

Aptamer microarray as a novel bioassay for protein–protein interaction discovery and analysis

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ABSTRACT

Aptamer microarray is investigated as a novel bioassay for protein–protein interaction (PPI) discovery and analysis. Assaying a mixture of fluorescence-labeled thrombin and *Escherichia coli* proteins with an aptamer microarray, we found that thrombin and an unknown protein of *E. coli* (protein X) formed a complex of PPI, which was captured by an anti-thrombin aptamer probe. The PPI observed on the microarray was double-checked by protein microarrays and confirmed by aptamer-baited co-immunoprecipitation (Co-IP) assays. Characterizing the Co-IP products, we identified protein X as an *E. coli* Dps protein (DNA-binding protein from starved cells). A SDS–PAGE analysis suggested that Dps should be a substrate for thrombin, a trypsin-like serine protease. A dose–response microarray experiment predicted an apparent dissociation constant of 1.33 μ M for the PPI. Moreover, an on-microarray competition assay revealed that the capture of the PPI by the anti-thrombin aptamer probe would be blocked by an *E. coli* aptamer via complementary base pairing. Thus, a network of protein–protein, protein–DNA, and DNA–DNA interactions and their interaction orders could be addressed in addition to simple PPI discovery.

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1. Introduction

Protein–protein interactions (PPIs) that result in stable or transient complexes between two or more proteins (Braun and Gingras, 2012) play crucial roles in various cellular functions of living organisms, such as DNA replication and transcription, protein modification and signal transduction, and transportation. Finding new PPIs can enhance the capability of life scientists to decipher the causes of chronic and difficult diseases and the solutions (Wells and McClendon, 2007). Physical association methods (such as affinity chromatography and immunoprecipitation) were employed to detect PPIs traditionally and are still used to couple with mass spectrometry (MS) for target identification. The dissociation constant of a PPI could be measured by gel filtration, fluorescence detection with tagged molecules, or surface plasmon resonance (SPR; Phizicky and Fields, 1995). Since the 1990s, there has been an increasing interest in functional genomics and proteomics studies that gives a vision of systems biology. To act upon the interest, high-throughput PPI screening tools are highly needed. Two-hybrid screen and tandem affinity purification tagging are two standard high-throughput methods for PPI discovery (Williamson and Sutcliffe, 2010). The former

uses *Escherichia coli lacZ* gene as a reporter for the bait protein–prey protein association, and the latter exploits protein A and a calmodulin-binding peptide for two-step purification. Later on, the advent of microarrays (Skena et al., 1995) and the post-genomics era have together brought in another powerful tool for high-throughput PPI discovery—a protein microarray. It is capable of quick, one-shot screening for a multitude of PPIs and requires only a tiny amount of sample, and it is a complementary approach for emerging nanotechniques-based analytical proteomics (Ray et al., 2010). There are two major classes of protein microarrays—proteome (function) microarrays (MacBeath and Schreiber, 2000; Zhu et al., 2001) and antibody (capture) microarrays (Haab et al., 2001; Sreekumar et al., 2001). A proteome microarray contains as many purified recombinant proteins (carrying affinity tags, e.g. 6xHis or GST) of an organism as possible. The best example was the first proteome microarray covering 5800 ORFs of the budding yeast *Saccharomyces cerevisiae* (Zhu et al., 2001). This kind of protein microarray is advantageous for detection of protein–DNA and protein–small molecule interactions in addition to PPIs, and it is able to detect post-translational modifications as well. An antibody microarray is spotted with a large number of monoclonal or polyclonal antibodies against a wide variety of proteins. The most famous applications reported to date are monitoring changes in protein abundance in carcinoma cells (Sreekumar et al., 2001) and identifying potential cancer biomarkers in serum (Miller et al., 2003). In theory, antibody microarrays can screen

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PPIs through on-microarray parallel co-immunoprecipitation (Co-IP) as well. Thus, both classes of microarrays are competent platforms for high-throughput PPI interactome analysis, but their production cost and stability issues have rendered them less affordable and less popular when compared to DNA microarrays. In this proof-of-principle work, a DNA aptamer microarray, combining the fabrication advantage of a DNA microarray and the function of an antibody microarray, is investigated as a novel tool for PPI discovery and analysis.

Systematic evolution of ligands by exponential enrichment (SELEX; Ellington and Szostak, 1990; Tuerk and Gold, 1990), an *in vitro* ligand selection process, can produce a panel of single-stranded oligonucleotide ligands that specifically and tightly bind to different target molecules ranging from small organics and proteins to whole cells. Such short DNA or RNA ligands are called aptamers. In particular, DNA and modified RNA aptamers are regarded as promising antibody rivals, since they are chemically stable, easy-to-synthesize, easy-to-modify, small, and low immunogenic. They have become important classes of molecules for biosensors (Sassolas et al., 2011; Citartan et al., 2012), biosimilar drugs, and drug carriers (Bunka and Stockley, 2006; Keefe et al., 2010). The first aptamer microarray for protein-profiling-based diagnostics was demonstrated in 1999 (Brody et al., 1999), where photoaptamer probes with photocross-linking functionality (e.g., 5-bromo-2'-deoxyuridine; Smith et al., 2003; Bock et al., 2004) were used. Such a microarray can tolerate stringent washes after cross-linking, so it can achieve multiplexed serum protein analysis with high specificity and sensitivity. Because stringent washes dissociate protein complexes and denature the captured proteins, photoaptamers are not suitable for PPI study. By contrast, the microarrays spotted with typical aptamers (Cho et al., 2006; Walter et al., 2008; Lao et al., 2009) are better formats for PPI identifications. Taking the advantages of high-density DNA chips, an aptamer microarray has been proven to be an ideal tool for massively parallel interrogation of as-selected aptamer sequences and their subdomains or variants (Fischer et al., 2008; Chang et al., 2009; Platt et al., 2009). This paves a systematic, reliable way for post-SELEX optimization. Recent papers further disclose the niches of this technology in functional proteomics. The feasibility of aptamer microarray mediated capture and high-throughput MS analysis of biomarker in serum samples has been demonstrated (Ahn et al., 2010; Zhang et al., 2012). A capture microarray comprising a large number of slow off-rate modified aptamers has been presented to measure 813 proteins with low detection limits (1 pM) and has been applied to disease's biomarker discovery (Gold et al., 2010). Although there is considerable progress in aptamer microarray R&Ds, there is still no literature reported relevant to PPI discovery and analysis. Presumably, the major reason is due to the lack of a library of aptamers selected for a whole organism proteome. To this end, we have performed complex SELEX against *E. coli* K12 total protein and identified a panel of *E. coli* protein fraction-specific ($p < 1\%$) DNA aptamers (< 40 mers) out of 384 candidate sequences by microarray assays (unpublished). This can be applied for development of aptasensors for detection of microbial pathogens as well (Torres-Chavolla and Alocilja, 2009). Here, we prove the viability of aptamer microarrays in PPI discovery and analysis using the example of thrombin–*E. coli* protein interaction. In the following text, we will detail how an aptamer microarray reveals the evidence of a PPI and how a protein microarray assay, aptamer Co-IP assay, differential PAGE analysis, and MS analysis are used to confirm the PPI. In addition, it will show that the aptamer microarray is capable of estimation of the PPI's apparent dissociation constant and allows on-microarray competition against the PPI capture by an *E. coli* aptamer. Finally, an interaction network among protein–protein, protein–DNA, and DNA–DNA molecules and their interaction orders will be depicted.

