7

Surface Plasmon Resonance Imaging Measurements of Protein Interactions With Biopolymer Microarrays

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Summary

The surface-sensitive optical technique of surface plasmon resonance (SPR) imaging is an ideal method for the study of affinity binding interactions of unlabeled biological molecules in a multiplexed format. This approach has been widely applied to monitor DNA–DNA, DNA–RNA, peptide–protein, and protein–protein interactions as well as surface enzyme reactions. The success of SPR imaging measurements relies on the robust attachment of biomolecules in an array format. In this chapter, we introduce two different surface attachment chemistries that covalently immobilize DNA and peptides onto gold surfaces through the modification of self-assembled alkanethiol monolayers. Array fabrication approaches for the creation of individually addressable elements through the use of either gold dot patterns or polydimethylsiloxane (PDMS) microchannels are detailed. The utility of SPR imaging for the study of protein interactions is demonstrated with two biological systems: the binding of response regulator proteins, VanR and OmpR, onto a DNA array, and the interaction of S protein with an array of S-peptide variants. Furthermore, the application of real-time SPR imaging to the multiplexed determination of S-protein adsorption/desorption kinetics is described.

Key Words: Surface plasmon resonance (SPR) imaging; DNA microarrays; peptide microarrays; polydimethylsiloxane (PDMS) microfluidic channels; VanR and OmpR; S protein; S peptide.

1. Introduction

The ability to detect proteins in an array format would be highly beneficial for many applications, such as epitope mapping ***(1)***, protein identification ***(2,3)***, screening of potential protein-binding inhibitors, and protein-binding kinetics ***(4,5)***. Typically, protein-binding studies make use of fluorescent tags or radiolabels for detection purposes. However, labeling presents the potential problem of altering the protein's active binding site and thus its biological activity. The technique of surface plasmon resonance (SPR) is ideally suited to protein-binding

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studies, as affinity interactions are detected by local changes in the index of refraction, and thus tagging of the target molecule is not required.

Surface plasmons are surface electromagnetic waves that propagate parallel to a metal/dielectric interface and are generated when energy from a photon of p-polarized light incident onto a thin metal film, such as gold (Au), is coupled into oscillating modes of free electron density. Surface plasmons have a maximum intensity at the metal/dielectric interface and decay exponentially away from the interface. This gives SPR a sensing depth of only 200 nm, making it a surface-sensitive technique ***(6,7)***.

SPR measurements can be made in a variety of formats including scanning-angle SPR, Fourier transform (FT) SPR, and SPR imaging. In a scanning-angle SPR experiment, the change in percent reflectivity is measured as a function of incident angle, while the wavelength of light is held fixed. This is the most common SPR method and has been popularized by the commercially available BIAcore instrument. In FT-SPR, the wavelength of light is scanned as the angle of incidence is held fixed. Both of these techniques are single-channel measurements and cannot be used in a high-throughput format. SPR imaging is a fixed-angle and wavelength technique where arrays of molecules can be analyzed in parallel over the entire array surface.

In an SPR imaging experiment, the intensity of reflected light from the metal surface is collected at an optimum angle just off of the plasmon angle. When a molecule is adsorbed onto the surface, the SPR curve will shift to a higher angle, as a result of a change in the index of refraction at the surface. This is observed as a change in the light intensity reflected from the metal surface, and is captured by a charge-coupled device (CCD) camera. Changes in percent reflectivity in individual array elements are determined by subtracting an image taken before (reference) from an image taken after exposure to analyte. In equilibrium measurements, sufficient time must be allowed between the two images to ensure that the reaction is complete. Alternatively, the surface reaction can be continually monitored in real time to provide multiplexed kinetic information on processes such as adsorption and desorption.

The key to applying SPR imaging for the study of biomolecular interactions is the development of robust surface-attachment chemistries. In addition, array fabrication procedures are necessary to create surfaces with spatially resolved detection elements for high-throughput studies. In this chapter, two covalent attachment strategies are presented for the creation of both short single-stranded oligonucleotide and peptide microarrays on thin gold films. SPR imaging is then employed in conjunction with DNA arrays to evaluate the binding specificity of the response regulator proteins VanR and OmpR to various DNA promoter regions ***(8)***. Finally, both equilibrium and real-time measurements of differential S-protein binding to an array of S-peptide variants ***(9)*** are demonstrated.

2. Materials

2.1. Thiol-Modified DNA Purification and Preparation

1. 1 *M* triethylammonium acetate (TEAA) (pH 7.01) is prepared by placing 800 mL of water in a 1-L flask (*see* **Note 1**). Place the flask in a fume hood, and add 140 mL of triethylamine while stirring on ice. Slowly add acetic acid over several hours while stirring on ice to adjust the pH to 7.01. Store at 4°C.
2. Buffer A: 4% acetonitrile (high-performance liquid chromatography (HPLC)-grade), 10% TEAA, 86% water.
3. Buffer B: 50% acetonitrile (HPLC-grade), 10% TEAA, 40% water.
4. 50 m*M* phosphate buffer (pH 8.4). Store at room temperature.
5. 200 m*M* dithiothreitol (DTT) in 50 m*M* phosphate buffer (pH 8.4).
6. 100 m*M* triethanolamine hydrochloride (TEA) buffer at both pH 7.0 and pH 8.0. Store at room temperature.
7. 4 mg of Ellman's reagent (0.4% 5,5′,-dithiobis[2-nitrobenzoic acid]) dissolved in 1 mL of TEA buffer (pH 8.0) and used immediately. This solution is light sensitive and should be made immediately before use.
8. C6 S-S 5′ thiol-modified DNA used for array fabrication is obtained commercially (*see* **Note 2**).

