

Reverse Phase Protein Arrays for Compound Profiling

Nathan Moerke¹ and Mohammad Fallahi-Sichani²

¹Harvard Medical School—ICCB-Longwood Screening Facility, Boston, Massachusetts

²Harvard Medical School—Department of Systems Biology, Boston, Massachusetts

Reverse phase protein arrays (RPPAs), also called reverse phase lysate arrays (RPLAs), involve immobilizing cell or tissue lysates, in small spots, onto solid supports which are then probed with primary antibodies specific for proteins or post-translational modifications of interest. RPPA assays are well suited for large-scale, high-throughput measurement of protein and PTM levels in cells and tissues. RPPAs are affordable and highly multiplexable, as a large number of arrays can readily be produced in parallel and then probed separately with distinct primary antibodies. This article describes a procedure for treating cells and preparing cell lysates, as well as a procedure for generating RPPAs using these lysates. A method for probing, imaging, and analyzing RPPAs is also described. These procedures are readily adaptable to a wide range of studies of cell signaling in response to drugs and other perturbations. © 2016 by John Wiley & Sons, Inc.

Keywords: antibodies • BRAF • cell signaling • fluorescence • melanoma • reverse phase protein arrays

How to cite this article:

Moerke, N. 2016. Reverse phase protein arrays for compound profiling. *Curr. Protoc. Chem. Biol.* 8:179-196.
doi: 10.1002/cpch.9

INTRODUCTION

Quantitative protein measurements are fundamental to all areas of biology. The analysis of proteins in cells and tissues involves determination of both total levels of proteins and the post-translational modification (PTM) states of proteins, such as phosphorylation on specific amino acid residues. An important application of quantitative protein measurements is the characterization of the response of cellular signaling networks to external perturbations such as nutrients and growth factors. System-wide studies of these cellular response networks are becoming increasingly important in understanding human diseases such as cancer and inflammatory disorders. As these studies involve the collection of thousands of data points resulting from combinations of cell lines, perturbations, and time points, it is frequently necessary to measure protein levels using automated high-throughput methods (Albeck et al., 2006).

Large-scale protein-profiling experiments are also important in understanding the mechanism of action and basis for clinical response of the drugs used to treat disease. For example, the response of tumors to kinase inhibitors involves variations or adaptations in multiple signaling pathways that frequently cause primary or acquired resistance to drug treatment (Zhang et al., 2009). Methods for profiling these adaptations by quantifying proteins and PTMs are important in understanding the basis for resistance and identifying possible targets for co-drugging. Furthermore, many drugs, especially kinase inhibitors,



exhibit significant polypharmacology and affect proteins other than their principal target (Knight et al., 2010). Protein-profiling methods allow the identification of pathways affected by these off-target activities, which can guide the development of co-drugging strategies and the repurposing of drugs for new therapeutic indications. In addition to existing drugs, multiplex protein-profiling methods are useful in characterizing the cellular activities and specificity of newly discovered lead and tool compounds.

A number of technologies have been developed for collecting quantitative protein measurements in a high-throughput format. These include high-throughput western blotting, ELISAs, high-throughput mass spectrometry, Luminex xMAP assays, various label-free technologies, and protein microarrays (Wolf-Yadlin et al., 2009). The three major classes of protein microarray are functional arrays, forward phase or antibody arrays, and lysate or reverse phase protein arrays (RPPAs). Functional arrays involve immobilization of peptides or whole proteins and are used for applications such as discovery of protein-protein interactions (Stiffler et al., 2006). In forward phase or antibody arrays, specific antibodies are immobilized on a surface and capture specific antigens from a cell lysate or biological fluid (Nielsen et al., 2003). The bound antigens are then quantified by either direct labeling of analytes or by a labeled detection antibody that recognizes a separate epitope from the capture antibody. RPPAs, also called reverse phase lysate arrays (RPLAs), involve immobilizing cell or tissue lysates, in small spots, onto solid supports which are then probed with primary antibodies specific for proteins or PTMs of interest (Luckert et al., 2012). These primary antibodies can be either directly labeled or probed with a labeled secondary antibody, with chemiluminescent, colorimetric, or fluorescent readouts used to quantify the proteins of interest.

RPPA assays are well suited for large-scale, high-throughput format measurement of protein and PTM levels in cells and tissues. The technology can readily accommodate large numbers of samples, as up to several thousand individual lysate samples can be printed in the area of a standard 75 × 25-mm slide. The process of generating and analyzing RPPAs can be automated to a significant extent, using robotic instrumentation for preparing and spotting samples and specialized software for imaging and extracting data from the arrays. RPPA is also a highly multiplexable technology, as a large number of arrays can readily be produced in parallel and then probed separately with distinct primary antibodies. Production of each array requires only a minute amount of lysate, with as little as 5 µl of 1 mg/ml solution of lysate being sufficient to print hundreds of spots on arrays (Luckert et al., 2012). Thus, the RPPA technology is compatible with small initial sample sizes, and lysates for arraying can readily be produced by growing and treating cells in microplates. The denaturing conditions used in RPPA lysate buffers allow detection of antigens that may be masked in other types of assay. Furthermore, lysates prepared for an RPPA experiment can be stored long term at –80°C for future use in validating the earlier findings or testing new antibodies. A final advantage of RPPA methods is their affordability (particularly for large-scale experiments), as cost per data point is significantly lower than other technologies such as bead-based sandwich assays.

The major disadvantage of RPPAs is their dependence on antibodies with very high selectivity. Unlike western blots, in RPPA assays there is no separation of the protein of interest from off-target proteins by molecular weight prior to detection. While sandwich-style immunoassays such as the Luminex xMAP technology achieve high specificity through the use of two antibodies that recognize distinct nonoverlapping epitopes on the target antigen, RPPA assays use a single antibody per analyte and are more susceptible to high background caused by nonspecific antibody binding. The availability of high-quality primary antibodies is thus the major limiting factor in data quality and the number of targets that can be measured in RPPA experiments.

A common strategy is to use RPPA in a large-scale screen monitoring multiple cell lines, perturbations (such as drugs or growth factors), and time-point combinations. For each condition tested in such a matrix, RPPAs enable collection of a large number of measurements of levels of different proteins and PTM states. An advantage of this approach is that it allows the generation of dose-response curves using multiple doses and replicates of each drug or perturbation. Analysis of these curves can better distinguish true signals from noise than can single-concentration measurements. After analysis of an RPPA dataset, lower-throughput and/or more expensive methods such as quantitative western blotting and high-content microscopy can be used to validate the findings of the RPPA assays.

STRATEGIC PLANNING

Scale of the Experiment

The first consideration in designing an RPPA study is the scale of the experiment. RPPAs can be produced on scales ranging from fewer than a hundred to thousands of spots per array. In order to obtain data of the highest possible quality, it is recommended to design experiments so that each data point has multiple biological replicates (corresponding to unique lysate samples) and technical replicates (corresponding to spots printed on the same or different arrays from the same lysate sample). Depending on the size of the experiment, slides or other solid supports can be used with multiple arraying surfaces (each which can be probed with a distinct primary antibody) or a single arraying surface. In designing a study it will be important to make sure that the desired number of spots (incorporating sufficient biological and technical replicates) can fit in the arraying surface that will be used.

Antibodies

A second component of RPPA study design is the selection of antibodies to be used. A relatively small subset of all antibodies has sufficient selectivity to work in this assay format, due to the problem of high background caused by off-target antigen binding. Several efforts have screened large collections of antibodies to identify those that can perform well in RPPA experiments (Hennessy et al., 2010; Sevecka et al., 2011). An approach used in these studies is to generate RPPAs containing samples from multiple “biological contexts,” representing specific combinations of cell types and treatment conditions. For those antibodies that give a significant signal in one or more contexts using RPPA, quantitative western blotting is used to confirm antibody performance. An antibody is considered to be validated if the data from the RPPA measurements is in agreement with the western blotting data (defined as having a Pearson correlation coefficient of greater than 0.75; Sevecka et al., 2011). For this analysis, the signal for two or more replicate array spots is averaged and compared to the signal in a western blot band corresponding to the protein of interest. The antibodies that have been validated in these studies include a large number of proteins and PTM states that include growth factor receptors, transcription factors, cell cycle regulatory proteins, and components of the major signaling networks such as the MAPK and PI3K/AKT/mTOR pathways.

