Reverse-phase protein lysate microarrays for cell signaling analysis

Brett Spurrier[1,2], Sundhar Ramalingam[3] & Satoshi Nishizuka[1,4,5]

[1]Molecular Translational Technology Section, Molecular Therapeutics Program, National Cancer Institute, National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892, USA. [2]Aushon BioSystems, 43 Manning Rd, Billerica, Massachusetts 01821, USA. [3]University of North Carolina at Chapel Hill, School of Medicine, Chapel Hill, North Carolina 27599, USA. [4]Laboratory of Proteomics and Analytical Technologies, SAIC-Frederick, Inc., NCI-Frederick, Frederick, Maryland 21702, USA. [5]Molecular Therapeutics Laboratory, Department of Surgery, Iwate Medical University, Uchimaru 19-1, Morioka, Iwate 020-8505, Japan. Correspondence should be addressed to S.N. (snishizu@iwate-med.ac.jp)

Published online 30 October 2008; doi:10.1038/nprot.2008.179

'Reverse-phase' protein lysate microarray (RPA) assays use micro-scale, cell lysate dot blots that are printed to a substrate, followed by quantitative immunochemical protein detection, known to be particularly effective across many samples. Large-scale sample collection is a labor-intensive and time-consuming process; the information yielded from RPA assays, however, provides unique opportunities to experimentally interpret theoretical protein networks quantitatively. When specific antibodies are used, RPA can generate 1,000 times more data points using 10,000 times less sample volume than an ordinary western blot, enabling researchers to monitor quantitative proteomic responses for various time-scale and input-dose gradients simultaneously. Hence, the RPA system can be an excellent method for experimental validation of theoretical protein network models. Besides the initial screening of primary antibodies, collection of several hundreds of sample lysates from 1- to 8-h periods can be completed in ~10 d; subsequent RPA printing and signal detection steps require an additional 2–3 d.

INTRODUCTION

'Reverse-phase' protein lysate microarrays (RPAs) are a micro-format dot western blot (WB) that does not separate protein samples using electrophoresis, allowing for the protein expression of hundreds to thousands of samples to be monitored simultaneously in a quantitative manner[1]. This technique entails printing whole-cell fraction lysates in a microarray format to detect proteins of interest by immunochemistry, as illustrated in **Figure 1**. Based on the concept that RPAs require only miniscule sample volume for multiplex protein detection, tissue lysates from various organs have also been analyzed for biomarker discovery and clinical diagnostics using this method[1–4]. Another powerful application for which RPA can be used is monitoring protein dynamics as a function of time in response to different input types and doses across many samples. Such high-dimensional data acquisition can contribute to the identification of accurate molecular drug targets, as well as the examination of protein network theoretical models[5,6]. RPAs are one of the few technologies that provide high-dimensional proteomic data in a prompt, quantitative and affordable manner[7–9].

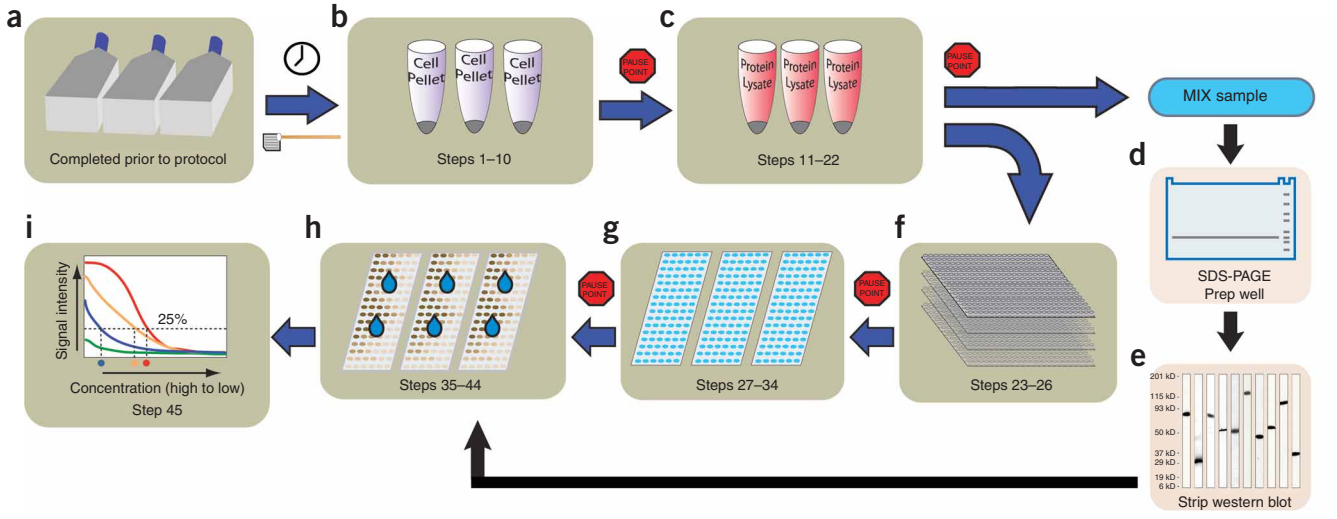

Figure 1 | Outline of reverse-phase protein lysate microarray protocol for quantitative protein expression analysis. (**a**) Cells are grown in T-25 flasks and stimulated or stressed. (**b**) Cells are collected into pellets at small time intervals. (**c**) The cell pellets are lysed with Pink buffer to generate the full-fraction protein lysate. (**d**) A portion of the collected samples are pooled and run on a 2D prep well western blot (WB), and (**e**) probed with various antibodies to determine specificity across the entire molecular weight range of collected samples[13]. This process may be necessary before large-scale sample collection with a smaller sample size under identical conditions. (**f**) A large number of samples are plated into 384-well or 1,536-well microtiter plates, which are (**g**) subsequently printed to nitrocellulose film slides using an Aushon BioSystems 2470 solid-pin microarrayer. (**h**) The printed slides are probed with specific primary antibodies screened (**e**) for specific protein detection. (**i**) Protein levels are quantified by using the DI_{25} algorithm implemented in the freely available software packages P-SCAN and ProteinScan.

Cell signaling is a continuous biochemical process that takes place in response to stimulation. One prominent biochemical role is played by phosphorylation, a posttranslational modification. When phosphorylation takes place in response to a particular input, an upstream molecule first responds, and then downstream changes in phosphorylated protein levels follow. To trace such dynamic events, frequent (short-time interval) sample collections must be performed[5,6,10–12]. To determine the correct window in which protein levels exhibit these dynamic changes, preliminary WBs should be performed with a small number of samples taken from a wide range of both times and doses[13]. Information from these preliminary experiments helps to determine more precise time- and dose-concentration windows in which samples should be collected using shorter intervals.

Seven stages are involved in this protocol as described in Experimental design. In principle, the stages are aimed at lysing cell pellets from various conditions followed by micro-dot printing to be used for quantitative output when detected with specific primary antibodies. One of the most important stages is antibody screening because a dot blot does not account for the antibody's specificity. Hence, the limited number of antibodies which give an analyzable signal is a major constraint of the technology. However, RPAs are extremely powerful when many antibodies, identified as *analyzable*, are available. Previously, we reported the utility of RPAs including: (i) proteomic profiling[14], (ii) diagnostic marker identification[15], (iii) protein kinetic monitoring[6,13] and (iv) experimental validation of theoretical models[5]. All these studies employ the same protocol, varying only by the type of samples used. Information for these applications may also be obtained by ordinary western blotting[16], but the results are not quantitative and the throughput is low. WB, however, resolves the sample protein fractions by molecular weight, so the band result is more specific. Alternatively, ELISA may yield more sensitive quantitative results when an appropriate antibody pair is available, which may be relatively limited[17]. Overall, the advantage of RPAs is the throughput in terms of sample number; the disadvantage, however, is the restriction of information learned by antibody specificity and performance. It is extremely useful when one needs to investigate protein expression levels changing over variables such as time or input strength.

