Video Article

Probing High-density Functional Protein Microarrays to Detect Protein-protein Interactions

Joseph Fasolo[1], Hogune Im[1], Michael P. Snyder[1,2]

[1]Department of Genetics, Stanford University

[2]Stanford Center for Genomics and Personalized Medicine, Stanford University

Correspondence to: Michael P. Snyder at mpsnyder@stanford.edu

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Abstract

High-density functional protein microarrays containing ~4,200 recombinant yeast proteins are examined for kinase protein-protein interactions using an affinity purified yeast kinase fusion protein containing a V5-epitope tag for read-out. Purified kinase is obtained through culture of a yeast strain optimized for high copy protein production harboring a plasmid containing a Kinase-V5 fusion construct under a GAL inducible promoter. The yeast is grown in restrictive media with a neutral carbon source for 6 hr followed by induction with 2% galactose. Next, the culture is harvested and kinase is purified using standard affinity chromatographic techniques to obtain a highly purified protein kinase for use in the assay. The purified kinase is diluted with kinase buffer to an appropriate range for the assay and the protein microarrays are blocked prior to hybridization with the protein microarray. After the hybridization, the arrays are probed with monoclonal V5 antibody to identify proteins bound by the kinase-V5 protein. Finally, the arrays are scanned using a standard microarray scanner, and data is extracted for downstream informatics analysis[1,2] to determine a high confidence set of protein interactions for downstream validation *in vivo*.

Video Link

The video component of this article can be found at https://www.jove.com/video/51872/

Introduction

The need to perform global analyses of protein biochemistry and binding activity *in vivo* has resulted in the development of new methods for profiling protein-protein interactions (PPIs) and the post-translational modifications of whole proteomes[1,3-8]. Protein microarrays are manufactured as functional protein microarrays using full-length functional proteins[4-6,8,9], or analytical protein microarrays containing antibodies[10,11]. They are engineered to contain a high-density of proteins arrayed onto microscope slides with a variety of surface chemistries to facilitate a variety of experimental conditions required for conducting wide-ranging biochemical analyses[12]. Nitrocellulose and aldehyde surface chemistries for chemical attachment through lysine or affinity attachment methods such as nickel- chelated slides for attaching His-tagged proteins and glutathione for affinity attachment among others[13].

The use of functional protein microarrays to detect protein-protein interactions requires access to a high-quality functional protein library[14]. *S. cerevisiae* is amenable to producing such a library through the pairing of high-copy affinity tagged protein constructs with high-throughput chromatographic purification techniques. The vast majority of the yeast genome has been sequenced and nearly the entire proteome can be expressed from a high-copy plasmid for purification and biochemical analyses[12]. Once the proteins are obtained and arrayed in 384-well format, they are printed onto a microscope slide allowing for rapid parallel multi-parametric biochemical analysis and bioinformatic interrogation[8,14-16]. Protein microarrays have been used for enzymatic assays and interactions with proteins, lipids, small molecules, and nucleic acids among many other applications. The accessibility of proteins on the surface of proteome arrays make them amenable to different types of analytical detection including, immune-affinity, Surface Plasmon Resonance, fluorescence and many other techniques. Moreover, it allows for fine control of the experimental condition where it might be hard to do *in vivo*.

The aim of this protocol is to demonstrate the appropriate use of functional protein microarrays to detect protein-protein interactions. This application enables the high-throughput parallel biochemical analysis of protein binding activities using a highly purified analyte (protein) of interest. A C-terminal(carboxy-terminal) tagged V5-fusion bait protein of interest is produced from a high-copy plasmid in a yeast strain optimized for protein purification. C-terminal tagging ensures that the full-length protein has been translated. The protein used in this study is Tda1-V5 fusion protein kinase, which is purified using nickel affinity resin via a His6X tag. The Tda1-V5 fusion construct is purified through serial elution using an imidazole gradient to elute the most highly enriched fraction for use in the assay.

