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Phosphoprotein Stability in Clinical Tissue and Its Relevance for Reverse Phase Protein Microarray Technology

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Abstract

Phosphorylated proteins reflect the activity of specific cell signaling nodes in biological kinase protein networks. Cell signaling pathways can be either activated or deactivated depending on the phosphorylation state of the constituent proteins. The state of these kinase pathways reflects the *in vivo* activity of the cells and tissue at any given point in time. As such, cell signaling pathway information can be extrapolated to infer which phosphorylated proteins/pathways are driving an individual tumor's growth. Reverse Phase Protein Microarrays (RPMA) are a sensitive and precise platform that can be applied to the quantitative measurement of hundreds of phosphorylated signal proteins from a small sample of tissue.

Pre-analytical variability originating from tissue procurement and preservation may cause significant variability and bias in downstream molecular analysis. Depending on the *ex vivo* delay time in tissue processing, and the manner of tissue handling, protein biomarkers such as signal pathway phosphoproteins will be elevated or suppressed in a manner that does not represent the biomarker levels at the time of excision. Consequently, assessment of the state of these kinase networks requires stabilization, or preservation, of the phosphoproteins immediately post tissue procurement. We have employed reverse phase protein microarray analysis of phosphoproteins to study the factors influencing stability of phosphoproteins in tissue following procurement. Based on this analysis we have established tissue procurement guidelines for clinical research with an emphasis on quantifying phosphoproteins by RPMA.

Keywords

cell signaling; kinase; phosphoprotein; pre-analytical variability; reverse phase protein microarray; stability

1.0. Introduction

The instant a tissue biopsy is removed from a patient, the cells within the tissue react and adapt to the absence of vascular perfusion, ischemia, hypoxia, acidosis, accumulation of cellular waste, absence of electrolytes, and temperature changes (1). It would be expected that a large surge of stress related, hypoxia related, and wound repair related protein signal pathway proteins and transcription factors will be induced in the tissue immediately following procurement (2, 3). Investigators in the past have worried about the effects of vascular clamping and anesthesia, prior to excision on the fidelity of molecular data in tissues. A much more significant and underappreciated issue is the fact that excised tissue is

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alive and reacting to *ex vivo* stress (1). The promise of tissue protein biomarkers to provide revolutionary diagnostic and therapeutic information will never be realized unless the problem of tissue protein biomarker instability is recognized, studied, and solved. There is a critical need to develop standardized protocols and novel technologies that can be used in the routine clinical setting for seamless collection and immediate preservation of tissue biomarker proteins, particularly those that have been postrationally modified such as phosphoproteins. This need extends beyond the large research hospital environment to the private practice, where most patients receive therapy. The fidelity of the data obtained from a diagnostic assay applied to tissue must be monitored and verified, otherwise a clinical decision can be based on incorrect molecular data. To date, clinical preservation practices routinely rely on protocols that are decades old, such as formalin fixation, and are designed to preserve specimens for histologic examination, not molecular analysis.

Tissue processing delays in clinical tissue procurement

Two categories of variable time periods that define biomarker stability during human tissue procurement are the a) post excision delay time, and b) processing delay time. The post excision delay time is the variable timeframe between specimen excision and the point at which the specimen is placed in a stabilized state, *e.g.*, immersed in fixative or snap-frozen in liquid nitrogen. During the post excision timeframe the tissue may reside at room temperature, or it may be refrigerated, either in a closed or open container. The second variable time period is the processing delay time. Common variables associated with processing delay time are the permeation rate of the fixative through the tissue and length of time to freeze the specimen.

In addition to the uncertainty about the length of these two time intervals, a host of known and unknown variables can influence the stability of tissue molecules during these time periods prior to measurement. These include 1) patient hypoxia, 2) tissue ischemia, 3) presence of imaging dyes and contrast media, 4) temperature fluctuations prior to fixation or freezing, 5) preservative chemistry and rate of tissue penetration, 6) size of the tissue specimen, 7) extent of handling, cutting, and crushing of the tissue, 8) fixation and staining prior to microdissection, 9) tissue hydration and dehydration, and 10) the introduction of phosphatases or proteinases from the environment at any time (1).

Formalin fixation may be unsuitable for quantitative protein biomarker analysis in tissue—Proteins can be extracted with variable yield from formalin fixed tissue (4). The yield depends on the time, chemistry of formalin fixation, and the tissue geometry and density. Formalin penetrates tissue at a variable rate, reported to be within the range of mm/hr (5-7). During this time the portion of the living tissue deeper than several millimeters would be expected to undergo significant fluctuations with regard to phosphoprotein analytes. When one considers the volume of a typical 16 gauge core needle biopsy (7mm × 1.6mm (volume=17.9mm³)) the cellular molecules in the depth of the tissue will have significantly degraded by the time formalin permeates the tissue (5, 8). Moreover, penetration rate is not synonymous with fixation. In aqueous solutions formaldehyde becomes hydrated, forming methylene glycol (5, 7). Methylene glycol penetrates the tissue, yet it is the small percentage of carbonyl formaldehyde component that covalently cross-links with proteins and nucleic acids and causes tissue fixation (5, 7). Formalin cross-linking, the formation of methylene bridges between amide groups of protein, blocks analyte epitopes as well as decreases the yield of proteins extracted from the tissue. Typically the dimensions of the tissue and the depth of the block from which samples are prepared are unknown variables. Consequently formalin fixation would be expected to cause significant variability in protein and phosphoprotein stability for molecular diagnostics (5, 9, 10).