We stress that the following protocol focuses on collecting 'many samples' for examining relative changes of proteins within a cell, not in a collection of tissue snapshots or a panel of cell lines. Following is a detailed description of our large-scale sample collection method for quantitative protein expression analysis, using RPA for experimental validation of protein network models. An overview of the process has been illustrated as a flowchart in **Figure 1**.

Experimental design

Cell culture and stimulation. Cells are grown in T-25 flasks at 37 °C in the presence of 5% CO_2 in the appropriate medium. Split the cells as many times as necessary to obtain the desired number of flasks. Once the cells have been split to the optimal number of flasks (when the cell population is ~70% confluent for each flask), prepare the stimulation medium or ready the stress equipment (e.g., γ-ray or U.V. irradiator) according to the study design. Stimulating agents can be drugs, growth factors, irradiations and others[6,12]. For time course studies, optimal stimulant is added to a set of flasks simultaneously and the flasks are then collected at different time points. For dose escalation studies, different doses of stimulus are added to a set of flasks and the set is collected at the same time. Although it is possible to culture cells in smaller format, cells must be cultured in a flask (allowing cells to be collected first as a pellet before lysis) in order to make as high a concentration as possible. Cells grown in a well of a microplate are not abundant enough to form a pellet, nor can be they lysed in a small lysate buffer volume because of the viscosity of resulting solution. To achieve high concentration of protein, we lyse 20 µl volume in a well—pellet from a T-25 flask (2–5 × 10^6 cells) with a 20 µl Pink buffer (PB) (described in PROCEDURE). Do not stimulate or stress the cells until you are fully ready to begin the sampling procedure.

Sample collection. We have found that each sample should generally be collected from a T-25 flask to minimize handling errors while yielding sufficient cell pellet volumes for the required concentrations used in RPAs. When the entire sample-collecting process is performed on ice, the protein levels and biochemical reactions are ideally preserved isentropically. To complete each sample collection process promptly, the apparatus and equipment should be arranged based on the individual experimentalist's ergonomics (e.g., PBS bottles should be placed at a minimum distance from the flask). It also becomes critical to build a mock sample collection to identify whether any physical limitations are significant.

Cell lysis. Collected cell pellets are lysed with PB[18]. PB was initially used for 2D gel electrophoresis, but it was found to be ideal for RPA use based on the following qualities: (i) the urea content keeps proteins denatured at room temperature (i.e., around 20 °C); (ii) DTT is less volatile than β-mercaptoethanol (a commonly used reducing agent); and (iii) CHAPS does not precipitate after repeated freeze-thaw cycles. The concentration of each chemical component is adjusted as appropriate in all wells of a microplate using 0.67× dilution buffer such that only the protein concentration changes when twofold serial dilutions of the sample are made[14].

RPA production. Although PB is advantageous for retaining protein status, the viscosity of the resulting lysate is a critical problem in RPA production using conventional arrayers. Moreover, sample evaporation is another obstacle in large-scale microarray printing because of the long sample exposure time in source plates. To address these issues, we used the Aushon BioSystems 2470 microarrayer, which can produce arrays with even the most viscous lysate material[7,19]. The Aushon 2470 uses a solid-pin configuration, a humidity/temperature environmental control and an automated continuous slide/microplate supply system. The throughput of the arrayer and the ability of the system to handle high-viscosity samples enabled the creation of a high-density lysate array with >10,000 features. Initially, RPAs were produced by a pin-in-ring format GMS/Affymetrix 417 arrayer that is no longer available in the market. The Aushon 2470 has been developed to retain the advantages of the 417 and improve pin architecture as well as printing environmental control. The solid-pin printing system eliminates the potential for clogging that can occur in quill pins or piezo electric array systems.

Our current Aushon BioSystems 2740 RPA production system uses a 32-pin configuration (maximum of 48) that can print 32 fields containing 20 rows and 20 columns. Sample placement within the microtiter plate and array layout should be carefully

designed, depending on the number of samples being studied and the purpose of the printing. Although the number of dilutions is completely up to one's study design, we choose 10-point twofold dilution series because: (i) more dilution points give a wide range of sample concentrations, which allow more accurate calculation of protein expression level; (ii) with 32-pin configuration, we can make up to 12 dilution points per microplate, but the 110-μm pin and 4.5-mm pin–pin spacing limits the maximum number of dilution points. A high-density, large-scale format of the RPA provides maximal use when the study design requires many different samples to be tested under identical conditions. Other formats are preferred for preliminary testing in terms of staining, printing and layout (**Table 1**). The present sample collection protocol is designed to be used with a large-scale, high-density array design, which is the most powerful application of RPA in the context of monitoring protein dynamics[5,6]. Options are presented for using 384-well microtiter plates as well as 1536-well microtiter plates, which are capable of holding four times as many extractions as 384-well plates.

Immunochemical signal detection. After the lysate is printed, an immunochemical method is performed for signal detection. RPA does not resolve protein fractions by molecular weight, so it is important to ensure that antibodies are specific to their target antigens in the protein lysate. To test many antibodies in a range of cellular conditions, we developed an efficient WB method, using strip membranes with pooled lysates from various conditions[13,20]. The result was a large dataset correlating WB results to the samples used and to antibody-probing conditions, which led us to develop a relational database to aid in proper antibody selection[13].

Antibody choice and screening. The selection of antibodies can be different for each study. Investigators must address the performance of a set of antibodies before each RPA experiment. For general use, we have published two open resource antibody databases (http://discover.nci.nih.gov/abminer[20] and http://mtt.uscancer.org[13]) to display WB results for antibodies that produced single bands at the expected ranges. Although samples were tested exhaustively, they are still limited and it is strongly recommended for each investigator to screen antibodies against their current samples by WB before RPA experiment. Specific primary antibodies are chosen only when they generate a single predominant band in the WB against the lysates to be used for RPA. However, it is important to note that not all such antibodies work well with RPA. Often, antibody concentrations need to be increased, while some antibodies may require a longer incubation time or a different type of blocking. For instance, we have found that ~50% of the commercially available antibodies yield quantifiable RPA output.

Data quantification. After immunostaining, protein expression is quantified using available software packages. Because most RPA signal detection methods use a specific primary antibody followed by catalyzed signal amplification colorimetric detection (CSA; DakoCytomation) (**Box 1**), the signal levels can be obtained using the reflective mode of an ordinary optical flatbed scanner[21]. Scanned images are then converted into numerical values by two freely available software packages, P-SCAN and ProteinScan (http://mttab.cancer.gov)[5,6,22]. The software quantifies the signal intensities of the scanned images for each spot and uses the DI_{25} algorithm to eliminate background effects by looking at the difference in the linear range of each dose–response curve and computing a single protein expression value for each sample (**Figs. 2a,b**). In contrast to other microarray formats, RPA requires additional processing because a single value for the expression of a protein needs to be calculated per protein per sample from a dose–response curve, which provides 10 data points per sample (**Figs. 2c,d**). It should be noted that normalization of protein expression could carry over noise to some extent because of the

TABLE 1 | Recommended 'reverse-phase' protein lysate microarray formats.

