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Cover letter

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May 10th, 2018

Dear Dr. Myers, Science Editor, JoVE

Following up our conversation on April 10th and after receiving your invitation to publish a full article, we are pleased to submit our manuscript entitled: “Extracellular Protein Microarray Technology for high throughput identification of low affinity receptor-ligand interactions” by Husain et al., to be considered for publication in JoVE.

Secreted factors and plasma membrane-expressed proteins (collectively referred to as extracellular proteins) essentially regulate multiple physiological and pathological processes, and therefore represent main targets for therapeutic development. In most cases, these proteins exert their functions through interaction with other proteins in the extracellular space. Nevertheless, despite their importance and abundance (more than 5,000 proteins in the human genome), the protein-protein interactions that take place in the extracellular environment remain poorly understood. This gap is mainly due to the challenges associated with the study extracellular proteins, and in particular detection of binding partners, which often establish low affinity interactions that are difficult to detect using common methodologies. We have previously developed the Protein Microarray Technology, which coupled to an extensive library of purified proteins, allows high throughput interrogation of binding partners for targets of interest. Furthermore, we have implemented a method based on increased protein multimerization using microbeads, an approach that has been shown to significantly improve detection of transient interactions, such as the ones often established between cell surface receptors. This technology has proven key for identification of novel interacting partners, which have opened new avenues to study the basic biology of multiple extracellular targets, from nervous cell receptors to viral immunomodulators. In this manuscript, we provide a detailed description of the methodology developed, from selection of protein candidates, to microarray slide printing, hybridization assays as well as data analysis for hit calling. Key elements for success, advantages over other methods and potential pitfalls are also discussed. This approach represents a robust, sensitive and high throughput method for binding partner discovery, focused on extracellular proteins, one the most challenging protein types.

Given the applicability of this technology to the elucidation binding partners for any extracellular protein under study, we are confident the experimental procedures described here will be of interest to a broad scientific audience, including Cell Biologists, Structural Biologists, Biochemists and any Scientists alike.

Thank you in advance for considering this manuscript for publication in *JoVE.*

Sincerely,

Nadia Martinez-Martin, Scientist, Receptor Discovery Group, Genentech, Inc.

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TITLE

Extracellular Protein Microarray Technology for High Throughput Detection of Low Affinity Receptor-Ligand Interactions

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KEYWORDS

Protein microarray technology, cell surface, receptor, extracellular protein, protein-protein interactions, library, low affinity, receptor discovery, multimerization

SUMMARY

Here, we present a protocol to screen extracellular protein microarrays for identification of novel receptor-ligand interactions in high throughput. We also describe a method to enhance detection of transient protein-protein interactions by using protein-microbead complexes.

ABSTRACT

Secreted factors, membrane-tethered receptors, and their interacting partners are main regulators of cellular communication and initiation of signaling cascades during homeostasis and disease, and as such represent prime therapeutic targets. Despite their relevance, these interaction networks remain significantly underrepresented in current databases; therefore, most extracellular proteins have no documented binding partner. This discrepancy is primarily due to the challenges associated with the study of the extracellular proteins, including expression of functional proteins, and the weak, low affinity, protein interactions often established between cell surface receptors. The purpose of this method is to describe the printing of a library of extracellular proteins in a microarray format for screening of protein-protein interactions. To enable detection of weak interactions, a method based on multimerization of the query protein under study is described. Coupled to this microbead-based multimerization approach for increased multivalency, the protein microarray allows robust detection of transient protein-protein interactions in high throughput. This method offers a rapid and low sample consuming-

approach for identification of new interactions applicable to any extracellular protein. Protein microarray printing and screening protocol are described. This technology will be useful for investigators seeking a robust method for discovery of protein interactions in the extracellular space.

INTRODUCTION

The method reviewed here describes the printing of a collection of extracellular proteins in a microarray format, followed by a method for screening of a target of interest against this library. We have identified protein multimerization as a crucial step for detection of interactions characterized by low binding affinities. To enhance detection of these interactions, we describe a protocol based on multimerization of the query protein of interest using microbeads.

Secreted and cell surface-expressed proteins (collectively termed extracellular proteins) along with their interacting partners are key regulators of cellular communication, signaling and interaction with the microenvironment. They are, therefore, essential in regulating many physiological and pathological processes. Approximately a quarter of the human genome (≈5,000 proteins) encodes for extracellular proteins, which, given their significance and accessibility to systematically delivered drugs, represent key targets for drug development[1]. Consequently, extracellular proteins represent more than 70% of the protein targets with known pharmacological action for approved drugs on the market, known as the "druggable proteome". Despite their importance and abundance, the extracellular protein-protein interaction (ePPI) networks remain remarkably underrepresented in the available databases. This is fundamentally due to the complex biochemical nature of the extracellular proteins, which precludes their characterization using most available technologies[2]. Firstly, membrane proteins are difficult to solubilize, a process that often involves harsh washing conditions and detergents; secondly, extracellular proteins often lack relevant post-translational modifications such as glycosylation that are absent when these proteins are expressed in commonly used heterologous systems. Finally, interactions between receptors, such as co-receptors expressed on immune cells, are often transient and characterized by very low affinities (K_D in the ~1 µM to >100 µM range). Altogether, the nature of these proteins and their binding partners render most widely utilized technologies, such as affinity purification/mass spectrometry (AP/MS) or yeast-two-hybrid, unsuitable for detection of interactions in the extracellular space[2,3].

