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In situ protein microarrays capable of real-time kinetics analysis based on surface plasmon resonance imaging

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ABSTRACT

In recent years, *in situ* protein synthesis microarray technologies have enabled protein microarrays to be created on demand just before they are needed. In this paper, we utilized the TUS-TER immobilization technology to allow label-free detection with real-time kinetics of protein–protein interactions using surface plasmon resonance imaging (SPRi). We constructed an expression-ready plasmid DNA with a C-terminal TUS fusion tag to directionally immobilize the *in situ* synthesized recombinant proteins onto the surface of the biosensor. The expression plasmid was immobilized on the polyethylene imine-modified gold surface, which was then coupled with a cell-free expression system on the flow cell of the SPRi instrument. The expressed TUS fusion proteins bind on the surface via the immobilized TER DNA sequence with high affinity ($\sim 3–7 \times 10^{-13}$ M). The expression and immobilization of the recombinant in situ expressed proteins were confirmed by probing with specific antibodies. The present study shows a new low cost method for *in situ* protein expression microarrays that has the potential to study the kinetics of protein–protein interactions. These protein microarrays can be created on demand without the problems of stability associated with protein arrays used in the drug discovery and biomarker discovery fields.

Continuous development in protein microarrays has provided new tools and methodologies that are playing an increasing role in functional proteomics. These methods became available by the rapid development of genomics databases, bioinformatics, and cDNA technologies that enable the simultaneous analysis of thousands of protein with better sensitivity than other conventional assays (ELISA and 2D gel) [1]. Unfortunately, the widespread use of protein microarrays has remained limited, largely due to the inherent cost and technical limitations including protein synthesis, immobilization, stability, and storage [2]. Despite these challenges, protein arrays are extensively used for various applications such as antibody profiling, biomarker identification, protein–protein interactions, and enzymatic assays [3–8].

In order to overcome some of the technical challenges, elegant methods for in vitro transcription and translation (IVTT)[2] protein arrays have enabled high-throughput and instant analysis of proteins which can be used to fabricate protein microarrays directly on the sensor chips from their expression plasmids. Several technologies for *in situ* protein microarrays such as PISA (protein *in situ* array) [9], NAPPA (nucleic acid programmable protein microarray) [10], DAPA (DNA array to protein array) [11], and TUS-TER protein array [12] have been developed. All these technologies include protein generation by immobilized cDNA or PCR fragments containing a C or N terminal fusion tag with a capturing agent on a solid support coupled with commercially available cell-free expression systems [13]. All these cell-free expression-based microarray technologies have been published using fluorescence detection, which can be limiting. Fluorescent labeling can affect the activity or function of certain proteins and fails to give kinetic parameters and real-time information. Therefore, the development of an alternative label-free detection technology can provide significant advantages. Surface plasmon resonance imaging (SPRi) is a label-free technique used for real-time identification of biomolecular interactions along with their kinetic parameters [14].

This paper shows for the first time the synthesis of *in situ* protein arrays in conjugation with SPRi, which allows real-time monitoring of the transcription and translation processes along with high-throughput measurement of kinetic information of

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[2] *Abbreviations used:* BS3, bis[sulfosuccinimidyl] suberate; BSA, bovine serum albumin; IVTT, in vitro transcription and translation; MUA, 11-mertcaptoundenaconic; PEI, polyethylene imine; SPRi, surface plasmon resonance imaging.

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interactions. As a proof of concept, the reaction mixture for cell-free protein synthesis was loaded onto a gold surface that self-assembled with polyethylene imine (PEI). A schematic representation of the whole process is shown in Fig. 1. In brief, the TUS fusion protein (C terminus) expression plasmids were arrayed and the IVTT mix was added to synthesize the fusion proteins. The *in situ* expressed recombinant proteins were instantly bound tightly via the TUS tag to the surface containing TER (20-bp DNA sequence involved in the regulation of DNA replication). In order to explore this high-throughput technique, five different plasmid constructs, GFP, P53, JUN, CDK4, and Base along with negative controls were arrayed in multiplex on the biosensor chip. A linear increase of the resonance signal was observed as the protein synthesis reaction proceeded. Moreover this platform facilitates the measurement of *in situ* synthesis of the protein as well as kinetics information of interactions.

