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A Microwell—Printing Fabrication Strategy for the On-Chip Templated Biosynthesis of Protein Microarrays for Surface Plasmon Resonance Imaging

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Supporting Information

ABSTRACT: A two-step templated, ribosomal biosynthesis—printing method for the fabrication of protein microarrays for surface plasmon resonance imaging (SPRI) measurements is demonstrated. In the first step, a 16-component microarray of proteins is created in microwells by cell free on chip protein synthesis; each microwell contains both an in vitro transcription and translation (IVTT) solution and 350 femtomoles of a specific DNA template sequence that together are used to create approximately 40 picomoles of a specific hexahistidine-tagged protein. In the second step, the protein microwell array is used to contact print one or more protein microarrays onto nitrotriacetic acid (NTA)-functionalized gold thin-film SPRI chips for real-time SPRI surface bioaffinity adsorption measurements. Even though each microwell array element contains only approximately 40 picomoles of protein, the concentration is sufficiently high for the efficient bioaffinity adsorption and capture of the approximately 100 femtomoles of hexahistidine-tagged protein required to create each SPRI microarray element. As a first example, the protein biosynthesis process is verified with fluorescence imaging measurements of a microwell array containing His-tagged green fluorescent protein (GFP), yellow fluorescent protein (YFP), and mCherry (RFP), and then the fidelity of SPRI chips printed from this protein microwell array is ascertained by measuring the real-time adsorption of various antibodies specific to these three structurally related proteins. This greatly simplified two-step synthesis—printing fabrication methodology eliminates most of the handling, purification, and processing steps normally required in the synthesis of multiple protein probes and enables the rapid fabrication of SPRI protein microarrays from DNA templates for the study of protein—protein bioaffinity interactions.

INTRODUCTION

The use of protein microarrays for the study of protein—protein interactions has become a key research tool for many modern biochemical, bioanalytical, and biomedical research laboratories.1−7 However, for many researchers the preparation of multicomponent protein microarrays via classical protein expression methods is often a significant hurdle, because the current labor-intensive methodologies require many synthesis and purification steps, in addition to the spotting of multiple proteins onto gold thin films. Furthermore, the amount of reagents required in this process is significant, and once fabricated, the protein microarray must be carefully handled to avoid denaturation. In contrast, DNA microarrays are much more easily fabricated. Inexpensive DNA, either single-stranded (ssDNA), double-stranded (dsDNA), or even modified with various chemical attachment handles or fluorescent tags, are readily available commercially, and DNA microarrays can be stored for long periods of time in the absence of water as compared to their protein-based counterparts.

For this reason, a number of research groups have developed various on-chip multiplexed templated biosynthesis methods that employ DNA microarrays to create protein microarrays using cell-free coupled in vitro transcription and translation (IVTT) methods. Some examples include the “DNA array to protein array” (DAPA)8 method that converts a dsDNA array to a protein array in situ through a nitrocellulose membrane; the “nucleic acid programmable protein arrays” (NAPPA)9,10 that directly converts a DNA array to a protein array using the in situ capture of GST-fusion proteins; and the protein in situ array (PISA), a well-based synthesis method in which proteins are captured in situ at the bottom of the well they are synthesized in.11 Each of these methods have been designed to create protein microarrays on glass substrates for analysis by fluorescence or by radioactivity and have been used in a variety of applications.4,5,12,13

While all of these methods are powerful, the applications to date have required the use of either fluorescently or radioactively labeled analytes. In comparison, surface plasmon resonance imaging (SPRI) is a multiplexed surface-sensitive method that detects the adsorption of unmodified biomolecules.
onto a protein microarray via changes in the local index of refraction and has been successfully employed in the real-time analysis of either single or multiple sequential bioaffinity adsorption processes. The use of unlabeled target analytes in SPRI eliminates any extra label conjugation and purification steps and also removes any concerns that labeling could perturb the naturally occurring bioaffinity and bioassembly interactions.

The extension of on-chip multiplexed templated biosynthesis methods that create protein microarrays for SPRI measurements would make it significantly easier for researchers to use SPRI in biosensing, biomedical research, and drug discovery applications. With this goal in mind, our group recently published a method for the fabrication of one- and two-component protein microarrays in a microfluidic format from DNA microarrays in an on-chip IVTT synthesis process for use in SPRI measurements. Because each IVTT-synthesized protein requires a unique microfluidic channel, the total number of proteins on a single chip was limited. Thus, there is a need for other methods that can fabricate microarrays with larger numbers of IVTT-synthesized proteins for SPRI measurements.