In an effort to overcome these technical challenges and accelerate the discovery of novel interactions for extracellular proteins, we have developed a high coverage extracellular protein microarray[4,5]. Microarrays offer the advantage of generating high-density surfaces with small amounts of sample, and are generally amenable to high throughput studies. Protein microarray-based studies have previously provided relevant insights into protein interactions for several model organisms, albeit mainly focusing on cytosolic interactions or on specific protein families[6-8]. In contrast, limited work has been done to investigate extracellular protein interactions using this technology. We have developed a protein microarray method to enable studies of ePPIs by building a comprehensive and highly diverse library of purified secreted proteins and single transmembrane (STM) receptors expressed as recombinant extracellular domains (ECD) fused to

common tags for affinity purification[4]. The success of the protein microarray screens relies heavily on the establishment of a high quality protein library. For expression of both the library and query protein, mammalian cells or insect cells were preferentially chosen as heterologous expression systems, to ensure proper addition of post-translational modifications such as glycosylation or disulphide bonds. SDS-PAGE, size exclusion chromatography and multi-angle laser light scattering are techniques commonly utilized to assess recombinant protein quality. The protein library is then spotted onto epoxysilane slides and stored at -20 °C for long-term use. Protein concentrations above of 0.4 mg/mL are recommended for the protocol described below. Therefore, low-expressing proteins may require a concentration step prior to sample printing and storage. Nevertheless, a main advantage of this technique is the small volume of protein required (50 µg of protein is sufficient to perform >2,000 screens), alongside minimal query protein consumption (20-25 µg per duplicates screen). Using the protocol and equipment described here, and provided libraries are available, results for individual query proteins can be generated within one working day.

A major challenge in detecting protein interactions in the extracellular environment arises from their characteristically weak or transient nature, which precludes identification by most commonly used methodologies. Increasing the binding avidity greatly improves sensitivity for detection of weak protein interactions[9-11]. Based on this principle we developed a method to multimerize the query proteins (expressed as Fc fusion) using protein A-coated beads[4,5]. To avoid any potential inactivation of the query protein by random labeling, we instead label an irrelevant human immunoglobulin G with Cy5 and add it along with the query protein to the protein A beads, thus eliminating any artifacts due to the direct conjugation of a dye to the protein of interest. Given the micromolar affinities of several co-receptor pairs, the multivalent complexes greatly enhance signal to noise ratio, compared to Fc-fusion query proteins screened as soluble proteins[4].

In summary, the goal of this protocol is to describe the preparation of microarray slides containing a pre-existing extracellular protein library for identification of receptor-ligand interactions. We review the steps for slide printing, followed by a protocol for screening of a protein of interest against the extracellular protein library. Moreover, we describe a method for enhanced detection of ePPIs based on microbeads to achieve increased avidity of the protein under study. The extracellular protein microarray technology described here represents a fast, robust and effective approach for screening and detecting novel ePPI with low false-positive ratios, and by utilizing only microgram quantities of the query protein under investigation. This technology has fueled multiple studies that have provided relevant insights into previously unknown cellular functions and signaling pathways for a variety of receptors[12,13], including viral immunoregulators[14], and can be utilized to de-orphanize any extracellular protein of interest.

PROTOCOL

1. Generation of a Library of Extracellular Human Proteins

1.1. Compile a list of cell surface receptors or secreted proteins of interest to build the protein microarray library. Specific protein families (for example, the immunoglobulin superfamily) or proteins selectively expressed in particular cell types can be selected for the study.

1.2. For cell surface receptors, determine the extracellular domain (ECD) boundaries by identifying the signal peptide and transmembrane regions using software tools. Some of the relevant tools, freely available online, are referenced in the **Table of Materials**[15-18].

1.3. Synthesize the ECD for the genes of interest and clone into the relevant vector. Secreted proteins or the ECD of STM receptors fused to a number of common affinity tags can be purified from the conditioned media of cells transfected with the appropriate vector, or from baculovirus-insect cell expression systems.

Note: Mammalian cell-based systems (such as HEK/293 or CHO cells) are recommended to maximize the likelihood of proper protein folding and addition of relevant posttranslational modifications such as glycosylation.

1.4. Purify the proteins by standard affinity purification methods. Previous efforts have described automated or semi-automated procedures suitable for purification of hundreds of proteins, which can be scaled to generate the set of proteins of interest[9,19-21].

Note: SDS-PAGE, size exclusion chromatography (S), or multi-angle laser light scattering (MALLS) are recommended to assess any non-covalent aggregation and to control for the overall quality of the protein preparations.

1.5. Adjust proteins to 200 or 400 µg/mL whenever possible, and dilute stocks with 80% glycerol for long-term storage in cryogenic vials at -20 °C. These represent master stocks and should be accessed only when necessary.

1.6. Transfer aliquots of each protein (100 µL) to 96-well plates (stock plates), seal with adhesive foil, and store at -20 °C until microarray slide preparation. Stock plates are generated to minimize freeze-thaw cycles and ensure protein stability.

2. Extracellular Protein Microarray Printing

2.1. Use a standard microarrayer for slide printing. The instrument utilized for the protocol described here has a capacity of 57 slides/run and uses a printhead holding 48 spotting pins. These pins generate spots of ~100 µm diameter separated by a spot-to-spot distance of ~350 µm and draws 0.25 µL per load. Under this configuration, up to 8000 spots per slide can be accommodated.

2.2. Generate working plates (384 well plates, 10 µL sample/well) from the stock plates. Perform this step manually prior to slide printing using the microarrayer. Then, spot proteins with quill-

type spotting pins onto epoxysilane slides at 60% humidity (to prevent dehydration of the protein spots).

Note: Cy3-labeled Bovine serum albumin (BSA) can be spotted in duplicates between each protein sample (5 µg/mL in PBS/40% glycerol) to visualize the array for mask fitting (see section 4). Although recommended, this step is optional.

2.3. Subsequent to printing, remove the protein microarray slides from the humidified environment and block them overnight with 5% milk in PBST to inactivate the surface.

Note: The preferred approach is to use an ultrasonic fogger to generate a fine mist of blocking solution that settles onto the slide surface.

2.4. Store slides at -20 °C in 50% glycerol to prevent freezing.

3. Preparation of Multivalent Bait Complexes

Note: Interactions between extracellular proteins are often characterized by low affinities. To enable detection of these interactions by increasing binding avidity, a multivalent approach based on capturing the query protein, expressed as Fc-tagged ECD, on protein A-coated microbeads was developed[4].

3.1. Label the carrier IgG used for detection with Cy5 monoreactive dye and separate the free dye using desalting columns. Determine the dye to protein ratios by measuring ultraviolet absorbance at 280 and 650 nm.

Note: Protein-dye ratios between 2.0 and 4.0 are normally used. It is recommended to spin the Cy5 conjugates at 100,000 x g for 15 min to remove potential soluble aggregates due to the labeling process.