2.2. Thiol-Modified DNA Array Fabrication

1. 100 m*M* triethanolamine hydrochloride (TEA) buffer at both pH 7.0 and pH 8.0. Store at room temperature.
2. A 9% Cytop (Bellex International, Wilmington, DE) solution is diluted to a working concentration of 1.5% using CT-180 perfluorosolvent (Bellex International, Wilmington, DE).
3. A 1 m*M* solution of 11-mercaptoundeclyamine (MUAM, Dojindo Laboratories) is prepared by dissolving 1.7 mg in approx 7 mL of absolute ethanol. Sulfosuccinimidyl 4-(*N*-malemidomethyl) cyclohexane-1-carboxylate (SSMCC, Pierce) is dissolved at a 1 m*M* concentration in 100 m*M* TEA buffer (pH 7.0).
4. 1 m*M* solutions of thiol-modified DNA in 100 m*M* TEA buffer (pH 7.0).
5. A thin stainless steel mask containing 700-µm-diameter holes spaced 550 µm apart (edge to edge) is used to vapor deposit gold spots onto Cytop-coated SF-10 (Schott Glass) glass slides.

2.3. PDMS Microfluidic Channels

1. Silicon wafers (3-in diameter; International Wafer Services Inc.) are used for the fabrication of three-dimensional (3D) silicon wafer masters for the production of PDMS microfluidic channels.
2. Rigid chrome masks containing the desired microfluidic features are designed using a CAD program and created using e-beam photolithography. High-resolution transparencies containing the desired microfluidic features can be used as an alternative to the chrome masks.
3. Negative photoresist (SU-8 50, Microlithography Chemical Corp.).
4. Propylene glycol methyl ether acetate (Developer, Microlithography Chemical Corp.).

5. Tridecafluoro-1,1,2,2-tetrahydroctyl-1-trichlorosilane (Gelest, Inc.).
6. PDMS prepolymer and curing agent (Sylgard 184, Dow Corning).

2.4. Peptide Array Fabrication

1. A 1 m*M* solution of MUAM is prepared by dissolving 1.7 mg in approx 7 mL of absolute ethanol.
2. A 6.4 m*M* solution of *N*-succinimidyl 3-(2-pyridyldithio) propionamido (SPDP; Pierce) is made in a 1:1 ratio of DMF and 0.1 *M* phosphate-buffered saline (PBS) solution (pH 7.4) immediately prior to use.
3. 2 m*M* solutions of cysteine-modified peptides are prepared in PBS buffer (pH 7.4) prior to immobilization.
4. 2 mg of *N*-hydroxysuccinimidyl (NHS) ester of methoxypoly (ethylene glycol) propionic acid MW 2000 (PEG-NHS, Nektar) is dissolved in 250 μL of TEA buffer (pH 8.0). Prepare fresh immediately before use.

2.5. Affinity Binding Measurements

1. Response regulator protein binding buffer: 10 m*M* phosphate buffer (pH 7.1), 5 m*M* $MgCl_2$, and 100 m*M* NaCl.
2. Bacterial response regulator proteins, VanR and OmpR, are phosphorylated in 100 μL of 50 m*M* HEPES (pH 7.2), 5 m*M* $MgCl_2$, and 50 m*M* acetyl phosphate prior to use.
3. 8 *M* urea, stored at room temperature.
4. S-protein binding buffer: 10 m*M* phosphate buffer (pH 7.4), 2.7 m*M* KCl, and 137 m*M* NaCl.

3. Methods

Successful SPR imaging measurements of protein interactions with biopolymer microarrays require three key components: robust surface chemistry for tethering biopolymers onto a gold surface, array fabrication methods, and surface biochemistry. A fabrication methodology that utilizes alkanethiol chemistry and gold patterned glass substrates to create arrays of DNA molecules is presented first. These arrays are used in conjunction with SPR imaging to measure the relative binding affinity of the bacterial response regulator proteins, VanR and OmpR ***(8)***, to various DNA sequences. In addition, an alternative approach employing the use of PDMS microfluidic networks ***(10)*** to prepare arrays of S-peptide variants onto chemically modified gold surfaces is discussed ***(9)***. SPR imaging is used for the acquisition of both equilibrium and real-time measurements of S-protein binding to the prepared arrays.

3.1. SPR Imaging of DNA–Protein Interactions

3.1.1. DNA Purification and Quantification

1. Dilute the thiol DNA with 50 μL of 50 m*M* phosphate buffer (pH 8.4) for every 10 OD_{260} of DNA concentration.

2. Take a 50-μL aliquot of the diluted DNA and mix it with 50 μL of a freshly prepared 200 m*M* solution of DTT diluted in phosphate buffer (pH 8.4). Let it react for 30 min to cleave the disulfide bond of the C6 thiol S-S modifier.
3. Purify the DNA using reverse-phase binary HPLC (*see* **Note 3**).
4. Collect the purified DNA in an Eppendorf tube. Elution time for the DNA occurs in approx 20–25 min.
5. Dry the DNA using a Speed-vac until all of the solution has evaporated.
6. Resuspend the dried DNA in TEA buffer (pH 7.0). Use approx 5 μL of buffer to resuspend the DNA collected from each HPLC purification run.
7. To calculate the concentration of the resuspended DNA, set the detection wavelengths of an ultraviolet (UV)-Vis spectrometer to 260 and 412 nm, and blank the system by placing 59.6 μL of TEA buffer (pH 8.0) into a cuvette.
8. Add 0.4 μL of Ellman's reagent to the cuvette and take a measurement. This will be used as the 100-fold dilution reference.
9. Add an additional 540 μL of TEA buffer (pH 8.0) to the cuvet and take another measurement. This will be used as the 1000-fold dilution reference.
10. In a clean cuvet, add 59 μL of TEA buffer (pH 8.0), 0.6 μL of the purified thiol DNA, and 0.4 μL of Ellman's reagent. Let this reaction proceed for 10 min before taking a measurement.
11. Add an additional 540 μL of TEA buffer (pH 8.0) to the cuvet and take another measurement. The 260-nm measurements are used to calculate the DNA concentration, while the 412-nm measurements are used to calculate the free thiol concentration (*see* **Note 4**).