In this paper, we demonstrate a method for creating two-dimensional SPRI protein microarrays via an on-chip ribosomal biosynthesis-printing process shown in Figure 1 that is a variation of the DAPA and PISA methods used to create protein microarrays for labeled analysis. We first create a protein microwell array by adding IVTT solutions and dsDNA templates to an array of 16 microwells; each microwell is approximately 0.9 mm in diameter and 0.5 μL in volume. The array is then incubated at 30 °C for 30 min to produce 16 different His-tagged proteins. This resulting protein microwell array is then used to directly print and capture the His-tagged proteins to SPRI chips that consist of NTA-functionalized microarray elements perfectly matched to the spacing and arrangement of the microwell array. As an initial demonstration of this technology, we use multiplexed on-chip templated biosynthesis to create 16 element “XFP” SPRI protein microarray chips that contain four elements each of wild type green fluorescent protein (GFP), yellow fluorescent protein (YFP), mCherry (RFP), and a control which contained only IVTT solution in the microwell. These microarray chips are then used in real-time SPRI adsorption measurements to study the binding strength and specificity of various antibodies specific for the related proteins.

## EXPERIMENTAL CONSIDERATIONS

### Chemicals.

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. 11-Mercaptoundecane (MUAM) was purchased from Dojindo. N-succinimidyl-S-acetylatedpropionate (SATP) was purchased from Pierce. Maleimide polyethylene glycol (350 Da) was purchased from NanoCS. Maleimido-C3-nitrilotriacetic acid was purchased from Dojindo. Rabbit monoclonal anti-wtGFP was purchased from Santa Cruz Biotechnology. Polyclonal anti-GFP and polyclonal anti-mCherry (anti-RFP) were purchased from Abcam.

### Fabrication of Microwell Arrays.

Microwell arrays were laser-cut into 0.2 mm thick polyolefin acrylate Nunc sealing tape (Thermo). The tape was laser cut using a VLS 2.30 laser cutter (Versa Laser) at 4% power. Wells were cut to 0.9 mm diameter with a 1.5 mm center-to-center distance between wells.

### IVTT Solution.

In vitro coupled transcription/translation (S30 T7 high-yield protein expression system) kits were purchased from Promega. The IVTT reactions were adapted from the manufacturer’s recommendation. All components were assembled on ice. S30 premix (5 μL), 4.5 μL of Escherichia coli extract, and 1 μL of glycerol were added to a PCR tube. Then 0.6 μg of DNA in water was added, followed by dilution to 12.5 μL with water. The reaction solution was gently mixed, then 0.5 μL of this solution was added to its respective well in the microwell array. This procedure was used for each protein examined in this study.

### Fluorescent Protein Synthesis.

DNA encoding His-tagged wtGFP was obtained from the T7 Rapid Translation System from SPrime. This vector was then mutagenized to obtain the 10C Q69K YFP variant carrying the following amino acid substitutions: S65G, V68L, Q69K, S72A, and T203Y. The mutations were introduced by PCR site-directed mutagenesis.

pET Biotin His6 mCherry LIC cloning vector (H6-mCherry) was a gift from Scott Gradia (Addgene plasmid #29722).

### Fluorescence Measurements.

Fluorescence spectra were obtained using a Jasco-6300 Fluorimeter. Fluorescence images were obtained using an Olympus IX-71 microscope and an Andor Neo 5.5 sCMOS detector. A 100 W xenon-arc lamp was used as the light source. Fluorescence filter cubes were purchased from ThorLabs. Images were processed and analyzed by ImageJ.

Wild-type GFP, YFP, and TXRED filter cubes were purchased from Thor Laboratories and used without further modification for the imaging of GFP, YFP, and RFP respectively.