	Small scale high-density array	Medium scale low-density array	Large-scale high-density array
Image			
Image contents	16 samples 1 replicate 10-time serial dilutions	16 samples 1 replicate 10-time serial dilutions	640 samples No replicate 10-time serial dilutions
Recommended scale	4–32 samples	16–128 samples	64–640 samples
How to make	8 × 384-microplate Use 2 pins of 32-pinhead	One or two 384-microplate Use 16 pins of 32-pinhead	20 × 384-microplate Use all 32 pins 5 × 1536-microplate
Recommended study	Quantitative test Image processing program test	Semi-quantitative test Pre-large scale sample positioning test	Large scale quantitative study
Advantage	Less uneven staining effect Smaller image size Less pin effect	Easy microplate preparation Shorter printing time	Hundreds samples can be tested under the same condition Large scale data output
Challenge	Many microplates with many empty wells Longer total spotting time	Uneven staining affects the results Larger scanning area without data Some degree of mechanical effect	High-dimension high throughput study Automated analytical program Sophisticated array design

dose–response curve calculation algorithm. Options for detection methods using fluorescence and near-infrared detection have been used[23,24], and their protocols are described in **Boxes 2–4**.

Controls/replicates. The number of replicates is determined by how robust the experimental design needs to be. It is clear that more replicates are better for statistical purposes. If an RPA is used for screening, perhaps no replicates are necessary; however, at least six replicates (three biological, replicated experiments and two technical, samples printed with different pins or on different positions for each) are recommended when the study involves rigorous quantitation for mathematical modeling, for example. Control lysate should be printed within each field of the array to monitor variation associated with pins, regions and antibodies. It has been demonstrated that pooled lysate of all printed samples on the RPA are useful as such controls[13,14,20]. Previous quality control studies have shown that the average CV (coefficient of variation) was 3.69% for DI_{25} values (DI_{25} is the concentration required to reach 25 percent intensity of all dose-response curves in a given stained slide) taken from experiments repeated 2,560 times[6].

MATERIALS

REAGENTS

- Cultured cells
- PBS without Ca^{2+} or Mg^{2+} (BioWhittaker, cat. no. 17-516Q)
- Urea (Fluka/BioChemika, cat. no. 51456)
- Ultra pure water (KD Medical, cat. no. RFG-3410)
- Pharamlyte pH 8–10.5 (Amersham Biosciences, cat. no. 17-0455-01)
- CHAPS (Calbiochem, cat. no. 220201)
- DTT (Amersham Biosciences, cat. no. 17-1318-02)
- DI Water
- Tris-Buffered Saline Tween-20 (TBST) 10× (DakoCytomation, cat. no. K3306)
- Casein (I-block; Tropix, cat. no. T2015-0507022)
- Tween-20 (Bio-Rad, cat. no. 170-6531)
- Colloidal Gold Total Protein Stain (Bio-Rad, cat. no. 170-6527) (see REAGENT SETUP)
- CSA kit (DakoCytomation, cat. no. K1500): H_2O_2; avidin block; biotin block; streptavidin–biotin complex; amplification reagent
- Diaminobenzodine (DAB) solution (DakoCytomation, cat. no. K3468)
  ! CAUTION An oxidizing element; the substrate buffer and chromogen should not be mixed until it is ready to be used.
- DAB substrate buffer
- DAB+ chromogen
- Biotinylated link, anti-Rabbit immunoglobins (DakoCytomation, cat. no. K1498)
- Avidin solution (Invitrogen, cat. no. 00-4303)
- Biotin solution (Invitrogen, cat. no. 00-4303)

BOX 1 | CATALYZED SIGNAL AMPLIFICATION

Take the slides from Step 38 and incubate with the following reagents and timing:

1. H_2O_2 (5 min)
2. Blow out residual solution
3. Avidin block (25 min)
4. Blow out residual solution
5. Biotin block (25 min)
6. Blow out residual solution
7. I-Block (blocking buffer) (10 min)
8. Blow out residual solution
9. Primary antibody (30 min)
10. TBST (TBS + 0.5% Tween-20) (15 min)
11. Blow out residual solution
12. Biotinylated secondary antibody (15 min)
13. TBST (5 min)
14. Blow out residual solution
15. Streptavidin-biotin-HRP complex (15 min)
16. TBST (5 min)
17. Blow out residual solution
18. Amplification reagent (biotinyl-tyramide) (15 min)
19. TBST (5 min)
20. Blow out residual solution
21. HRP (5 min)
22. TBST (5 min)
23. Blow out residual solution
24. DAB (5 min)

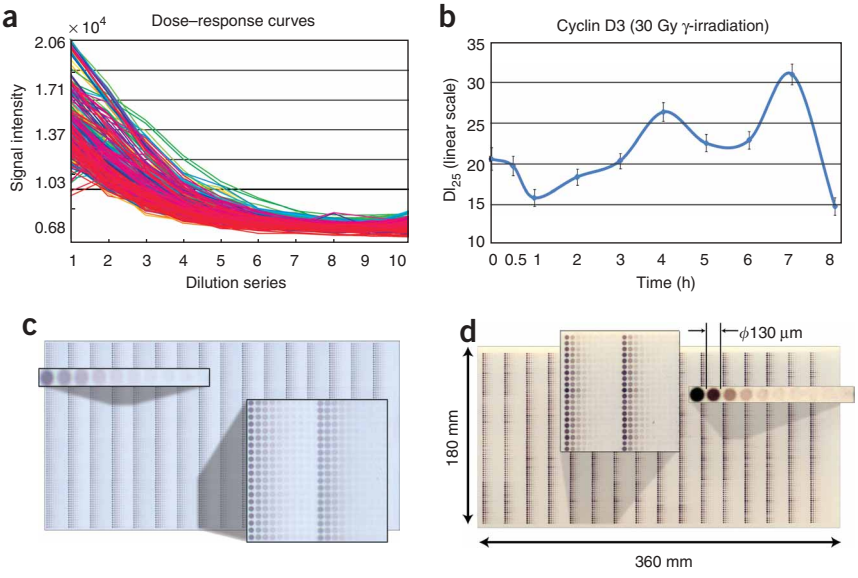

Figure 2 | 'Reverse-phase' protein lysate microarray (RPA) images and data output. (**a**) Dilution curves are generated from the signal intensities of the stained serial dilutions for each sample. A diaminobenzodine-coupled colorimetric method allows RPAs to be read by an optical flatbed scanner, yielding a dilution curve based on signal intensity. (**b**) The dose interpolation algorithm DI_{25} reads the value of the dilution series point that is located in the 25th percentile of the intensity range of a given RPA, where most curves are in the linear range. (**c**) Total protein stain of a high-density RPA. The left inset shows a magnified view of the 10 features comprising the 10-point dilution curve for a single sample. The right inset depicts all the features printed by a single pin. (**d**) Epidermal growth factor receptor is stained on a high-density RPA by the CSA colorimetric detection method. The left inset depicts all the features printed by a single pin. The right inset shows a magnified view of the 10 features comprising the 10-point dilution curve for a single sample.