Protocol

1. Probe Preparation

1. Culture and purify the V5-fusion kinase probes used to examine interactions with other proteins as follows:
    1. Use freshly streaked yeast strain Y258 (MATa pep4-3, his4-580, ura3-53, leu2-3,112) containing V5-fusion protein (expressed from GATEWAY vector pYES-DEST52). Use the isolated protein as probe on the microarrays. Plate the yeast on synthetic complete-uracil (Sc-Ura)/2% Dextrose/Agar and grow at 30 °C for 3 days from frozen culture (-80 °C glycerol stock).
    2. Inoculate starter cultures (5–20 ml) from a single colony and grow overnight in Sc-Ura/2% Dextrose on shaking platform (220-250 rpm) or wheel at 30 °C.
    3. The following morning, inoculate 400 ml of the Sc-Ura/2% raffinose culture with sufficient starter culture to a final OD_{600} of 0.1.
    4. Grow the inoculums to OD_{600} of 0.6 followed by galactose induction of the V5-kinase fusion construct expression by adding a solution of 3x Yeast Extract/Peptone (YEP) supplemented with 6% Galactose, by adding enough to dilute the induction media by a factor of 3 so that final concentration of galactose is 2%.
    5. Induce cells at 30 °C for 6 hr on a shaking platform. Use a 2 L Erlenmeyer flask to ensure appropriate aeration.
    6. Harvest cells using a JA-10 (or comparable) rotor by spinning 400 ml of cell suspension at 1,000 x g for 5 min at 4 °C.
    7. Wash the cells once with 50 ml of ice cold PBS buffer and transfer to a 50 ml conical tube. Wash the pellet again in ice cold PBS buffer (without detergents or other additives) used for lysis and transfer to 2 ml snap-cap tubes for lysis.
    8. Spin the cells at 20,000 x g for 1 min at 4 °C to a pellet and pipette away the buffer.
    9. Place tubes on ice and proceed with lysis step.
    10. Lyse the cells (250-350 µl pellet) with 0.5 mm zirconia beads in a 1:1:1 volume of cell pellet, beads, and Phosphate-buffered saline (PBS) lysis buffer and vortex the mixture using a agitation platform 3 times at 2 min intervals at 4 °C.
    11. Centrifuge the lysate at 20,000 x g in a tabletop microfuge for 10 min at 4 °C.
    12. Pipette supernatant into polycarbonate high speed centrifuge tube and clarify by ulracentrufugation for 30 min at 150,000 x g at 4 °C.
    13. Transfer the clarified lysate to a tube containing prewashed Ni^{2+} affinity resin (~100 µl) and incubate on a nutator for 2 hr at 4 °C to capture Histidine(His)6X tagged V5-fusion protein.
    14. Wash the resin three times for 10 min at 4 °C with Wash Buffer. Pellet the resin using a table top centrifuge for 5 min at 1,000 x g at 4 °C and aspirate the supernatant. Add fresh wash buffer for each wash and return the tube the nutator for agitation during the wash.
    15. After the wash steps are complete, apply the washed resin to a fresh G-25 column for elution.
    16. Apply 25 µl of elution buffer in a step-wise manner beginning with 100 mM Imidazole to 500 mM imidazole in 50 mM increments for a total of 9 fractions.
    17. Assay the collected fractions using sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and coomassie staining to identify the most highly enriched fraction(s) for use in the assay and add glycerol to 30% and store at -80 °C until used in the assay.

2. Probing the Arrays

NOTE: Please see step 2.6 of this section to prepare the antibody solution before beginning the assay.