Choice of Equipment

Additional considerations in the planning of RPPA experiments are the instruments used in producing and imaging arrays. When choosing an arraying method, one option is a contact printer, in which pins are dipped into samples and transfer a defined volume by directly touching the arraying surface. Examples of contact printers that have been successfully used for RPPA production include the Aushon 2470, the Spot Bot and Nano Print (ArrayIT Corporation), the OmniGrid and MicroGrid (Genomics Solutions), and the Q-array (Genetix). The other main type of arraying instrument is a non-contact printer,

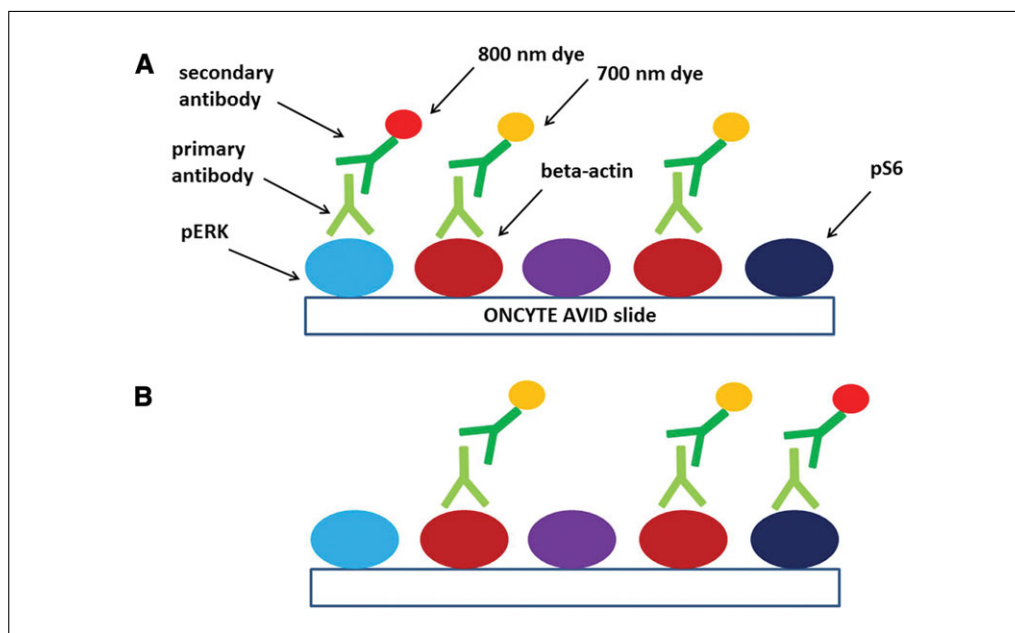


Figure 1 Principle of RPPA experiments. **(A)** Simplified diagram of the detection scheme in a typical RPPA assay. A lysate-deposited slide coated with a nitrocellulose membrane (ONCYTE AVID) contains a complex mixture of denatured proteins including phosphorylated ERK (light blue), phosphorylated S6 (dark blue), and β -actin (dark red). The array is probed with primary antibodies (light green) that are specific for phosphorylated ERK and β -actin but that do not bind to phosphorylated S6 or off-target proteins (purple). Following primary antibody incubation, the array is probed with secondary antibodies conjugated to near-IR fluorescent dyes that emit near 700 nm (orange) or 800 nm (red). **(B)** A view of an identical array as in panel (A), but probed with primary antibodies specific for phosphorylated S6 and β -actin.

which may use syringes or piezoelectric technology: examples of these that have been used for generation of RPPAs include the NanoPlotter (GeSiM) and sciFLEXARRAYER (Scienion AG). In general, contact printers are faster, less expensive, and simpler in design than non-contact arrayers. In choosing the instrument to be used in imaging arrays, the major consideration is the detection method to be used: luminescence, fluorescence, or colorimetric. For fluorescence detection, it is important that the array scanner be capable of measuring signal at the desired excitation and emission wavelengths with adequate sensitivity.

Detection Method

A detection method with key advantages is the use of dyes that fluoresce in the near-IR (far-red) region of the spectrum (at 700 nm or 800 nm). These labels can provide higher signal-to-noise ratios than shorter-wavelength visible fluorescent dyes, as they exhibit less light scattering, lower signal loss due to absorbance, and reduced autofluorescence from proteins, lysate buffer components, and array materials. Instruments that can measure fluorescence at the near-IR wavelengths and have sufficient resolution to image RPPAs include the Odyssey (LI-COR) and the InnoScan 710-IR (Innopsys). A further advantage of using far-red fluorophores is that the measurement of fluorescence at 700 and 800 nm allows the simultaneous detection of the protein or PTM state of interest as well as a second signal (typically a constitutively expressed housekeeping protein) that can be used to normalize the signal of interest to total protein content in the lysate. Figure 1 shows a diagram of this detection scheme in RPPA experiments.

The protocols in this unit describe a previously published RPPA study (Fallahi-Sichani et al., 2015) designed to characterize alterations in cell signaling networks in melanoma during treatment with BRAF kinase inhibitors. At least 50% of human melanomas contain

an activating mutation in BRAF (most frequently V600E) that drives constitutively activated proliferation and survival signaling. Small-molecule inhibitors that specifically inhibit the mutated form of BRAF such as PLX-4032 (vemurafenib) have shown great potential in treatment of melanoma, with most patients exhibiting rapid initial tumor regression (Bollag et al., 2012). However, the development of lethal drug resistance in the majority of patients has posed a significant challenge for this type of therapy (Flaherty, 2010). A comprehensive profile of the effect of BRAF inhibitors on the state of cell signaling pathways is necessary to understand the molecular basis for resistance and develop co-drugging strategies that can overcome resistance.

In this study, a panel of BRAF mutant melanoma cell lines was treated with a panel of BRAF inhibitors: cells were treated with eight different doses of each inhibitor and analyzed at five time points post-treatment (1, 5, 10, 24, and 48 hr). For each cell line, arrays were printed using four biological replicates and two technical replicates for each drug/concentration/time point combination, and each probed with a different primary antibody for a specific protein or PTM state of a protein. The arrays in this study were generated using a contact printing method on an Aushon 2470 pin arrayer, and imaged using near-IR wavelengths with an InnoScan 710-IR slide scanner. To illustrate the expected results of RPPA experiments, representative data is shown in this unit for the response of the BRAF V600E melanoma line RVH-421 to increasing doses of PLX-4032. This data includes measurements of phosphorylated ERK (which is downstream of BRAF and MEK in the MAPK signaling cascade) and phosphorylated ribosomal protein S6 (which is activated by both the MAPK pathway and parallel signaling pathways). Basic Protocol 1 describes a procedure for treating cells and preparing cell lysates, and Basic Protocol 2 describes a procedure for generating RPPAs using these lysates. In Basic Protocol 3, a method for probing, imaging, and analyzing RPPAs is described. We expect that the procedures described in this unit will be readily adaptable to a wide range of studies of cell signaling in response to drugs and other perturbations.

GENERATION OF LYSATES FOR REVERSE PHASE PROTEIN ARRAYS

This protocol describes a method for preparation of lysates from cells treated with drugs or tool compounds that is suitable for preparation of RPPAs, which was used in a study of BRAF kinase inhibitors in human melanoma cell lines (Fallahi-Sichani et al., 2015). The protocol was used for dose-response measurements in BRAF V600E mutant cell lines. Cells were treated with compounds in 96-well tissue culture plates, with each well used to produce a unique lysate sample for arraying. For each cell line, samples were collected at five time points post-treatment (1, 5, 10, 24, and 48 hr) using a mini-library of five inhibitor compounds. At each time point, four replicates of eight concentration points were measured, along with 32 DMSO-treated control wells, for a total of 960 samples. A set of 96 control wells with untreated cells at the start of the experiment were also collected to make a total of 1056 unique biological samples, taken from a total of 11 96-well plates.

