Optimizing antibody microarrays for high resolution SPR microscopy

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ABSTRACT

A high resolution surface plasmon resonance (SPR) microscopy technique based on the combination of Lumera’s polymer and proprietary optic technologies offers parallel high-throughput analysis of biomolecular binding kinetics on high-density antibody microarrays ( > 1,500 spots in 1.4 cm²). The high resolution SPR microscopy also shows potential as a quantitative drug screening tool (Binding kinetics of a 1500 Dal. peptide has been experimentally demonstrated). However, the high resolution SPR microscopy requires optimal surface modification and dense sample addressing technology. Another constraint for the high density biomolecular microarray experiment is maintaining the activity of proteins during the microarray fabrication process. In this report, various spotting solutions combined with different surface modification chemistries are evaluated for the uniformity and integrity of protein spots in microarrays. The effects of various spotting solution compositions on the protein activity resulting from the microarray fabrication process are also investigated.

Keywords: SPR, microarray, immunoassay, biosensor,

1 BACKGROUND

Recent development in optical sensors based on evanescent wave propagation allows biomolecular interactions within sub-micron thickness from the sensor surfaces. Among them, surface Plasmon Resonance (SPR) spectroscopy is a powerful method capable of measuring molecular binding events by detecting changes in the effective refractive index with no-labeling required. A limitation of SPR spectroscopy is that a limited number of points can be monitored at a given time. The changes of SPR signal upon environmental disturbances have been obstacles in designing a cost-effective and robust analytical tool. Simultaneous analysis of multiple samples in high through-put mode has recently been available with high sensitivity using the SPR microscopy technique. One of the additional advantages of the SPR microscope over traditional SPR spectroscopy is in simultaneous monitoring of multiple adsorption sites over a relatively large sensing area with the same sensitivity. An SPR microscope with larger sensing area compatible to fluorescence-based techniques is demanding because simultaneous measurements of multiple analytes can be done with less labor and cost, and sample volume is dramatically reduced down to nanoliters. Measurements with real-time referencing minimize all variations such as different bulk characteristics and environmental noises.

Recently, a high resolution SPR microscope has been introduced by Lumera Corporation, which can monitor thousands of biomolecular interactions with a sensitivity of sub-femtograms per spot detection in a microarray. In order to take full advantage of this high resolution SPR microscopy, immobilization of biomolecules on SPR active substrates is necessary while still retaining the specific binding sites intact in a relatively compact area.

Surface immobilizations of proteins on glass slides are widely used in many applications. One of the obstacles in covalent immobilization of biomolecules on solid substrates such as on glass slides is loss or reduction of biological activity of proteins. The short distance of active sites in biomolecules, especially proteins, to solid substrates can impede the binding events on the surface. Many studies have shown the binding affinity is decreased on these solid surfaces. Homogeneity of immobilized proteins is also an important factor in obtaining uniform binding affinity on surfaces. Therefore designing optimal immobilization methodology enhances the detection limit in biological sensing.

Among the variety of approaches to effectively immobilizing biomolecules in compact areas, one approach based on contact printing methods involving a robotic arm with metal pins is a well established and commonly used technique in order to generate micron-sized spots in an array format for high through-put analysis such as a high resolution SPR microscope. It is critical to maintain protein activity in microarray fabrication along with other challenges. Wettability or hydrophobicity of substrate surfaces is known to play a major role in increasing the density of spots in microarray in order to separate each spot after printing due to the nature of water-based spotting solutions.

There have already been numerous efforts devoted to improving reproducibility in fabricating microarrays on glass slides utilizing large sensing areas in fluorescence based applications. Lumera’s proprietary optical technology brings this high resolution SPR microscope, which can evaluate much of the previous research aimed towards improvement of fluorescence labeled-microarray thanks to its scalable view area up to 400 mm².

