parameters of nanohole arrays have dramatic influence on the spectral features of EOT, therefore the sensing capability. These design parameters include hole diameter (d), periodicity (p, hole-to-hole distance), and number

of nanoholes. (Ebbesen, Lezec et al. 1998; Thio, Ghaemi et al. 1999; Wang, Yi et al. 2003; Van der Molen, Segerink et al. 2004) To characterize binding events, we are interested in the change of EOT intensity induced by binding. Given a binding event, a nanohole array design that gives the most EOT signal change would allow most sensitive and detailed monitoring of the event. We therefore fabricated a series of nanohole arrays in 100nm thick gold film with different designs and studied their response to refractive index changes, probing for optimum array design and test conditions.

Figure 1a shows a CCD image of a transducer containing 13 nanohole arrays spaced at 16 μ m apart. Since calculated surface plasmon propagation length on gold along the X-Y direction is less than 3 μ m for excitation wavelength shorter than 630nm, (Homola, Yee et al. 1999) 16 μ m spacing ensures sufficient distance between individual arrays to avoid interference. Our later studies also indicate that spacing at 16 μ m is necessary to allow enough room for microwriting. The transducer in Figure 1a was fabricated to study the effect of periodicity on EOT signal change induced by surface refractive index (RI) change. All the arrays on this transducer contain a 16 by 16 array of holes with diameters of 150nm, while their periodicity varies from 250nm to 550nm. As an example, a Scanning Electron Microscope (SEM) image of a nanohole array with periodicity of 400nm is shown in Figure 1b. To study the effect of periodicity, we recorded the transmission spectra of all the nanohole array designs by scanning the wavelength of incident light and recording the output of all 13 arrays through a CCD camera simultaneously. The nanohole arrays were submerged in two different solutions, phosphate buffer saline (PBS, RI ~ 1.33) and 21% NaCl solution in water (RI = 1.37), and their spectra recorded subsequently. Figure 2a shows the spectra of one nanohole array with periodicity of 400nm submerged in two different solutions. Spectra of other arrays were removed for clarification. As predicted, the different refractive indexes of the two solutions induced a shift in the spectra of the nanohole array. Plotting the percentage change of the EOT signal against wavelength revealed two EOT response minima at 542nm and 602nm, and one EOT response maximum at 570nm (as shown in Figure 2b). The EOT signals had the most significant changes at those wavelengths, which means using incident light at one of these wavelengths would offer better probing capability to record protein bindings events.

We repeated the same analysis to all 13 nanohole arrays and obtained Figure 2(C) where the percentage changes of EOT signal of arrays with all the different designs are plotted against the wavelength of incident light. For a clear presentation, average signals obtained from duplicate arrays were shown in the graph. Interestingly, arrays with different periodicities displayed dramatically different EOT response maxima and minima. When the wavelength of the incident light is 602nm, we discovered a unique situation where arrays with periodicity of 400nm displayed EOT response minima while arrays with periodicity of 300nm displayed EOT response maxima. This finding indicates that, on one transducer, we can fabricate different nanohole arrays that exhibit dramatically different response to protein bindings. Combined responses from different nanohole arrays could provide more information on the composition or purity of target analyte, providing cross-evaluation for improved accuracy and confidence.

As proof of concept, we performed a real time testing where the wavelength of incident light was set at 602nm. The transducer containing 13 arrays with various designs was placed in a custom-made flow cell to allow continuous flow and sample injection. As Figure 3 shows, changing the solution in the flow cell, i.e., refractive index on the transducer surface, prompted dramatically different responses from all 13 nanohole arrays. Arrays with periodicity of 300nm displayed ~15% EOT increase while arrays with periodicity of 400nm displayed ~15% EOT decrease. Arrays with periodicity of 450nm displayed nearly no EOT change. For our further studies on protein binding, we chose the two types of arrays with maximum sensitivities: the arrays with periodicity of 300nm and 400nm, respectively. These two types of arrays have been

shown in the above study (Figure 2 and 3) to display opposite signal changes responding to refractive index changes on the transducer, providing a distinctive profile of the RI change.