Materials and methods

Reagents

The TER DNA oligo (5′ amino-C6 linker-CCGGCCACTTTAGTT-ACAACATACTTATT 3′) was purchased from SBS (Beijing, China). The pDEST-TUS-His plasmid DNA (Supplementary Fig. 1) was kindly donated by Deb K. Chatterjee from the Protein Expression Laboratory, National Cancer Institute (USA). Methionine and rabbit cell lysate were purchased from Promega. RNAase-free H_2O was purchased from Takara (Japan). Monoclonal anti-His was purchased from Origene. The monoclonal anti-p53 and GFP antibodies were purchased from RayBiotech, Inc. PBS was purchased from Solarbio (Beijing, China). BSA (bovine serum albumin), MUA (11-mertcaptoundenaconic), and PEI were purchased from sigma Aldrich. BS3 (bis[sulfosuccinimidyl] suberate) was purchased from Thermo Scientific. An E.Z.N.A. HP Plasmid Maxi Kit for plasmid extraction was purchased from Omega Bio-Tek.

DNA preparation

Bacteria harboring the expression vector plasmids (pDEST-TUS-His vector sequence, Supplementary Fig. 2) were cultured in 250 ml of Luria-Bertani medium containing 100 μg/ml ampicillin in a 500-ml flask for 16 h, and then pelleted by centrifugation at 3500*g* for 15 min. DNA was prepared according to the E.Z.N.A. HP Plasmid Maxi Kit (Omega Bio-Tek) and concentration was measured by Nanodrop.

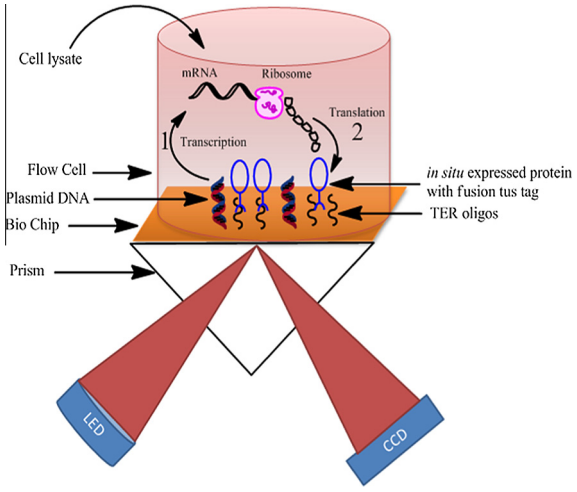

Fig.1. Schematic representation of *in situ* protein array fabrication on SPRi. Full-length cDNA including TUS tag encoded at the C-termini and TER DNA sequence are coprinted on the sensor chip. At the time of the experiment the corresponding proteins are expressed using rabbit cell lysate, a reticulocyte-based cell-free expression system. The *in situ* synthesis proteins are captured by surface-bound TER DNA sequence.

Microarray fabrication

SPRi gold chips fabricated by evaporating gold film on optical glass, BK5 substrate (75 × 25 × 1 mm with a thin layer of gold 47.5 nm) were obtained from Plexera LLC. Sensor chips were functionalized with PEI as described elsewhere [15]. In brief, the gold surface was thoroughly rinsed with ethanol and then washed with ultrapure H_2O. A self-assembled MUA (Sigma) monolayer was chemisorbed onto the gold surface by immersing the chip in a 1 mM solution of MUA overnight at 4 °C. Then, the MUA-coated chip was immersed in a PEI (Sigma) solution (2.5 mg/ml in 1X PBST) for 60 min, thoroughly rinsed with ultrapure water, and then dried by using N_2 gas. The PEI layer is electrostatically bound to the MUA-coated surface by the interaction between the amino groups of the PEI and the carboxylic end of the MUA. PEI fabricated SPR sensor chips were then stored in a dry container at 4 °C until ready for printing.