Materials

- Compounds being studied: e.g., PLX-4032
- Dimethyl sulfoxide (DMSO)
- Melanoma cells grown in dishes: e.g., RVH-421
- Phosphate-buffered saline (PBS; see recipe)
- 0.25% trypsin/EDTA (available from many suppliers)
- Complete medium (see recipe)
- RPPA lysis buffer (see recipe)

Polypropylene 384-well compound storage plates (Thermo Scientific, cat. no. AB-1056)

BASIC PROTOCOL 1

Reverse Phase Protein Arrays

183

Centrifuge
Clear 96-well tissue culture plates
Pin transfer apparatus (V&P Scientific, <http://www.vp-scientific.com>; Rudnicki and Johnston, 2009)
24-pin aspiration wand (Drummond)
Matrix WellMate (or other automated liquid dispenser for multiwall plates; Rudnicki and Johnson, 2009)
WellMate manifold (sterile, optional)
Multichannel pipettor
Orbital shaker
Metal foil seals for microtiter plates (Corning, cat. no. 6570)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise indicated.

Preparation of compound stocks

1. Before the experiment, prepare source plates of small molecules dissolved in DMSO for the determination of dose-response curves for each compound of interest.

Compound stocks are prepared in polypropylene 384-well storage plates.

The stock plate should contain compounds at 720× the desired final concentration in each well (assuming a pin transfer volume of 250 nl and an assay volume of 180 nl). A number of wells should contain only DMSO as a control. Small-molecule stock plates can be stored long-term at −20°C. See Figure 2 for a representative layout of the 384-well compound storage plate.

Plating of cells

2. Prepare melanoma cell suspension for plating as follows:
 - a. Trypsinize the contents of one or more 10-cm dishes of cells by first aspirating the medium from the plates and briefly washing with sterile PBS. The cells should be harvested under optimal growth conditions in which the cells are subconfluent, have recently received fresh medium, and are actively dividing. After the wash, add 2 ml of 0.25% trypsin/EDTA and incubate 5 min at 37°C. Add 10 ml complete medium (to stop the trypsinization), centrifuge 5 min at 1000 × g, room temperature, aspirate the supernatant, and resuspend the cell pellet in fresh medium.
 - b. Resuspend the cells at a final density of 111,111 cells/ml in complete medium.
3. Using a multichannel pipettor, add 180 μl of cell suspension to each well of a clear 96-well tissue culture plate. Prepare duplicate plates for each time point, and an additional plate for the pre-treatment controls (for 11 plates total if assaying 5 timepoints). Transfer the plates into the tissue culture incubator and incubate at 37°C for 24 hr to allow the cells to adhere to the plates.

If other assays will be run in parallel with the RPPAs, it is convenient to seed additional plates for these at the same time. It may be advantageous to use an automated liquid dispenser to prepare plates if the total number is large.

Compound treatment

4. If necessary, thaw the small-molecule stock plates (from step 1) 1 hr prior to transferring compounds to plates.

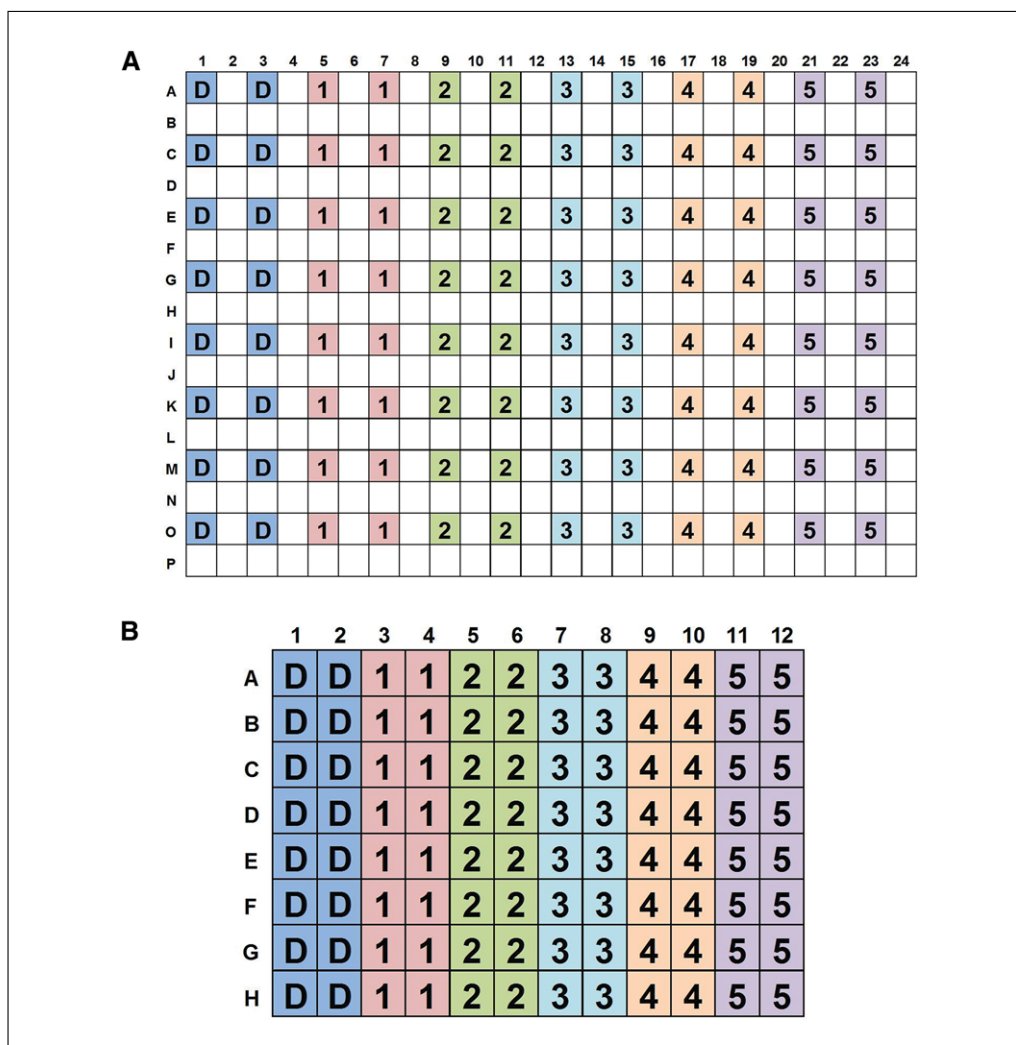


Figure 2 Layout of compound transfer plates (with five test compounds). **(A)** Layout of 384-well compound source plate, with wells filled with DMSO indicated as D, and compound wells indicated with numbers 1 to 5. Typically, each compound will have 8 concentration points in duplicate on this source plate. **(B)** Layout of 96-well assay plate showing the positions of DMSO and compounds transferred from the source plate in panel (A).

- After the cells have grown for 24 hr, remove the plates from the incubator and pin transfer the compounds to the plate using the pin transfer robot and a 250-nl pin array.

At this point, if additional assays will be run in parallel, it is convenient to treat these plates with compounds at the same time.

- For the drug-treatment time-point plates, return the plates to the tissue culture incubator and incubate for 1, 5, 10, 24, and 48 hr, as appropriate, before proceeding to step 7.
- For the untreated plate (and for the time-point plates after the appropriate incubation time), aspirate tissue culture medium using the 24-pin aspiration manifold. Transfer 100 μ l of ice cold PBS to each well using a multichannel pipettor, rinse briefly by gentle manual agitation of the plate, and aspirate again using the manifold.
- Add 30 μ l of RPPA lysis buffer equilibrated at room temperature to each well of the tissue culture plate, and incubate on an orbital shaker for 5 min.

9. *Optional:* Seal plates with opaque foil and store at -80°C until ready to array samples.
10. Proceed to Basic Protocol 2.