3.1.2. DNA Array Fabrication

1. Cover a 1.8 × 1.8 cm SF-10 slide with 1.5% Cytop solution.
2. Spin coat the slide at 500 rpm for 5 to 10 s, and then manually ramp the speed up to 5000 rpm and allow it to spin for at least 30 s (*see* **Note 5**).
3. Place the slides Cytop face up into a Pyrex® Petri dish, cover, and bake at 70°C for 50 min.
4. Remove the slides from the oven and place them in a different oven preheated to 190°C for 1 h. This temperature is above the boiling point of the CT-180 perfluorosolvent.
5. Place the Cytop-coated glass slides face up into a sample holder and cover with the mask containing the 700-μm holes. Mount the sample assembly into a vapor deposition chamber.
6. Vapor deposit 1 nm of chromium and then 45 nm of gold onto the Cytop-modified slides though the stainless steel mask. This will create gold patches on the hydrophobic glass surface (*see* **Note 6**).
7. Place the gold dot slide into an ethanolic MUAM solution for at least 4 h to form a well packed amine-terminated monolayer on the gold dots.
8. Remove the gold dot slide from the MUAM solution and rinse with absolute ethanol, then with water, and dry under a stream of nitrogen.
9. A PV830 Pneumatic Pico Pump (World Precision Instruments) is used to deliver 40- to 100-nL volumes of a freshly prepared SSMCC solution to the individual

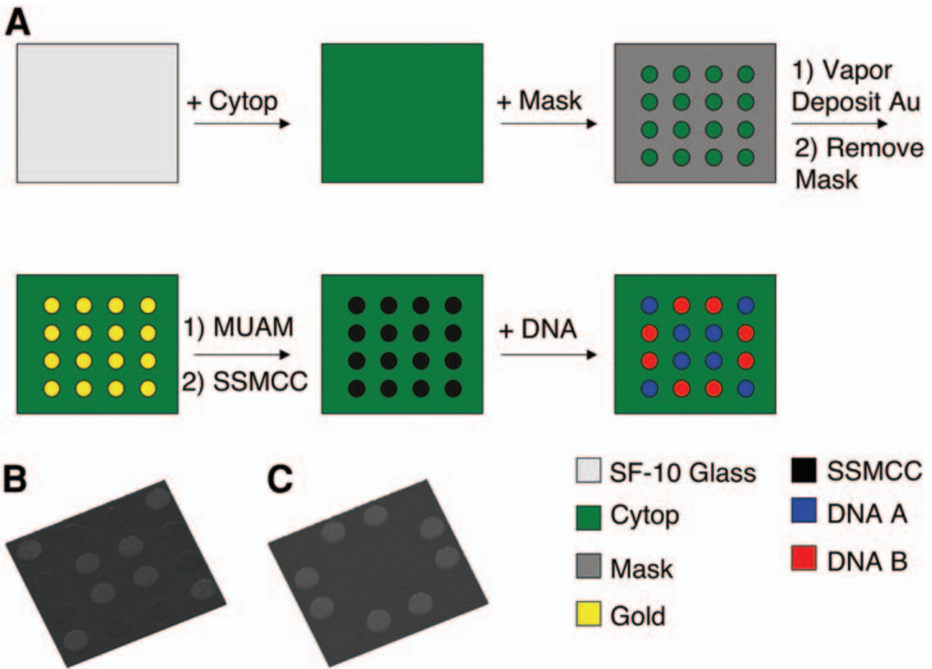

Fig. 1. **(A)** Schematic showing the DNA array fabrication process. **(B)** Surface plasmon resonance (SPR) difference image of DNA probe A binding to its complementary DNA sequence. The array is then denatured using 8 *M* urea to regenerate the single-stranded DNA array surface. **(C)** SPR difference image of DNA probe B binding to its complementary DNA sequence on the same array.

MUAM array elements within the Cytop-coated glass background. Allow the SSMCC to react with the surface for 20 min in order to form a maleimide surface that is thiol reactive.

10. Wash the slide with water and then dry under a stream of nitrogen. Using the pico pump, spot the thiol-modified DNA sequences onto the maleimide-terminated array elements to create an array of DNA molecules on the surface. Leave the thiol-modified DNA to react with the surface overnight.
11. Clean the surface with water to wash away the excess DNA solution, and dry under a stream of nitrogen. The DNA array should then be immediately mounted into the sample holder of the SPR imager or stored in a humidity chamber. An overview of the array fabrication process can be seen in **Fig. 1A**. **Figure 1B,C** shows SPR difference images for the sequence specific binding of complementary DNA sequences to a two-component DNA array. When the array is exposed to a DNA sequence complementary to DNA probe A, only those array elements are visible in the SPR difference image, indicating a hybridization/adsorption event (**Fig. 1B**). The hybridized DNA is then denatured with 8 *M* urea, and the same array is used

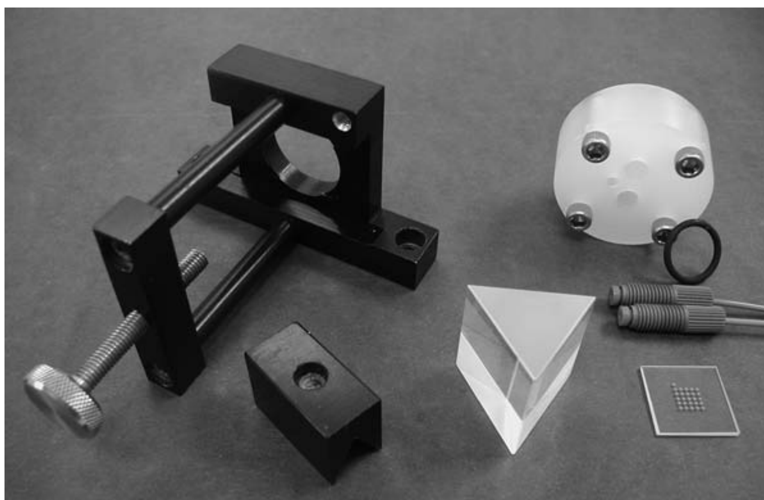

Fig. 2. Sample holder/flow cell assembly components.

to detect a DNA sequence complementary to probe B. When the array is exposed to the complementary sequence of probe B, only changes in the probe B elements are visible in the SPR difference image (**Fig. 1C**).