Sample preparation and array printing

Ready to use expression plasmid DNA was precipitated as shown by a previously described method [16], by addition of 0.6 vol isopropanol, followed by centrifugation at 5000 rpm for 30 min. The DNA pellet was washed with 200 μl of 80% ethanol, centrifuged at 5000 rpm for 15 min, and dried. The master mix solution was prepared for printing, containing 1 mg/ml of plasmid DNA, 1 μmol of capture amino-modified TER DNA sequence, and 1 mM BS3. BS3 linker was used as a coupling structure for amino-amino coupling of TER DNA sequence and polyethylene imine surface. The master mix was transferred to a 384-well microtiter plate. The master mix plate and PEI-modified sensor chips were loaded to a robotic microarray spotter (Smart Arrayer48; Capital Bio) and each plasmid was spotted in multiplex (7 × 6) on a chip with contact mode of printing which produces 500 μm features to generate the microarray. The printing volume and distance between two spots for each plasmid were set to 5 nl and 0.5 mm, respectively. After printing, the sensor chips were stored overnight at 80% humidity in chamber at room temperature.

In situ expression of proteins on SPRi

The printed sensor chips were washed with 1X PBST at pH. 7.4, 0.05% Tween 20 for 15 min with gentle agitation and then a quick washing step with deionized water for 2 min and placed in 2% BSA for 1 h to block the array surface. After blocking, the arrays were washed with deionized water for 5 min and dried with N_2.

The IVTT (Promega) was performed in the flow cell (Plexera, LLC). Briefly, the flow cell was applied to the sensor chips. The IVTT lysate was prepared with 40 μl of master mix supplemented with methionine. This mix was added directly to the flow cell after the sensor chip was mounted on the SPRi instrument (KX5 Plexera, LLC) [17]. Once the sensor chip was installed, the minimal angle and regions of interest (ROIs) were set up. The ROIs were set for 9 × 8 pixels of ROIs which are equal to 360 × 320 μm in size which covered approx 70% area of the array element (500 μm) in our experiments to ensure uniform expression of each protein. The protein synthesis reactions were performed for 90 min at 30 °C and 120 min at 15 °C to capture the fusion proteins by the immobilized TER DNA oligo. The arrays were then washed by injecting

1X PBST at the rate of 3 μl/s for 30 min and blocked with BSA (5% in PBST, 25 °C for 60 min).

SPRi method

The expressed proteins were captured on the array due to the interaction of TUS tag with the TER DNA sequence. The detection of *in situ* expressed proteins was performed by anti-His antibody (mouse anti-His; Origene). The expression of specific protein GFP and p53 was confirmed by specific anti-GFP monoclonal antibody (goat anti-GFP; Ray Biotech Inc.) and anti-p53 monoclonal antibody (mouse anti-p53; Ray Biotech Inc.). CDK4-p16 was used to test the *in situ* protein microarray system with SPRi for protein–protein interaction study. The P16 protein was purified as previously described [18]. The purified p16 protein was diluted in running buffer at a final concentration of 10 μg/ml. Flow rate for all SPRi experiments was set to 3 μl/s and association and dissociation time was set to 300 s for each injection. For data analysis we choose two software packages, ORIGIN Lab and Data Analysis Module (DAM) of Plexera. All data from SPRi reported here are after subtraction of the background intensity/signal by DAM software. In short, the entire concentration of analyte was fitted with a 1:1 Langmuir interaction integrated rate equation to obtain the equilibrium association constant (K_A) and the equilibrium dissociation constant (K_D) [17].