We have also studied the impact of hole diameter and number of holes on EOT response (Yang, Ji et al. 2008). Our studies revealed that although large hole diameter led to high EOT intensity of an array, it did not have significant impact on the EOT response maxima or minima. The EOT intensity of nanohole arrays was proportional to the number of nanoholes in an array, but it did not shift the EOT response maxima or minima. Based on these studies, we chose hole diameter of 150nm and hole number of 256 (16 by 16 square array) for all the arrays presented in this studies. The decision on the diameter and number of holes in the arrays was based on easy fabrication, strong EOT signal, and small footprint of these arrays.

3.2. Microwriting Process Development

To demonstrate the use of nanohole arrays in simultaneous and multiple protein binding studies, we developed a microwriting process to deposit different protein solutions on to different nanohole arrays. Studies have shown that depending on the relative humidity and substrate wetting properties, aqueous solutions would either be transported from an AFM-type of capillary tip on to a solid substrate or vice versa. In the latter case, micrometer-scale even nanometer-scale patterns could be formed on a substrate from thin layers of solutions deposited. (Piner and Mirkin 1997) If the transported molecules could anchor themselves to the substrate through chemisorption, the deposited droplets could be stabilized on the substrate. (Piner, Zhu et al. 1999) To realize stable deposition of proteins on gold nanohole array structures, we first developed a chemical scheme to activate nanohole array surface to allow for chemisorption of protein. We then developed a microwriting process that applies DPN method to deposit femtoliters of one component of the binding pairs on to nanohole arrays to obtain individually modified nanohole arrays. For proof of concept, we chose three different antigen-antibody binding pairs to study: Human IgG and antihuman IgG, Rat IgG and anti-Rat IgG, and GST and anti-GST. Antigens from all four binding pairs would be nanodeposited and chemically bound onto individual nanohole arrays to function as capturing agents.

The chemical activation of nanohole array was achieved through modifying gold nanohole surface with amine-reactive groups through forming a self-assembled monolayer (SAM) of amino-functionalized thiols. Detailed process is described in the supporting materials. This developed chemical modification scheme is robust and insensitive to prolonged process time, allowing hours of operation time for microwriting process.

A microwriting process was then developed to deposit antigens of the three binding pairs of interests onto the amine-reactive nanohole arrays for covalent immobilization. We chose a Nano enabler (from Bioforce Nanosciences) that applied a DPN-type capillary writing tip to transport minute amount of protein solutions onto the nanohole arrays. The system includes a variable intensity laser and position-sensitive photodetector to monitor the precise movements of the writing tip. The tip we used was a 10 μ m wide cantilever with its tip narrowing down to 1 μ m. Our data show that the extremely small dimension of the cantilever was necessary to produce fine droplets that covered the densely packed nanohole arrays without cross-contamination. However, to pass through an extremely small cantilever, a protein solution had to overcome the large surface tension between the liquid and the cantilever wall. This dictated a spotting buffer to be included to ensure the deposition of protein solutions. Since the proteins were to be deposited onto amine-reactive nanohole arrays for covalent attachment, any spotting buffer added to help the deposition should not interfere with the covalent reaction. The spotting buffer should also be biocompatible with the protein solution, but not impacting the folding and binding characteristics of the proteins. We studied a few spotting buffers including glycerine and n-octyl glucopyranoside. We found that by adding into protein solution less than 5% of glycerine, a wetting agent that has been used in DNA microarray studies (MacBeath and

Schreiber 2000), we were able to ensure deposition of protein solutions through a small cantilever while retaining both chemical and biological activity of the protein.

To switch from one protein to another protein deposition, the cantilever was hand-changed to a fresh one loaded with the next protein solution to be deposited. Used cantilevers were refreshed by washing with deionized water followed by cleaning with UV/ozone. Although automated cantilever washing and reloading could be developed, we opted for mechanical cantilever changing for this study as proof of concept.