3.2. Determine the optimal microbead-to-protein ratio by titration of protein A against a constant amount of the query protein. The minimal saturating volume of beads where no free Fc-tagged protein remains, as measured using a competitive assay determined by biolayer interferometry, is used for the screening.

3.3. Form the microbead-protein complexes by incubating the Fc-tagged query and the Cy5-IgG with protein A microbeads and mix on a tube rotator in PBS for 30 min at room temperature protected from light.

3.4. To form these complexes, use the query protein at a final concentration of 20 µg/mL. To calculate the molar ratio of the query protein and Cy5-IgG, divide the molecular weight of the query protein by the molecular weight of the IgG (150,000 Da) and multiply by 40 µg/mL to determine the concentration of Cy5-IgG needed.

3.5. Supplement samples with soluble protein A (1 mg/mL) immediately prior to incubation with the microarray slides (see 4.2 section below) to prevent binding of any free protein A beads to the Fc fusion proteins that may be present on the array.

Note: The final reaction volume may vary depending on the hybridization station or incubation chamber utilized. The instrumentation described in the **Table of Materials** allows sample incubation using relatively small volumes (~200 µL per slide).

4. Extracellular Protein Microarray Screening and Processing.

Note: There are a number of manufacturers that provide automatic processing platforms. If a hybridization station is not available, the following steps can be performed manually, ensuring that there is sufficient volume of buffer to keep the slides submerged at all times.

4.1. Warm the slides at room temperature and rinse with PBS/0.1% Tween 20 (PBST) to remove residual glycerol before loading onto the hybridization station.

4.2. Use the following screening protocol:

4.2.1. Wash with PBST for 1 min.

4.2.2. Load 200 µL of 1 mg/mL protein A in PBS/5% milk and incubate for 30 min to prevent uncomplexed protein A microbeads from binding to Fc-tagged proteins that may be present in the microarray.

4.2.3. Wash five times with PBST for 1 min.

4.2.4. Load 200 µL of the query:microbeads complex in the presence of 1 mg/mL protein A and incubate for 30 min.

4.2.5. Wash with PBST for 1 min.

4.3. After hybridization, rinse slides with water, place in individual 50-mL conical tubes, and dry by spinning at 900 x g for 5 min.

4.4. Finally, scan the slides with a microarray scanner appropriate to detect Cy3 (if BSA-Cy3 has been printed) and Cy5 fluorescence by exciting at 532 and 635 nm, respectively.

4.5. Perform data analysis using the accompanying microarray data analysis. The relevant array list (as a .gal file) is loaded into the data extraction software. At this stage, utilize the Cy3-BSA spots to find blocks and use the auto-fitting options in the software, followed by manual alignment of the features when necessary. Save files as .gpr files for further analysis.

5. Data Analysis

5.1. Save data as GPR files and process in R using the limma package, commonly utilized for analysis of microarray data[4,5].

5.1.1. For preprocessing, do background correction and within-slide normalization. Since each protein is printed in duplicated spots, combine both replicate measurements to create a single score for that protein in the library.

5.1.2. Calculate scores for each slide and analyze results for the intersection of high-scoring candidates between slides.

5.1.3. Use duplicate microarrays from separate print-runs to control for slide variability and use intersection plots representing data from both screens to call final hits.

5.1.4. Finally, perform an additional level of filtering to exclude promiscuous proteins within the array. Such non-specific binders are identified as proteins exceeding a specific threshold hit frequency as defined by the user across independent screens.

Note: This protocol is described in detail in Tom *et al.* 2013[5]. In our case the non-specific binder calling is based on the determination of the cumulative prey hit rate, and a data-driven elimination threshold of 5% is used.

REPRESENTATIVE RESULTS

A schematic of the workflow for the extracellular protein microarray technology is shown in **Figure 1**. Once the microarray slides containing the extracellular protein library are available, the screening of the protein of interest and data analysis can be completed within one day. Many physiologically relevant interactions between membrane-embedded receptors are characterized by very weak binding strengths (K_D in the micromolar range). To improve detection of such interactions, a method based on query protein (expressed as Fc fusion) multimerization on protein A-microbeads was developed. This protocol is described in the text, and a representative example of the performance of this approach for enhanced detection of PPI is shown in **Figure 2**.

A representative example of a protein microarray screen is presented in **Figure 3**. In the example shown, an orphan human adenovirus immunomodulatory protein is screened for interactions against a library consisting of more than 1,500 receptor ECD or secreted proteins[14.] As described, the ECD of the viral protein is recombinantly expressed as an Fc fusion protein, and then incubated with protein A-coated microbeads in order to form multivalent complexes. The protein complexes were screened using a hybridization station to minimize manual operations, however, similar screens can be performed using a hybridization chamber or similar devices. It is advised to run duplicate screens using slides printed in separate spotting runs in order to control for any variability during the print run. The figure shows the resultant intersection plots from duplicate, independent screens. Positive interactions and overall slide background are readily visualized upon scanning of the plates (Cy5 fluorescence), facilitating hit calling. Note that it is

recommended to spot each receptor in the library in duplicates so as to increase hit confidence. For most query proteins, none, one, or few hits are observed, demonstrating the specificity of the method for detection of PPIs.

Certain query proteins show high background as detected by binding to an unusually high number of proteins within the array and/or to the slide. In our experience, such non-specific background is observed for only a small number of proteins. Different factors relating to the nature of the protein could influence non-specific interactions, such as recognition of glycosylation motifs. **Figure 3** shows an example of a viral protein screened against the microarray library that showed high background, precluding identification of specific hits. While minor modifications in the protocol can be considered to decrease background (such as increased salt or detergent concentration), in these instances it is recommended to explore alternative methods for deorphanization of the protein under study.

FIGURE LEGENDS

Figure 1. **Extracellular protein microarrays for rapid and robust identification of receptor-ligand interactions**. (Step 1) A library of extracellular proteins of interest is compiled. (Step 2) The purified proteins are printed on epoxysilane slides using a microarray printer and stored at -20 °C. (Step 3) The query protein of interest is multimerized on microbeads for increased avidity and detection of transient protein-protein interactions. A fluorescent IgG is complexed with the query:microbeads as inert labeled carrier. (Step 4) Screening for binding partners of a query protein. The fluorescent query protein complex is screened against the extracellular protein microarray using a hybridization station for automated slide processing. (Step 5) Slides are then visualized using a microarray scanner. The BSA-Cy3 spots can be observed in the 535 nm channel and the hits yielded from the screen can be observed as duplicate spots at 635 nm. Data analysis for hit calling is performed by creating intersection plots of two independent experiments. Hits are called based on mutually high scores above background, as described in the protocol section.