1. To detect interactions, dilute the V5-fluorophore conjugated antibody (*i.e.* AlexaFluor 647) to 260 ng/ml in probe buffer and mix thoroughly by shaking.
   NOTE: Prepare this antibody solution 30 min prior to use and place the tube on a nutator or wheel to ensure complete mixing and a homogenous suspension of antibody.
2. Dilute the V5-fusion protein probe over a concentration range of 5–500 µg/ml.
   NOTE: Optimized for each protein–protein interaction assay, using probe buffer. Optimization involves adding more probes to probe buffer. Typically 10 µg/ml is used as starting point and adjusted accordingly based on the signal strength.
3. Remove the protein microarrays from the freezer (−20 °C) and bring to 4 °C in the refrigerator just prior to use.
4. Add blocking buffer directly to the slide holder containing the protein micorarrays and cover the top with parafilm to prevent leakage. Block the arrays in blocking buffer for 1 hr by shaking at 50 rpm on a stage at 4 °C.
5. After blocking, transfer the arrays to a humidified chamber chilled to 4 °C, and add 90 µl of diluted probe directly to the array surface. Overlay the arrays with a raised lifter slip and incubate static (no shaking) in the humidified chamber at 4 °C for 1.5 hr.
6. Wash the arrays 3 times for 1 min each in probe buffer in three 50 ml conical tubes. Add the slide to conical tubes containing enough pre-chilled probe buffer to completely envelope the slide. Allow the lifter slip to gently slide off of the protein microarray (do not force it off as this could result in damage to the array surface).
7. Apply the antibody solution directly to array immediately after completing the wash (step 2.5) and overlay with a raised lifter slip as before. Incubate the arrays for 30 min at 4 °C in the humidified chamber.
8. Perform the same wash step as before (3 times 1 min in probe buffer), and spin in a 50 ml conical tube at 800 x g in a tabletop centrifuge for 5 min at room temperature. Air-dry the arrays in a slide holder in the dark for 30 min prior to scanning the array at 647 nm.

Representative Results

The protein-protein interaction activity was observed using a standard chip reader to evaluate the Tda1-V5 protein kinase fusion construct as a bait protein against a yeast functional protein microarray containing approximately 4,200 unique *S. cerevisiae* GST-fusion proteins. Further interrogation with the Genepix software revealed a multitude of binding events of varying intensities. The affinity was gauged from the graded intensity of the signal derived from a monoclonal V5-Fluorophore conjugated antibody bound to its target (V5-kinase). ProCat scoring algorithm[2]

was used to identify protein-protein interactions across two separate protein microarrays for each of the proteins assayed in duplicate (each protein is spotted twice on the array), then a threshold cut-off was determined for scoring the observed interactions.

By comparing two arrays one obtains 4 technical replicates (n = 4) that can use to determine inter-assay variation through correlation (R^2) of the two spots for each protein on the arrays. A total of 9 protein-protein interactions were identified between the Tda1-V5 bait protein and the proteins on the microarray using a predefined threshold for identifying unique protein kinase interactions[1]. Moreover, the same proteins are spotted in different positions throughout the arrays as a control to assess surface artifacts that may contribute to false positives. The comparison of multiple binding profiles of different kinase-V5 fusion proteins enables the identification of kinase specific protein-protein interactions for testing *in vivo*.

In this experiment we tested the binding activity of Tda1-V5 and identified several statistically significant PPIs based on ranked by p-value compared to other kinases tested. Of particular interest is Rim11, which has been identified as a phosphorylated target of Tda1 in a previous published study[9]. Once targets have been identified, they can be tested for biochemical and functional activity *in vivo*[1].