2. Experimental section

2.1. Preparation of protein samples

E. coli K12 MG1655 total protein fractions and human α -thrombin (Haematologic Technologies, Essex Junction, VT) were studied here. To obtain *E. coli* total proteins, bacterial cells were cultured in an LB medium at 37 °C overnight. Prior to lysis, the cells were washed with low salt (50 mM NaCl) phosphate buffer saline (PBS) twice to remove the residual medium. The B-PER™ bacterial protein extraction reagent (Thermo/Pierce, Rockford, IL) was used to lyse *E. coli* cells. Two batches of total protein fractionated samples were prepared. The first batch was prepared by two-dimensional fractionation: (i) ammonium sulfate (AS) fractionation (0–35% AS, 35–70% AS, and 70–100% AS) followed by (ii) anion-exchange FPLC fractionation (performed by an FPLC system, AKTApurifier 10, GE Healthcare, Piscataway, NJ) using a Source 15Q AIX column (GE Healthcare) with an elution gradient ranging from 20 to 500 mM NaCl. The second batch was prepared (for a double check of the assay results) using the FPLC fractionation only. The protein fractions of the first batch assayed with microarrays were Fraction L (denoted as Frac L, 35–70% AS and 100–150 mM NaCl) and Frac S (70–100% AS and 20–50 mM NaCl). The second batch yielded Frac 1A (flow-through and 20–50 mM NaCl), Frac 1B (50–100 mM NaCl), Frac 1C (100–150 mM NaCl), Frac 1D (150–250 mM NaCl), Frac 1E (250–500 mM NaCl), and Frac 1F (> 500 mM NaCl) where Frac 1A (covering Frac S) was the main fraction studied. Thrombin (Thr) was used as a positive control sample in aptamer microarray assays and used as a bait protein for PPI study.

2.2. Aptamers

Two anti-thrombin DNA aptamers, HT3 (AGT CCG TGG TAG GGC AGG TTG GGG TGA CT, 29 mer and exosite II-specific) and HT5 (GGT TGG TGT GGT TGG, 15 mer and exosite I-specific; called HTDQ29 and HTQ15 in our previous paper (Chi et al., 2011)), and *E. coli* K12 total protein fraction-specific DNA aptamers (see Supporting information, S1) generated from the complex SELEX against *E. coli* K12 total protein were studied. The key *E. coli* aptamers were 9mFC12 (AGC CGC ACA GGG AGG TGG TTG GGT CGC GCT ACT TAA AGT G, 40 mer and Frac S specific) and 14mG53 (AGC CGC ACA GGG AGG TGG TTG GGT CGC GCT ACT TAA AGT G, 40 mer and Frac L specific). Most aptamers were synthesized by Purigo Biotech, Inc. (Taipei, Taiwan), while some of them were in-house synthesized. A 5'-amino C6 linker was added for microarray spotting and fluorescent labeling, while a 5'-biotin C6 linker was added for coupling to streptavidin (SA) magnetic beads (Merck Estapor, Darmstadt, Germany) in the aptamer-based Co-IP assays.

2.3. Aptamer microarray assays

Unless otherwise noted, aptamer microarray fabrication with Corning epoxide slides (Corning Inc., Lowell, MA), post-arraying slide treatments, fluorescent protein (and aptamer) labeling with Cy3 and Cy5 NHS ester reagents (GE Healthcare), labeled protein assays and microarray analyses with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA) were performed according to our reported method (Lao et al., 2009) and the vendors instructions. (Note: since fluorescence labeling might possibly lead to a bias in PPI study, we utilized different assay conditions and distinct experiments including protein microarray and co-immunoprecipitation to validate the PPI discovery from the aptamer microarray as described later.) The position, ID, and sequence of each 5'-amino-C₆-modified aptamer

printed on the microarray are summarized in Table S1 (see Supporting information, S1). Each microarray slide was arrayed with three identical but separate printed “fields” for distinct assay conditions, and each probe was printed in quadruplicate in a field to examine probe-to-probe variation. For a standard assay, a 20 μ l protein solution comprising designated amount(s) of labeled protein(s), 1% Roche BM blocking reagent (Roche Applied Science, Upper Bavaria, Germany) and 1% PEG 8000 in a TBSM buffer (20 mM Tris, pH 7.5, 150 mM NaCl, and 5 mM $MgCl_2$) was drop-coated on the test field and then covered with a 18×18 mm² microscope coverslip. Then the microarray slide was put in a humidified chamber (with a steady RH% maintained by a saturate KCl aqueous solution) for 1-h room temperature incubation. A post-assayed slide was briefly washed with PBS (50 mM phosphate, pH 7.4, and 150 mM NaCl) in a 50 mL centrifugal tube to remove the coverslip(s) and unbound labeled protein solutions, and then it was washed with PBST (500 mM NaCl; 50 mM phosphate, pH 7.4, 500 mM NaCl, and 0.05% Tween 20) in a 9-cm Petri dish for 20 min under gentle shaking at room temperature three times. After a final brief rinse with PBS and then Q-water, the slide was spun to dry and then scanned using the GenePix 4000B microarray scanner and analyzed with GenePix Pro 6.0 (Axon Instruments, Union City, CA).

2.4. Protein microarray assays

Protein microarray fabrication and assays were similar to those of aptamer microarrays. The second batch of *E. coli* fractions (Frac 1A–1F) and thrombin were printed in duplicate onto Corning epoxide microarray slides (arraying environment: 67% RH, 25 °C and subsequent overnight room temperature prehydration in a saturate KCl humidified chamber). The position, ID, and printing concentration of each protein probe printed on the protein microarray are summarized in Table S2 (see Supporting information, S2). Just after the overnight prehydration was done, the protein microarrays were used. The non-protein-spotted area of a microarray slide was blocked with 0.2% Roche BM blocking reagent in TBS (20 mM Tris, pH 7.5 and 150 mM NaCl) at room temperature for 1 h. Then two protein microarray slides were assayed with 1 μ g Thr–Cy5 and 1 μ g Frac 1A–Cy3 in 20 μ l (with 1% Roche BM blocking reagent and 1% PEG 8000 in a TBSM buffer). The assay and post-assay protocols were the same as

those of aptamer microarrays except for the use of PBST (150 mM NaCl) instead of PBST (500 mM NaCl) for washes.

2.5. Aptamer co-immunoprecipitation (Co-IP) assays

We coupled 5'-biotinylated HT5 (at 16.7 μ M) with 50 μ l streptavidin (SA) coated magnetic Estapor[®] microspheres (1% solid concentration, 1 μ m in diameter) for the bait preparation for each Co-IP assay. The coupling was done according to the vendor's instruction. Uncoated SA-coated magnetic beads were also used as the bait for negative control. Four Co-IP assays were carried out with the following 1 h incubation conditions at room temperature: (A) bead-SA-biotin HT5+4.5 μ g Frac 1A–Cy3, (B) bead-SA-biotin HT5+2.5 μ g Thr–Cy5, (C) bead-SA-biotin HT5+4.5 μ g Frac 1A–Cy3+2.5 μ g Thr–Cy5, and (D) bead-SA+4.5 μ g Frac 1A–Cy3+2.5 μ g Thr–Cy5. All incubation mixtures were placed in a 200 μ l PCR tube with a 50 μ l TBSM buffer containing 1% Roche BM blocking reagent and 1% PEG 8000 and under vigorous vortex mixing. After incubation, the four bead baits with the pull-down proteins were briefly rinsed with 200 μ l of PBS twice and collected with magnets. Then the sequential elution of the “pull-down” proteins with an increasing ionic strength was performed with 50 μ l of PBST (100 mM NaCl), PBST (250 mM NaCl), PBST (500 mM NaCl) and PBST (1000 mM NaCl) at room temperature, with 10-min incubation for each. The eluted protein samples were analyzed with a standard SDS–PAGE but scanned by a Typhoon 9410 variable mode imager (GE Healthcare) to obtain two-color (Cy3 vs. Cy5) differential gel electrophoresis images. Later, the thrombin associated protein was identified by the gel images and was analyzed using MALDI-TOF mass spectrometry (Voyager-DETM STR, Life Technologies (ABI), Carlsbad, CA).