3.1.3. SPR Imaging Flow Cell Assembly

1. These instructions assume the use of an SPR imager from GWC Technologies. It is important that the three optical sides of the prism be thoroughly cleaned with methanol and lens paper. There should be no visible marks of any kind on the three faces of the prism. (*See* **Fig. 2** for an overview of the cell assembly components.)
2. Using tweezers, place the prepared array into the sample holder with the gold side facing down, using the four corners cut into the holder as a guide. Hold the array only by the corners to avoid damaging the functional array surface with the tweezers.
3. Clean the glass side of the gold slide using a cotton swab and methanol to remove any dust or smudges from the surface.
4. Without touching the surface, put one drop of index-matching fluid (Cargille, RI = 1.720) onto the center of the chip (*see* **Fig. 3**). Gently lower one of the optical sides of the prism on top of the chip at a slight angle to avoid bubble formation (*see* **Note 7**). Be sure to center the prism over the chip.
5. Place the prism wedge onto the top of the prism, and align the hole in the prism wedge with the tightening screw. If the hole in the prism wedge is not aligned with the tightening screw, gently move the wedge with tweezers until they are properly aligned. Hand-tighten the thumbscrew so that the chip and prism are held firmly in place.
6. Assemble the flow cell onto the chip surface, being careful to align the four screw holes of the flow cell with those in the sample holder. It is preferable that the inlet

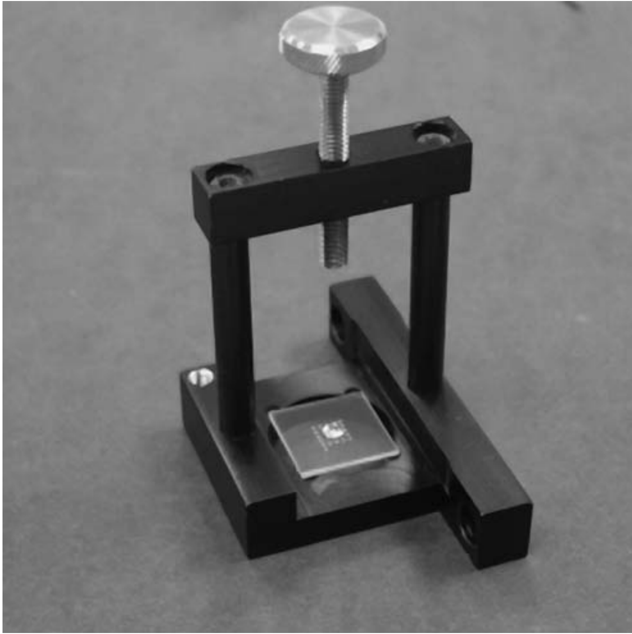

Fig. 3. Prepared array placed in the sample holder with the gold side facing down. The four corners cut into the holder are used as a guide. A drop of index-matching fluid is then placed onto the center of the glass side of the array.

and outlet ports be positioned vertically when the assembled sample holder is placed into the SPR imager. Screw the flow cell into the sample holder while applying even pressure by incrementally tightening the screws diagonally from one another. Tighten the flow cell to the sample holder until the o-ring is visibly compressed (as viewed through the prism) against the gold slide.

7. Screw the inlet and outlet ports into the flow cell openings by hand. The fully assembled sample holder/flow cell assembly (shown in **Fig. 4**) can then be placed onto the SPR imager.

3.1.4. Mounting the Flow Cell and Angle Adjustment

1. Place the assembled flow cell onto the rotation stage so that the screw holes in the flow cell are aligned with the mounting holes in the rotation stage. Align the sample holder so that the light reflected from the gold surface will be directed toward the CCD camera. Fasten the flow cell to the rotation stage.
2. Connect the tubing from the pump to the lower inlet port of the flow cell. A section of tubing is connected to the top port and is used to deliver the waste solution to a beaker.
3. Place the end of the pump tubing into a small beaker of water, and turn the pump on to deliver water to the array surface.

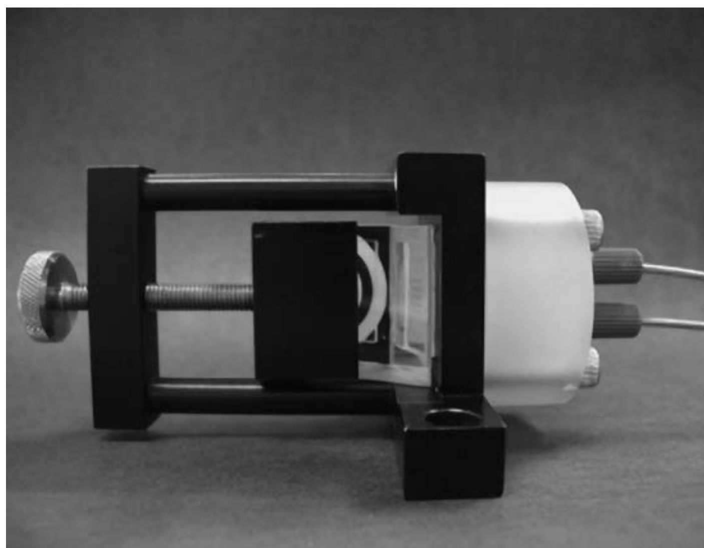

Fig. 4. Fully assembled sample holder/flow cell assembly.