### Preparation of NTA-Functionalized Gold SPRI Chips.

Nitrilotriacetic acid (NTA)-modified SPRI chips were created using a procedure adapted from previous work. The 16-element SPRI chips were created by treatment with Sigmacote, using the manufacturer’s protocol, then by thermally evaporating (Denton DV 502-A evaporator) 1 nm of chromium.
as an adhesion layer, then 45 nm of gold onto SF10 glass slides (18 × 18 mm; Schott Glass). These chips were submerged in a 1 M ethanolic solution of 11-mercaptoundecanamide (Dojindo) for 12 h, creating a self-assembled monolayer of terminal amines. The chips were then washed with ethanol and nanopure water and dried under N2. A solution of 30 mM N-hydroxysulfosuccinimide (NHS), 150 mM 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) and 14 mM N-succinimidyl-S-acetyltiolipropionate (SATP) was spotted onto each element. After incubation for 12 h in a humidity chamber, the chip was washed with nanopure water and dried under N2. A deprotection solution of 25 mM ethylenediaminetraacetic acid (EDTA), 500 mM hydroxylamine, and 50 mM dithiothreitol (DTT) in nanopure water was then added to the SPR chip, and the sample was incubated in a humidity chamber for 30 min. This solution removes the terminal S-acetyl group to reveal a free thiol surface. After an additional washing and drying step, a solution of 11.25 mM maleimide PEG 350 Da and 3.75 mM maleimido-C3-NTA in PBS and 10% PBS/DMF, respectively, was added to each element, and the sample was incubated in a humidity chamber for 12 h. This step results in an NTA/PEG functionalized surface. Before protein capture, each element is exposed to a 40 mM CuSO4 aqueous solution for 15 min, which is required for His6-tag affinity purification.

**On-Chip Synthesis and Protein Microarray Printing.** In vitro coupled transcription/translation (S30 T7 high-yield protein expression system) kits were purchased from Promega. The IVTT reactions were adapted from the manufacturer’s recommendation. All components were assembled on ice. S30 premix (5 µL), 4.5 µL of E. coli extract, and 1 µL of glycerol were added to a PCR tube. Then 0.6 µg of DNA in water was added, followed by dilution to 12.5 µL with water. The reaction solution was gently mixed, then 0.5 µL was added to its respective well in the microwell array. The microwell array was sealed in a PDMS chamber and incubated at 30 °C for 30 min. The PDMS chamber was removed, and the array was placed in a humidity chamber. The array and chamber were incubated at 4 °C for at least 2 h to allow for protein folding.

A 0.25 mm thick, square spacer (1.0 × 1.0 cm²) (ThermoFisher) is adhered to the protein microwell array. The array is then inverted and contact printed onto an NTA-PEG functionalized surface. 1 mM ethanolic solution of 11-mercaptoundecamine (Dojindo) (18 µL) was spotted onto the chip. Each microwell was spotted with 0.5 µL of a solution that contained both the IVTT mix and approximately 350 femtomoles of a dsDNA template (Figure 1b). These dsDNA templates encode a specific protein modified with a N-terminal His6-tag that will be used for surface affinity capture onto the SPRI chip. The microwell array was then sealed in a poly(dimethylsiloxane) (PDMS) chamber to prevent evaporation, and protein synthesis was performed by incubating the sealed microwell array at 30 °C for 30 min followed by incubation at 4 °C for 12 h to allow for protein folding (Figure 1c). The PDMS chamber was then removed and the resultant protein microwell array was either checked directly with fluorescence microscopy or employed in the printing of SPRI chips (Figure 1d).

**Demonstration Part 1: Fabrication of XFP Protein Microwell Microarrays.** To track the effectiveness of this method, an “XFP” protein microwell array of three related and well-studied fluorescence proteins were chosen: GFP, YFP, and RFP. Choosing three proteins each with a unique fluorescence spectrum allowed us to easily confirm successful on-chip IVTT protein synthesis and the correct folding of the IVTT-synthesized proteins. The fluorescence spectra of these three IVTT-synthesized proteins are shown in Figure 2. The multiplexed on-chip IVTT method described above was used to synthesize a 16-element, four-component “XFP” microwell array containing GFP, YFP, and RFP, along with non-fluorescent control wells. A series of fluorescence images of an XFP microwell array utilizing different excitation and emission filters is shown in Figure 3; the quantitative analysis (bar graphs) of these images are shown in the figure as well and verify the efficient multiplexed biosynthesis of these fluorescent proteins. The solutions were removed from the wells, and by comparing with fluorescence from IVTT standards (see Experimental Considerations) we estimate that approximately 40 ± 10 picomoles of protein was synthesized in each microwell from 350 femtomoles of DNA templates; the variation in this number is attributed to differences in the efficiency of synthesizing the various proteins from dsDNA templates. Forty picomoles in 0.5 µL corresponds to an 80 µM protein solution in each well that was used for the printing of SPRI chip microarrays.