- Stepavidin-Alexa Fluor 647 conjugate (optional) (Invitrogen, cat. no. S-32357)
- IRDye-680 Goat anti-Mouse secondary antibody (optional) (Li-Cor, cat. no. 926-32220)
- IRDye-800CW Goat anti-Rabbit secondary antibody (optional) (Li-Cor, cat. no. 926-32211)

EQUIPMENT

- Cell incubator (Sanyo UV SafeCell)
- T-25 flasks (BD Falcon, cat. no. 353808)
- 1.5 ml Centrifuge tubes, 2 per sample collected (Daigger, cat. no. FX42654RA)
- Cell scraper (Corning/Daigger, cat. no. FX8612B)
- 0.2 µm Nalgene sterile filter (Nalgene/Daigger, cat. no. FX8221D)
- Genetix 384-microplate (Genetix, cat. no. X6004)
- Bio-Rad mini-incubation trays (Bio-Rad, cat. no. 170-3903)
- 16-Channel pipette, 4.5 mm spacing (Matrix, cat. no. 2080)
- Centrifuge (Eppendorf centrifuge 5417C)
- Rocking platform (Bio-Rad UltraRocker)
- Parafilm
- Ziploc bags
- Nitrocellulose film slides (Grace Bio-Labs)
- Microarrayer (2470; Aushon BioSystems) ▲ **CRITICAL** The Aushon 2470 arrayer is strongly recommended because of the quality of pins, throughput and printing environmental control. Any type of automated device for immunochemistry may be used if complex signal development procedure is employed.
- Autostainer (DakoCytomation)
- Fluorescent scanner (optional) (428; Affymetrix)
- Near-infrared scanner (optional) (Li-Cor Odyssey)

REAGENT SETUP

PB For the preparation of a 40-ml scale, take 21.6 g of urea and add 22.5 ml Ultra Pure Water, mixing with a magnetic stirrer. Stir for 30 min or until the solution becomes clear. The total volume should be ~40 ml. Filter the solution with a 0.2-µm sterile filter. Add 0.8 ml Pharmalyte, pH 8–10.5, and 1.6 g CHAPS; mix gently in this order. ! **CAUTION** Do not shake. Add 0.4 g DTT and mix gently. ! **CAUTION** Do not shake. Bring the resulting solution up to 40 ml with Ultra Pure Water if necessary. Make aliquots of 0.5–1 ml. Store at −20 °C up to 1 year. ! **CAUTION** Once thawed (at room temperature), any unused solution should be discarded. Scale the Pink buffer recipe to any required solution volume as necessary.

0.67× Dilution buffer For the preparation of a 300-ml scale, take 108 g of urea and add 168 ml Ultra Pure Water, mixing with a magnetic stirrer. Stir for 30 min or until the solution becomes clear. Filter the solution with a 0.2-µm sterile filter. Add 3.9 ml Pharmalyte, pH 8–10.5, and 8.1 g CHAPS; mix gently in this order. ! **CAUTION** Do not shake. Add 1.8 g DTT and mix gently. ! **CAUTION** Do not shake. Bring the resulting solution up to 300 ml with Ultra Pure Water if necessary. Make aliquots of 10–15 ml. Store at −20 °C up to 1 year. ! **CAUTION** Once thawed (at room temperature), any unused solution should be discarded. Scale the 0.67× dilution buffer recipe to any required solution volume as necessary.

Blocking buffer Several blocking buffers may be used, but we primarily begin with I-Block solution. Make 0.1% wash buffer by mixing 250 µl of Tween-20 with 250 ml PBS. Mix 0.5 g of Tropix I-Block (powder form) with 250 ml of wash buffer. Heat in a microwave for 30 s, then stir with a magnetic stirrer until the powder I-Block has dissolved. Filter the solution with a 0.2-µm sterile filter and store it at 4 °C for 2 weeks.

Colloidal Gold Total Protein Stain The staining reagent should be stored at 4 °C before use. The Colloidal Gold waste should be discarded in a hazardous waste disposal.

Catalyzed signal amplification reagents DakoCytomation provides a kit (cat. no. K1500) that requires marginal setup. The Streptavidin–biotin complex is prepared by adding 40 µl (approximately one drop) of Streptavidin–biotin complex reagent A for each 1 ml of Streptavidin–biotin complex diluent. Then, add 40 µl (approximately one drop) Streptavidin–biotin complex reagent B for each 1 ml of Streptavidin–biotin complex diluents and Reagent A. Gently invert the solution to ensure adequate mixing. It is often best to perform this step near

BOX 2 | FLUORESCENT TYRAMIDE SIGNAL AMPLIFICATION

Take the slides from Step 38 and incubate with the following reagents and timing:

1. H_2O_2 (5 min)
2. Blow out residual solution
3. Avidin block (25 min)
4. Blow out residual solution
5. Biotin block (25 min)
6. Blow out residual solution
7. I-Block (blocking buffer) (10 min)
8. Blow out residual solution
9. Primary antibody (30 min)
10. TBST (TBS + 0.5% Tween-20) (15 min)
11. Blow out residual solution
12. Biotinylated secondary antibody (15 min)
13. TBST (5 min)
14. Blow out residual solution
15. Streptavidin–biotin complex (15 min)
16. TBST (5 min)
17. Blow out residual solution
18. Streptavidin-HRP (15 min)
19. TBST (5 min)
20. Blow out residual solution
21. Amplification reagent (biotinyl-tyramide) (15 min)
22. TBST (5 min)
23. Blow out residual solution
24. Streptavidin-AlexaFluor-647 (15 min)

BOX 3 | SINGLEPLEX NEAR-INFRARED DETECTION

Take the slides from Step 38 and incubate with the following reagents and timing:

1. I-block (blocking buffer) (10 min)
2. Blow out residual solution
3. Mouse or rabbit Primary Antibody (30 min)
4. TBST (15 min)
5. Blow out residual solution
6. IRDye-680 Goat anti-Mouse or anti-Rabbit IgG Secondary Antibody (15 min)

BOX 4 | MULTIPLEX NEAR-INFRARED DETECTION

Take the slides from Step 38 and incubate with the following reagents and timing:

1. I-block (blocking buffer) (10 min)
2. Blow out residual solution
3. Mouse primary antibody (30 min)
4. TBST (15 min)
5. Blow out residual solution
6. IRDye-680 Goat anti-mouse secondary antibody (15 min)
7. TBST (5 min)
8. Blow out residual solution
9. Rabbit primary antibody (30 min)
10. TBST (15 min)
11. Blow out residual solution
12. IRDye-800CW Goat anti-rabbit secondary antibody (15 min)

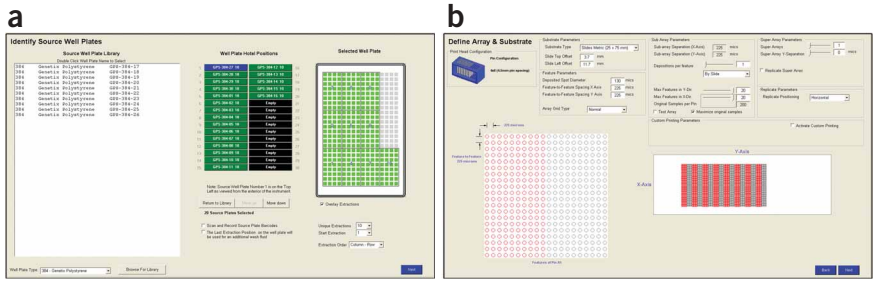

Figure 3 | Screenshot of the Aushon BioSystems 2470 Microarray Graphical User Interface (GUI). (**a**) Aushon 2470 GUI for defining a plate library. While 384-well plates are shown here, the GUI also allows for the selection of 1,536-well plates. (**b**) The GUI allows users to define array design including feature-to-feature spacing (225-µm spacing yields 12,800 features per slide) and replication patterns.

the end of the setup. The DAB solution (DakoCytomation, cat. no. K3468) is prepared by adding 20 µl of liquid DAB+ chromogen for every 1 ml of DAB substrate buffer. ▲ CRITICAL Because DAB is an oxidized element, this step should be performed directly before its use.