4. When the flow cell is completely filled with water, turn the pump off and transfer the pump tubing to an Eppendorf tube containing the buffer to be used in the SPR imaging experiment. Turn the pump on to deliver the buffer to the array surface.
5. Using the line profile function in the SPR imaging software, draw a line across the displayed image. This line on the image determines the points from which intensities are measured and displayed in the right-hand side of the window.
6. Turn the knob on the rotation stage until a minimum is observed in the line profile window, as determined from the pixel intensity values. This angle is known as the plasmon angle, and is the angle at which all incoming light is converted into surface plasmons. Turn the angle adjustment knob counterclockwise until the pixel value reaches a maximum. The angle is then adjusted to a point that is approximately one-third of the way from the minimum to the maximum pixel value. A background pixel intensity of approx 60–80 is a typical value, with respect to a maximum pixel intensity of 220.
7. This is a fixed-angle technique, and therefore it should not be readjusted during the course of the experiment.

3.1.5. SPR Imaging of DNA–Protein Binding

1. Fill the flow cell with response regulator protein-binding buffer and take an SPR image with an average of 30 capture frames (*see* **Note 8**). This will be the background image.
2. Flow complementary single-stranded DNA (500 n*M*) diluted in response regulator protein-binding buffer onto the array surface. Allow the DNA to hybridize to the

array elements for 15 min, and then flush the array with analyte-free buffer to remove nonspecifically adsorbed DNA from the background.

3. Take an SPR image (30 capture frames) of the array. Subtract the background image from this image to obtain an SPR difference image. The SPR difference image will show a change in percent reflectivity at those array elements in which hybridization has occurred. The double-stranded DNA array can then be used to detect double-stranded DNA binding proteins.
4. Flow response regulator protein-binding buffer over the array surface and take an SPR image. This image will serve as the background reference image.
5. Introduce a solution of transcription factor proteins, OmpR or VanR, diluted in response regulator protein-binding buffer to the array surface. These proteins can be imaged over a concentration range from 1 to 500 n*M*. The protein solution is allowed to sit on the surface for 15 min.
6. An SPR image is taken, and the background image (taken in **step 4**) is subtracted from this image. The resulting SPR difference image shows the specific binding of the transcription factor proteins to the surface. **Figure 5A** shows the specific binding of a 100 n*M* solution of OmpR to the double-stranded DNA array surface. Differential binding of the protein is observed for three of the probe sequences, with their relative binding affinities indicated by the relative signal intensity at each of the array elements (i.e., signal intensity increases as binding affinity increases). Changes in the SPR signal are not observed for the array elements designed to bind the protein VanR, and therefore they are not visible in the SPR difference image. The array was denatured with 8 *M* urea and then exposed to a 500 n*M* solution of VanR. **Figure 5B** shows the differential binding of the protein VanR to the VanR probe array elements. VanR shows the highest binding affinity to the Van H2 and Van R1 array elements, and significantly less binding to the Van H1 array elements. Slight nonspecific adsorption is observed at the OmpF1 array elements, whereas no change in the SPR signal is observed for the other array elements or to the PEG background.
7. Turn the polarizer 90 degrees, so that s-polarized light is striking the array surface. Take an SPR image, and then turn the polarizer back 90 degrees to return to p-polarized light. The value obtained for the s-polarized light will allow for the conversion of pixel intensity to change in percent reflectivity (*see* **Note 9**).
8. Wash the surface with 8 *M* urea, and allow it to remain on the surface for 15 min. This step will remove all of the complementary DNA and protein from the surface. Rinse the surface with water to wash away the urea. This process regenerates the single-stranded DNA array surface.
9. Repeat **steps 1–8** for each additional protein to be analyzed.

3.2. SPR Imaging of Peptide–Protein Interactions

3.2.1. Creation of 3D Silicon Wafer Masters and PDMS Microchannels

1. Pipet approx 4 to 5 mL of the negative photoresist onto the center of a silicon wafer and spin-coat at 5000 rpm for 60 s so that the wafer is evenly coated. Bake the wafer at 65°C for 5 min.

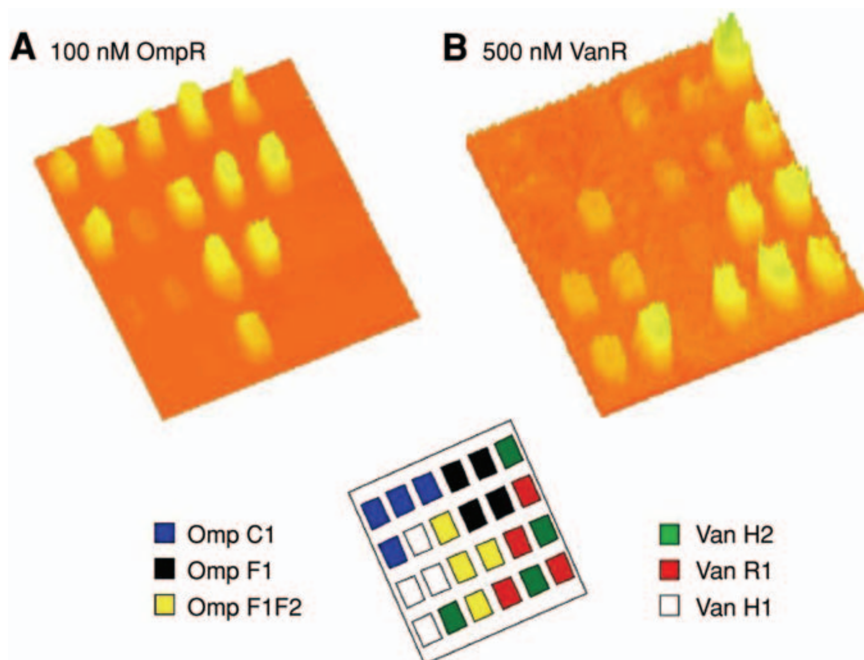

Fig. 5. Surface plasmon resonance (SPR) difference images of a six-component DNA array containing three sequences specific for OmpR and three specific for VanR. A double-stranded DNA array was first formed by exposing the array surface to a solution containing DNA complementary to all of the DNA probe sequences. **(A)** SPR difference image showing the binding of a 100 n*M* solution of the protein OmpR to the double-stranded DNA surface. Only the array elements specific for OmpR can be seen. **(B)** SPR difference image showing the binding of a 500 n*M* solution of the protein VanR to the double-stranded DNA array surface. Only the array elements specific for VanR show changes in the SPR difference image. Differences in binding intensity at each probe sequence are attributed to differences in their binding affinity to the specific protein. (Reprinted from **ref.** ***8***, with permission of the American Chemical Society.)