Results

In situ protein array synthesis on SPRi biosensor chips

We adopted an existing TUS-TER capturing chemistry to monitor the *in situ* protein expression as well as kinetics study of biomolecular interactions. TUS protein has been shown to bind with the TER DNA sequence as a monomer with very high affinity of ~3–7 × 10^{-13} M [12]. The design of the expression construct (pDEST-TUS-His plasmid) and basic concept of the array platform with SPRi are shown in Fig. 1. The expression vector is based on the Gateway (Invitrogen, Carlsbad, CA) cloning system that makes it easier to generate libraries of constructs. The TUS protein sequence was inserted as a C-terminal fusion tag along with 6X histidine (6X His). The addition of a C-terminal TUS protein tag to each protein enabled its capture to the array through the TER DNA sequence. In order to validate the TUS-TER, DNA-binding protein system for the development of an *in situ* self-assembling protein array on SPRi, a master mix was printed on the array surface as described under Materials and methods. The protein expression and capture were verified in real time and an anti-His antibody was used to confirm the expression and immobilization of the recombinant proteins.

Real-time monitoring of protein expression during cell-free protein synthesis on a SPRi chip

Here we show that SPRi has been successfully used for *in situ* protein microarray and real-time biomolecular interaction analysis. The first step in this method is the immobilization of the plasmid DNA on the SPRi sensor chip. Through testing of a variety of plasmid DNA printing schemes, we found that an optimum balance between DNA binding potency and conformation that support an efficient transcription and translation process is required. A well-known DNA immobilization strategy based on electrostatic interaction between a negative backbone of plasmid DNA and a positive charge of PEI was used [15].

To test the system, expression plasmids encoding four genes (GFP, P53, JUN, and CDK4) were printed in multiplex onto a sensor chip along with a base plasmid (without protein encoded gene), empty plasmid (nonexpression construct), and purified protein (GFP-6X His) were used as a negative and positive control, respectively. The expression of target proteins was confirmed with an anti-6X His antibody as all plasmid construct encoding with C-terminal TUS-6X His. As shown in Fig. 2, significant increases in spot intensity of all four genes and base plasmids were recorded but not in the case of the empty plasmid. The changes in spot intensities before (Fig. 2A) and after expression (Fig. 2B) are clearly indicating the progress of cell-free protein expression onto the sensor chip. It is important to note that array before expression (Fig. 2A) is more uniform compared to the one after expression (Fig. 2B) due to its exposure to cell lysate for long time during the transcription and translation process which results in an inconsistency of spots. The process of protein expression, protein capture, washing, and detection on SPRi is shown for one of the spots in Fig. 3A. In parallel, a slight increase in background intensity of the surface after expression was observed due to cell lysate adsorption. The final data are presented after background subtraction. The relative amount of protein produced and retained by each construct varied modestly, presumably due to characteristic differential transcription/translation efficiencies. Real-time SPRi responses were plotted in Δ% normalized in AU of each expressed protein. The values of Δ% 5.17 ± 0.7% for GFP, 5.11 ± 0.7 for p53, 2.1 ± 0.41 for JUN, and 3.8 ± 0.5 for CDK4, 5.6 ± 0.78 for base, and 0.53 ± 0.15 for empty plasmid (Fig. 3B) were obtained. All experiments were repeated three times to ensure reproducibility of the method.