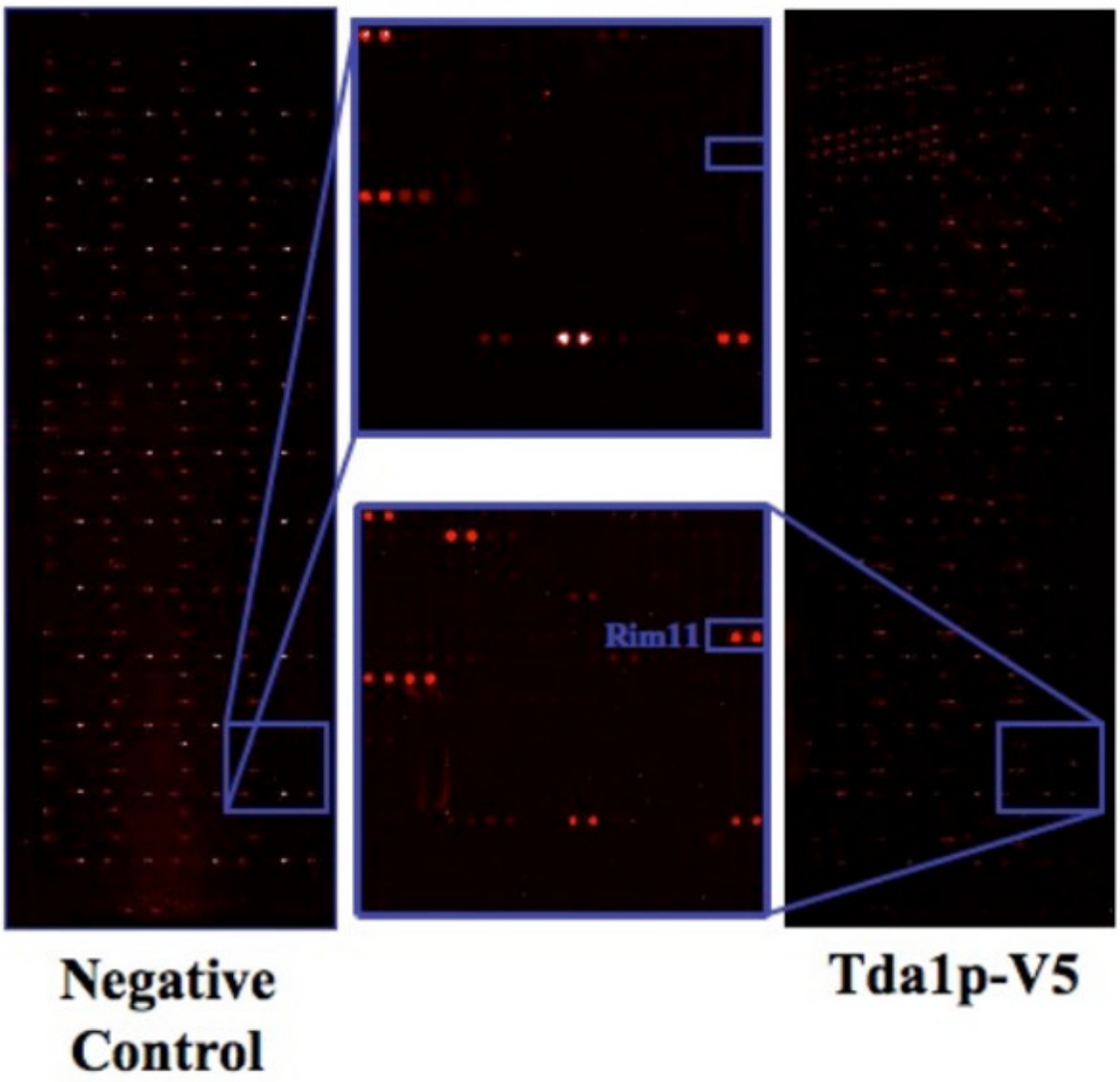

Figure 1: Tda1-V5 compared to empty vector control. Yeast protein microarrays spotted in duplicate with ~4,200 full length GST-fusion protein was incubated with control or Tda1–V5 probes. Inset panel compares the identical region in both arrays. Protein-protein interaction was detected (blue boxes) between Rim11 and Tda1-V5.

Discussion

The protocol presented was originally performed using 85 unique yeast protein kinase-V5 fusion proteins to compare binding activity across distinct and related families of yeast protein kinases resulting in the identification of new kinase interaction networks *in vivo*[1]. As an emerging proteomic profiling technology, the development of High Throughput (HTP) screening of proteomes using protein microarrays relied on peptide libraries, and eukaryotic and prokaryotic model organisms[8,17]; later, this assay platform was applied to higher eukaryotes such as plant and

human[5,7]. Currently there is no commercially available yeast protein array. However, sequence verified cDNA collections are available from multiple resources

Although the technique can be powerful, one caveat to keep in mind is that the assay is done *in vitro,* and, thus results will have false positives. Thus, optimizing assay condition and strategic filtering scheme is advisable to retrieve meaningful data. Next generation protein microarray technologies contain a much larger number of highly purified functional proteins for biochemical analysis across a multitude of organisms including *A. thaliana, S. cerevisiae*, and *Homo Sapien*.

The level of sophistication that the new protein microarrays have engineered into them enables an expansive variety of biochemical analyses that can be performed. Nearly the entire proteome has been cloned and purified for *S. cerevisiae* using a C-terminal tagged library to ensure proper post-translational modification of the proteins[14]. Moreover, the rapid parallel analyses offered using protein microarrays enables an unbiased approach to protein-protein interaction and post-translational modification profiling.

Disclosures

The authors declare that they have no competing financial interests.

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