3. Results and discussion

3.1. Does anti-thrombin aptamer capture an *E. coli* protein?

Fig. 1(a) shows an aptamer microarray assayed with a mixture of 2 μ g Frac L–Cy3 and 2 μ g Thr–Cy5 in 20 μ l. The green (Cy3) and red (Cy5) fluorescences occur on 14mG53 and HT5 aptamers, respectively. This suggests that aptamers generated from a complex SELEX against *E. coli* K12 total protein are “protein fraction specific”

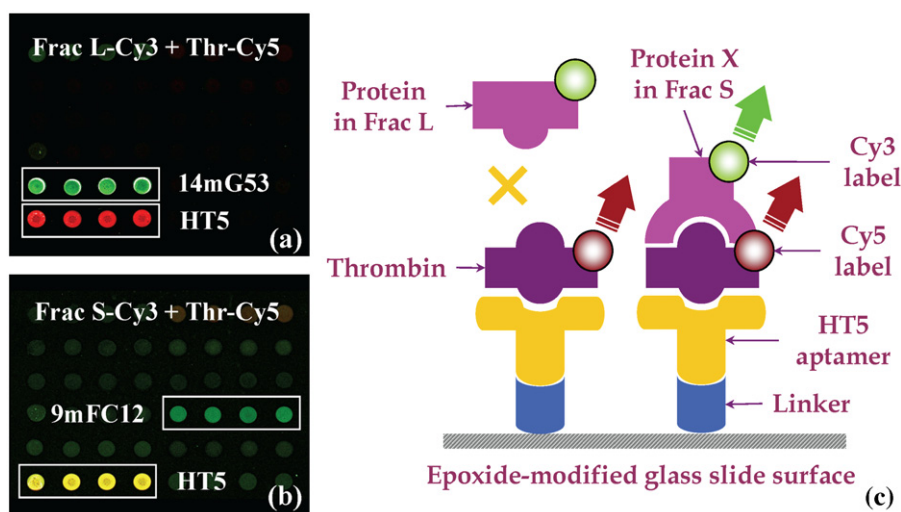


Fig. 1. Aptamer microarrays assayed with (a) 2 μ g Frac L–Cy3 + 2 μ g Thr–Cy5 and (b) 2 μ g Frac S–Cy3 + 2 μ g Thr–Cy5 in 20 μ l. (c) Schematic illustration of the PPI observed on the aptamer microarray. Cy3 and Cy5 fluorescence were excited at 532 nm and 635 nm, respectively. The emission maxima of Cy3 and Cy5 were at 570 nm and 670 nm, respectively. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

(e.g. 14mG53 for Frac L). The HT5–thrombin complex can be a positive control for aptamer microarray assays (Lao et al., 2009). (Note: the HT3 aptamer was observed to bind Thr–Cy3 only, whereas HT5 interacted with both Thr–Cy3 and Thr–Cy5.) When we assayed the microarray with a mixture of Frac S–Cy3 and Thr–Cy5, it was indeed only 9mFC12 (Frac S-specific) out of the *E. coli* aptamers that was lit with green fluorescence. However, to our surprise, the highly specific and well-proven anti-thrombin HT5 aptamer displayed yellow fluorescence, as shown in Fig. 1(b), which was a sign indicating that HT5 captured both Frac S–Cy3 and Thr–Cy5 at the same time. This finding brings in an intriguing question—“Does the anti-thrombin HT5 aptamer also recognize an *E. coli* protein?” Believing that HT5 should be highly specific, we propose a model (Fig. 1(c)) to postulate that there would be a protein X in Frac S that forms a complex with thrombin via PPI; in comparison, all proteins in Frac L would not associate with thrombin. To test this postulation, we perform a series of aptamer microarray assays and PPI assays, as described and discussed in the following sections.

3.2. Double-checking of the PPI with a new aptamer microarray assay

To verify Fig. 1(c), an aptamer microarray assay with mixtures of 2 μ g Frac S–Cy3 and varied amounts of Thr (non-labeled: 0, 1.3, or 2.6 μ g) in 20 μ L was performed. The microarray images are presented in Fig. 2(a) and (b). The mean green fluorescence on 9mFC12 and that on HT5 in response to Frac S–Cy3 are compared in Fig. 2(c). The raw data of the fluorescence intensities and the coefficients of variation of the quadruplicate spots are summarized in Table S3 (see Supporting information, S3). Although the presence of labels could affect the protein recognition, Figs. 1(b) and 2(b) basically tell the same story that the presence of thrombin, no matter whether it is labeled or not, would lead to a Frac S–Cy3 response appearing on the HT5 probe. (Note: two microarray images might look slightly different because different PMT settings were used in these two separate assays.) It is found that HT5 does not capture Frac S–Cy3 when the thrombin is absent. By contrast, after addition of 1.3 μ g thrombin, Frac–Cy3 is captured on HT5.

The Cy3 signal on HT5 is even higher than that on 9mFC12, a Frac S-specific aptamer. When thrombin increases to 2.6 μ g, HT5's Cy3 signal (a measure of Frac–Cy3 binding) is further increased. On the other hand, the thrombin addition decreases the Cy3 signal on 9mFC12. It is presumed that the reversed trend of 9mFC12's response to Frac S–Cy3 is due to a competitive binding effect between 9mFC12 and thrombin. The results shown in Fig. 2 support the proposed model of the on-microarray PPI in Fig. 1(c). It is also found by the dose-dependent Frac S–Cy3 signals on 9mFC12 and HT5 that thrombin is an even stronger recognition element (or ligand) for protein X than is 9mFC12. This finding can explain why there is no observable Thr–Cy5 signal on 9mFC12 in Fig. 1(b), since thrombin would take Protein X (in Frac S) away from 9mFC12 instead of binding onto the Frac S–9mFC12 complex. This competitive binding effect can be further confirmed using a real-time, label-free system (Li et al., 2007; Gronewold et al., 2009; Chang et al., 2011) by sequential interactions of proteins with the aptamer microarray.

3.3. Direct PPI confirmation on a protein microarray

To confirm the PPI observed on the aptamer microarray, we arrayed newly prepared *E. coli* K12 protein fractions (Frac 1A–1F, where Frac 1A covers all of the proteins in Frac S) and thrombin onto the epoxide slides (see Supporting information, S2) for protein microarray-based PPI investigation (Zhu et al., 2001). Two identical protein microarrays were assayed with 1 μ g Thr–Cy5 in 20 μ L and 1 μ g Frac 1A–Cy3 in 20 μ L. The assay results are shown in Fig. 3. Fig. 3(a) shows that Thr–Cy5 interacts with Frac 1A and Thr spotted on the microarray, while Fig. 3(b) shows that Frac 1A–Cy3 interacts with Frac 1A, Frac 1B, and Thr. Although the protein microarray is not optimized like the aptamer microarray, it still provides informative results and discloses the following three types of protein–protein interactions—(i) Thr–Frac 1A interaction: the specific thrombin–protein X (in Frac 1A or Frac S) complex; (ii) Thr–Thr interaction—a same-same interaction; (iii) Frac 1A–Frac 1B interaction—a similar-similar interaction between neighbor fractions. This not only confirms Fig. 1(c) from a different dimension of protein microarray but also infers that a protein complex rather than a single protein would be captured by an