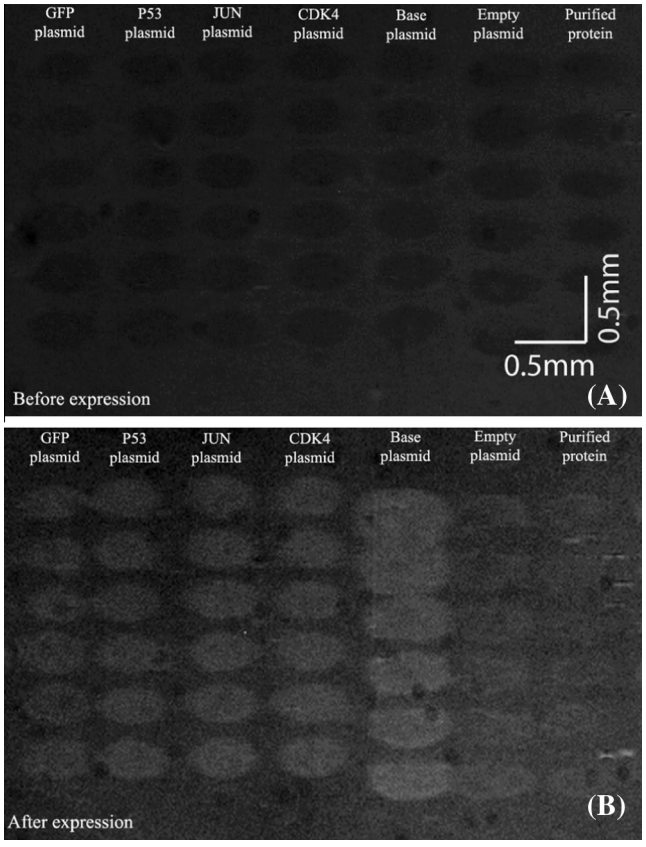

Fig.2. Images from an SPRi instrument at 20% normalized reflection of the SPR curve before and after protein expression. (A) Image showing array of 6 plasmids and one purified protein in multiplex (7 × 6) before protein expression. (B) The increase in spot intensity after 180 min of protein expression and capture on individual spots. The image also shows washing with 1X PBST to remove nonspecific adsorption of cell lysate proteins.

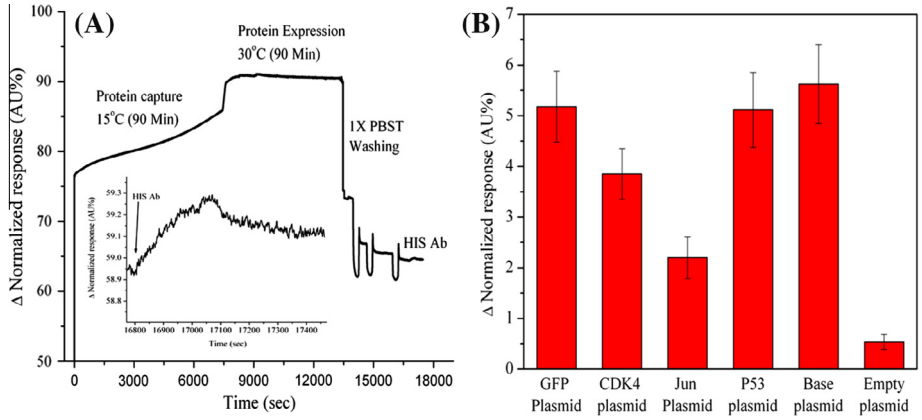

Fig.3. Real-time label-free monitoring of one spot protein expression on SPRi. (A) Sensogram showing one spot synthesis process of protein array includes protein expression, protein capture, washing, and detection by anti-His Ab by SPRi. (B) Diagram representing the increase in response after protein expression at individual spots. The spot corresponding to the empty plasmid does not change.

Confirmation of protein expression by SPRi

The expression and immobilization of the target proteins were confirmed by injecting an anti-6X His antibody (5 μg/ml) into a flow cell as analyte. The anti-6X His antibody was found to bind to all the constructs encoding protein with the 6X His moiety and purified protein, but none of the negative control (empty plasmid) as shown in Fig. 4. Binding events from different experiments were reproducible. Undoubtedly, the increase in signal response generated from the binding of the expressed proteins with antibody to 6X His is enough to prove the instant capturing of expressed proteins onto the sensor chips.