Application of this high resolution SPR microscope to quantitative biological assays unveils the ability to monitor
thousands of antibodies interacting with antigens in parallel with different specificities while requiring only a minimal volume of samples. In contrast traditional labor-intensive and time consuming ELISA-based tests require large sample quantities.

Recent research of array-based antibody screening has demonstrated large numbers of antibodies covalently bound on glass slides and interacting specifically. Development of high density antibody microarrays for this high resolution SPR microscope could provide an efficient means to quantify thousands of different protein interactions in research applications. In this report, we will evaluate the effect of the functionalized surfaces on the spot profile and shape by using an antigen:antibody model binding system. The composition of spotting solutions are also studied to evaluate the effects in the spot profile.

2 EXPERIMENTAL

Surface modification
The gold-coated glass slides purchased from GenTel Biosurface, Inc. (Madison, WI) were cleaned with a piranha solution at 80°C for 15 seconds, rinsed with distilled (>18 MΩ·cm) water and absolute ethanol, and dried in N₂. They were then functionalized with amine-reactive organic headgroups in a proprietary linker layer and functionalized as described previously. DMSO was purchased from EMI science. BSA and glycerol were purchased from Sigma (St. Louis, MO). Betaine and PEG (Molecular weight of 2K Dal.) were purchased from Fluka.

Instrument
The high resolution SPR microscopy used in this report was developed by Lumera Corporation (Bothell, WA) in collaborative work with Prof. Charles T. Campbell at the University of Washington (Seattle, WA). This SPR microscope is capable of monitoring binding kinetics of approximately 30,000 antibody molecules binding on 1,000 microarray spots, simultaneously.

Data was collected using multiple image movie acquisition by area integration of the intensity in any number of pre-selected regions of interest. The intensity from regions of interest was averaged over a selected time to improve the sensitivity.

Microarray fabrication
Protein microarrays were solution spotted onto these SPR active slides using a robotic pin microarrayer. These microarrays were fabricated with a benchtop QArraymini microarrayer (Genetix USA, Inc., Boston, MA) on modified gold-coated glass surfaces. The various spotting solutions and concentration used were were 100 mM PBS, 10% (v/v) of DMSO, 5% (v/v) DMSO in PBS, 1% (v/w) poly(ethylene glycol) (PEG) in PBS, 1.5 M betaine, and 5% glycerol (w/v) mixed in 100 mM PBS.

The protein concentrations of these spotting solutions ranged from 0.5 mg/ml to 1 mg/ml and the arrayer delivery volume ranged from 0.3 to 0.6 nl. Delivery destinations were programmed to form spots within a 1.4 cm² area to produce up to 1,020 separate sample spots. Immobilization of the deposited proteins was achieved by covalent coupling between the functionalized SPR active slide and the protein, forming the stable linkage. The arrays were rinsed with distilled water and dried in N₂.

3 RESULTS

3.1 Spot Density

Numerous microspotting parameters can affect the reproducibility of protein microarrays. Humidity, evaporation of solutions, surface tension, and wettability of surfaces are among important factors to be optimized.

![Protein microarray printed on amine-reactive gold-coated slides. Center-to-center spot spacing was 280 µm. Entire spotting process takes place in a humidity-controlled chamber.](image)

Amine-reactive hydrophobic modifications on SPR slides were evaluated to increase the spot density on the protein microarray for this high resolution SPR microscope. Figure 1 shows an SPR image of a protein microarray fabricated with a 150-µm pin head on gold surfaces presenting amine-reactive hydrophobic surface modifications. Hydrophobic alkyl chains on gold surfaces provide well separated protein spots printed with a 150-µm-diameter pin head. Hydrophilic surface modifications, however, poorly defines spacing between spots (Data not shown).

Increasing diameters of spots can improve the signal-to-noise ratio by increasing the number of pixels per spot. 300-µm pin head was used to print protein spots on the hydrophobic modifications as shown in the following table.