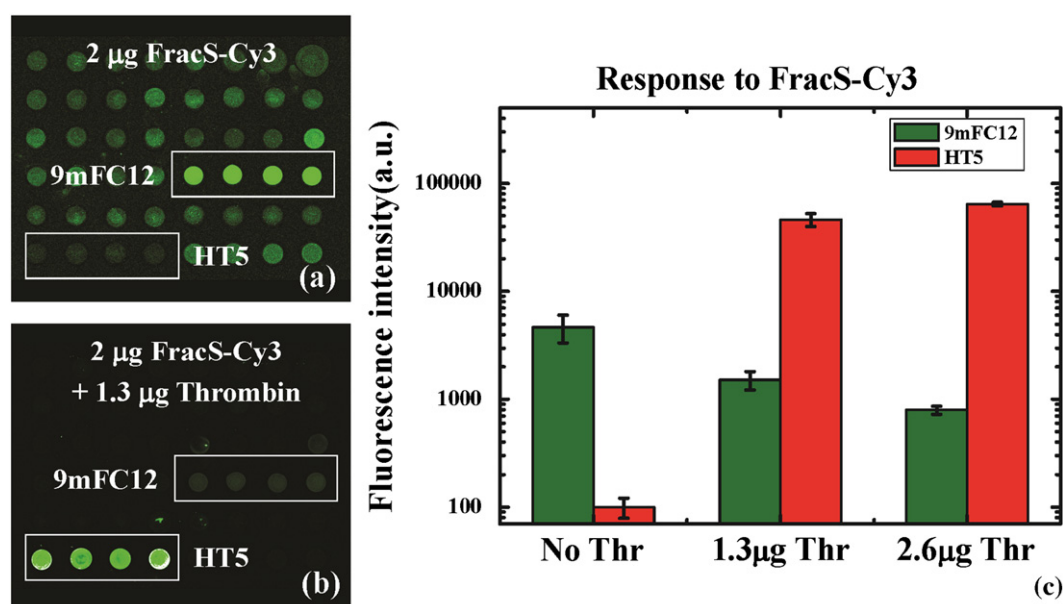


Fig. 2. Aptamer microarrays assayed with (a) 2 μ g Frac S–Cy3 and (b) 2 μ g Frac S–Cy3 + 1.3 μ g Thr in 20 μ L. (c) Comparison between the mean fluorescence signals of 9mFC12 and HT5 probes in response to 2 μ g Frac S–Cy3 + different amounts of Thr in 20 μ L. The raw data of (c) and the coefficients of variation of the quadruplicate spots are given in Table S3 in Supporting information, S3. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

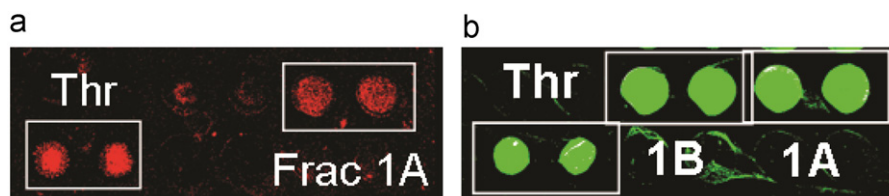
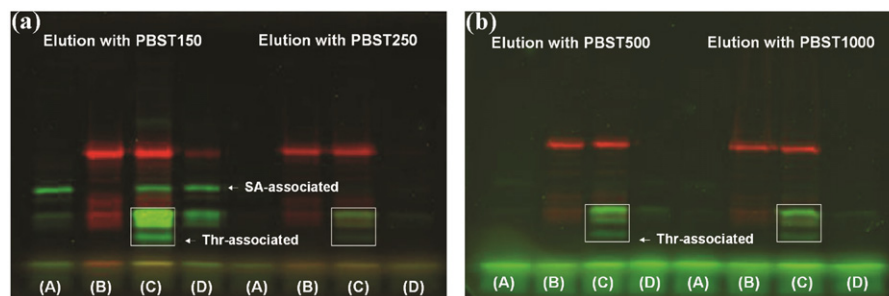


Fig. 3. Protein microarrays assayed with (a) 1 µg Thr–Cy5 and (b) 1 µg Frac 1A–Cy3 in 20 µl.



* Co-IP conditions: (SA = streptavidin; Thr = thrombin)

(A) Bead-SA-biotin-HT5 + Frac 1A–Cy3; (B) Bead-SA-biotin-HT5 + Thr–Cy5

(C) Bead-SA-biotin-HT5 + Frac 1A–Cy3 + Thr–Cy5; (D) Bead-SA + Frac 1A–Cy3 + Thr–Cy5

** Elution buffer PBST = 50 mM Phosphate + X mM NaCl + 0.05% Tween 20 (X = 150 – 1000)

Fig. 4. Two-color fluorescence SDS–PAGE analysis for the Co-IP assays to identify the thrombin-associated *E. coli* protein as well as protein X in Fig. 1(c). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

aptamer probe—this is important but seldom mentioned in the literature of aptamer-based assays or biosensors.

3.4. Identification of protein X by Co-IP assays

Co-immunoprecipitation assays were done for further proving the thrombin–Frac 1A (Frac S) interaction and for identifying protein X. The bait used to pull down (or “fish”) the protein target from the mixture was 5′ biotinylated HT5-coupled SA magnetic bead, and the uncoupled SA bead was used as “negative” bait. The protein samples used for the Co-IP assays are (A) Frac 1A–Cy3 for HT5-coated beads, (B) Thr–Cy5 for HT5-coated beads, (C) Frac 1A–Cy3+Thr–Cy5 for HT5-coated beads, and (D) Frac 1A–Cy3+Thr–Cy5 for blank SA beads. Fig. 4, a set of two-color differential SDS–PAGE images, exhibits the sequential elution of the pull-down proteins, from the low salt to the high salt condition. In Fig. 4, the major red bands eluted from (B) and (C) in all NaCl concentrations are thrombin. The upper green band appearing only in the PBST (150 mM NaCl) elution from (A), (C) and (D) is supposed to be a weak interaction between SA and Frac 1A. Comparing lanes (C) with others in four elution conditions, we find a group of green bands specifically associated with thrombin as indicated in the PAGE images. In particular, it is clear that the lowest one is even more specific to thrombin, totally independent of SA (refer to lane (D) of PBST150). We consider that it must be protein X as suggested in Fig. 1. We also performed a similar Co-IP assay to double-check the results and purified protein X (non-labeled) from the PAGE gel for peptide fingerprinting with MALDI-TOF mass spectrometry (see Figs. S1 and S2 in Supporting information, S4). It was then identified that protein X is a DNA protection during starvation protein (Dps) in *E. coli* cells (Almiron et al., 1992; Nair and Finkel, 2004). It is a starvation-inducible DNA-binding protein (19 kDa). *In vitro* Dps forms extremely stable complexes with DNA, without apparent sequence specificity. When complexed with Dps, DNA is rendered DNase resistant (Almiron et al., 1992). To date there is no literature reporting the PPI between thrombin and Dps. In another batch of Co-IP assays using non-labeled protein samples and analyzed by typical

Coomassie blue-stained SDS–PAGE (see Fig. S3 in Supporting information, S4), we found that the nonlabeled Dps protein in Frac 1A would be degraded in the presence of non-labeled thrombin. As a result, the thrombin-associated Dps elution could not be observed in this case. This suggests that Dps should be a substrate more than a simple ligand for thrombin, a “trypsin-like” serine protease protein. Moreover, the comparison between, Fig. 4 and Figs. S1 and S3, implies that Cy3-labeled Dps could not be degraded by Cy5-labeled thrombin at lower concentration. This might be due to the fact that the labels affect the enzymatic functionality of thrombin, although the labels do not seem not affect the formation of the PPI from microarray assays. This reminds readers that the possible labeling effect on PPIs should be noticed when using the present label-based microarrays. Besides, Fig. S3 also shows that HT5 can capture Dps in the absence of thrombin, if Dps is present in a large quantity and/or non-labeled. This might be because of Dps’ DNA-binding capability (Almiron et al., 1992; Nair and Finkel, 2004). A detailed discussion of the PPI between thrombin and Dps is beyond the scope of this work, which aims at a proof-of-principle study about how to discover and analyze PPIs with an aptamer microarray. However, it is worthwhile to note here that the possibility for Dps as an intervention in thrombin signalling and protease-activated receptors (Coughlin, 2000) should be an interesting topic for further study.