Furthermore, we investigated the expression of specific proteins through GFP and p53 antibody. To demonstrate specific protein expression efficiency, specific monoclonal antibodies against GFP and p53 proteins were injected into the flow cell. The binding of these antibodies to GFP and p53 at the spots where the specific plasmid construct were seeded confirmed the presence of the specific proteins. The specific binding responses of anti-GFP and anti-p53 are shown in Fig. 5A and B, respectively. A response of Δ%AU of 0.51 ± 0.19 and 0.7 ± 0.26 was observed for the GFP and p53 detector elements after 300 s for the GFP and p53 proteins. The calculated amount of proteins produced from Δ*R*% on each feature is around ~16 pg which is already higher than detection

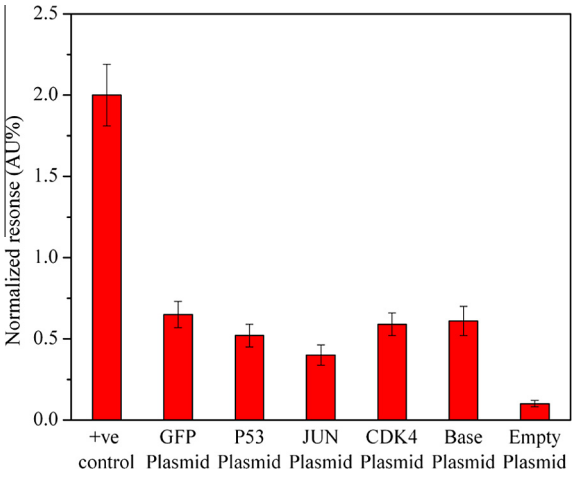

Fig.4. Real-time label-free detection of protein. The indicated proteins with C-terminal TUS-6X His tags were expressed. The proteins were probed with anti-His and individual increases in responses were recorded on the basis of their location on the array. Response corresponding to empty vector did not exhibit any signal.

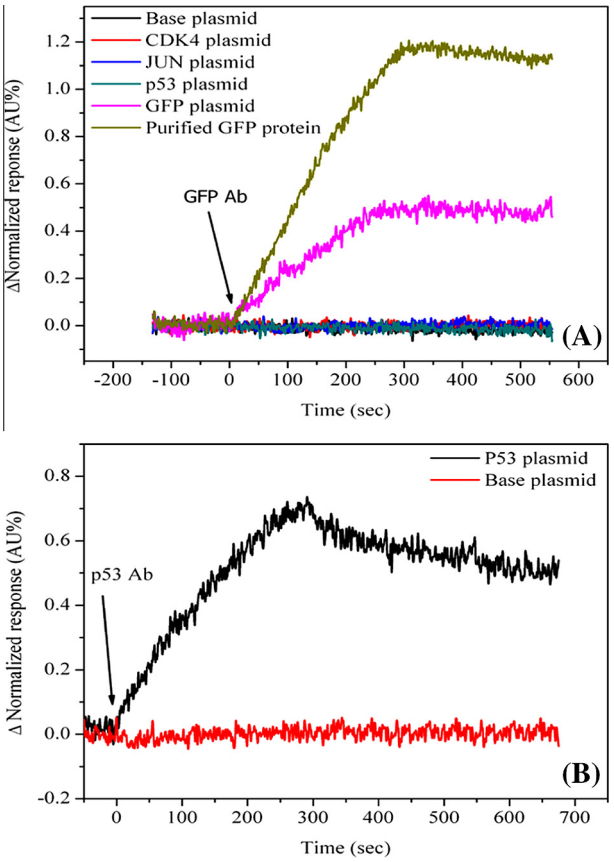

Fig.5. Specific protein detection by relative antibodies. (A) Monoclonal anti-GFP was injected as indicated and revealed binding only to its relevant target including detection of purified GFP protein (B) showing detection of expressed p53 protein by anti-p53. No response of both antibodies was detected with unrelated proteins or spots.

limits of ~0.48 pg [19]. These experiments clearly verify that the multiplexed, *in situ* synthesis method can be used for rapid creation of protein arrays for SPRi bioaffinity binding experiments directly from arrayed cDNA constructs.