**Strategy for Fabricating SPRI Protein Chips from Protein Microwell Arrays.** As shown in Figure 1, from the protein microwell arrays, gold thin-film SPRI chips that had a matching pattern of 16 gold thin-film spots (1 mm diameter, 45 nm gold thickness) on SF10 glass substrates were created in a

Antibodies were diluted to 1 nM in PBS and used without further modification.

**RESULTS AND DISCUSSION**

**Strategy for Fabricating Protein Microwell Arrays for SPRI.** Our overall strategy for obtaining SPRI bioaffinity adsorption measurements with protein microarrays created by on-chip templated biosynthesis and contact printing is outlined in Figure 1. The first step is to create a protein microwell array using on-chip IVTT. This preparation methodology is similar to previously reported methods for the fabrication of protein microarrays for fluorescence microscopy, DAPA, NAPPA, and PISA. The 16-element microwell array was created by laser-cutting 16 0.9 mm diameter holes in a 4 × 4 grid into polyolefin sealing tape that was subsequently attached to a clean microscope slide (Figure 1a). These chips were then used for simultaneous on-chip synthesis of multiple proteins using commercially available IVTT solutions. Each microwell was spotted with 0.5 µL of a solution that contained both the IVTT mix and approximately 350 femtomoles of a dsDNA template (Figure 1b). These dsDNA templates encode a specific protein modified with a N-terminal His6-tag that will be used for surface affinity capture onto the SPRI chip. The microwell array was then sealed in a poly(dimethylsiloxane) (PDMS) chamber to prevent evaporation, and protein synthesis was performed by incubating the sealed microwell array at 30 °C for 30 min followed by incubation at 4 °C for 12 h to allow for protein folding (Figure 1c). The PDMS chamber was then removed and the resultant protein microwell array was either checked directly with fluorescence microscopy or employed in the printing of SPRI chips (Figure 1d).

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**Strategy for Fabricating SPRI Protein Chips from Protein Microwell Arrays.** As shown in Figure 1, from the protein microwell arrays, gold thin-film SPRI chips that had a matching pattern of 16 gold thin-film spots (1 mm diameter, 45 nm gold thickness) on SF10 glass substrates were created in a
contact printing process. The His-tagged IVTT synthesized proteins are simultaneously captured onto 16 Cu$^{2+}$-NTA-functionalized gold microarray elements. Maleimide-functionalized NTA was attached to the gold thin-film microarray elements using a SATP strategy shown in Figure 4 that has been described and characterized previously22 (Figure 4). The SPRI chips also include a thin hydrophobic Sigmacote film on the glass areas surrounding the gold microarray elements to prevent crosstalk during the fabrication process.19 The NTA-functionalized chips were exposed to an aqueous 40 mM CuII solution for 15 min and then rinsed with nanopure water. A 0.25 mm thick, square spacer (1.0 × 1.0 cm$^2$) (ThermoFisher) was placed around the microwell array to prevent crosstalk between wells by reducing droplet compression. The microwell array was then placed, wells facing down, on top of a 16-element, NTA-functionalized SPRI chip such that each well spotted a single solution onto a single SPRI element. The array-chip sandwich was incubated in a humidity chamber at 25 °C for 15 min to allow for NTA-capture of the proteins onto the SPRI chip. The chip was subsequently washed with various solutions outlined in Experimental Considerations and then used in SPRI experiments. Multiple (up to 3) SPRI chips could be fabricated from a single protein microwell array.

**Figure 2.** Fluorescence spectra of GFP, YFP, and RFP. Normalized fluorescence emission spectra of GFP, YFP, and RFP. Emission was measured at 395 ± 10 nm, 516 ± 10 nm, and 570 ± 10 nm, respectively. The proteins were synthesized using a cell free coupled transcription/translation system then diluted into 60 μL of PBS. These solutions were then measured for fluorescence by a fluorometer.

**Figure 3.** Fluorescence images of the XFP protein microwell array. Fluorescence images of (a) RFP, (b) GFP, and (c) YFP were obtained after on-chip synthesis and incubation for 12 h at 4 °C. Images were obtained via fluorescence microscopy using the appropriate fluorescence filter cubes for each protein with a 2X objective. Quantitative data was obtained by measuring average fluorescence intensity for each protein under each fluorescence filter. (Filters used are outlined in Experimental Considerations.) Melting during the laser cutting process caused the shape distortion observed in the microwell arrays. This distortion did not affect IVTT or the contact printing process.