3.5. Dose–response analysis of PPI on the aptamer microarray

Basically, the fluorescence signal observed on a microarray probe is proportional to the amount of the target captured. Thus, it is able to quantitatively analyze the dose-dependent behavior of the protein–protein interaction by the aptamer microarray. Fig. 5 plots the (a) Cy5 and (b) Cy3 fluorescence signals observed on HT5 against thrombin’s concentration for assay with a mixture of 1 µg Frac 1A–Cy3 in 20 µl and a varied concentration of Thr–Cy5. It is seen in Fig. 5(a) that the Cy5 signal is linearly correlated to the thrombin concentration, which indicates that the assayed thrombin

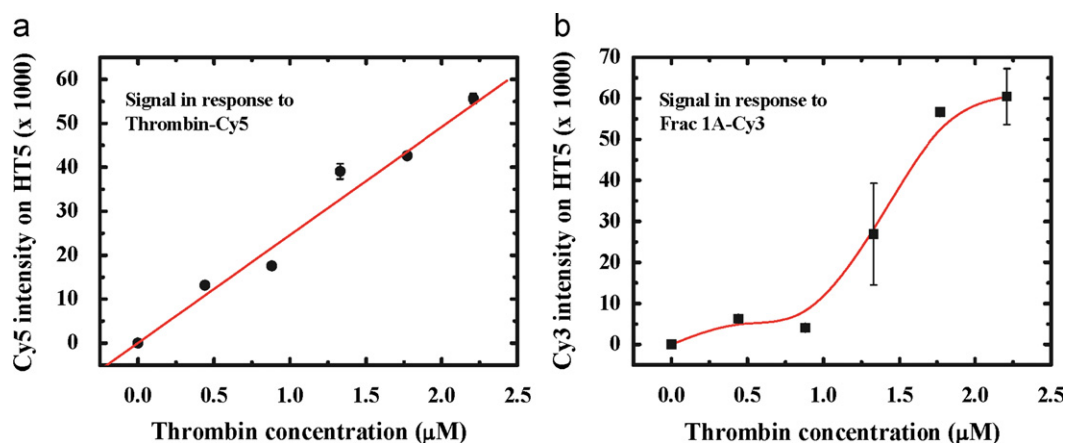


Fig. 5. Fluorescence signals on the HT5 probe of the aptamer microarray assayed with 1 μg Frac 1A–Cy3 and different amounts of Thr–Cy5 in 20 μL . (a) Cy5 signal on HT5 as a function of thrombin concentration. (b) Cy3 signal on HT5 as a function of thrombin concentration. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

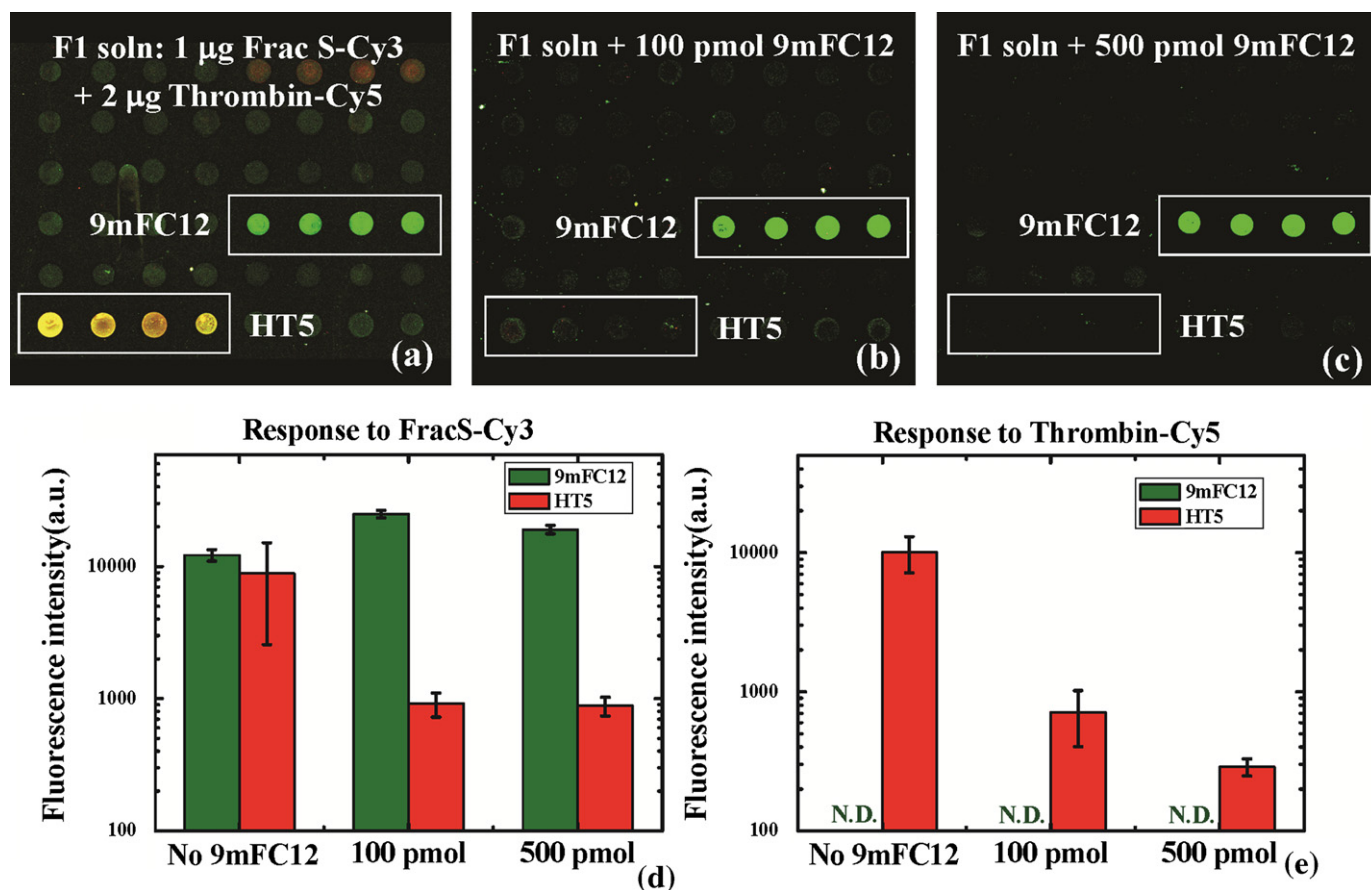


Fig. 6. Effect of the presence of free 9mFC12 on the microarray detection of 1 μg Frac S–Cy3 and 2 μg Thr–Cy5 in 20 μL . (a)–(c) The microarray images. (d)–(e) Comparison between the mean fluorescence signals of 9mFC12 and HT5 probes in response to (d) 1 μg Frac S–Cy3 and (e) 2 μg Thr–Cy5. The raw data and the coefficients of variation of the quadruplicate spots in (d) and (e) are given in Tables S4 and S5, respectively, in Supporting information, S3.

concentrations fall in the linear dynamic range of microarray detection with HT5. By contrast, the Cy3 signal, as can be seen in Fig. 5(b), is a sigmoid function of thrombin concentration. When Thr–Cy5 is less than ca. 1 μM ($=0.75 \mu\text{g}/20 \mu\text{L}$; molecular weight of thrombin is 37.5 kDa), the interaction between Frac 1A–Cy3 and Thr–Cy5 is not significant. On the other hand, when Thr–Cy5 exceeds 1.75 μM ($=1.31 \mu\text{g}/20 \mu\text{L}$), the Frac 1A–Cy3's response on HT5 is saturated. The sigmoid curve give an apparent dissociation constant of ca. 1.33 μM ($=1 \mu\text{g}/20 \mu\text{L}$) for the thrombin–Frac 1A

complex. The K_d value ($1.33 \times 10^{-6} \text{ M}$) is similar to that of a cognate enzyme/substrate pair of yeast peroxidase/yeast cyt c ($K_d=1.6 \times 10^{-6}$) and a noncognate antigen/antibody pair of flu HA(T1311)/fab HC19 ($K_d=4 \times 10^{-6} \text{ M}$; Kastritis et al., 2011). Comparing Fig. 5(b) with 5(a), we infer that the HT5:Thr complex has a stronger binding strength than that of Thr–Frac 1A. This is because the onset of Cy5 signal occurs at a lower dose of thrombin than that of Cy3 signal. Besides, the binding between Thr and Frac 1A seems to not compete with the recognition of Thr on HT5 at all

concentrations tested. This suggests that protein X and HT5 bind to different sites of thrombin.