Figure 2. Enhanced signal through formation of multivalent complexes for the query protein. (A) Multimerization of query proteins**.** Query proteins are expressed as recombinant Fc fusions and coupled to protein A microbeads to form multimerized complexes. **(B)** High-avidity leads to detection of low affinity interactions. The avidity afforded by the multimerized query protein results in the stabilization of weak interactions on the protein microarray, leading to higher signal to noise ratios and enhanced hit calling. Plots shows screening results for CD200, screened as a microbead complex, or a soluble protein directly labeled with Cy5 dye to enable visualization of the hits. Multimerization (red bars) allows robust detection of the low affinity binding partner CD200R1, present in the protein microarray library, with significant signal/noise ratio in comparison with the same protein screened as a soluble product (blue bars). Grey bars indicate non-specific binders. Only the top 10 interactions have been represented in the plot.

Figure 3. **Protein microarray Technology for identification of novel receptor-receptor virus-host interactions. (A)** Exemplary receptor discovery results for an orphan adenoviral immunomodulatory protein. Images represent actual microarray scans, showing hits from

proteins spotted in duplicates (red). Assays are performed in duplicates to control for any slide variability, and results represented as intersection plots. In the plots, the red and blue dots represent hits called only on one array replicate, whereas black dots represent intersecting hits from both independent runs. The entire microarray library is printed on two slides for ease of use given the number of proteins present in the collection, and to ensure enough separation for each individual protein spotted on the array. **(B)** Viral protein screen exhibiting high background that precludes hit calling. Certain query proteins may display high background, as detected by binding to multiple proteins and/or the slides, therefore posing challenges for identification of high scoring hits.

DISCUSSION

A significant number of orphan receptors remain in the human genome, and novel interacting partners continue to emerge for extracellular proteins with previously characterized ligands. Defining the receptor-ligand interactions in human and model organisms is essential to understand the mechanisms that dictate cellular communication during homeostasis, as well as dysregulation leading to disease, and therefore inform new or improved therapeutic options. Nevertheless, detection of extracellular protein interactions by widely used technologies has represented a significant barrier mainly due to the technical challenges associated to the biochemistry of the cellular receptors and their interacting partners. In this report we describe the use of an extracellular protein microarray for screening of query proteins of interest, with emphasis on utilization of microbead complexes for enhanced detection of PPIs.

Probing receptor-receptor interactions is particularly challenging, as many physiologically relevant pairs are characterized by very weak interaction with binding affinities in the micromolar range[2,3]. Avidity enhancement through multimerization has proven to be a useful strategy to increase sensitivity for detection of low affinity PPIs. We have developed a method based on Fc fusion proteins, for efficient formation of multivalent microbead-protein query complexes[4]. Multimerization of the query protein is a key step to detecting low affinity PPIs. As shown in the example in **Figure 2**, this method significantly enhances signal in comparison with the same query protein screened as a soluble (non-complexed) analyte, while retaining minimal background. Alternatively, the protein microarray screens can be performed with soluble Cy5-labeled query protein, an approach that is compatible with any fusion tag and that may suffice for identification of more stable, higher affinity interactions, such as those between some soluble ligands and their receptors. It should be noted that because the avidity is increased through multimerization of the query on microbeads, the microarray signal is not a quantitative measure of the interaction strength. Rather, binding affinities should be calculated with the monomeric versions of the proteins under evaluation. In addition, it is highly advisable that any hits identified through the microarray technology are validated through independent methods. We routinely use surface plasmon resonance as a gold standard biophysical method for PPI studies and measurement of the binding kinetics.

Although out of the scope of this report, it should be noted that the success of any screening efforts using the microarray technology heavily relies on selection and availability of a

high quality protein library. Relevant criteria for selection of extracellular proteins have been previously published[4,22]. A number of useful tools for prediction of relevant protein features (such as transmembrane helices or signal peptides) are freely available online, including the following servers: SignalP[15], TMHMM[16], Phobious[17], and TOPCONS[18]. Excellent methods papers describing affinity purification methods are available elsewhere. It should also be mentioned that the microarray protein library generation relies on the production of protein ECDs as soluble recombinant products, and therefore this approach allows studies of type I, type II and GPI-anchored cell surface receptors and secreted proteins, but is generally not suitable for multitransmembrane-containing proteins such as G protein-coupled receptors. The purification of hundreds of proteins as recombinant soluble proteins demands significant logistic efforts and can be very resource- and time-consuming. Nevertheless, with the decreased costs of gene synthesis and increased throughput of the protein purification systems, library generation is now relatively faster and more affordable. Alternatively, commercial sources offer purified extracellular proteins that can be purchased in microgram amounts, which suffice for generation of a durable protein microarray library given the small requirements for slide printing. These limitations notwithstanding, microarray technology offers great advantages such as minimal consumption of protein reagents, fast readouts (new PPIs can be identified within one day) and affordable instrumentation. In addition, once the constructs and purification conditions have been established, small-scale purifications can produce enough material to prepare thousands of arrays.

Finally, in some instances, relatively high background is observed, appearing as binding to many proteins on the microarray library. There are multiple factors that might influence non-specific binding. For example, some proteins may be highly charged, or interact with carbohydrates, accounting for non-specific background via recognition of common motifs. Similarly, non-specific binding may also be due to the nature of the query protein, such as for example recognition of sialic acid motifs, found in multiple other proteins. As the microarray libraries expand, it is more likely that that a common list of non-specific binders might be present. Such non-specific binders can be identified by tracking their behavior across various screens against unrelated query proteins. It is advisable to generate a list of non-specific binders across screens, to improve specific hit calling. In our case, we have found that applying a 5% cutoff (*i.e.,* a protein is flagged as non-specific if detected as binder is 5% or more of unrelated query protein screens) is important to reduce false positives. It should be noted that if the main purpose of the assays is to run only a few selected screens, it may not be worthwhile to develop a statistical analysis. In this case, it may not be feasible to detect false positives due to a limited dataset, and therefore we recommend that special emphasis is placed on confirmation of the hits using alternative methods.