2. Assemble the photoresist-covered silicon wafer and patterned chrome mask onto the sample stage of a UV aligner. Shine 365-nm UV light though the assembly for 40 s to transfer the pattern from the mask to the silicon wafer surface.
3. Bake the silicon wafer at 90°C for 5 min and then place the wafer in developer solution for 15 min at room temperature. This forms protruding shapes on the silicon wafer surface at regions that were previously exposed to the UV light in **step 2**.
4. Silanize the silicon master by storing it in a desiccator under vacuum for 2 h with a vial containing a few drops of tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane. This last step is necessary to ensure the easy removal of the PDMS replicas from the 3D silicon master. These wafers can be reused for several months.

5. Mix the PDMS prepolymer with curing agent in a 10:1 ratio and pour it onto a 3D silicon wafer that has been placed into a plastic Petri dish. Be sure to pour enough PDMS onto the silicon master to cover the entire surface to a depth of 1 to 3 mm.
6. Allow the PDMS to de-gas for 20 min, until no air bubbles are visible, and then put the 3D silicon master and PDMS into an oven at 70°C for at least 1 h (*see* **Note 10**).
7. Take the PDMS and 3D silicon master from the oven and cut around the outside of the silicon master with a scalpel so that it can be removed from the Petri dish.
8. Carefully peel the PDMS microchannels from the surface of the silicon master and place them into a clean Petri dish.
9. Using a scalpel, cut out the desired microchannel so that it will fit onto the gold thin film (1.8 × 1.8 cm maximum).
10. Punch out reservoir holes at the ends of the PDMS microchannels so that target solution can be delivered through the microchannel (*see* **Note 11**). PDMS microchannels should be used only once and made just prior to use.

3.2.2. S-Peptide Array Fabrication

1. Soak a gold slide in an ethanolic solution of MUAM for at least 4 h to form a self-assembled monolayer of amine-terminated alkanethiols. Take the slide from the ethanolic solution, wash with ethanol, then with water, and dry under a stream of nitrogen.
2. Attach a set of microchannels featuring multiple parallel channels to the surface as a means to deliver reagents to the chemically modified gold surface.
3. Flow a solution of the bifunctional linker SPDP through the microchannels and allow it to react for 2 h. The NHS ester of the SPDP forms a covalent amide linkage with the MUAM monolayer, forming an active disulfide-terminated surface.
4. Wash the microchannels with water and then flow a 2 m*M* solution of either S peptide or modified S peptide down each individual microchannel. Let the thiol-disulfide immobilization reaction proceed overnight. **Figure 6A** shows a schematic of the patterned S-peptide variants on the array surface.

3.2.3. Flow Cell Assembly for Use With S-Peptide Array

1. Follow **Subheading 3.1.3., step 1**.
2. Mold a set of PDMS microchannels from the aluminum master containing a serpentine design (*see* **Note 12**).
3. Treat the PDMS microchannels with oxygen plasma for 10 s immediately prior to flow cell assembly.
4. Place a set of serpentine PDMS microchannels into a specially designed sample holder with prefabricated inlet and outlet ports that seal to the microchannels.
5. Place the gold slide onto the microfluidic channels face down, making sure to orient the immobilized lines of peptides perpendicular to the serpentine channels. SPR detection regions (300 µm × 670 µm) are formed where the immobilized peptide lines intersect with the PDMS microchannel. **Fig. 6b** shows a schematic of the serpentine PDMS microfluidics used to deliver analyte to the array surface throughout the SPR imaging experiment.