3.6. On-microarray aptamer competition against the protein complex

Fig. 6(a) represents a repeated experiment for Fig. 1(b). The result is reproducible. It is interesting that the fluorescence signals on HT5 are diminished after nonlabeled 9mFC12 aptamer is incorporated into the protein sample, as can be seen in Fig. 6(b) and (c). Comparisons between the mean microarray signals on 9mFC12 and HT5 probes in response to Frac S–Cy3 and Thr–Cy5 are shown in Fig. 6(d) and (e), respectively. The relevant raw data are summarized in Tables S4 and S5 (see Supporting information, S3). The results of Fig. 6 are repeated by assay with 1 µg Frac S–Cy3 and 1.3 µg Thr+ different amounts of 9mFC12–Cy5 in 20 µl. The results are shown in Fig. S4 (see Supporting information, S5). It is observed in Fig. 6(d) that the presence of 9mFC12 in the protein sample diminishes the Frac S–Cy3 response on HT5. It also diminishes the Thr–Cy5 response on HT5, as shown in Fig. 6(e). This suggests that the 9mFC12 addition competes with the complex of Frac S–Thr away from HT5. The mechanism of such on-microarray aptamer competition is disclosed by Fig. S4, which shows obvious Cy5 signals on HT5, HT3, and 14mG53 in response to the addition of 9mFC12–Cy5. In particular, the replacement of Thr–Cy5 by 9mFC12–Cy5 on HT5 implies an attractive interaction between HT5 and 9mFC12. By sequence alignment (see Fig. S5 in Supporting information, S5), we find that 9mFC12 covers a sequence region that is complementary to 11 bases of the 15-mer HT5. (9mFC12 also has a complementary region for HT3 and 14mG53.) Accordingly, we realize that the base-pairing interaction between 9mFC12 and HT5 is stronger than thrombin–HT5 interaction and thus can compete against the thrombin binding. This evidences that an aptamer microarray is an ideal surrogate for protein microarrays in analysis of DNA(aptamer)–protein, protein–protein, and DNA–DNA interactions simultaneously. It is also observed in Fig. 6(d) that the addition of 9mFC12 in the protein sample enhances the FracS–Cy3 binding on the 9mFC12 probe. This phenomenon is found in Fig. S4 as well. In Fig. S4, the slight increase of Cy5 response on HT5 along with the addition of 9mFC12–Cy5 suggests a sandwich capture of Frac S–Cy3 by the top and the bottom aptamers. We presume there is a cluster of Frac S–Cy3 formed by the similar–similar interaction as suggested by Fig. 3. Hence there are same “exposure epitopes” of Frac S–Cy3 for both 9mFC12 (binding from top and down). This suggests that sandwich recognition of protein can be done by two identical aptamers from distinct directions.

4. Conclusions

Through the above results and discussion, an interaction network of interest disclosed on the aptamer microarray can be depicted (see Fig. S6 in Supporting information, S6): (A) the strongest interaction is the DNA–DNA base-pairing interaction (9mFC12:HT5), (B) The second strongest interaction is the aptamer–protein interaction (HT5:thrombin; 9mFC12: FracS/1A), (C) the interaction between thrombin and protein X (Dps) in Frac S/1A is moderately strong, which can stand for stringent PBST (500 mM NaCl) washes, (D) Frac S/1A must associate with protein X through a similar–similar protein interaction and (E) the weakest interaction is the DNA binding protein effect (Dps: HT5) which occurs at a higher protein X concentration. Through the Co-IP assay and MS characterization, protein X is identified as Dps, which might be the substrate for thrombin (see Fig. S3).

In conclusion, we have successfully demonstrated PPI discovery and analysis by aptamer microarray based on the aptamers obtained from *E. coli* total protein SELEX and hope to inspire the experts in the fields of large-scale label-free sensing technologies (Li et al., 2007; Gronewold et al., 2009; Chang et al., 2011) and high-throughput microarrays to a new way for PPI studies.

Acknowledgment

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2012.10.082>.

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< S1 >

Supporting information for the article: “**Aptamer Microarray as a Novel Sensor for Protein-Protein Interaction Discovery and Analysis**”

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S1. Information of the Aptamer Microarray Composition.

Table S1. The position, ID, and sequence of each aptamer probe printed on the aptamer microarray. The aptamer printing concentration was 20 OD (=660 ng/μl = 50 μM for a 40 mer).

Position ^a	Aptamer ID	Aptamer Sequence (5'→3')
R1C1-R1C4	04mG13 ^e	TCTCAGCTAAATGGACGGATCAATGTAGCATAACTCTAAA (40mer)
R1C5-R1C8	HT3 ^b	AGTCCGTGGTAGGGCAGGTTGGGGTGACT (29mer)
R2C1-R2C4	7mD109 ^e	GGCCCCCAGCTTTTCCTTTGCCACGACCAATTGCGCCCA (39mer)
R2C5-R2C8	5DAC06 ^e	CTGGACCTGCAAAAAGGAACTCCCGATCGTACGCGAGACGA (40mer)
R3C1-R3C4	9mFC08 ^e	GGTATAGTGCCGAGACTCGCCACCCCTGCCAACCCTAAATA (40mer) ^f
R3C5-R3C8	7mDD09 ^e	GCAGACCTGCCCTGTGCGCTCCGCTGGATTTGCCAAC (37mer) ^f
R4C1-R4C4	14mG38 ^e	AGCGATAATACTATTAATCTAACGGTACCCGTAA (36mer)
R4C5-R4C8	9mFC12 ^d	TCTGCATCCGACCACCCTAACGTCCGCCAGCTCTCACCGG (40mer) ^f
R5C1-R5C4	14mG53 ^c	AGCCGCACAGGGAGGTGGTTGGGTCGCGCTACTTAAAGTG (40mer)
R5C5-R5C8	14mG49 ^e	TATCTAGTTACTGCTATCGTAATAGGTAGGCTATATACG (39mer)
R6C1-R6C4	HT5 ^b	GGTTGGTGTGGTTGG (15mer)
R6C5-R6C8	14mG55 ^e	ACTTGTGGCGCGCGCTAGCATATATGCGGCACAACCCCCC (40mer)

^a. The top row (R) of a microarray is defined as R1, while the bottom row is R6. The most left column (C) is defined as C1, while the most right column is C8.

^b. Anti-thrombin aptamers: HT3 = HTDQ29; HT5 = HTQ15.

^c. Aptamer targeted to Fraction L (Frac L) of *E. coli* K12 total protein.

^d. Aptamer targeted to Fraction S (Frac S) of *E. coli* K12 total protein.

^e. Aptamers targeted to other fractions of *E. coli* K12 total protein.

^f. Aptamers were synthesized with 50% BrdU and 50% dT.