**Figure 4.** Surface attachment chemistry for printing SPRI protein microarrays from the protein microwell array. Surface attachment chemistry was adapted from previous methods.22 (a) A self-assembled monolayer of 11-mercaptoundecamine was formed on a gold thin-film SPRI element. (b) The resulting amine surface was modified with SATP, a heterobifunctional linker, using EDC/NHSS chemistry. (c) The surface terminal S-acetyl group is removed using a hydroxylamine solution, revealing a free thiol. (d) The revealed thiol surface is treated with a solution of maleimido-nitrilotriacetic acid and maleimido-polyethylene glycol (350). (e) This surface is then exposed to a 4 mM solution of Cu$^{2+}$, which primes the surface for His-tag capture.
In the process of optimizing the conditions for this parallel on-chip IVTT microwell biosynthesis and subsequent SPRI chip printing, several hurdles needed to be overcome to create a successful and repeatable fabrication process: the reduction of evaporation losses, mass transfer (“coffee-ring”) effects, cross-talk between microarray elements, and inefficient His-tag protein adsorption—desorption kinetics onto the SPRI elements. Losses from evaporation were prevented by both the use of a humidity chamber and the addition of glycerol (~10% of the total solution) to the protein microwell solutions before the incubation step. The use of glycerol to minimize evaporation issues has been employed previously in various drop-based microarray synthesis methods.\textsuperscript{25−27} The addition of glycerol coupled with a short reaction time (30 min) also alleviated the formation of nonuniform adsorption of protein molecules into a ring at the edges of the wells, which also has been observed previously and explained as a “coffee-ring” effect by other researchers.\textsuperscript{28,29} To prevent cross-talk due to bleeding of adjacent protein microwells into each other by droplet compression, a 0.25 mm thick spacer was placed between the microwell array and the surface of the SPRI chip. Additionally, a 1.5 mm center-to-center distance between the 0.9 mm diameter microarray elements was used; this spacing/diameter ratio yielded the maximum microarray element density while still completely preventing cross-talk. Finally, because the His-tag capture of the proteins onto the SPRI microarray elements is itself a bioaffinity adsorption process, losses due to slow Langmuir adsorption kinetics were avoided by using a high (80 \(\mu\)M) protein solution. Moreover, to avoid the reversible desorption of His-tagged proteins that has been observed previously from Ni-NTA modified surfaces,\textsuperscript{30} we substituted Ni\textsuperscript{2+} with Cu\textsuperscript{2+}, which has been shown to form much less labile affinity capture complexes with His-tagged proteins.\textsuperscript{31}