EQUIPMENT SETUP

Aushon Biosystems 2470 Microarrayer Turn on the arrayer and set the humidity control on the control view to 80%. Set the number of plates and change the extraction setting to 10 sectors (**Fig. 3a**). Modify the pin diameter and spacing to accommodate the sample set (i.e., for a high-density matrix set: pin diameter to 130 µm, *x*-axis spacing to 225 µm, *y*-axis spacing to 225 µm). This step does not change the actual pin size; rather, it is used to properly set the spacing between features. Set the printing replicate type to reflect the study design (**Fig. 3b**). We use the setting *horizontal* with *2* replicates. Choose to use slide 10 as a blot slide to ensure that pin tips do not carry residual material. Follow by setting the number of slides to be printed on each platen according to the sample layout. Once the parameters are set, empty the carboy waste water and fill the wash fluid with DI water. Place the set number of slides on the platens and place them into the arrayer. ! CAUTION Use gloves to avoid getting fingerprints on the slide and for general safety.

DAKO Autostainer The parameters of the DAKO Autostainer setup should be modified to the number of slides being stained. Fill the TBST and DI water containers. Keep all the reagents at 4 °C when they are not in use. Once the reagents are set up as described in the protocol below, keep them covered as long as possible during the setup. ▲ CRITICAL Always keep the slides in a wet condition, either with blocking buffer or DI water until staining starts.

PROCEDURE

Sample collection after stimulation or stress

● TIMING **15 min for preparation, 6 min per sample collection point**

1| Before sample collection, label 1.5-ml centrifuge tubes for each sample to be collected.

▲ CRITICAL STEP Label time point, stimulation or stressed condition, dosage and replicate number for organization. The tubes are used as an intermediary step.

2| Set up 1,000-µl pipettes and tips, ice bucket and cell scraper.

▲ CRITICAL STEP The protocol is a time-sensitive procedure; therefore, all materials should be set up before sample collection and placed within reach for an ergonomically effective position.

3| Put several 50-ml tubes of PBS on ice. Approximately 50 ml for 3 T-25 flasks is required for hand-pour rinsing.

4| When you reach the proper time point, remove the flask from its stimulation or stressed condition and *immediately* place on ice.

▲ CRITICAL STEP All processes after taking flasks out of incubator need to be completed on ice or in a cold room. A slow transition from stimulation or stress point to the ice will result in inconsistent protein sampling.

5| The sample washing and cell collection procedure is different depending on whether the cells are adherent or grow in suspension. Use option A if the collected cells are adherent or option B if the collected cells are growing in suspension.

(A) Cell collection for adherent cells

(i) Aspirate the medium from the flask.
(ii) Wash cells with ~5 ml of chilled PBS and aspirate.
! CAUTION You do not need to use a pipette.
(iii) Wash cells a second time with ~5 ml of PBS and aspirate.
(iv) Wash a third time with PBS and aspirate exhaustively to minimize residual medium for making a cell pellet.
(v) Pipette 1.5 ml of chilled PBS into the flask.
(vi) Use a cell scraper to remove cells from the bottom of the flask, which yields a suspension of cells within the 1.5 ml of PBS.

(B) Cell collection for suspended cells

(i) Centrifuge cells to a pellet at 4 °C for 30 s at 6,000*g*.
(ii) Aspirate the medium.
(iii) Wash cells with ~5 ml of chilled PBS by pipetting several times to thoroughly resuspend the cells, and repeat Steps 5B(i) and 5B(ii).
(iv) Wash the cells a second time with ~5 ml of chilled PBS by pipetting several times to thoroughly resuspend the cells.
(v) Centrifuge cells to a pellet at 4 °C for 30 s at 5,000*g*.
(vi) Aspirate the medium.

(vii) Wash the cells a third time with ~5 ml of chilled PBS by pipetting several times to thoroughly resuspend the cells, and repeat Steps 5B(v) and 5B(vi).
(viii) Pipette 1.5 ml of chilled PBS into the flask.
(ix) Create a 1.5-ml cell suspension by pipetting several times.

6| Pipette the 1.5-ml cell suspension from Step 5A(vi) or 5B(ix) into a prelabeled centrifuge tube from Step 1.

7| *Immediately* place centrifuge tube on ice.

8| Centrifuge the chilled centrifuge tube at 6,000*g* for 30 s at 4°C.
! CAUTION Make sure you see the cell pellet formed at the bottom of the tube.

9| Use a pipette to remove the PBS and immediately place the tube containing the cell pellet on dry ice.
! CAUTION Do not use a vacuum pump for aspiration.

10| Repeat this process for each sample.
▲ CRITICAL STEP This process should be performed with multiple flasks simultaneously when biological replicates are being made. As each sampling process takes 6 min, a maximum of four flasks can be handled at once.
■ PAUSE POINT The cell pellet may be stored at −80 °C for 2–3 months before proceeding.

Cell lysis ● TIMING 1 h, 20 min

11| Label a new set of 1.5-ml centrifuge tubes as 'protein lysate' tubes.

12| Bring Pink buffer to room temperature.
▲ CRITICAL STEP Make sure that Pink buffer remains at room temperature for a long enough period of time. Gently mix the tube by inverting it to make sure all the contents are dissolved.

13| Bring cell pellet to room temperature.

14| Centrifuge the cell pellet briefly at 2,500*g* for 30 s at room temperature to make sure there is no residual PBS. If there is, remove it with a pipette.

15| Estimate the cell pellet volume by comparing it with a known volume using 5, 10, 20 and 40 µl of visible material (i.e., food coloring) in an identical tube.

16| Lyse the cell pellet by pipetting an equal volume of Pink buffer (the volume amount estimated in the previous step) into the cell pellet tube.
▲ CRITICAL STEP Constant cell pellet volumes can be obtained with a fine degree of accuracy as long as they come from the same flask and the confluency of the cells is the same. However, the ratio of cell pellet volume to Pink buffer volume needs to be consistent throughout all the samples collected for the study. An ideal ratio would be 1:1, Pink buffer volume to cell pellet volume, which yields ~10–20 µg μl^{-1} of lysate and equivalent to 0.67× buffer component concentration.
? TROUBLESHOOTING

17| Close the tube lid and carefully flick the tube continuously until the visible cell pellet has dissolved in the buffer.
! CAUTION Be sure not create bubbles while flicking the tube.
? TROUBLESHOOTING

18| Place the resulting lysate tubes on ice until all the cell pellets have been lysed with Pink buffer.

19| Repeat the cell lysis steps for each sample.

20| Centrifuge all the cell lysates at 16,000*g* for 30 min at 4 °C to spin down the larger fractions of the molecules. At completion, there will be a small pellet visible.
? TROUBLESHOOTING

21| Pipette the supernatant containing the protein lysate into the corresponding new 'protein lysate' tube from Step 11.
? TROUBLESHOOTING

22| Place in −80 °C for storage.
■ PAUSE POINT The protein lysate may be stored at −80 °C for at least 1 year.