We anticipate the extracellular protein microarrays, especially in combination with multimerization methods for detection of transient receptor-ligand interactions, will continue to offer a unique platform for rapid and robust identification of novel PPI in the extracellular space.

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DISCLOSURES

B.H., S.R-R and N.M-M. are Genentech employees and own shares in the Genentech Inc./Roche group.

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Figure 1

Click here to access/download;Figure;Husain_Fig1_JoVE_2018.tiff

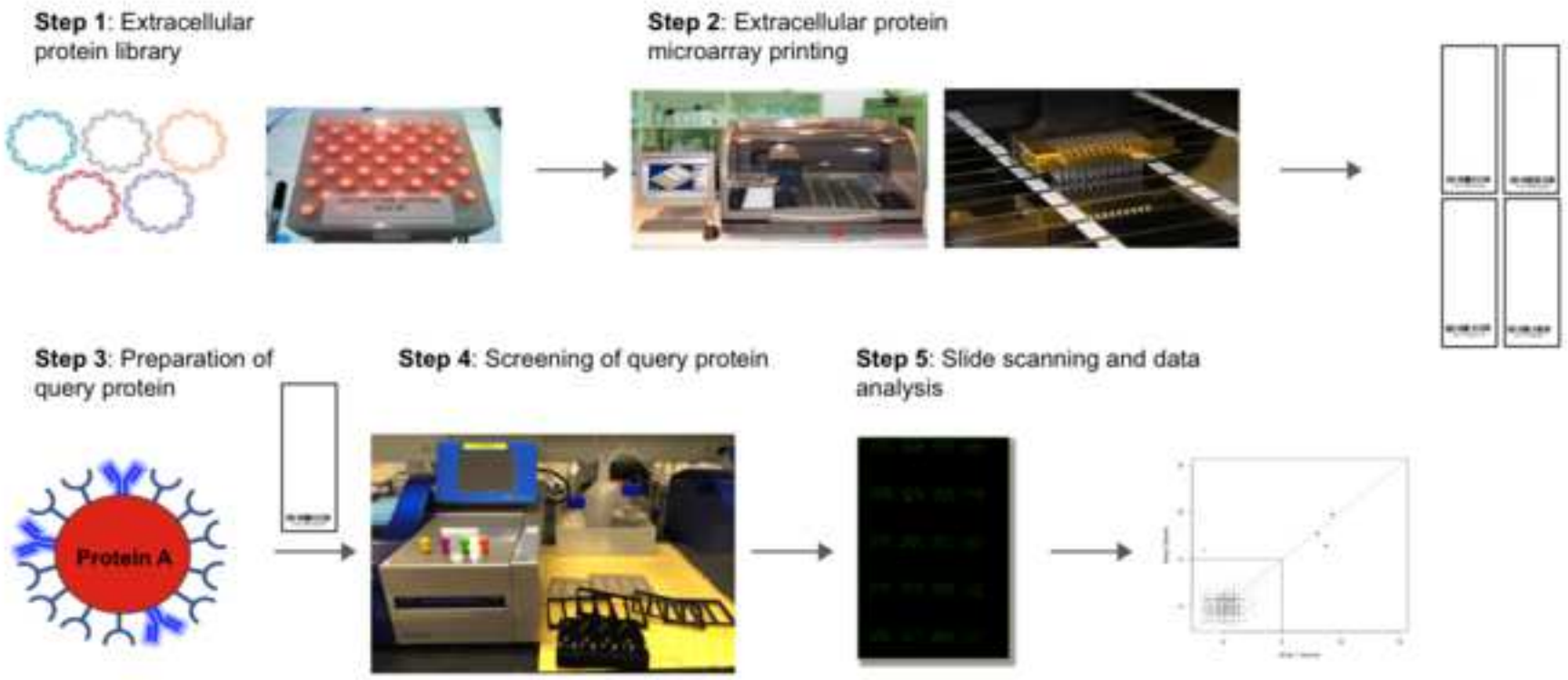


Figure 2

Click here to access/download;Figure;Husain_Fig2_JoVE_2018.tiff

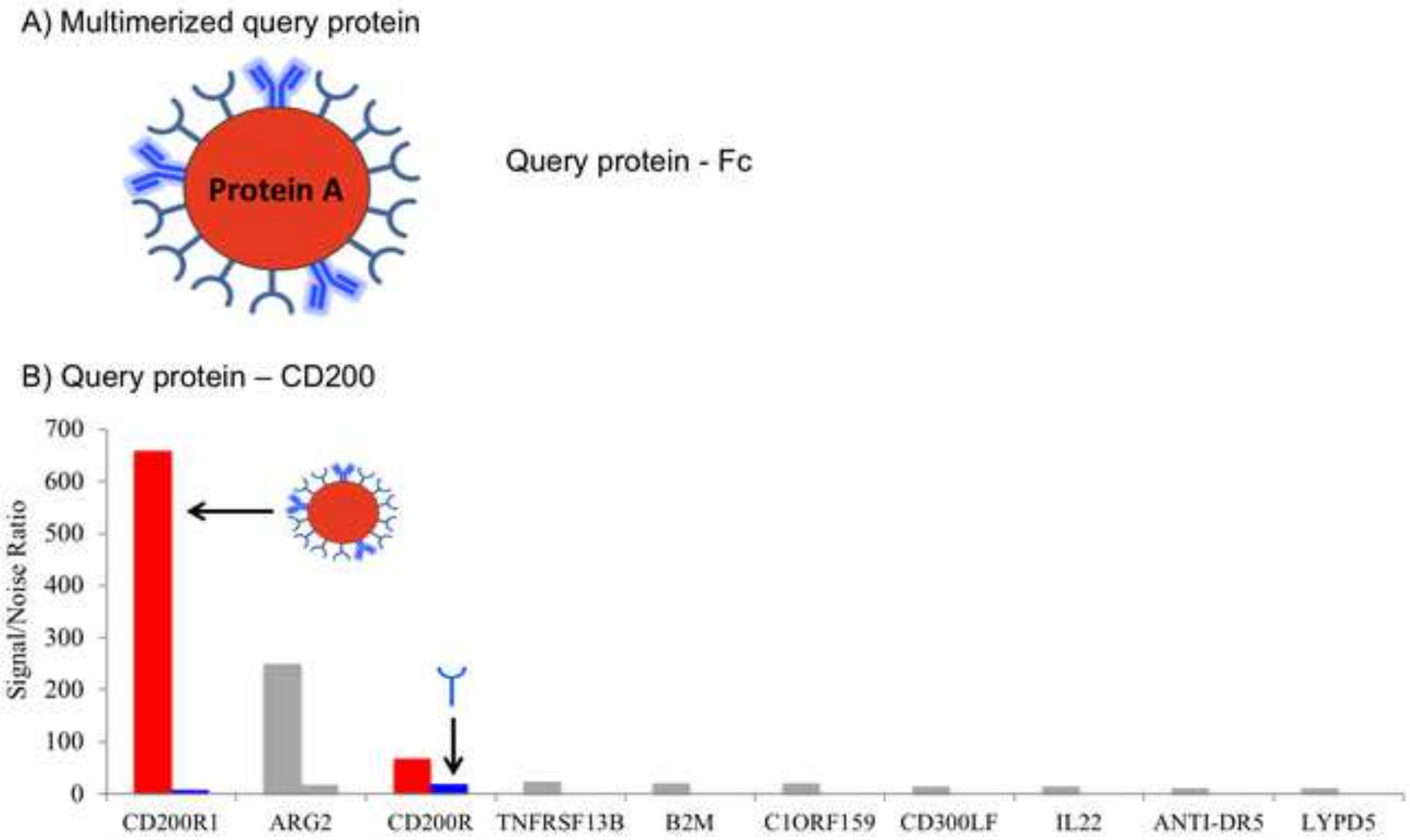
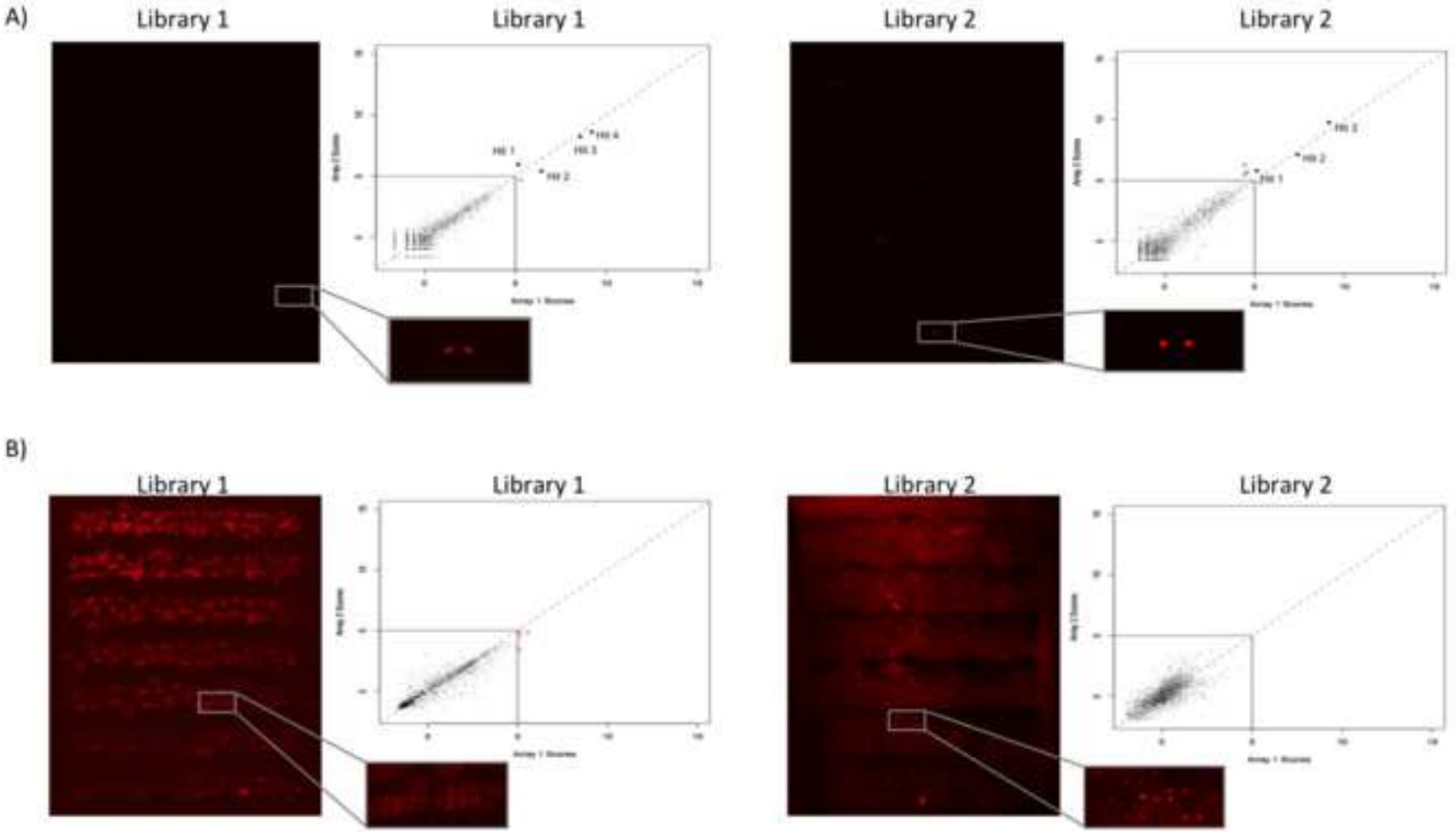