PRODUCTION OF REVERSE PHASE PROTEIN ARRAYS

This protocol describes a procedure for producing RPPAs from cell lysates generated in Basic Protocol 1. This procedure has been used to print a set of replicate nitrocellulose-coated slides, each containing an array of 2112 lysate spots generated by printing duplicates of the 1056 unique lysates produced in Basic Protocol 1. The combination of 2 technical replicates and 4 biological replicates yielded 8 array spots for each compound/concentration/time point combination (1600 total), 64 array spots for DMSO controls at each time point (320 total), and 192 array spots corresponding to untreated cells at the start of the experiment. Prior to printing, it is necessary to filter the lysates generated in Basic Protocol 1 to remove nucleic acids and cell debris

Materials

- Lysate plates (Basic Protocol 1)
- 96-well filter plates (Pall, cat. no. 5042)
- 96-well lysate collection plates (Corning, cat. no. 3870)
- Multichannel pipettor
- Centrifuge with microtiter plate carrier
- Polypropylene 384-well lysate storage plates (ABgene storage plates; Thermo Scientific, cat. no. AB-1056)
- Arraying robot (e.g., Aushon 2470)
- ONCYTE AVID nitrocellulose coated single-pad slides (Grace Biolabs, cat. no. 305177)

Filtration and formatting of cell lysates

1. If necessary, thaw the lysate plates generated in Basic Protocol 1.
2. For each 96-well lysate plate, place a 96-well filter plate on top of a 96-well collection plate. Use a small piece of plastic tape on each side to hold the two plates together
3. Using a multichannel pipettor, carefully transfer the 30 μl of lysate from each 96-well lysate plate into a 96-well filter plate stacked on top of a collection plate.

The lysates will be highly viscous prior to filtration. Thus, if a pipetting robot is used instead of a multichannel pipettor, it will be important to confirm that is capable of handling viscous solutions.

4. Spin the stacked filtration/collection plates for 10 min at $2000 \times g$, room temperature, in a centrifuge to filter the lysates.
5. Remove the clarified lysates in collection plates from the centrifuge, detaching and discarding the filtration plates.
6. Using a multichannel pipettor, transfer the clarified lysates into 384-well ABgene plates.

The volume will be reduced after centrifugation and filtration. Four 96-well plates of lysates can be merged into one 384-well plate to maximize the efficiency of arraying, assuming the user is careful to track how the wells are combined. Figure 3 shows an example of how 96-well plates can be mapped onto a 384-well plate. This mapping should be captured in a spreadsheet or other document so that the samples can be tracked. At this stage, the clarified lysate plates can be stored long-term at -80°C until arraying. If desired, the lysates can be initially stored frozen in the 96-well collection plates and transferred into 384-well plates before arraying.

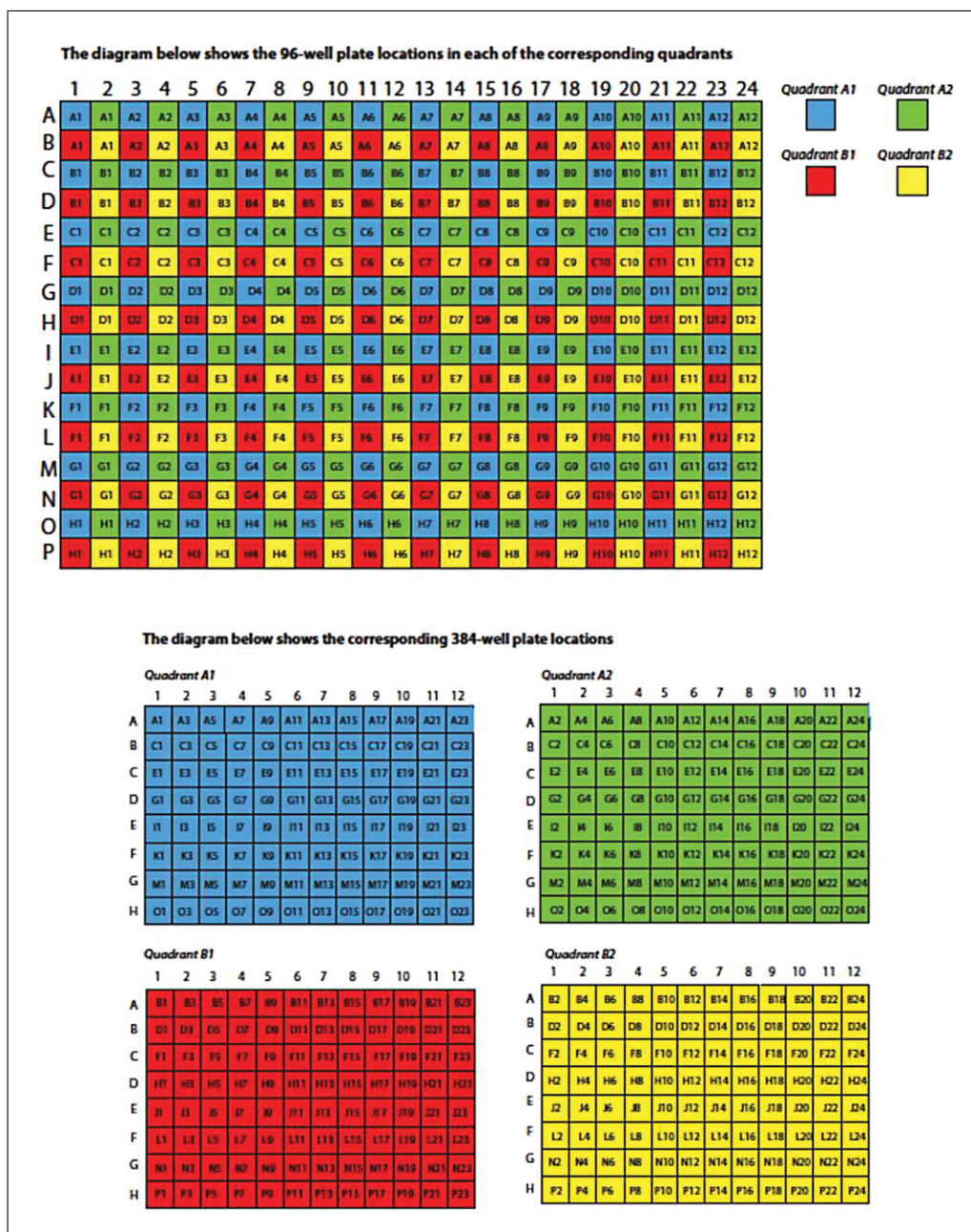


Figure 3 Mapping of 96-well plates combined into a 384-well plate. Schematic by which four 96-well lysate plates can be merged into a single 384-well source plate for arraying, with each 96-well plate mapping into a separate quadrant.

Printing of arrays

7. Prepare the arrayer to be used for printing slides (e.g., Aushon 2470) by adjusting the pin configuration in the print head, emptying the waste container, and filling the wash container with distilled deionized water.
8. Set up the desired printing configuration within the arrayer and arrayer software.