S2. Information of the Protein Microarray Composition.Table S2. The position, ID, and printing concentration of each protein probe printed on the protein microarray.^a

Position^b	Protein ID^c	Printing Concentration
R1C1-R1C2	Frac 1C	1.28 $\mu\text{g}/\mu\text{l}$ (1x) ~ 0.32 $\mu\text{g}/\mu\text{l}$ (4x dil.)
R1C3-R1C4	Frac 1B	1.07 $\mu\text{g}/\mu\text{l}$ (1x) ~ 0.27 $\mu\text{g}/\mu\text{l}$ (4x dil.)
R1C5-R1C6	Frac 1A	0.78 $\mu\text{g}/\mu\text{l}$ (1x) ~ 0.20 $\mu\text{g}/\mu\text{l}$ (4x dil.)
R2C1-R2C2	Thr	1.49 $\mu\text{g}/\mu\text{l}$ (1x) ~ 0.37 $\mu\text{g}/\mu\text{l}$ (4x dil.)
R2C3-R2C4	Frac 1F	3.13 $\mu\text{g}/\mu\text{l}$ (1x) ~ 0.78 $\mu\text{g}/\mu\text{l}$ (4x dil.)
R2C5-R2C6	Frac 1E	3.13 $\mu\text{g}/\mu\text{l}$ (1x) ~ 0.78 $\mu\text{g}/\mu\text{l}$ (4x dil.)

^a. The protein microarray fabrication was not optimized but printed at a serial dilution fashion. Frac 1E and 1F were printed at higher concentrations for non-specificity check. The best protein microarray images are present in Fig. 3. (A: the array was printed at 4x dilution. B: the array was printed at 1.33x dilution.)

^b. The top row (R) of a microarray is defined as R1, while the bottom row is R2. The most left column (C) is defined as C1, while the most right column is C6.

^c. Frac D is skipped due to lack of significant microarray signal.

S3. Raw Data for Microarray Fluorescent Intensities Plotted in Fig. 2 and Fig. 6.Table S3. The raw data of microarray fluorescent intensities^a (in response to 2 µg Frac S-Cy3 + different amount of thrombin in 20 µl) plotted in Figure 2(c).

[Thr]	+ 0 «g Thr		+ 1.3 «g Thr		+ 2.6 «g Thr	
Probe ID	9mF12	HT5	9mF12	HT5	9mF12	HT5
Spot 1	2963	75	1381	51392	781	65297
Spot 2	5394	113	1761	39829	876	61119
Spot 3	5101	111	1189	41083	746	65307
Spot 4	5291	102	1715	52605	767	65316
Avg.	4687	100	1512	46227	793	64260
Stdev.	1327	21	291	6344	67	2415
CV(%)	28.3%	21.3%	19.3%	13.7%	8.5%	3.8%

^a. The intensity for each microarray feature (spot) has subtracted its background value. The 16-bit fluorescent intensity ranges from 1 to 65536 (arbitrary unit).

Table S4. The raw data of microarray Cy3 fluorescent intensities^a (in response to 1 µg Frac S-Cy3 and 2 µg Thr-Cy5+ different amount of non-labeled 9mFC12 probe in 20 µl) plotted in Figure 6(d).

[9mFC12]	+ 0 pmol 9mFC12		+ 100 pmol 9mFC12		+ 500 pmol 9mFC12	
Probe ID	9mF12	HT5	9mF12	HT5	9mF12	HT5
Spot 1	11210	16148	22790	1158	18980	1021
Spot 2	11510	6720	25359	945	18011	960
Spot 3	13436	4249	25717	778	16278	748
Spot 4	12677	8272	25916	779	22908	800
Avg.	12208	8847	24946	915	19044	882
Stdev.	1208	6279	1597	190	1369	143
CV(%)	9.9%	71.0%	6.4%	20.8%	7.2%	16.2%

^a. The intensity for each microarray feature (spot) has subtracted its background value. The 16-bit fluorescent intensity ranges from 1 to 65536 (arbitrary unit).

Table S5. The raw data of microarray Cy5 fluorescent intensities^a (in response to 1 μ g Frac S-Cy3 and 2 μ g Thr-Cy5+ different amount of non-labeled 9mFC12 probe in 20 μ l) plotted in Figure 6(e).

[9mFC12]	+ 0 pmol 9mFC12		+ 100 pmol 9mFC12		+ 500 pmol 9mFC12	
Probe ID	9mF12	HT5	9mF12	HT5	9mF12	HT5
Spot 1	-115	15073	-31	1240	-36	290
Spot 2	-117	9069	-66	602	-10	248
Spot 3	-83	7370	-36	516	-59	262
Spot 4	-154	8886	-68	488	-6	355
Avg.	-117	10100	-50	712	-28	289
Stdev.	25	2946	17	308	21	41
CV(%)	-21.4%	29.2%	-33.5%	43.2%	-77.1%	14.2%

^a. The intensity for each microarray feature (spot) has subtracted its background value. The 16-bit fluorescent intensity ranges from 1 to 65536 (arbitrary unit).

S4. Aptamer-based Co-Immunoprecipitation Assays for Protein X Identification

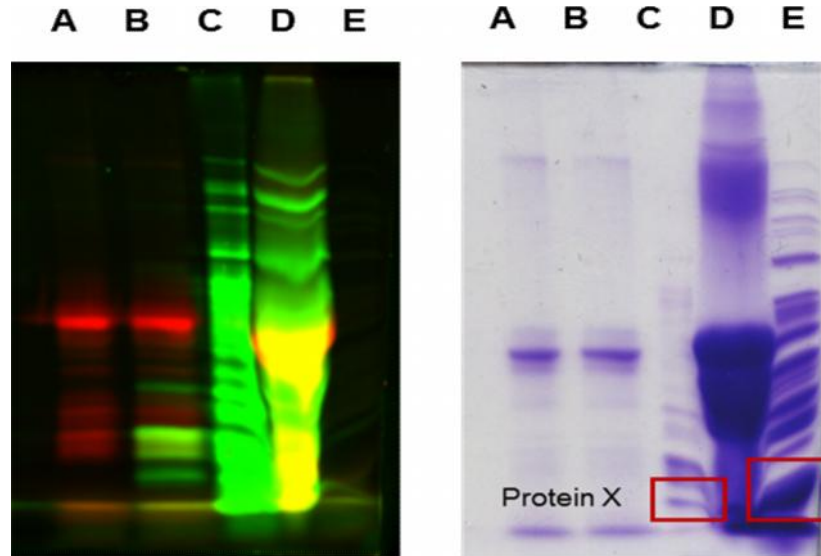


Figure S1. Analysis of Co-IP assays (two-color fluorescent SDS-PAGE, left vs. Coomassie blue-stained SDS-PAGE, right). A: The proteins eluted from the HT5 conjugated streptavidin (SA) beads interacted with a mixture of Thr-Cy5 and Roche BM blocking reagent. B: The proteins eluted from the HT5 conjugated SA beads interacted with a mixture of Thr-Cy5, Frac 1A-Cy3, and BM blocking reagent. C: Frac 1A-Cy3. D: The unbound proteins in assay B. E: Concentrated Frac 1A (without labeling for isolation of thrombin associated protein (protein X) and subsequent protein digestion/MALDI-TOF MS analysis).

MS ID of Protein X = Dps

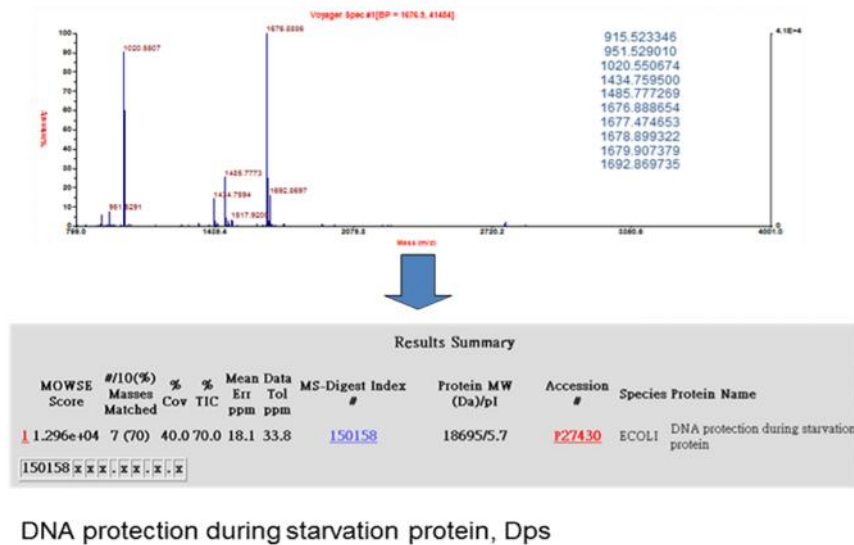


Figure S2. MALDI-TOF MS analysis of thrombin-associated protein, protein X, in line E in Figure S1. Protein X is identified as an *E. coli* DNA protection during starvation protein (Dps) as well as a DNA binding protein.