Figure 3

Click here to access/download;Figure;Husain_Fig3_JoVE_2018.tiff



Click here to access/download;Table of Materials;JoVE58541_Table_revised2.xlsx

Name of Material/ Equipment
Ultra Pure MB Grade glycerol
SeptoMark blocking buffer
Bovine serum albumin
Polypropylene multiwell plates
Polypropylene multiwell plates
NanoPrint LM60, or similar contact microarrayer
Micro spotting pins
ZeptoFOG blocking station
Skim milk powder
Epoxysilane-coated glass slide
Glass holder and slide rack set
Cy5 monoreactive dye
Cy5 monoreactive dye
Pro-spin desalting column
Adhesive aluminum foil seal
Polypropylene cryogenic vials
Protein A microbeads
Human IgG
Protein A
Hybridization station, a-Hyb or similar
GenePix 4000B scanner or similar
GenePix Pro or equivalent data extraction software
Signal P4.1
TMHMM 2.0 server
Phobius
TOPCONS

Company
USB Corporation
Zeptosens
Roche
Greiner Bio One
Arrayit
Arrayit
Arrayit
Zeptosens
Thermo Fisher
Nextrion Slide E
Wheaton
GE Healthcare
GE Healthcare
Princeton Separations
AlumaSeal
Corning
Miltenyi
Jackson Immunoresearch
Sigma
Miltenyi
Molecular Devices
Molecular Devices
DTU Bioinformatics, Technical University of Denmark
DTU Bioinformatics, Technical University of Denmark
Stockholm Bioinformatics Center
Stockholm University

	Catalog Number
56-81-5	
BB1, 90-40	
03-117-957-001	
82050-678	
MMP384	
NanoPrint LM60, or similar contact microarrayer	
Micro spotting pins	
ZeptoFOG blocking station, 1210	
LP0031	
1064016	
900303	
PA23031	
PA25001	
CS-800	
F-384-100	
430658	
120-000-396	
009-000-003	
P7837	
Hybridization station, a-Hyb or similar	
GenePix 4000B scanner or similar	
GenePix Pro or equivalent data extraction software	
online software	
online software	
online software	
online software	

Comments/Description

Protein storage
Blocking buffer microarray slides
Slide control for mask fitting (optional)
Protein storage
Slide printing
Slide printing
Slide printing
Block slides after printing
Blocking solution
Microarray slides
Slide storage
Albumin labeling
Human IgG labeling
Remove free dye
Seal stock plates
Master vials for protein library storage
Query protein multimerization
Irrelevant IgG for labeling
Microarray slide blocking
Automated microarray processing (optional)
Slide scanning
Data processing
Prediction tool to determine presence and location of signal peptide cleavage sites
Prediction of transmembrane helices in proteins
A combined transmembrane topology and signal peptide predictor
Prediction of membrane topology and signal peptides

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Title of Article: **Extracellular Protein Microarray Technology for high throughput identification of low affinity receptor-ligand interactions**

Author(s): **Bushra Husain, Sairupa Paduchuri, Sree R. Ramani and Nadia Martinez-Martin**

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CORRESPONDING AUTHOR:

Name:	**Nadia Martinez-Martin**
Department:	**Microchemistry, Proteomics and Lipidomics**
Institution:	**Genentech**
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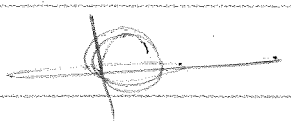
Signature:	 Date: 05.10.18

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Rebuttal Letter

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July 18th, 2018

Dear Dr. Steindel,

Please find attached our manuscript entitled “Extracellular Protein Microarray Technology for high throughput detection of low affinity receptor-ligand interactions”, which has been revised following the Reviewers suggestions and Editorial comments.

We hope you will now find the manuscript suitable for publication in JoVE.

Sincerely,

Nadia Martinez-Martin

Scientist, Genentech

Dear Dr. Martinez-Martin,

Your manuscript, JoVE58451 Extracellular Protein Microarray Technology for high throughput detection of low affinity receptor-ligand interactions, has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300 dpi.

Your revision is due by **Jul 11, 2018**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision".

Best,

Phillip Steindel, Ph.D.
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_____

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We apologize for the typographical errors, which we have corrected.

2. Please provide an email address for each author.

Response: Please find the contact information below. The email address of the corresponding author is also listed in the manuscript.

Corresponding Author:
Nadia Martinez-Martin
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Co-authors:
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Sairupa Paduchuri (paduchus@gene.com)
Sree R. Ramani (ramani.sree@gene.com)

3. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

Response: The short abstract (page 1, line 17) has been edited following the Editor's suggestions.

4. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

Response: The Abstract (page 2, line 32) and Introduction (page 2, line 46) have been modified to more clearly state the goals of the methods presented.

5. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Response: The text has been revised, units are now shown using SI abbreviations.

6. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Response: The text has been revised, and a space included between numbers and their corresponding units.

7. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Signal P4.1, TMHMM 2.0 servers, Phobius, NanoPrint LM60, Arrayit, etc.

Response: Commercial language has been removed from the main text. The relevant items are now referenced in the Table of Materials and Reagents.

8. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: The text has been revised and edited to avoid the use of personal pronouns.

9. Please revise the protocol so that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Response: The text has been modified following the Editor's suggestions.

10. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Response: The protocol has been simplified, avoiding large paragraphs between sections and shortening individual steps.

11. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: The text that describes essential steps for the video has been highlighted in blue font, including Sections 2, 3 and 4.

12. Discussion: Please also discuss critical steps within the protocol.

Response: Critical steps are reviewed in the Discussion, including: Multimerization for detection of challenging interactions (page 13, line 365); advantages and alternatives for labeling of query protein (page 13, line 372); note for quantitation of binding affinities (page 14, 375); incompatibilities with certain protein types (page 14, line 391); background due to non-specific binders (page 15, line 409), etc.

Critical steps and representative examples are also illustrated in Figures 2 and 3.

Following the Editor suggestions and in response to Reviewer 1 comments, we have further emphasized in the text the scope of the current methods paper, which is focused on the array printing and screening, rather on the generation of the protein library itself (page 2, line 31; page 2, line 46; page 5, line 116; page 14, line 384; page 14, line 400). Following Reviewer 2 suggestions we have added references for the relevant prediction tools (page 14, line 389).

13. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

14. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

15. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

Response: References have been edited as per the Editor instructions.

Reviewers' comments:

Reviewer #1:
Extracellular protein microarray technology for high throughput detection of low affinity receptor-ligand interactions.