A typical configuration, which was used in this study, involves a 4 × 6 array of pins on the arrayer head that dips into the 384-well source plate. The deposition onto the slide of lysate by this set of pins will generate an array of 24 smaller sub-arrays, as shown in Figure 4A. In this study, each sub-array contained 44 spots, with 1056 spots in the larger main array (the same as the number of unique wells). This array was printed in duplicate on the left and right sides of the same slide for a total of 2112 spots. Figure 4B shows the

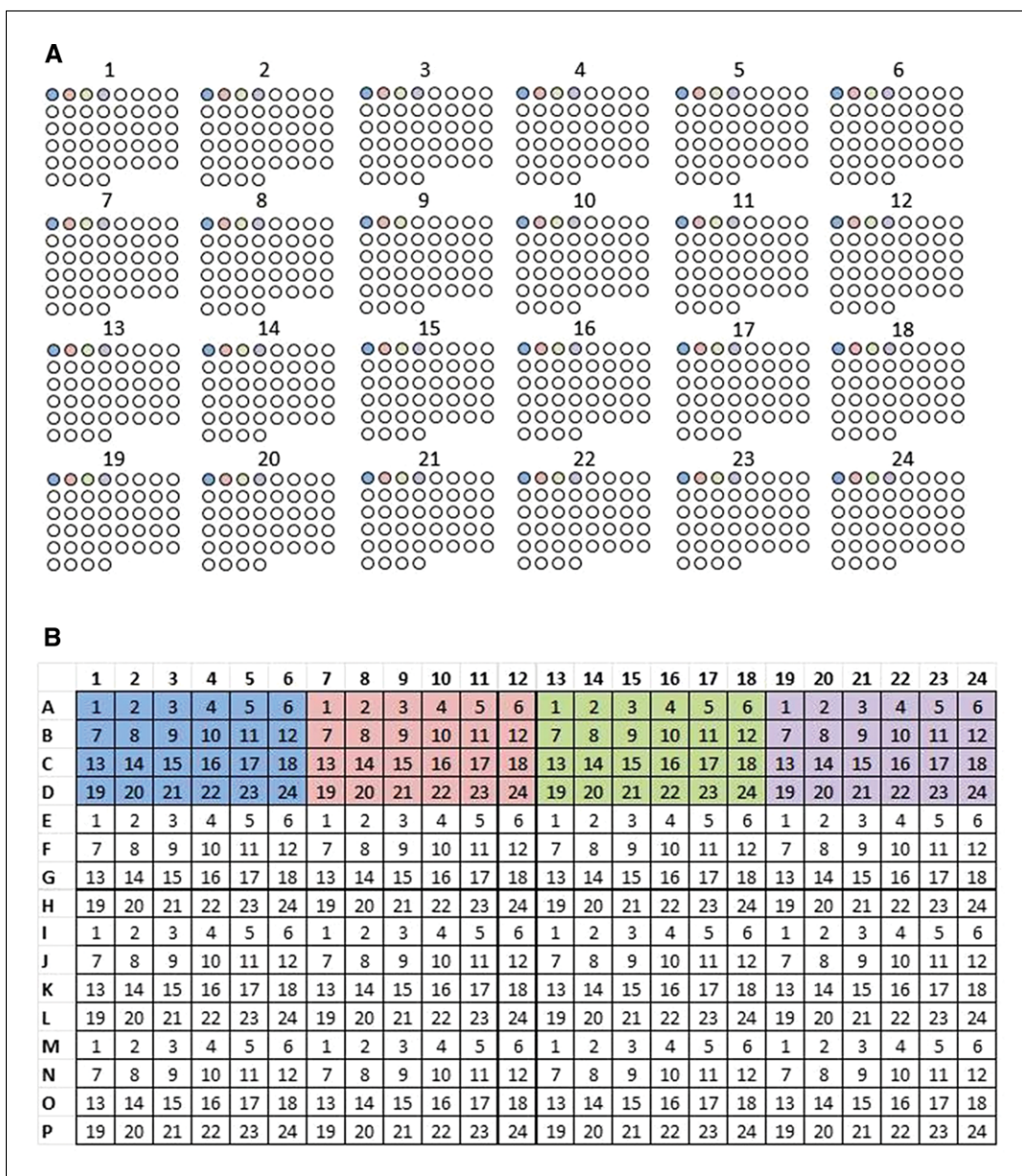


Figure 4 Example of array design using Aushon 2470 printer. **(A)** Configuration of array spots on slide using a 4×6 array of pins on the Aushon 2470 printer. There are 24 sub-arrays, each containing 44 spots for 1056 spots total. This array is replicated on the left and right sides of a single slide to generate duplicate spots from each sample well. Spots generated from the first four deposition steps are color coded to correlate positions on the array with positions in the source plate shown in panel **(B)**. **(B)** Configuration of a lysate source plate used to generate the array shown in panel **(A)**. The wells used in the first four deposition steps are color coded to correlate with the position of spots in the array, with each deposition step sampling from 24 contiguous wells at the same time.

pattern by which the 4×6 set of pins dips into a 384-well source plate. It is important to track the positions of each well on the final array in a spreadsheet so that the spot intensity information can be accurately correlated with the identity of the samples.

- Transfer the 384-well lysate source plates and ONCYTE AVID slides into the appropriate racks inside the arrayers.
- Print the desired number of slides on the arraying robot.

For the configuration used in this study on an Aushon 2470, each slide will take approximately 30 min to print. If necessary, the printing of slides can be run overnight. In an

experiment such as this, in which a single array is printed in duplicate on each slide, it is recommended to print enough slides for each antibody to be tested, plus at least five to ten extra slides in case any antibody needs to be repeated.

11. Transfer the printed arrays to a sealed container that is protected from moisture and store at room temperature in the dark until ready to proceed with Basic Protocol 3.

IMMUNOSTAINING, IMAGING, AND ANALYSIS OF REVERSE PHASE PROTEIN ARRAYS

BASIC PROTOCOL 3

This protocol describes a procedure for immunostaining the RPPAs generated in Basic Protocol 2 using specific antibodies. This is followed by quantitative imaging and analysis to measure total or post-translationally modified protein levels in the sample lysates that were generated in Basic Protocol 1. This procedure has been used to measure a number of key signaling pathway components including phosphorylated ERK and S6.

Materials

Arrays to be immunostained and imaged (see Basic Protocol 2)

PBS-T (see recipe)

RPPA wash buffer: 100 mM Tris·Cl, pH 9.0

Odyssey Blocking Buffer (LI-COR, cat. no. 927-40010)

Primary antibodies:

Rabbit anti-Erk1/2 phospho-T204/T205 antibody (Cell Signaling Technologies, cat. no. 4370)

Rabbit anti-rpS6 phospho-S235/S236 antibody (Cell Signaling Technologies, cat. no. 4848)

Mouse anti- β actin antibody (Sigma, cat. no. A1978)

Secondary antibodies:

Goat anti-mouse IgG antibody, DyLight 680 conjugated (Pierce, cat. no. 35518)

Goat anti-rabbit IgG antibody, DyLight 800 conjugated (Pierce, cat. no. 35571)

Perfect Western 6-Chamber Incubation Tray (GenHunter, cat. no. B131)

Orbital shaker

15- and 50-ml conical tubes (e.g., Corning Falcon)

Forceps

Centrifuge

Near-infrared-capable scanner (e.g., InnoScan 710-IR, Innopsys)

Microarray analysis software (e.g., Mapix from Innopsys)

Spreadsheet software (e.g., Microsoft Excel)

Multidimensional data visualization software (e.g., Matlab and DataPflex)

Washing of slides

An initial washing procedure is necessary to remove potentially fluorescent buffer components that can interfere with analysis of the slides.

1. Transfer the array slides to the 6-chamber incubation trays, and wash three times, each time for 5 min with 10 ml PBS-T in each chamber using an orbital shaker at room temperature.

Remove the buffer between washes by careful decantation from the incubation tray. The edges of slides can be held by forceps or a gloved hand to prevent them from falling out of the tray.

2. Add 10 ml per slide of RPPA wash buffer to the trays and incubate on an orbital shaker at room temperature. Wash the slides for a total of 48 hr, with at least four changes of RPPA wash buffer during this time.
3. After 48 hr, wash the slides three times, each time for 5 min with 10 ml PBS-T at room temperature.
4. Carefully transfer the slides to 50-ml conical polypropylene tubes using forceps (with one slide per tube). Spin the slides dry in the tubes by centrifugation for 5 min at $33 \times g$, room temperature.
5. Remove the slides from the Falcon tubes and briefly air dry on a flat, clean surface. Optionally, at this time, scan the slides for residual autofluorescence from the lysate buffer at 700 nm on an available near-infrared capable scanner.

Blocking the arrays

6. Transfer the washed slides to 6-chamber incubation trays and add at least 5 ml of LI-COR Odyssey blocking buffer to each slide.
7. Incubate the slides in the blocking buffer on an orbital shaker for 1 hr at room temperature.
8. Just before the end of the blocking period, prepare appropriate dilutions of primary antibodies (a typical dilution factor is 1:1000, used for both pERK and pS6 antibodies) in LI-COR Odyssey blocking buffer using 15-ml conical tubes, pooling each rabbit primary antibody against a protein or PTM of interest with a 1:1000 dilution of mouse anti- β actin antibody.