Kinetic analysis

In order to prove that our system is capable of real-time kinetics measurements, we analyzed antibody-based protein interactions

between GFP/anti-GFP and p53/anti-p53 antibody as well as tested non-antibody-based protein interactions by examining the binding of the cyclin-dependent kinase CDK4 (target on the array) to its inhibitor p16 (query) (Supplementary Table 1). We compared the antibody-based kinetic measurements of *in situ* expressed GFP protein with those of the purified GFP protein. We estimated that the K_D values for *in situ* expressed and purified GFP protein for the GFP antibodies were comparable at 7.3 ± 1.1 and 8.34 ± 1.2 nM, respectively. The K_D for the p53 protein and its antibody was 13.7 ± 1.2 nM. To further evaluate this technology, protein–protein interaction of CDK4-p16 was evaluated (Supplementary Fig. 3). Although there is no reported K_D value of this interaction, the IC50 of CDK4-p16 was previously published at a value of 72 ± 15 nM [20]. The K_D from our SPRi experiments gave us an equivalent value of 86.4 ± 1.5 nM. This confirmed the feasibility of this methodology for analyzing kinetic measurements for protein–protein interactions.

Discussion

In situ protein arrays lend themselves to high-throughput formats that are desirable for the study of proteins which are hard to express and purify in living organism. A number of research papers have been published in this area [21,22]. However many issues concerning these methodologies are yet to be solved. To date, no integrated system for *in situ* protein arrays has been reported that allows high-throughput analysis and kinetic evaluation of biomolecular interactions on a single platform. To address all these issues, we have conjugated surface plasmon resonance imaging with *in situ* protein arrays that provides kinetic measurement of interactions in a high-throughput manner. A number of limitations are associated with previously published techniques such as the leaky His tag for protein capture on a Ni-NTA surface [14]. In the NTA-based capturing experiments, the captured proteins could dissociate from the chip surface during the injection/monitoring period of the measurement cycle, because the histidine-tagged proteins are not tightly immobilized on the NTA surface. The dissociating behavior of a protein generally depends not only on the type of tag sequence but also on the protein itself [23]. This is also enhanced by the nonspecific binding that leads to a high background while applying complex mixes such as total cell extracts or serum. Secondly, a specifically designed microfluidic requirement and two spot methods for *in situ* protein array generation may lead to deficiencies in the methodology [22]. We solved some of these limitations by using the TUS tag which possesses a high affinity of $\sim$3–7 $\times$ 10^{-13} M toward the TER DNA, which is very stable for storing for long periods of time. High-throughput and real-time monitoring of the whole expression and immobilization process on each spot is a strong advantage of our methodology and allows us to analyze hundreds of spots in parallel on single sensor chip. A time-dependent increase of the SPRi signal was observed in the processes encoded from different plasmids. The kinetics values obtained from our SPRi experiments were comparable to those of normal antibody–protein affinities. The specific binding of the antibodies onto the expected protein microarray spots was a clear confirmation of the accurate expression and immobilization with negligible nonspecific adsorption onto the control elements of the microarray. Finally, we demonstrated that not only can we express well all the designated proteins, but that each captures its encoded protein without detectable diffusion or "bleeding" to adjacent features on the microarray.

In conclusion, our data support the utility and potential of this high-throughput *in situ* protein microarray platform for the study of protein–protein, protein–DNA/RNA, and protein–drug interactions, and therapeutic antibody profiling. Despite all these advantages, we can still envision further improvements that facilitate the functionality and creation of protein microarrays by using three-dimensional surface chemistries [24], integration with a microfluidic system [25], and addition of synthetic chaperones [26]. The use of three-dimensional surfaces will provide not only higher density of protein but also lower the nonspecific adsorption of nonrelevant protein and improve the sensitivity. Recently, we reported a trilayered silver surface with enhanced sensitivity and comparable stability with a standard gold chip for SPRi application [17]. But due to the low dynamic range of silver, this platform is not suitable for the presented study. We believe that these advancements in *in situ* protein microarrays will expand their applications for testing multiple protein variants generated by single nucleotide polymorphisms. Another possibility is the application of these microarrays to personalized medicine [27] or antibody profiling, which is another major area where this methodology can make a positive contribution. We believe that the combination of SPRi with *in situ* protein microarrays can help in the development of several high impact applications in the proteomics field in the future.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2014.06.002.

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