**Demonstration Part 2: SPRI Measurements of Antibody Binding to the XFP Chips.** To demonstrate the utility of these contact printed, multiplexed protein SPRI microarrays, we examined the adsorption of antibodies onto various XFP SPRI chips with real-time SPRI measurements. We first examined the efficiency of our contact printing method with a 16-element, two-component protein microarray that contained 8 elements of IVTT-synthesized GFP and 8 control elements of IVTT solutions alone. The SPRI real-time reflectivity change (\(\Delta R\)) was measured over the course of 900 s upon exposure of the microarray to a one nanomolar solution of monoclonal anti-GFP; Figure 5 shows the two average \(\Delta R\) curves for the eight GFP and eight control elements. An increase in \(\Delta R\) of up to 1.6% after 600 s was observed for the GFP elements, and a small increase (~0.1 increase in \(\Delta R\)) was observed from the control elements. These results match well with our previous SPRI on-chip biosynthesis using channels.\textsuperscript{20} A final \(\Delta R\) value of 1.6% from a 1 nM anti-GFP solution compares favorably with previous SPRI measurements of microarrays with adsorbed probe protein surface densities of approximately \(10^{12}\) molecules cm\(^{-2}\).\textsuperscript{32,33} Using this number as an upper limit, we estimate that approximately 100 femtomoles of GFP adsorbed onto each gold SPRI chip element. The SPRI difference image shown as an inset in Figure 5 also verifies by SPRI measurements of the specific adsorption of anti-GFP to the printed GFP elements that there is no cross-talk between the elements due to the printing process. In a second set of real-time SPRI measurements we monitored the adsorption of various antibodies onto XFP chips created from the protein microwell arrays in the section **Demonstration Part 1: Fabrication of XFP Protein Microwell Microarrays.** Sixteen-element SPRI microarray chips which contained four elements each of GFP, YFP, RFP, and a no-protein control were contact-printed. These XFP chips were then used in real-time SPRI reflectivity measurements to examine the specific adsorption of three antibodies: monoclonal anti-GFP, polyclonal anti-GFP, and polyclonal anti-RFP. The results of these antibody binding measurements are plotted in panels a, b, and c of Figure 6, respectively. One nanomolar antibody solutions in PBS were used in each of these experiments. As seen in Figure 6a, monoclonal anti-GFP preferentially adsorbed to the GFP elements (after 1100 s, \(\Delta R\) values of 0.8 ± 0.2%, 0.20 ± 0.08%, and 0.03 ± 0.08% for GFP, YFP, and RFP, respectively). The 4X preference for this antibody to GFP elements over YFP elements is significant given the similarity between the two proteins: GFP and YFP differ by only six residues, and many of these amino acids are buried within the beta-barrel structure.\textsuperscript{35,33,34} In contrast, the real-time SPRI measurements plotted in Figure 6b of polyclonal anti-GFP binding onto XFP chips show similar adsorption to both GFP and YFP elements (after 1100 s, \(\Delta R\) values of 0.3 ± 0.1%, 0.3 ± 0.1%, and 0.00 ± 0.09%, for GFP, YFP, and RFP, respectively). Finally, the real-time SPRI measurements in Figure 6c demonstrate that the exposure of the XFP chip to 1 nM polyclonal anti-RFP leads only to specific adsorption to RFP elements (after 1100 s, \(\Delta R\) values of 0.06 ± 0.03%, 0.01 ± 0.07%, and 0.6 ± 0.2% for GFP, YFP, and RFP, respectively). This specificity is attributed to RFP’s unique primary structure as compared to GFP and YFP, even though the three share a similar beta-barrel structure.\textsuperscript{24,34}

We attribute the 4X preference of monoclonal anti-GFP to GFP over YFP to a change in the tertiary structure of YFP upon introduction of the six amino acid substitutions described in Experimental Considerations. Upon examination of the crystal structure of wtGFP, we find the peptide backbone of position 203 is solvent exposed. We suspect that upon the T203Y change, a change in the tertiary structure of YFP must cause the observed monoclonal GFP specificity. This selectivity was not observed with polyclonal anti-GFP serum because it is a heterogeneous mixture of antibodies that interact with a variety of epitopes; some of these interactions are insensitive to the structural differences between YFP and GFP.
This set of SPRI measurements of antibody adsorption on the XFP chips clearly demonstrates that the SPRI protein microarrays created via the biosynthesis−printing methodology described in this paper can be used in multiplexed protein−protein SPRI bioaffinity measurements.

**CONCLUSIONS**

The two-step methodology described in this paper for fabricating protein microarrays for SPRI measurements via a combination of multiplexed templated IVTT biosynthesis of His-tagged proteins in a microwell array followed by contact printing−adsorption onto an NTA-modified gold thin-film SPRI chip is a simple and attractive method for any researcher interested in quickly producing a variety of different protein microarrays for SPRI measurements with a minimum of reagents, processing, and purification steps. The surface density of the proteins for SPRI protein microarrays needs to be higher than for the DAPA or PISA protein microarrays used previously for fluorescence imaging experiments; the high concentration (80 μM) of the synthesized proteins in each microwell insures a significant surface coverage of captured and active his-tagged proteins. The high concentration of the microwell array also permits the fabrication of multiple SPRI chips as each SPRI microarray element requires only approximately 100 femtomoles of adsorbed protein.

As compared to our previous microfluidic method for the fabrication of SPRI microarrays with IVTT templated biosynthesis, this contact printing method is easier to implement for larger microarrays (16 or more) of proteins. However, both methods suffer from the fact that we use only one type of protein adsorption chemistry, namely, the adsorption of His-tagged proteins onto a Cu²⁺ NTA-modified gold surface. The ultimate goal of creating self-assembled SPRI microarrays in a single on-chip templated IVTT biosynthesis will require the future development of simultaneous multiple adsorption chemistries, perhaps through the addition of a ssDNA capture tag to each type of synthesized protein.

**ASSOCIATED CONTENT**

* Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcc.6b03307.

Line profiles of microwell fluorescence images (PDF)

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**Notes**
The authors declare no competing financial interest.

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**REFERENCES**