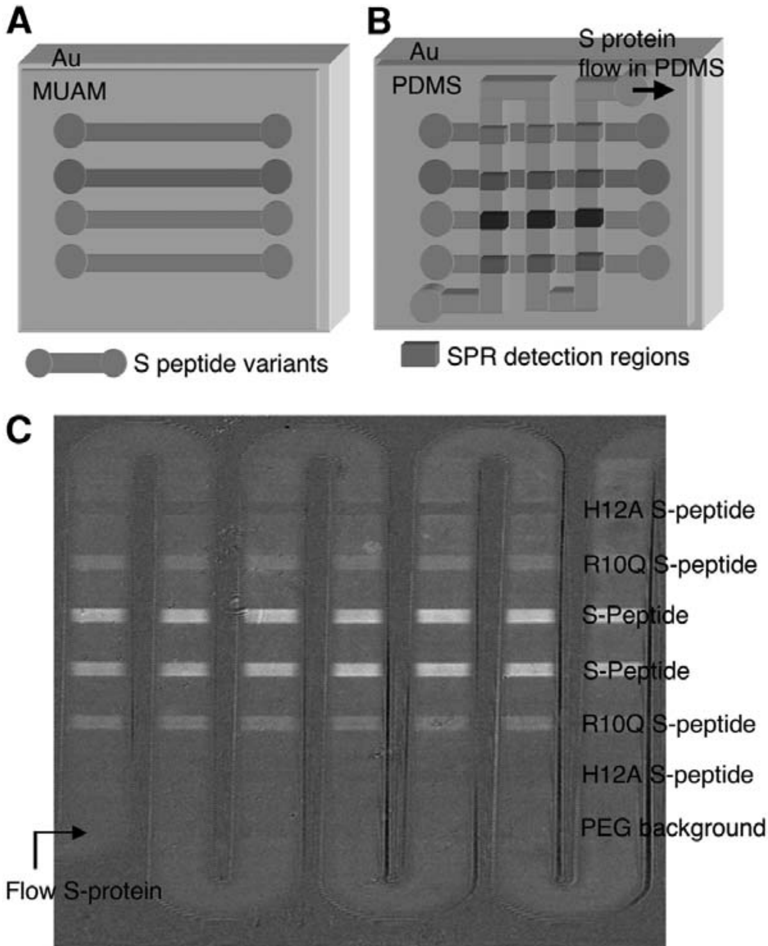

Fig. 6. **(A)** Schematic of S-peptide immobilization onto the chemically modified gold surface. **(B)** Schematic showing the flow direction of S protein through the polydimethylsiloxane microfluidics onto the peptide array. **(C)** Surface plasmon resonance difference image for a 75 n*M* solution of S protein binding to various S-peptide variants. Native S peptide shows the highest amount of binding to S protein, whereas the R10Q S-peptide variant containing a substitution of an amino acid at position 10 in the S-peptide sequence shows a much lower binding affinity. The H12A S-peptide variant shows no binding affinity to the S protein.

6. Without touching the surface, put one drop of index matching fluid (Cargille, RI = 1.720) onto the center of the back of the chip. Gently lower one of the optical sides of the prism onto the top of the chip at a slight angle to avoid bubble formation. Center the prism over the chip and tighten.

3.2.4. Mounting the PDMS Microfluidic Flow Cell and Angle Adjustment

1. Place the assembled flow cell onto the rotation stage so that the screw holes in the sample cell are aligned with the mounting holes in the rotation stage. Align the sample holder so that the light reflected from the gold surface will be directed towards the CCD camera. Fasten the flow cell to the rotation stage.
2. Connect the inlet port to a syringe pump for continuous sample delivery.
3. Flow PEG-NHS solution through the microchannnel and allow it to react for 1 h. The PEG-NHS will react with the surface amine groups in the regions surrounding the pattern of S peptides. This will result in a background that is resistant to the nonspecific adsorption of biomolecules.
4. The microchannel is then flushed with water to remove the solution of PEG-NHS.
5. Flow S-protein binding buffer through the PDMS microchannel using a syringe pump.
6. Using the line profile function in the SPR imaging software, draw a line across the displayed image. This line on the image determines the points from which intensities are measured and displayed in the right hand side of the window.
7. Turn the knob on the rotation stage until a minimum is observed in the line profile window, as determined from the pixel intensity values. This angle is known as the plasmon angle, and is the angle at which all incoming light is converted into surface plasmons. Turn the angle adjustment knob counterclockwise until the pixel value reaches a maximum. The angle is then adjusted to a point that is approximately one-third of the way from the minimum to the maximum pixel value. A background pixel intensity of approx 60–80 is typical.
8. Once the angle is adjusted, do not change it over the course of the experiment.

3.2.5. SPR Imaging of S Peptide–S Protein Equilibrium Binding

1. Flow a solution of S protein-binding buffer across the array surface through the PDMS microchannels using the syringe pump. Take an SPR image (30-frame average). This image will serve as the reference background image.
2. Flow a solution of S protein through the serpentine microchannel. Let the protein solution sit on the surface for 5 min to allow for equilibrium binding.
3. Take an SPR image with the protein still present in the microchannel. Subtract the background image (*see* **step 1**) from this image to obtain an SPR difference image for the affinity binding interaction. Changes in percent reflectivity are observed only where there is specific binding of S protein to the immobilized S peptide (*see* **Note 13**). **Figure 6C** shows an SPR difference image for the detection of S protein onto an array of S-peptide variants. Differential binding of the protein is clearly visible with a range of intensities observed at the various peptide array elements. Native S peptide shows the highest amount of binding to S protein (i.e., the peptide elements which appear to be the brightest in the image). The R10Q S-peptide variant containing a substitution of an amino acid at position 10 in the S-peptide sequence has a much lower binding affinity for the S protein. A negligible binding affinity of the S protein onto both the H12A S-peptide variant and the PEG background was observed (i.e., no changes in the SPR intensity).
4. Turn the polarizer 90 degrees, so that s-polarized light is striking the array surface. Take an SPR image, and then turn the polarizer back 90 degrees to return to

p-polarized light. The value obtained for the s-polarized light will allow for the conversion of pixel intensity to change in percent reflectivity.

5. Flush the array with buffer containing no analyte to regenerate the peptide array surface. The array can be used multiple times.

3.2.6. Real-Time SPR Imaging Measurements of S-Protein Binding

1. Fabricate the S-peptide array as outlined in **Subheading 3.2.2.**
2. Assemble the array onto the flow cell and mount onto the SPR imager as per **Subheadings 3.2.3.** and **3.2.4.**
3. Using custom-written software, a desired number of regions of interest (ROIs) on the array surface are defined. **Figure 7A** shows an SPR image with dotted lines indicating the boundaries of the ROIs on the chip. These ROIs correspond to different peptide array elements and PEG background correction regions.
4. Begin running the real-time data collection software prior to flowing the solution of S protein across the array surface. The collection macro calculates the change in average pixel intensity with respect to a corresponding reference value for each ROI and updates a graphical display in real-time. Typically, five frames are averaged with a time resolution of approx 1 s.
5. Flow analyte-free S-protein binding buffer over the array surface through the microfluidic channel to monitor the desorption curve of S protein from the S-peptide array surface.
6. These measurements can be repeated over a range of S-protein concentrations (i.e., 10 n*M* to 300 n*M*). From these measurements, values for the adsorption and desorption coefficients and binding affinity can be extracted.