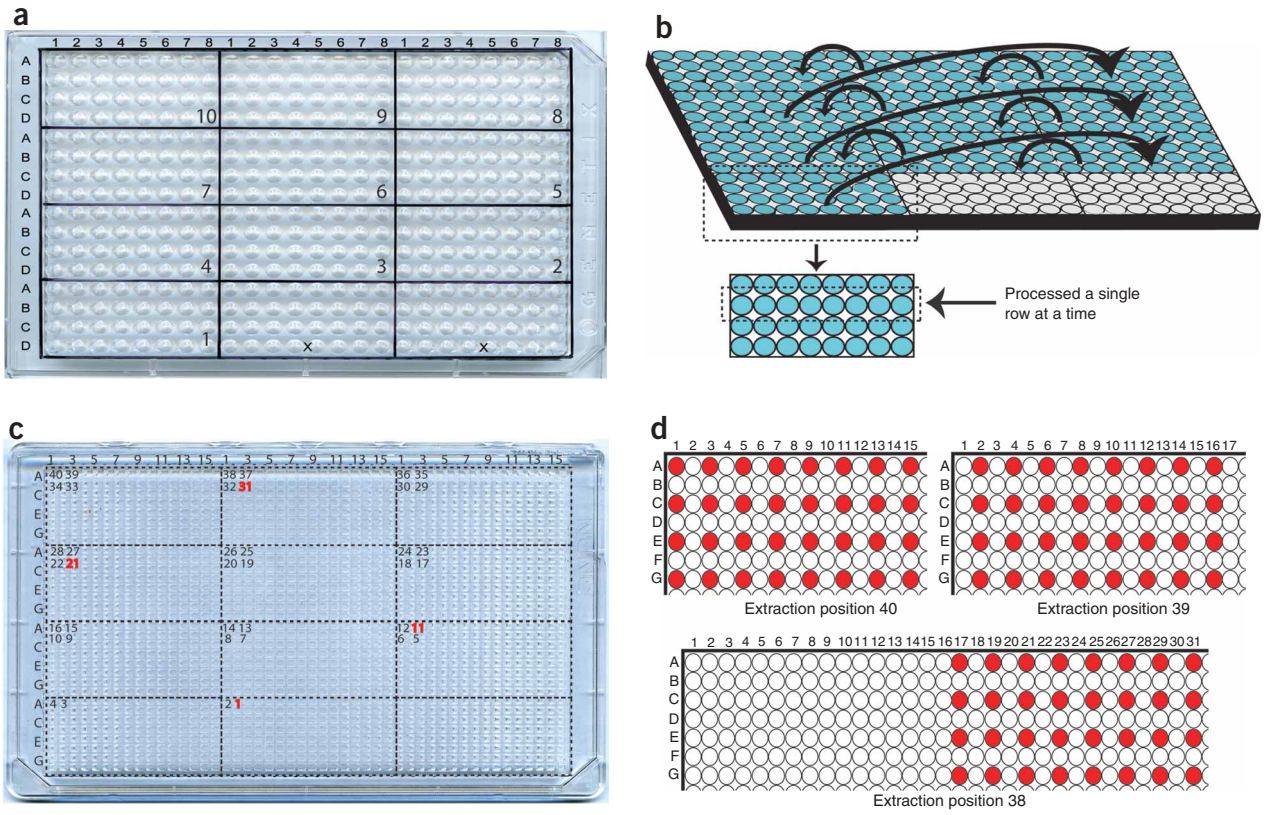

Figure 4 | Preparation of twofold serial dilutions in a 384-and 1,536-well microtiter plate. (**a**) Image of a sectioned Genetix 384 V-Well Microplate divided into 12 sectors (1–10 used). (**b**) Dilution setup of the 384-well microplate. Each of the sectors is numbered from 1 to 10, and has four rows and eight columns. The sector number represents the sample dilution series. For instance, the 10th point dilution curve for the sample located in well A1 (upper left corner) is located in the same position of all sectors 10 through 1, decreasing in concentration through the series. (**c**) The upper-left hand pin ('relative' pin A1) is marked for each extraction position labeled 40 through 1. Neat samples will be placed in extractions 31, 21, 11 and 1. (**d**) Positions of the first three extractions 40, 39 and 38 are shown as an example of the interweaving extraction pattern used with the 1,536-well plates. The pattern continues in this format such that all wells of the plate may be used.

Microtiter plate preparation ● TIMING **1 h, 25 min**

23| Design the overall layout of the microtiter plates such that at least three biological replicates are located in different rows, columns and plates (see Experimental design).

24| The microtiter plate setup is different depending on whether 384-well plates or 1536-well plates are used. Use option A when the layout is designed for 384-well plates and option B when using 1536-well plates.

(A) 384-Well microtiter plate setup

(i) Mark all 384-well plates in 4 by 8 well blocks, sectioning the plate into 12 sectors (**Fig. 4a**).
! **CAUTION** Designate the plate with a name or number as not to confuse it with other plates.

(ii) Place ~8 ml of dilution buffer in a single well of a mini-incubation tray.
! **CAUTION** Tape the mini-incubation tray to the bench-top to secure its location.

(iii) Use a multichannel pipette (4.5-mm spacing) to transfer 20 µl of dilution buffer from the mini-incubation tray to each well in sectors 2 through 10.
! **CAUTION** Tape the microtiter plate to the bench-top to secure its location.

(iv) Pipette 40 µl of neat samples (collected cell lysate) from Step 22 into each well of the starting sector, sector 1, where 20 of the 40 µl will be transformed to dilution series.
? **TROUBLESHOOTING**

(v) Create twofold dilution series for each sample (see Experimental design) by using the middle eight channels of the multichannel pipette to extract 20 µl of sample from the 8 wells in each row of sector 1 to the corresponding wells in each of the subsequent sectors, as follows: start with sector 1, row D, pipette 3 times for mixing. Take 20 µl of the neat

sample from sector 1 with the multichannel pipette. Transfer the 20 µl to the corresponding row in sector 2, in which the volume will total 40 µl, resulting in a twofold dilution or proteins.

(vi) Mix gently 3–5 times, and repeat the transfer sequentially until sector 10 (**Fig. 4b**).

! CAUTION Do not create bubbles.

? TROUBLESHOOTING

(vii) Discard 20 µl of mixed sample from sector 10 so that there will only be 20 µl remaining.

(viii) Repeat the dilution series process for the next neat sample remaining, starting with sector 1 and ending at sector 10.

▲ CRITICAL STEP Eight sets of sample dilution can be made at single dilution series preparation process (micropipetter handles eight samples at once).

? TROUBLESHOOTING

(B) 1536-Well microtiter plate setup

(i) Mark all 1536-well plates in 8 by 16 well blocks, sectioning the plate into 12 sectors (**Fig. 4c**).

! CAUTION Designate the plate with a name or number as not to confuse it with other plates. The extraction locations (where pins go into to take samples out) for a 1536-well plate interweave with every other well so each of the 10 sectors that are used will contain a total of four extraction locations.

(ii) Mark the location of the upper left-hand well on the lid of the plate for each extraction numbering them from 40 to 1 (**Fig. 4c**).

(iii) Place ~8 ml of dilution buffer in a single well of a mini-incubation tray.

! CAUTION Tape the mini-incubation tray to the bench-top to secure its location.

(iv) Use a multichannel pipette (4.5-mm spacing) to transfer 10 µl of dilution buffer from the mini-incubation tray to each well in the set of sectors numbered 2 through 10 (**Fig. 4d**).

! CAUTION Tape the microtiter plate to the bench-top to secure its location.