Each dilution should be prepared so there is at least 4 ml total volume per slide.

9. Carefully remove the blocking buffer by decantation without washing.

Probing arrays with antibodies

10. Add primary antibody dilutions to the slide chambers, and incubate overnight on an orbital shaker at 4°C
11. The next day, wash the slides four times, each time for 5 min with 10 ml PBS-T at room temperature
12. During the final wash, prepare dilutions of secondary antibodies. Dilute both the DyLight 680 conjugated goat anti-mouse IgG antibody and the DyLight 800 conjugated goat anti-rabbit IgG antibody together in the same solution at a 1:7500 dilution for each, in LI-COR Odyssey buffer using 15-ml conical tubes. Prepare at least 4 ml total volume per slide.
13. After decanting the final wash, add secondary antibody dilutions to slides and incubate for 2 hr on an orbital shaker at room temperature.
14. Wash the slides four times, each time for 5 min with 10 ml PBS-T at room temperature
15. Briefly rinse the slides two to three times, each time with 10 ml of distilled, deionized water to remove residual salt from the PBS-T wash buffer.
16. Carefully transfer the slides to 50-ml conical tubes using forceps. Spin the slides dry in the tubes by centrifugation for 5 min at $33 \times g$, room temperature.
17. Remove the slides from the tubes and briefly air dry on a flat, clean surface. Keep the probed slides in a sealed container protected from light until ready to image.

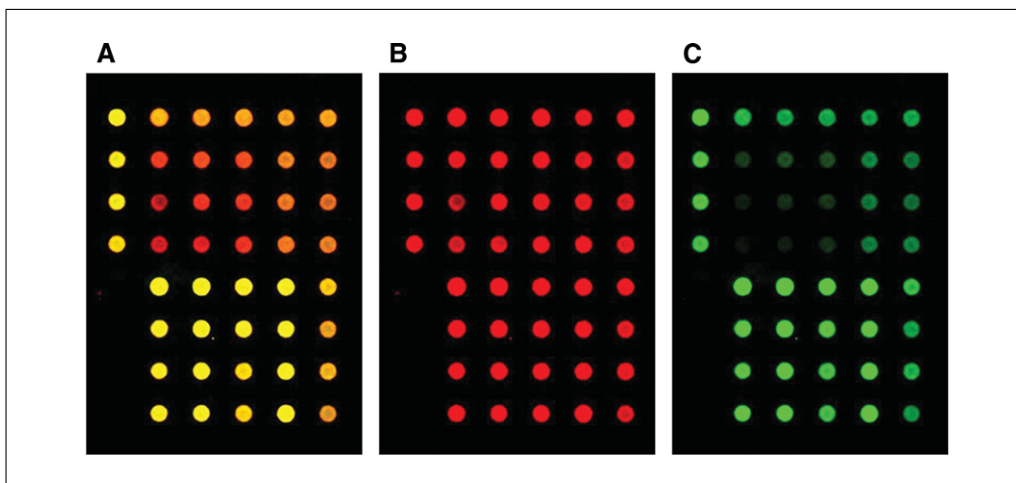


Figure 5 Fluorescent Images of RPPA slides. **(A)** Fluorescent image of part of an RPPA slide scanned on the InnoScan 710-R (Innopsys) at 700 nm (red) and 800 nm (green), showing the two channels merged. The slide was probed for phosphorylated S6 and for β -actin as a normalization control. **(B)** Same image as part (A), but only showing the 700 nm channel. **(C)** Same image as part (A), but only showing the 800 nm channel.

Imaging slides and data analysis

18. Scan the slides using a fluorescence-scanning instrument capable of measuring fluorescence at 700 and 800 nm (e.g., an Innopsys InnoScan 710-IR).

Figure 5 shows representative images of arrays scanned at these two wavelengths.

To obtain optimal signal, it is important to optimize the focus offset position of the scanner, the resolution of the scanning, and the intensity settings for both the 700 and 800 nm channels. The details will depend on the instrument used.

19. Using the instrument software, export the raw images of the scanned arrays to a format that is compatible with the image analysis software to be used.

On some scanners (such as the InnoScan 710-R), image analysis is integrated with the instrument software, and can be performed automatically as soon as slides are imaged.

20. Using image analysis software (e.g., Mapix from Innopsys), extract the fluorescence intensity values for the arrays from the raw images.
21. Check the results of the analysis to make sure no parts of the arrays are misassigned, and re-do the analysis if necessary.
22. Export the data from the image analysis into a format usable by Microsoft Excel or other suitable spreadsheet software.

At this stage, the array spot data can be integrated with the identity of each lysate sample using the mapping generated in step 6 of Basic Protocol 2.

23. For each spot in the array, calculate the normalized signal intensity for the protein or PTM state of interest by dividing the mean spot intensity measured in the 800 nm channel by the mean spot intensity measured in the 700 nm channel (to control for total protein content).
24. Plot the data using spreadsheet software, or if desired, a software package for multidimensional data visualization. See Figure 6 for an example of typical dose-response curves generated by this experiment.

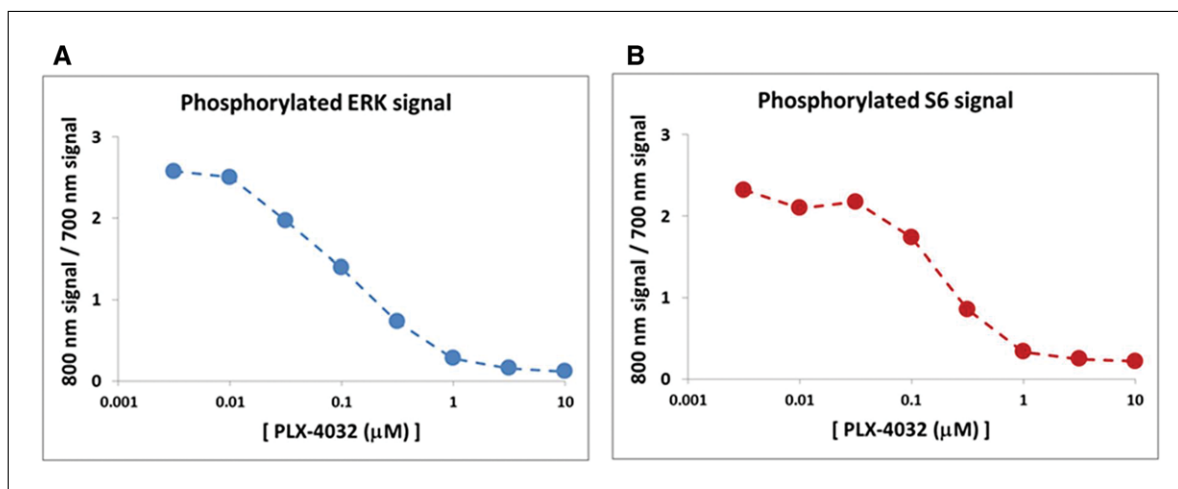


Figure 6 Representative RPPA data for RVH-421 melanoma cells treated with PLX-4032. **(A)** RPPA dose response curve of phosphorylated ERK at 10 hr post-treatment with increasing doses of the BRAF kinase inhibitor PLX-4032, as determined by the fluorescence intensity ratio of the 800 nm channel (used for the anti-pERK primary antibody) and the 700 nm channel (used for the anti-β actin primary antibody). **(B)** RPPA dose response curve of phosphorylated S6 at 24 hr post-treatment with increasing doses of the BRAF kinase inhibitor PLX-4032, as determined by the fluorescence intensity ratio of the 800 nm channel (used for the anti-pS6 primary antibody) and the 700 nm channel (used for the anti-β actin primary antibody).

REAGENTS AND SOLUTIONS

Use Milli-Q purified water or equivalent in all recipes and protocols steps unless otherwise specified.