Figure 4(a) shows a photograph of protein droplets deposited on 20 individual 16 by 16 nanohole arrays with periodicity of 300nm or 400nm. Image of the arrays before microwriting is also shown in Figure 4(b). In Figure 4(a) and (b), top two rows of the arrays have periodicity of 400nm, while the periodicity of lower two rows is 300nm. The size of a 16 by 16 nanohole array with the periodicity of 300nm is 4.65 μ m by 4.65 μ m. Such individual nanohole array with the periodicity of 400nm occupies an area of 6.15 μ m by 6.15 μ m. Droplets deposited onto the nanohole arrays exhibited uniform shapes and sizes. The diameter of each droplet is ~5 μ m, sufficient to cover individual arrays. The spacing between arrays is 16 μ m. Precise location of the protein droplet is realized by automatic precision stage controller, while the volume of the protein droplet is controlled by contact time between the cantilever and nanoarray surface. The contact time for this study is set at 1s unless otherwise stated, where the volume of a protein droplet is calculated to be roughly 13 femtoliters. To quantify the consistency of the protein droplets, we also performed a separate test where we deposited fluorescent protein droplets on to nanohole arrays and compared the fluorescent intensities of individual protein droplets. The coefficient of variation (CV) of the fluorescent intensities of 1024 individual droplets was less than 10%, indicating a high consistency of the volume of the droplets. Effect of different cantilevers on protein droplet volume is found to be negligible.

3.3. Multiple Protein Binding Events Using Nanohole Arrays and Microwriting

To demonstrate the use of microwriting and nanohole arrays in simultaneous, multiple protein binding studies, we fabricated a transducer containing 12 arrays with periodicity of 400nm (Array400). As discussed before, Array400 is one of the two best arrays that exhibited most sensitive response to refractive index changes, i.e., bindings. Using this particular array, any biological binding would increase the refractive index of the array surface, triggering drops in EOT intensity (see Figure 3). Using the microwriting process described above, we covalently immobilized three different antigens, human IgG, GST, and rat IgG, onto the 9 of 12 individual nanohole arrays (three replicas for each antigen). Three nanohole arrays were untreated to serve as references. The transducer was then placed in the custom-made flow cell to allow sample injection and real time signal monitoring.

Based on earlier studies using salt solutions, we understand that any bindings occur on an Array400 would incur refractive index increase of the Array400 surface, therefore inducing EOT decrease. Figure 5 records the multiple binding events occurring on all 12 nanohole arrays simultaneously in real time by monitoring the EOT intensities of the arrays. Without any antibodies in the flow cell, all arrays exhibited stable EOT intensities with standard deviations (σ) < 0.0015. The spikes observed in all curves originated from the noise of un-cooled CCD used. Introductions of three different antibodies induced waves of binding responses from respective, individual nanohole arrays. At $t = 15.8$ min, we introduced 50 μ g/ml of anti-GST into the flow system. Significant signal drops were found on all three arrays written with GST antigen, indicating binding events occurred on those arrays. All other arrays showed inert to the introduction of antiGST, indicating that no binding events occurred. At $t = 27.5$ min, introducing anti-Human IgG antibodies induced significant signal decreases from three arrays modified with Human IgG, while other arrays showed negligent responses. Similar response pattern were observed when introducing anti-Rat IgG at $t = 46.5$ min into the flow cell where

only three arrays written with rat IgG antigen responded. Signals of three arrays that were left unmodified to serve as references remained unchanged throughout the whole process, indicating the binding observed on all other arrays were specific bindings induced by introductions of various antibodies, but not non-specific protein absorptions. Introducing regeneration solution (10mM glycine, pH 2.2) at $t = 60.8$ min dissociated all the antigen-antibody binding pairs, bringing the signals back to baseline. Normalized EOT changes from replica arrays also showed high quantitative consistency, with standard deviations (σ) < 0.005 . Unless otherwise stated. All data presented throughout the paper are raw, unfiltered data.