The paper by Husain et al. reports a method for screening for novel extracellular protein interactions using a protein microarray technology.
Overall this is well written and in an interesting subject area but I would question whether the ambitious very broad-ranging protocol as described would actually be useful to other scientists. Instead of describing the whole process in a generic manner from start to finish at a very high level, it might be of more value to focus on one important aspect of the procedure that can be described in much more detail so that others can benefit from the authors' expertise. For example, it is a shame that there is not more space to describe how the protein library is created. This is a huge amount of work and requires significant expertise and is critical to start this protocol and yet is given only cursory treatment in points 1.1 to 1.4. For example, in point 1.2 they state that they use software prediction tools and list them but this brief statement leaves many important questions unanswered: How are the software tools implemented and used? Which thresholds they use? Do the constructs contain endogenous signal peptide, or one encoded in the expression plasmid? How do they design the synthesized gene expression constructs? Are they codon-optimised or use endogenous sequences? These (and the many others that are missing) are the details that a good protocol paper should really address to be of value to others.

Also, this method is useful for screening hundreds to thousands of extracellular receptor proteins and therefore requires significant resource which, while it might be available at large pharmaceutical companies such as the authors' institution, it is not clear how useful this will be for the average academic laboratory which does not have access to such protein libraries.

Section 3.2 needs further explanation so that this step could be repeated by other scientists. I was confused by the amounts / order in which reagents were used. Similarly for section 3.3 "Samples are supplemented with soluble protein A immediately prior to incubation with the microarray slides…". There is no mention of volumes or concentrations. These protocol sections are not helpful without these specific details.

Response: We are thankful to Reviewer 1 for her/his critical assessment of this methods paper and for highlighting the interest of the topic.

We certainly agree with this Reviewer that the generation of the protein library constitutes a significant effort that is key for the success of the microarray technology reviewed in this paper. As indicated by the Reviewer, the selection, cloning and purification of the relevant proteins can be described in much more detail. We acknowledge that a detailed description of these methods merits a separate report. Similarly, we think that that a protocol for printing of the microarray slides, multimerization for detection of transient binders, and microarray screening are key elements of a PPI screening effort that require a detailed explanation. We have focused the current method papers on these aspects, as we consider these as relevant steps that are also best suited for a visual protocol –the main scope of this report- given the multiple steps and instrumentation involved. The video protocol, should the manuscript be accepted, will focus on sections 2, 3 and 4, describing slide printing, and formation of query complexes for screening against the library. We hope other investigators will benefit from a protocol describing these steps in the form of a video.

A thorough description of the criteria used for annotation of extracellular proteins, including the bioinformatics tools used for classification, has been published and is referenced in this manuscript (Genome Res. 2003 Oct;13(10):2265-70. Epub 2003 Sep 15). Additional details on the purification of part of the protein library is also presented in a previous study describing the extracellular protein microarray technology, included in the References (Anal Biochem. 2012 Jan 15;420(2):127-38. doi: 10.1016/j.ab.2011.09.017). We hope the Readers will refer to these studies as well as protein purification methods papers published elsewhere to guide preparation of the libraries for screening using microarray technologies. In response to this Reviewer comments and following the Editor suggestions, we have added some notes to the text to further clarify the scope of the current methods paper (page 2, line 31; page 2, line 46; page 5, line 116; page 14, line 384; page 14, line 400), as well as additional references for prediction tools that we normally utilize and that may be of interest to the Reader (page 14).

We agree with this Reviewer that the generation of a large collection of purified proteins requires significant resources, as acknowledged in the text. However it should be noted that the methodology described here can be applied to any library of proteins, such as specific protein families, protein isoforms, etc., regardless of the size of the collection. The screening procedure, including the multimerization strategy for enhanced detection of PPI, can also be useful for more focused screening efforts, and could be applied, for example, for interrogation of commercial protein microarrays, readily accessible to any Academic and Industry Researcher. As such, we believe the protocols reviewed here may be of interest to researchers generally interested in receptor-ligand discovery.

We have edited section 3 to mention additional experimental details, and apologize if this section was not clear enough in the initial manuscript.

We thank this Reviewer for her/his suggestions.

Reviewer #2:
Manuscript Summary:
This manuscript by Husain et al. provides comprehensive information about materials and methods and representative results for Extracellular Protein Microarray Technology for high throughput detection of low affinity receptor-ligand interactions.

Response: We are thankful to Reviewer 2 for reviewing this manuscript, and her/his very useful feedback and insights into tools for transmembrane helix prediction.

We have incorporated all references suggested by this Reviewer (page 5, line 139; page 14, line 387). To comply with manuscript style, the list of prediction tools is also listed in the Materials and Reagent Table.

Major Concerns:
None

Minor Concerns:
On page 3, Protocol 1.2, I suggest providing details of the websites and references for the prediction tools SignalP, TMHMM and Phobius, as given below.
Signal P4.1 (http://www.cbs.dtu.dk/services/SignalP/) (Emanuelsson et al., 2007; Petersen et al., 2011; Nielsen, 2017):
-O. Emanuelsson, S. Brunak, G. von Heijne, and H. Nielsen, Locating proteins in the cell using TargetP, SignalP and related tools. Nat Protoc 2 (2007) 953-71.
-T.N. Petersen, S. Brunak, G. von Heijne, and H. Nielsen, SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8 (2011) 785-6.
-H. Nielsen, Predicting secretory proteins with SignalP. Methods Mol Biol 1611 (2017) 59-73.

TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001):
-A. Krogh, B. Larsson, G. von Heijne, and E.L. Sonnhammer, Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol 305 (2001) 567-80.

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I also suggest that the authors consider using and mentioning in the text the consensus tool TOPCONS (http://topcons.cbr.su.se/) (Bernsel et al., 2009) for predicting transmembrane helices, which has been reported as the best performing (Tsirigos et al., 2012) and was updated to efficiently distinguish signal peptides and transmembrane helices (Tsirigos et al., 2015). Furthermore, the performance of TOPCONS was recently tested on 235 integral membrane proteins of known high-resolution structure and had a success rate of 94.8% for identifying tramsmembrane helices (Saidijam et al., 2018).
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The manuscript also contains grammatical errors throughout that should ideally be rectified before publication.