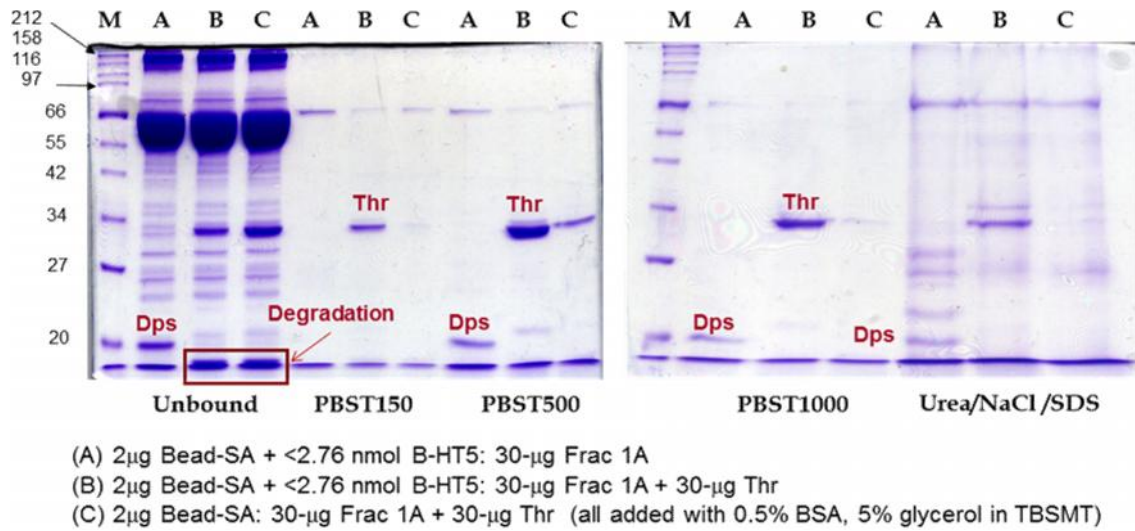


Figure S3. Coomassie blue-stained SDS-PAGE analysis for a series of HT5 Co-IP assays using non-labeled protein samples. The assay conditions are summarized below the gel images, where B-HT5 is 5' biotinylated HT5 and TBSMT means TBSM plus Tween 20. The beads after protein interaction and brief washes were sequentially eluted with PBST solutions containing 150 mM NaCl, 500 mM NaCl, and 1000 mM NaCl, respectively, and finally with a mixture of urea, NaCl and SDS.

S5. On-Microarray Aptamer Competition against the Protein Complex

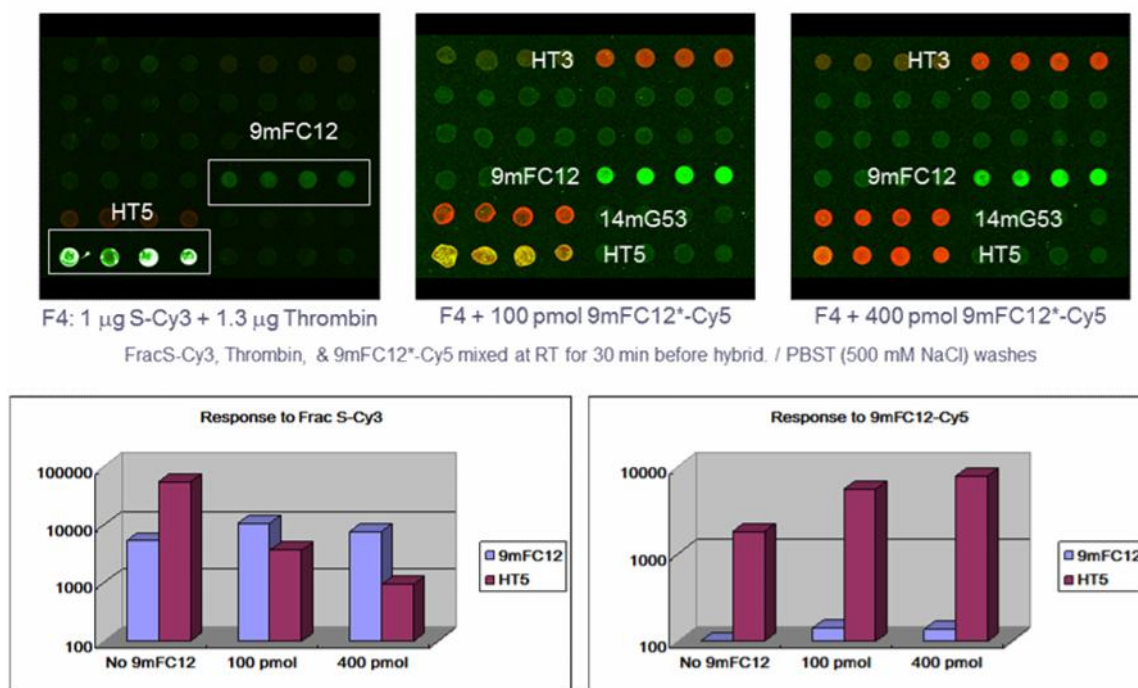


Figure S4 The aptamer microarray assayed with 1 µg Frac S-Cy3 and 1.3 µg Thr + different amount of 9mFC12-Cy5 in 20 µl. The assay conditions were the same as those of Fig. 6 except that (i) Thr was not labeled and (ii) 9mFC12 was labeled with Cy5 instead.



Figure S5 Multiple DNA sequence alignment analysis for 9mFC12 and its complimentary pairings to HT5, HT3, and 14mG53. 9mFC12-R represents the complimentary sequence of 9mFC12. All of the sequences are listed from 5' to 3' (from left to right).

S6. An Interaction Network Disclosed by the Aptamer Microarray Assay

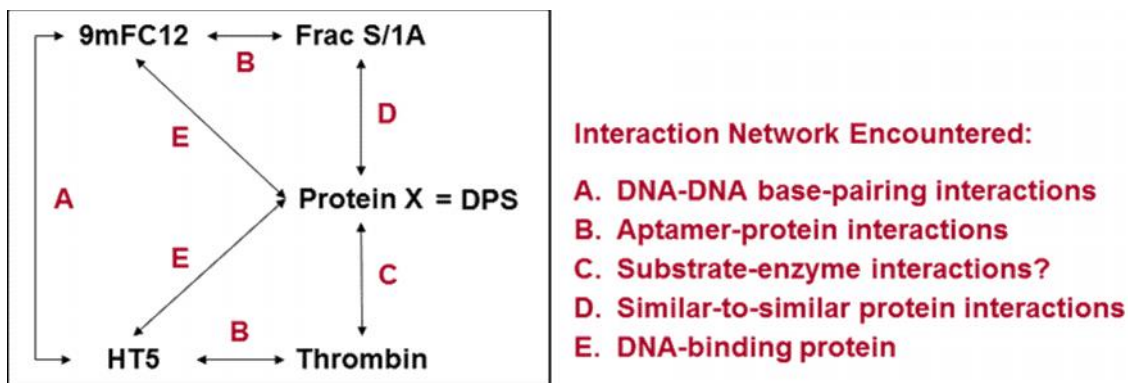


Figure S6 A proposed interaction network disclosed in this work. (Protein X represents the thrombin-targeting protein in Frac S or Frac 1A.) A further study to confirm if the PPI is a substrate-enzyme interaction is required.