To further explore using periodic nanostructures to monitor biological bindings in an accurate and high-through fashion, we studied the concept of using mixed designs of the nanohole array on one transducer to provide improved confidence level. We chose two types of arrays, arrays with periodicity of 300nm (Array300) and arrays with periodicity of 400nm (Array400) for this purpose. As shown in Figure 3, these two arrays not only showed the most sensitivity to refractive index changes, but also *opposite* changes, making them ideal candidate for possible tools for improved confidence level. We fabricated a transducer that contained four Array300s and four Array400s. Two Array300s and two Array400s were modified with human IgG through the aforementioned microwriting process, while the rest two Array300s and two Array400s were untreated to serve as references. Using this transducer, we carried out a study where we introduced anti-Human IgG labeled with Cy5 into the system. Cy5 is one of the reagents that are often used as protein labels in proteomic studies. It is a luminescent dye that absorbs light of a broad range peaking at 650nm, and emits light at 670nm. Interesting responses from two Array300s and two Array400s were shown in Figure 6. All data were normalized for clear comparison. To verify the observation, normalized responses from all four references (two Array300 references and two Array400 references) were also shown in the graph. Two Array300 written with Human IgG indicated negligible signal changes after the antibody introduction suggesting no binding or weak binding, while two Array400 written with Human IgG displayed large signal change suggesting a strong binding occurrence. Based on the combined responses from both types of arrays, we believe a strong binding event has occurred on both Array300 and Array400. The significant difference in the array response was induced by the absorption of Cy5 on anti-Human IgG, which partially absorbed incident light (605nm). The absorbance offset the signal increase in Array300 while magnified the signal decrease in Array400. Without the information provided from Array400, one could interpret that no binding or weak binding had occurred. Interpretation based on mixed types of the arrays eliminated potential false negative, providing improved accuracy.

We also investigated the use of nanohole arrays as an approach to quantify the binding interactions and derive binding kinetic constants. Discuss of this part of work can be found in the Supporting Materials.

4. Conclusions

In summary, we have demonstrated the use of microwriting process and mixed nanohole array designs to monitor multiple protein binding events simultaneously in real time. Based on nanohole arrays and the intensity of Extraordinary Optical Transmission, our approach presents a highly flexible platform to study protein-protein binding events. Using EOT intensity as a probe, a dramatic shift from commonly studied EOT spectra, we were able to monitor simultaneous and multiple binding events in real time, without the need to scan and evaluate binding one-by-one. This offers our approach extremely high temporal resolution, limited only by the camera speed and exposure time. The development of a microwriting process and robust chemical modification scheme allowed us to modify individual nanohole arrays, realizing simultaneous monitoring various binding events with extremely high spatial resolution. The small footprint of nanohole array and the small size of the droplets microwriting delivers

allowed us to space binding events only 16µm apart, the highest spatial resolution that has been achieved in real-time studies of protein bindings. This also provides us with an extreme potential of high throughput. In a previous study we demonstrated that our approach could be easily scaled up to monitor 20 164 redundant binding events simultaneously with a spatial resolution of 56µm.(Ji, O'Connell et al. 2008) Implementing microwriting process, our modest prediction is that we can at least monitor 20 164 independent, different binding events simultaneously in real-time. We also demonstrated the concept of fabricating nanohole arrays of various designs on one transducer to provide more information for binding events, compared to using one type of arrays. As proof of concept, we evaluated two types of nanohole arrays that showed opposite responses to binding events. The mixed designs proved to provide improved accuracy and confidence level, eliminating misinterpretation of the binding events induced by artifacts such as protein intrinsic signals.

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Fig. 1.

Images of nanohole arrays. (a) CCD image of a transducer containing 13 nanohole arrays with various designs. Spacing between arrays: $16\mu\text{m}$. (b) Scanning-electron microscope (SEM) image of an individual nanohole array that contains 256 (16×16) holes. Magnification: 35kx; Hole diameter: 150nm ; Hole-to-hole distance: 400nm .

Fig. 2.

Spectral analysis of nanohole arrays with various designs. (a) Spectral comparison of one nanohole array when submerged in two solutions: PBS and 21% NaCl. Black line: in PBS solution; red line: in 21% NaCl. Periodicity of the array: 400nm; (b) Percentage change of the spectra vs. wavelength. (c) Spectral comparison of nanohole arrays with seven different designs. Data points on all lines except for line 550 represent average readings from two replica nanohole arrays.

Fig. 3.
Response of 13 nanohole arrays containing seven different designs to 21% NaCl solution.

Fig. 4. Photo of DPN-deposited protein droplets and nanoholes arrays. (a) Photo of 20 deposited protein droplets covering individual nanohole arrays; (b) Photo of individual nanohole arrays before nanowriting process. Array-to-array spacing: 16μm.

(b)

Fig. 5.
Simultaneous, specific binding responses of 12 individually nanowritten nanohole arrays to three different antibodies.

Fig. 6. Dramatically different responses from Human IgG modified Array300s and Array400s. Responses from Array300 references and Array400 references were also shown as verification.