(v) Pipette 20 µl of neat sample (collected cell lysate) into each well sector 1.

! CAUTION 1536-Well plates hold a maximum of 22 µl per well.

? TROUBLESHOOTING

(vi) Create twofold dilution samples using the middle eight channels of the multichannel pipette to transfer 10 µl from each of the 8 wells in each row of sector 1 to the corresponding wells in each of the subsequent sectors.

▲ CRITICAL STEP Start dilution series with row D of sector 1, and pipette three times for mixing. Take 10 µl of the neat sample from sector 1 with the multichannel pipette and transfer the 10 µl to the corresponding row in sector 2, in which the volume will be a total 20 µl resulting in a twofold dilution of lysate. The final volume of is 10 µl.

(vii) Mix gently 3–5 times, and repeat transfer sequentially until sector 10.

! CAUTION Do not create bubbles.

? TROUBLESHOOTING

(viii) Repeat the dilution series process for each of the neat samples remaining starting with sector 1, ending at sector 10, creating a 10-time twofold dilution series for each sample.

? TROUBLESHOOTING

(ix) Repeat Steps (iv)–(viii) for sectors 11 through 20, 21 through 30 and 31 through 40.

25| To store the plates properly, wrap the plate in Parafilm and place on dry ice for 5–10 min, until the lysate is frozen.

26| Place the chilled plate in a plastic Ziploc bag and store the plates in −80 °C.

■ PAUSE POINT The prepared microtiter plates may be stored for 1 year at −80 °C.

RPA printing ● TIMING Up to 18 h

27| Make sure the arrayer humidity is at 80%.

28| Take the plates from −80 °C and let them thaw for 10 min in a Ziploc bag and Parafilm.

29| Remove Ziploc bag and let sit for 15 min.

30| Remove Parafilm and let the plates reach room temperature (about 10 min).

! CAUTION Do not remove the lid.

31| While the microtiter plates are thawing, fill the water reservoir with DI water, making sure the waste water is empty.

32| Load the plates according to the array layout. Use option A when using 384-well plates, and use option B for 1536-well plates.

(A) Microarray printing with 384-well plates

(i) Select the 384-well plate library before loading any initial plates (**Fig. 3a**).

(ii) Load the required number of source plates by double-clicking on the plate interface.

(iii) Set the number of extractions to 10 for each source plate loaded.

(B) Microarray printing with 1536-well plates

(i) Select the 384-well plate library before loading any initial plates.

(ii) Load the required number of source plates by double-clicking on the plate interface.

(iii) Set the number of extractions to 40 for each source plate loaded.

33| Load up to 50 slides, including five blotting slides. Print the lysates with the microarrayer.

■ **PAUSE POINT** Depending on the parameters used, this process can take up to 17 h, which may be done overnight. Once the print is completed, the plates may be re-stored in −80 °C in the manner described above.

BOX 5 | COLLOIDAL GOLD TOTAL PROTEIN STAIN

1. Wash the printed slide from Step 34 with DI water for 15 min with a slight agitation.
2. Refresh the water and wash the slide again for 15 min in DI water with a slight agitation.
3. Incubate the slide with colloidal gold total protein stain (2 ml per slide in a 2 × 1–inch container) with a slight agitation for 1 h at room temperature.
4. Briefly rinse the slide with DI water and allow it to air dry.
5. The slide may be viewed under an optical microscope or scanned with an optical flatbed scanner. The features would be expected to be a shade of purple, varying proportionally to the total protein concentration.

34| Remove the slides from the plates (optional protocols in **Boxes 5** and **6** can be used at this point to estimate the total protein content of the slides).

■ **PAUSE POINT** It is possible to store the printed slides in a sealed container containing desiccant at −20 °C.

? TROUBLESHOOTING

Preparation of slides for antibody staining ● TIMING 1 h, 20 min or 13 h (overnight)

35| Remove the printed slides from storage and wash for 20 min in 2 ml of TBST in a 2 × 1 inch container (or 4 × 2 inch container if four slides are washed at the same time).

36| Refresh the TBST and wash for another 20 min with TBST.

37| Incubate slides in I-Block (or chosen blocking buffer) with a slight agitation for a minimum of 1 h at room temperature or at 4 °C overnight. Cover the slides to avoid evaporation.

? TROUBLESHOOTING

Staining slides with specific primary antibodies ● TIMING 5 h

38| Using an autostainer, set up the parameters to stain the appropriate number of slides using the recommended procedure and timing described in **Box 1** (optional protocols in **Boxes 2–4** can alternatively be used at this point to stain slides instead of the standard protocol in **Box 1**—see Experimental design for further information about the choice of staining and detection systems).

39| Empty water waste carboy and fill up DI water and TBST carboys.

! CAUTION The Autostainer GUI tells how much DI water and TBST volume is required; however, it is best to supply extra.

40| Prime the water pump.

41| Prime the buffer pump, and start the autostainer run (as described in **Box 1** or **Boxes 2–4**).

▲ **CRITICAL STEP** : Although this procedure is slightly complex, it may alternatively be handled step by step manually.

? TROUBLESHOOTING

42| When finished, rinse the slides with TBST and allow to air dry.

43| Label the slides with the antibody used and other pertinent information.

44| Scan the probed slides with either an optical flatbed scanner (colorimetric detections), an Affymetrix 428 scanner (fluorescent detection) or a Li-Cor Odyssey scanner (near-infrared detection).

? TROUBLESHOOTING

BOX 6 | SYPRO RUBY TOTAL PROTEIN FLUORESCENT STAIN

1. Wash the printed slide from Step 34 in a solution of 7% acetic acid and 10% methanol for 15 min at room temperature.
2. Wash the slides in DI water for 5 min.
3. Refresh the DI and wash for 5 more min.
4. Refresh the DI a third time and wash for 5 min.
5. Incubate the slides in SYPRO Ruby blot stain (2 ml per slide in a 2 × 1–inch container) for 30 min at room temperature.
6. Wash the slide in DI water for 10 min, refreshing the DI water every 2 min.
7. Allow the slides to air dry.
8. Scan the slides with a fluorescent slide scanner at 635 nm (Affymetrix 428) to see total protein level.

Image analysis and data quantification ● TIMING **15 min per slide**

45| Quantify scanned spots (ideally >2,400 dpi or 10-μm pixel in resolution saved as TIFF file) by taking pixel-by-pixel intensities using P-SCAN software, which allows the dose–response curves visualization. The curves are analyzed by Proteinscan software to generate the number that represents each dose–response curve. Both programs are available at http://mttab.cancer.gov[7,14].