Complete medium

RPMI 1640 medium supplemented with:
 10% fetal bovine serum (FBS)
 1× penicillin/streptomycin (add from 100× stock; Invitrogen)
 5 μg/ml Plasmocin (Invivogen)
 Store up to 1 week at 4°C
 Warm up to 37°C in a water bath before using

PBS-T washing solution

To PBS (see recipe), add Tween-20 to a final concentration of 0.2% (v/v). Store up to 1 month at room temperature.

Phosphate-buffered saline (PBS)

37 mM NaCl
 2 mM KCl
 10 mM sodium phosphate dibasic
 2 mM potassium phosphate monobasic
 Adjust to pH 7.4 using NaOH and/or HCl
 Filter-sterilize or autoclave
 Store indefinitely at room temperature

RPPA cell lysis buffer

Make the following buffer stock:
 50 mM Tris hydrochloride
 2% (w/v) SDS
 5% (v/v) glycerol
 5 mM disodium EDTA
 1 mM NaF

Adjust pH to 6.8 using NaOH and/or HCl
Store with the above ingredients up to several months at room temperature
To the above stock, add:
1 × protease inhibitor tablets (Complete Mini EDTA-Free Protease Inhibitor Cocktail, Roche cat. no. 11836170001)
1 × phosphatase inhibitors (Phosphatase Inhibitor Cocktail 2; Sigma, cat. no. P5726)
10 mM β -glycerophosphate
1 mM PMSF
1 mM Na_3VO_4
1 mM DTT
Divide the buffer into aliquots, and freeze at -80°C (stable for up to 1 year)

COMMENTARY

Background Information

RPPAs have been successfully used in numerous studies of human disease and cellular pharmacology. In experiments using cultured mammalian cell lines, RPPAs have facilitated cell signaling applications such as classification of receptor tyrosine kinases (Wagner et al., 2013), characterization of the rewiring of apoptotic pathways in response to EGFR inhibition (Lee et al., 2012), and a study of canonical and non-canonical Wnt signaling (Luckert et al., 2012). The technology is also suitable for lysates prepared from tissue samples: for example, RPPAs have been used to profile signaling networks that are altered in breast cancer using samples from tumor biopsies and matched normal tissue (Gujral et al., 2013). Thus, biomarkers of disease state or drug response identified in RPPA experiments with cultured cell lines can readily be monitored in clinical studies using the same technology. RPPAs can also be used as a method for screening compound libraries by identifying molecules that induce a specific state of cell signaling, as determined by measuring the phosphorylation state of multiple proteins (Sevecka and MacBeath, 2006).

Critical Parameters

Antibody selection

When possible, antibodies to be used should be selected from those previously validated for RPPA. In addition to several published studies (Hennessy et al., 2010; Sevecka et al., 2011), the RPPA Core Facility at the MD Anderson Cancer Center maintains a list of validated antibodies (see <http://www.mdanderson.org/education-and-research/resources-for-professionals/scientific-resources/core-facilities-and-services/functional-proteomics-rppa-core/antibody-lists-protocols/functional-proteomics-reverse-phase-protein-array>

-core-facility-antibody-lists-and-protocols.html). It is important to note that an antibody validated for RPPA in one or more biological contexts may not perform as well in other cell lines, or under different growth and treatment conditions. This is due to the fact that the expression levels of proteins responsible for off-target interactions with a given antibody can vary significantly between cell lines (or in some cases, under different growth conditions in the same cell line). In large studies including many cell lines and perturbations, it is likely not feasible to re-validate antibodies in every new biological context. Thus, the data from these studies must be interpreted with care, and should be validated by follow-up experiments using complementary methods such as high-content microscopy and western blotting.

Choice of normalization method

A key parameter in RPPA experiments is the specific method used to normalize the signal of interest to the total amount of protein in each spot in the array. Normalization is commonly accomplished by incubation with a primary antibody specific for a generally expressed housekeeping protein, followed by an appropriate fluorescently conjugated secondary antibody. The detection of a reference protein in RPPA experiments can be considered analogous to the use of a loading control in a western blot. β -Actin is a popular and commonly used normalization signal for RPPA experiments, and a number of highly specific primary antibodies from multiple species including mouse and rabbit are available for this protein. Other housekeeping proteins that can be used for normalization include α - and β -tubulin, COX IV, and GAPDH. The choice of normalization protein will be influenced by the specific biology being studied: for example, GAPDH would be preferable to β -actin in

biological contexts where actin stability is expected to be altered. In most cases, the protein used for normalization will be more abundant in lysates than the protein or PTM of interest. Since the 700 nm channel is less sensitive than the 800 nm channel in fluorescence scanners, this channel is generally used to detect the normalization signal. A potential alternative to antibody staining for normalization is the use of a total protein stain that fluoresces in the 700 nm channel, such as IRDye Blue Protein Stain (LI-COR).

Arraying conditions

Proper selection of arraying substrates and printing conditions are necessary for obtaining high-quality data using the RPPA method. For the array surface, two key parameters are the protein binding capacity and the background autofluorescence of the material. One of the most commonly used arraying substrates is nitrocellulose, due to its high binding capacity and use in other immunoassay methods such as western blotting. The total surface area available for protein binding can be enhanced by using nitrocellulose surfaces with a porous three-dimensional structure, as in ONCYTE AVID substrates (Grace Biolabs). ONCYTE AVID slides also have relatively low background fluorescence compared to other materials. When designing arrays, it is necessary to optimize the spacing and configuration of spots to allow analysis of the maximum number of samples without overlap. In addition, the size of array spots, which is affected by the number of depositions and the protein concentration of the lysates, must be optimized to ensure adequate signal. A general rule of thumb for imaging arrays is that the pixel size should be no more than 1/10th of the spot diameter. Therefore, the resolution of the instrument to be used in scanning arrays should be kept in mind when adjusting the spot size.

Analysis software

The software used to analyze array images needs to automate a number of processes in order to extract useful data:

1. Detection of the overall grid of array spots in the image
2. Accurately segmenting the array spots
3. Quantifying the signal in spots and subtracting the background
4. Flagging any artifacts in the array image such as dust or misformed spots

A number of commercial software packages can be used to analyze array images, such

as Image Studio from LI-COR and MicroVigene from VigeneTech. The software used in this study, Mapix from Innopsys, has the capability of analyzing images from a batch of arrays as soon as they are acquired, increasing the throughput of the process.

Troubleshooting

Missing or misaligned spots in arrays

Problems with the printing process can result in missing or misaligned array spots. The quality of arrays can be assessed immediately after printing by scanning in a fluorescent imager using the 700 nm channel, as autofluorescence of buffer components will permit visualization of the spots. If spots are missing, the source plates should be checked to make sure no wells are empty or have very low volume of lysate. Assuming there are no issues with the source plates, a common cause of missing or misaligned spots for contact arrayers is damage to the pins. The integrity of pins can be checked using a microscope or stereo microscope, and any that are bent or broken should be replaced in the print head and sent to the manufacturer for repair if possible. Missing array spots can also result from the deposition of salts or other materials onto pins during the printing process. This problem can be resolved by thoroughly cleaning the pins and optimizing the washing protocol during print runs, following the procedures recommended by the instrument manufacturer.

Overlapping spots in arrays

If the diameter of printed spots is too large relative to their spacing, overlapping will interfere with analysis of the array images. This is particularly likely to occur if the sample is highly concentrated and/or viscous. This problem can be resolved by reducing the number of depositions made by the arrayer pins, diluting samples with additional lysate buffer to lower the protein concentration before printing, or reprinting the arrays with a lower grid density and greater spacing between spots.

Improper alignment of print head

Improper alignment of the print head for a contact arrayer can cause less lysate than expected to be spotted on arrays (if the head is too high), or damage the arraying surface, causing misformed spots (if the height is too low). If either issue is suspected, the alignment of the print head should be recalibrated as specified by the manufacturer of the instrument.