4. Notes

1. Unless stated otherwise, all solutions should be prepared in water that has a resistance of 18.2 MΩcm. This standard is referred to as "water" in the text.
2. Commercially available DNA can be obtained with a wide variety of 5′ or 3′ modifications for surface-attachment chemistry. This method assumes the use of 5′ C6 S-S thiol-modified DNA or 3′ C3 S-S thiol-modified DNA.
3. The HPLC gradient used for DNA purification is as follows:
   a. Hold at 10% buffer B for the first 5 min.
   b. Gradually ramp to 70% buffer B over the next 45 min.
   c. Ramp to 100% buffer B in the next 2 min to wash the column.
   d. After 15 min, return to 10% buffer B.
4. The extinction coefficient to be used for the thiol concentration calculation is 13,600 L/(mol·cm).
5. The estimated thickness of the Cytop layer is less than 20 nm. In this particular application, it is important to achieve a Cytop layer as thin as possible to avoid losing SPR sensitivity.
6. Gold dot patterned slides should be immediately immersed and stored in an ethanolic solution of MUAM after vapor deposition. These arrays are also commercially available (GWC Technologies).
7. If a bubble forms in the index-matching fluid between the prism and array, carefully remove the slide from the prism using a pair of tweezers, taking care not to

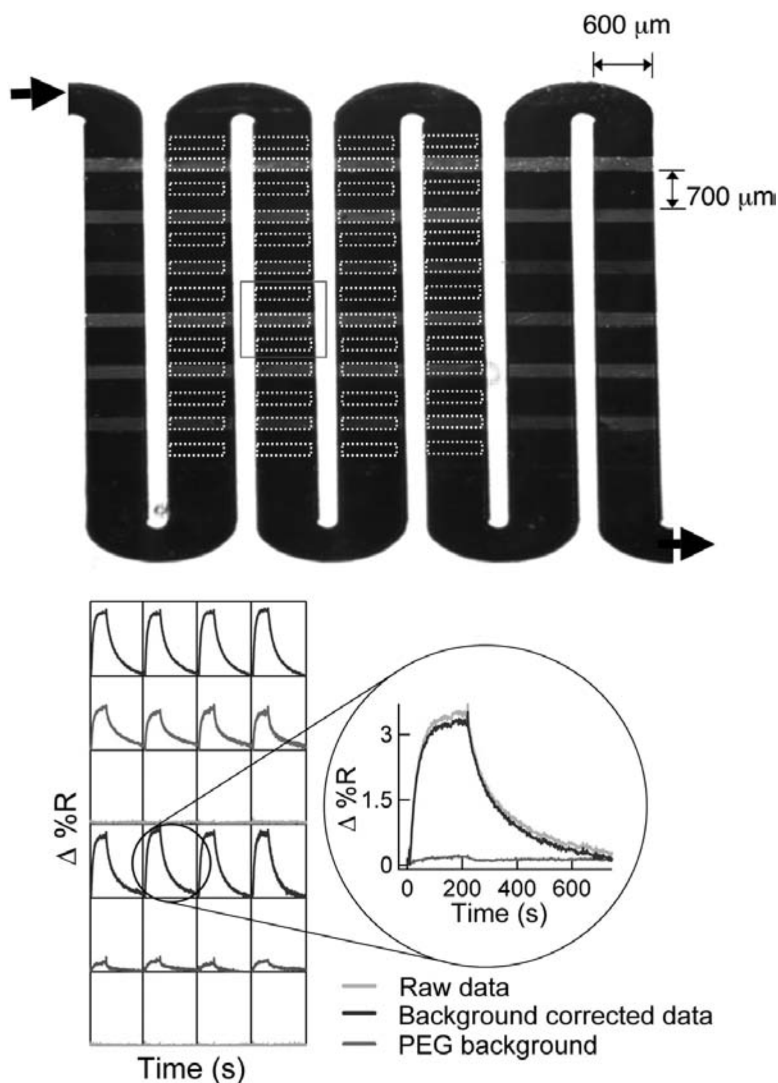

Fig. 7. **(A)** Representative raw surface plasmon resonance image of an S-peptide array with regions of interest (ROIs) drawn as dotted boxes on the array surface. The ROIs designate the position on the array where changes in percent reflectivity are to be measured as a function of time using a charge-coupled device camera. **(B)** Graphs showing the kinetics information obtained at each peptide array element when exposed to a solution of S protein. All of the curves were normalized with respect to adjacent polyethylene glycol regions. (Reprinted from **ref. *9***, with permission of the American Chemical Society.)

contaminate the array surface with the index-matching fluid. Using a cotton swab dipped in methanol, clean the glass side of the array to remove the index-matching fluid. Clean the prism and then remount the array and prism into the SPR sample holder.

8. An initial washing of the DNA probe surface with urea and water through the flow cell prior to beginning an experiment helps to clean the surface and ensure the best SPR imaging results.
9. Pixel intensity can be converted to change in percent reflectivity by the following equation:

$$\frac{0.85\,I}{S_{pol}} \times 100 = \Delta\%\ \text{Reflectivity}$$

where I is the SPR pixel intensity and S_{pol} is the pixel intensity measured using s-polarized light.
10. For both array fabrication and microfluidic flow cell preparation, it is preferable to cure the PDMS in the oven overnight.
11. When punching the reservoir holes in the PDMS, it is preferable to have the channel side facing up to avoid contaminating the channels with small PDMS particulates.
12. The serpentine microchannels fabricated from the aluminum master have a size of 670 μm width, 9.5 cm total length, 200 μm depth, and 400 μm spacing between folds, and are prepared in the same way as those fabricated on the silicon master (*see* **Subheading 3.2.1., steps 5–10**).
13. The solution of S protein must not be removed from the surface when taking an SPR measurement, because the S protein quickly disassociates from the immobilized S-peptide surface under nonequilibrium conditions.

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