? TROUBLESHOOTING

● TIMING

Steps 1–3: 15 min; Steps 4–10: 6 min for each time-point collection; Step 11: 10 min; Steps 12 and 13: 30 min; Steps 14–16: 5 min; Steps 17–19: 1 min; Step 20: 30 min; Steps 21 and 22: 5 min; Step 23: 15 min; Step 24: 1 h per plate; Steps 25 and 26: 10 min; Steps 27 and 28: 10 min; Step 29: 15 min; Step 30: 10 min; Steps 31 and 32, 15 min; Steps 33 and 34: overnight (up to 17 h); Steps 35 and 36: 40 min; Step 37: 1 h or 12 h (overnight); Step 38: 20 min; Steps 39 and 40: 10 min; Step 41: 4 h; Steps 42 and 43: 10 min; Step 44: 5 min; Step 45: 15 min per slide.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Problem	Possible reason	Solution
In sample collection, the cell pellets are not all the same size (Step 16)	Collection scraping was not consistent across samples	Mark the centrifuge tubes that are visibly different volumes. When you add lysing buffer, change the buffer volume to match the ratio of buffer to samples volume of the entire collection of samples
	Sample confluence was not consistent across samples	When sampling, make sure the confluency of the flasks are approximately equal across all samples
Air bubbles are present in the cell lysate (Step 17)	Violent 'flicking' or pipetting when lysing the cell pellet	Spin the protein lysate for 1 min with a bench-top centrifuge
Don't see any small pellet after the postlysing spin (Step 20)	Not enough light to see the contrast	Because the heavier elements including the spun down DNA fragments are very small, it is difficult to see the pellet. Hold the centrifuge tube up to the light and there should be a hazy film attached to the bottom of the tube
	Not enough cellular material	When sampling, make sure the confluency of the flasks is approximately equal across all samples
The postlysing pellet comes in suspension with the protein lysate (Step 21)	Not spun long enough or fast enough	Re-spin the centrifuge tube before extracting any protein lysate. Spin at a faster speed if possible
There is not enough sample cell lysate to fill 40 μl in each well of sector 1 of a 384-well plate or 20 μl of a 1,536-well plate (Step 24A(iv) and Step 24B(v))	Not enough sample volume was collected for each condition	Include 20 μl in each well of sector 1 of the 384-well microtiter plates. Then add 20 μl of sampled cell lysate to the appropriate well of sector 1 for each plate. *Note*: All the protein lysate must be the same volume across all the plates
		Move the plate design to a smaller volume well plate, such as a 1,536-well format
Air bubbles in the microtiter plates (Step 24A(vi) and Step 24B(vii))	Mishandling of the well plates	Spin the well plates at 1,000*g* for 5 min

(continued)

TABLE 2 | Troubleshooting table (continued).

Problem	Possible reason	Solution
Too difficult to see the lysate in the wells of the plate (Step 24A(viii) and Step 24B(viii))	Small volumes of clear lysate and buffer are difficult to see with the naked eye	Bromophenol-blue may be added to the 0.67× dilution buffer. When the plate is placed on a white surface, the buffer becomes very visible
Printed features are slightly different sizes (Step 34)	Microarray pins may have accumulated dust particles	Perform a pseudo-print run with 100% ethanol in sector 10, 70% ethanol in sector 2, DI water in sector 3, 10% SDS in sector 4, and DI water in sector 5. Leave the remaining sectors of the plate empty. Print with several replicates and several sample depositions to clean the pins
Blocking buffer is known not to work with the antibody (Step 37)	Some proteins require the use of different blocking buffers	Although the protocol calls for I-Block, the slides may be blocked and stained with whichever blocking buffer works best for the protein of interest
When autostaining slides, the slides begin to dry out (Step 41)	Low ambient humidity	Place a portable humidifier next to the autostainer
Staining does not give high enough signals (Step 44)	Primary antibody concentration may be too low. CSA reagents may be old	Increase primary antibody concentration. Use newer CSA reagents
	Sample is not treated for the induction of the protein	Confirm by WB with the same sample. However, it should be noted that some antibodies do not work in RPA despite showing a good signal with other methods
		Pour excess reagent over the slides
When using a fluorescent scanner for detections, it is difficult to see any printed spots (Step 44)	Gain on the scanner is too low	Increase the gain, and rescan the slide using the Cy5-channel
Replicated samples vary (Step 45)	Biological or technical errors introduced, by experiment and printing, respectively	Identify the error sources. Increase the number of replication of sample collection. Distribute samples to be printed to cancel these error sources (e.g., pins, rows, columns)

ANTICIPATED RESULTS

When the procedure is performed as described, the pellet volumes across all samples collected are expected to be approximately equal. In general, the process yields about 40 µl of lysate from 2–3 million adherent cells in a T-25 flask (~70–80% confluency), resulting in a total protein concentration of ~10–25 µg µl^{-1}. A Colloidal Gold Total Protein stain (**Box 5**) or SYPRO Ruby Total Protein Stain (**Box 6**) helps visualize the printed dilution curves when used on slides from Step 34. Detailed feature morphology can be seen with either an optical or a fluorescent microscope when the slides are stained in either one of these methods, allowing for quick visual inspection.

Detection limit or sensitivity seems to be dependent on antibody affinity and how the epitope is presented in the nitrocellulose membrane, which cannot be measured precisely. Hence, it is hard to generalize the level of sensitivity as a technology specification. Linearity in a dose–response curve is often different depending on which antibody and which sample is used. We have shown the dose–response curves previously[6,14], as well as in **Figure 2a**. Since they are not a typical linear or logistic model, we employ DI_{25} algorithm, which extracts the most representative protein expression value from each curve. On the basis of DI_{25} values, we previously reported CVs between slides ranging from 5 to 10.5% and within a run at 3–4.4% among those which produced analyzable signals[6]. We eliminate low signal experiments (not necessarily a 'low abundance') from the analysis.

The final results are generated as quantifiable protein levels for many samples as detected by the specific antibodies used in the study. Note that protein signal detection should only be performed using antibodies known to work based on expected protein level changes[13,20]. For instance, epidermal growth factor (EGF)-stimulated cells should show an initial increase in EGF-receptor phosphorylation briefly after stimulation using antibodies qualified to work against the same sample in WB

(Spurrier, B.S. and Nishizuka, S., unpublished data). When the signal levels derived by the specific primary antibodies are normalized to total protein content, protein levels across slides may be comparable with one another; however, this approach can often be complicated. Such pitfalls can be largely avoided when the study is designed to investigate changing protein levels within a cell line based on time and dose, relative to one another on a single slide (**Fig. 2d**).

Our recent study demonstrated use of this protocol to show quantitative monitoring of the p53-Mdm2 feedback loop in response to γ-irradiation, which was used to generate a kinetic model of the loop[5]. A set of differential equations were derived to express the feedback loop as a function of time. To evaluate the mathematical model, we used quantitative protein expression data from RPA. Quantitative p53 and Mdm2 (including phosphorylated p53 and phosphorylated Mdm2) protein expression over 8 h showed expected pulses in wild-type cells, whereas no pulses were seen in p53-knockout cells.

First, we determined mathematical parameters based on the result in wild-type cells. Subsequently, the p53 gene was knocked out *in silico,* allowing us to compare the behavior of Mdm2 *in silico* with its actual behavior *in vitro*. Interestingly, Mdm2's behavior in the *in silico* p53-knockout was quite similar to the actual experimental behavior. The results of this study suggest that the fundamental mechanism underlying the p53-Mdm2 feedback loop can be described in part by mathematical models and that quantitative protein data are an excellent reference for validating theoretical models of signal transduction. Such an approach may provide new means for the validation of *in silico* models, particularly in the context of identifying the molecular targets of drugs.

In summary, RPAs provide a unique opportunity to validate theoretical models, provided a large enough number of samples are collected properly. A well-described theoretical molecular network model always lays the groundwork for understanding signal transduction pathways. Rapid development of molecularly targeted drugs will increasingly necessitate a description of their detailed mechanisms at the protein network level. Clearly, the multidimensional proteomic data obtained by RPA will be a precious reference for theoretical biology and drug discovery.

ACKNOWLEDGMENTS We thank Drs. John Austin, Toni Holway and Peter Honkanen of Auslon BioSystems for excellent protein microarray technical support, and Dr. Lynn Young for customizing image processing programs.

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