Weak antibody staining on arrays

When low signal causing a poor signal-to-noise ratio is observed when scanning one or more of the arrays in an experiment, the first step to take should be increasing the intensity settings on the scanner. If this does not improve the signal, it may be necessary to optimize the primary and secondary antibody concentrations to improve the sensitivity of detection. In the case where low signal is observed for multiple arrays using different antibodies, it may be necessary to increase the amount of protein in each array spot. This can be achieved by using a higher number of depositions per spot when printing, or preparing lysates using a higher cell density in each well.

High array background staining

In the case of high background staining on arrays, it may be necessary to further optimize the blocking and washing procedures. This process can include testing different blocking reagents and assay diluents for the primary and secondary antibodies. Optimization of assay diluents can include testing different protein additives, detergents, and salt concentrations in order to minimize nonspecific binding interaction. It may also be necessary to increase the number of washes during array processing and lower the concentration of primary and/or secondary antibodies used.

Saturated fluorescent signal

If any array spots show saturated pixels in either the 700 or 800 nm fluorescence channels (saturated pixels are colored white on many instruments), the arrays should be rescanned using lower intensity settings until there is no longer saturation. In some cases it may be necessary to use a lower concentration of the labeled secondary antibody and/or primary antibody to reduce the fluorescent signal to within the linear range.

Errors in image analysis

Occasionally the algorithms used by array analysis software will incorrectly assign individual spots or entire regions of the array, leading to bad data points or systematic errors in the data. Errors in the image analysis process are more likely in cases of high background, overlapping spots, or irregularities in the arraying pattern. If these are observed, it will be necessary to optimize parameters in the algorithm used to detect spots and the array pattern. In the case where only a few spots are incorrectly assigned in an area, it may be possible to manually correct these using the analysis software.

Anticipated Results

The protocols described in this unit will produce a set of protein and post-translational state measurements, as illustrated in Figure 6 for phosphorylated ERK and S6 ribosomal protein in RVH-421 cells treated with PLX-4032.

Time Considerations

Once all of the necessary reagents are in hand, the procedures described in this unit can be completed in approximately two standard work weeks: the first week to seed cells, treat with compounds, prepare lysates, and print arrays; and the second week to wash, probe, and scan arrays followed by analysis of the images. In practice, the length of time required to complete an RPPA experiment will depend on the scope of the data set, the time points for which data is collected, the number of protein levels and PTM states measured, and the type of data analysis required.

Acknowledgements

We would like to thank Innopsys for valuable technical discussions as well as early use of a demo model of their InnoScan 710-IR scanner to generate the data in this study, and Grace Bio-Labs for valuable discussions regarding the use of ONCYTE AVID slides for arraying. We also thank Cyril Benes and Anahita Dastur of the Center for Molecular Therapeutics at MGH for providing the melanoma cell lines used in this study. Finally, we thank Caroline Shamu and the members of the ICCB-Longwood Screening Facility for laboratory automation support. This work was supported by NIH LINCS grant U54 HG006097.

Literature Cited

- Albeck, J.G., MacBeath, G., White, F.M., Sorger, P.K., Lauffenburger, D.A., and Gaudet, S. 2006. Collecting and organizing systematic sets of protein data. *Nat. Rev. Mol. Cell Biol.* 7:803-812. doi: 10.1038/nrm2042.
- Bollag, G., Tsai, J., Zhang, J., Zhang, C., Ibrahim, P., Nolop, K., and Hirth, P. 2012. Vemurafenib: The first drug approved for BRAF-mutant cancer. *Nat. Rev. Drug. Discov.* 11:873-886. doi: 10.1038/nrd3847.
- Fallahi-Sichani, M., Moerke, N. J., Niepel, M., Zhang, T., Gray, N.S., and Sorger, P.K. 2015. Systematic analysis of BRAF(V600E) melanomas reveals a role for JNK/c-Jun pathway in adaptive resistance to drug-induced apoptosis. *Mol. Syst. Biol.* 11:797. doi: 10.15252/msb.20145877.
- Flaherty, K.T. 2010. Narrative review: BRAF opens the door for therapeutic advances in

- melanoma. *Ann. Intern. Med.* 153:587-591. doi: 10.7326/0003-4819-153-9-201011020-00008.
- Gujral, T.S., Karp, R.L., Finski, A., Chan, M., Schwartz, P.E., MacBeath, G., and Sorger, P. 2013. Profiling phospho-signaling networks in breast cancer using reverse-phase protein arrays. *Oncogene* 32:3470-3476. doi: 10.1038/onc.2012.378.
- Hennessy, B.T., Lu, Y., Gonzalez-Angulo, A.M., Carey, M.S., Myhre, S., Ju, Z., Davies, M.A., Liu, W., Coombes, K., Meric-Bernstam, F., Bedrosian, I., McGahren, M., Agarwal, R., Zhang, F., Overgaard, J., Alsner, J., Neve, R.M., Kuo, W.L., Gray, J.W., Borresen-Dale, A.L., and Mills, G.B. 2010. A technical assessment of the utility of reverse phase protein arrays for the study of the functional proteome in non-microdissected human breast cancers. *Clin. Proteomics* 6:129-151. doi: 10.1007/s12014-010-9055-y.
- Knight, Z.A., Lin, H., and Shokat, K.M. 2010. Targeting the cancer kinome through polypharmacology. *Nat. Rev. Cancer* 10:130-137. doi: 10.1038/nrc2787.
- Lee, M.J., Ye, A.S., Gardino, A.K., Heijink, A.M., Sorger, P.K., MacBeath, G., and Yaffe, M.B. 2012. Sequential application of anticancer drugs enhances cell death by rewiring apoptotic signaling networks. *Cell* 149:780-794. doi: 10.1016/j.cell.2012.03.031.
- Luckert, K., Gujral, T.S., Chan, M., Sevecka, M., Joos, T.O., Sorger, P.K., Macbeath, G., and Pötz, O. 2012. A dual array-based approach to assess the abundance and posttranslational modification state of signaling proteins. *Sci. Signal* 5:pl1. doi: 10.1126/scisignal.2002372.
- Nielsen, U.B., Cardone, M.H., Sinsky, A.J., MacBeath, G., and Sorger, P.K. 2003. Profiling receptor tyrosine kinase activation by using Ab microarrays. *Proc. Natl. Acad. Sci. U.S.A.* 100:9330-9335. doi: 10.1073/pnas.1633513100.
- Rudnicki, S. and Johnston, S. 2009. Overview of liquid handling instrumentation for high-throughput screening applications. *Curr. Protoc. Chem. Biol.* Dec 1:43-54. doi: 10.1002/9780470559277.ch090151.
- Sevecka, M. and MacBeath, G. 2006. State-based discovery: A multidimensional screen for small-molecule modulators of EGF signaling. *Nat. Methods* 3:825-831. doi: 10.1038/nmeth931.
- Sevecka, M., Wolf-Yadlin, A., and MacBeath, G. 2011. Lysate microarrays enable high-throughput, quantitative investigations of cellular signaling. *Mol. Cell Proteomics*. 10:M110005363. doi: 10.1074/mcp.M110.005363.
- Stiffler, M.A., Grantcharova, V.P., Sevecka, M., and MacBeath, G. 2006. Uncovering quantitative protein interaction networks for mouse PDZ domains using protein microarrays. *J. Am. Chem. Soc.* 128:5913-5922. doi: 10.1021/ja060943h.
- Wagner, J.P., Wolf-Yadlin, A., Sevecka, M., Grenier, J.K., Root, D.E., Lauffenburger, D.A., and MacBeath, G. 2013. Receptor tyrosine kinases fall into distinct classes based on their inferred signaling networks. *Sci. Signal* 6:ra58. doi: 10.1126/scisignal.2003994.
- Wolf-Yadlin, A., Sevecka, M., and MacBeath, G. 2009. Dissecting protein function and signaling using protein microarrays. *Curr. Opin. Chem. Biol.* 13:398-405. doi: 10.1016/j.cbpa.2009.06.027.
- Zhang, J., Yang, P.L., and Gray, N.S. 2009. Targeting cancer with small molecule kinase inhibitors. *Nat. Rev. Cancer* 9:28-39. doi: 10.1038/nrc2559.