<table>
<thead>
<tr>
<th>Pin head diameter</th>
<th>Spots per mm²</th>
<th>Spots per slides</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 µm</td>
<td>16</td>
<td>1024</td>
</tr>
<tr>
<td>300 µm</td>
<td>7</td>
<td>400</td>
</tr>
</tbody>
</table>

Increased spot sizes decrease the spot density in half with minimum spacing between separated spots.
3.2 Reproducibility

Reproducibility of printing was evaluated by repeatedly printing protein A and BSA on hydrophobic surface modifications. A 300-μm diameter pin head was used to better judge the line-profiles of spots as shown in figure 2. Figure 2a represents the protein array exposed to PBS. The collected signal intensity showed a variance of 3 % from spot to spot. After the stable signal intensity was established, human IgG in PBS solutions were injected into the fluidic cell. The difference image after the microarray was exposed to human IgG is shown in figure 2b.

Figure 2. SPR microscope images from the microarray of protein A and BSA. The top two rows were printed with protein A and the bottom two rows were printed with BSA in PBS with 5% DMSO. (a) represents the raw image of the protein microarray after fabrication. The difference image after binding of human IgG is shown in (b).

As expected, human IgG selectively binds to protein A spots as shown in the top two rows of bright spots in figure 2b due to the signal increase upon binding, while BSA spots show no significant increase. The maximum coverages were varied within 3% of saturation coverage of the signal intensity in human IgG binding onto protein A spots. The line profile along the spots on the top row of protein A after human IgG binding is shown in Figure 4.

Figure 3. Simultaneous monitoring of binding kinetic curves for human IgG on 20 protein A spots in a microarray with a high resolution SPR microscope. Human IgG was reconstituted in PBS and then injected on to the microarray in the following concentrations (a) 0.5 ng/ml, (b) 5 ng/ml, (c) 50 ng/ml, (d) 500 ng/ml, (e) 5 μg/ml, (f) 50 μg/ml, and then (g) 500 μg/ml, respectively.

Figure 4. Line profile across the protein A spots in the top row.

3.3 Solution Effects

It is important to reproduce homogeneous protein spots in order to analyze binding events on each spot representing bulk characteristics in a microarray as a high throughput bioassay. A reproducibility measurement was performed by measuring the standard deviation in the intensity across each spot. The variation across spots was 0.6 % of the spot intensity when proteins were printed from PBS mixed with 5% DMSO.

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Spot intensity</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG</td>
<td>8.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Glycerol</td>
<td>7.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Betaine</td>
<td>8.2</td>
<td>0.3</td>
</tr>
<tr>
<td>5% DMSO</td>
<td>8.6</td>
<td>0.2</td>
</tr>
<tr>
<td>10% DMSO</td>
<td>8.6</td>
<td>0.1</td>
</tr>
<tr>
<td>PBS</td>
<td>9</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Previous studies have shown that mixing protein solutions with different constituents can improve the uniformity of microarray spots as well as better retain activity of protein during the immobilization or incubation process.
Figure 5. SPR microscope image of protein spots printed with PBS mixed with different constituents.

Figure 5 shows the SPR microscope image of protein spots in the microarray printed with mixing constituents commonly used in fluorescence based microarray techniques. It is shown that spotting solutions mixed with 5% or 10% DMSO produce more uniform protein A spots on this hydrophobic surface modification.

SUMMARY

This report demonstrates the reproducible protein microarrays fabricated on modified gold surfaces for use in an SPR microscope. Some of the previously studied spotting solutions mixed with PBS were also evaluated. The application of microarray techniques with various printing solutions allowed the high resolution SPR microscope to generate high density antibody arrays suitable for simultaneous monitoring of thousands of antigen:antibody interactions in real-time with the same efficiency as the traditional fluorescence based techniques. This high resolution SPR microscope can provide a cost effective robust sensing method for high throughput analyses. Further optimization of the protein microarray with this SPR microscope is currently underway in order to minimize labor and sample consumption.

REFERENCES