Phosphoprotein stability is a balance between kinase and phosphatase activity—Kinases phosphorylate a substrate amino acid and phosphatases remove the phosphate group from the amino acid (Figure 1). At any point in time within the tissue cellular microenvironment, the phosphorylated state of a protein is a function of the local stoichiometry of associated kinases and phosphatases specific for the phosphorylated residue. Thus in the absence of kinase activity, proteins may be dephosphorylated by phosphatases, reducing the level of a phosphoprotein analyte causing a false negative result. This can be prevented by a variety of chemical- and protein-based phosphatase inhibitors (11, 12). However if the kinase remains active, then the addition of a phosphatase inhibitor alone will result in an augmentation of the phospho-epitope, generating a false positive result. Optimally, a stabilizing chemistry should arrest both sides of the kinase / phosphatase balance in order to prevent positive or negative fluctuations in phosphorylation events as the excised tissue reacts to the *ex vivo* conditions (1).

During the *ex vivo* time period, because the tissue cells are alive and reactive, phosphorylation of certain kinase substrates may transiently increase due to the persistence of functional signaling, activation by hypoxia, or some other stress-response signal (1, 13-15). While these reactive changes would be expected to increase protein phosphorylation, the availability of ubiquitous cellular phosphatases would be expected to ultimately decrease phosphophorylation sites, given enough time (1-3). These imbalances will significantly distort the molecular signature of the tissue compared to the state of the markers *in vivo*. This physiologic fact must be taken into consideration for tissue protein biomarker analysis in the hospital or clinic, where the living, reacting tissue may remain in the collection container for hours (Figure 2).

Application of RPMA phosphoprotein analysis to freshly collected tissue (1, 13-17) emphasized that excised tissue is reactive. The guidelines below illustrate methods for the reducing pre-analytical variables (Adapted from (1)).

1. Tissue should be stabilized as soon as possible after excision. Taking into consideration the average time for procurement in a community hospital, the recommended maximum elapsed time is 20 minutes from excision to stabilization (e.g. flash freezing, thermal denaturation, or chemical stabilization).
2. Tissue stabilization and preservation methods should be compatible with the intended downstream analysis. Preservation of tissue histology and morphology is essential for verification of tissue type and cellular content.
3. Documentation of the sample excision/collection time, elapsed time to preservation/stabilization, and length of fixation time are essential data elements for sample quality control.
4. Kinase pathway stabilization methods should block both sides of the kinase/phosphatase kinetic reaction. Blocking only phosphatases can cause false elevation of an analyte's phosphorylation level.

In this chapter, we describe tissue collection and processing for analysis of phosphoproteins by reverse phase protein microarray.

2.0. Materials

2.1. Tissue procurement and cell lysis

1. Tissue samples obtained by surgical resection, fine needle aspiration, or biopsy, not to exceed 10mm × 5mm (see **Note 1**).

2. Tissue lysis buffer: 450 μ L T-PER™ Tissue Protein Extraction Reagent (Pierce), 450 μ L 2X SDS Tris-glycine loading buffer (Invitrogen), and 100 μ L TCEP Bond Breaker™ (Tris(2-carboxyethyl)phosphine (Pierce)).
3. Mortar and pestle: for snap frozen tissue pulverization.
4. Tissue homogenizer: for fresh tissue disruption (*see Note 2*).

2.2. Frozen section preparation

Frozen sections may be used for laser capture microdissection prior to printing reverse phase protein microarrays. Alternately, frozen sections may be used to assess overall tissue morphology and to prepare whole slide lysates.

1. Cryomolds
2. Optimal Cutting Temperature (O.C.T.) compound (Sakura Finetek)
3. Dry ice
4. Cryostat with appropriate blade, temperature setting -20°C or colder, and chucks
5. Plastic slide box
6. Pre-cleaned glass microscope slides
7. Bitran storage bags, 2 $\times$ 4cm (Fisher Scientific) and/or aluminum foil

2.3. Reverse phase protein microarray

1. Aushon 2470 arrayer (Aushon Biosystems, Billerica, MA, USA)
2. 384 well microtiter plates with lids (*see Note 3*)
3. Nitrocellulose coated slides (GE Healthcare/Whatman FAST™ slides, Schott Nexterion® NC-C slides, or ONCYTE® Nitrocellulose Film Slides, Grace Bio-Labs) (*see Note 4*)
4. 70% Ethanol
5. Commercial cell lysates, such as HeLa+Pervanadate or A431+EGF (*see Note 5*). A minimum of 3-20 μ L of each individual lysate is needed to construct a two-fold dilution sequence on the array, in a 384 well microtiter plate (*see Note 6*).
6. Desiccant (Drierite, anhydrous calcium sulfate)

2.4. Reverse Phase Protein Microarray Immunostaining

1. I-Block blocking solution: 2.0g I-Block (Applied Biosystems), 1000mL Phosphate Buffered Saline 1X without calcium or magnesium, 1.0mL Tween-20. Dissolve I-Block Protein Blocking powder in PBS on a hot plate with constant stirring (*see Note 7*). Cool the solution to room temperature and add Tween 20. I-Block solution can be stored at 4°C for one week.
2. Re-Blot™ Mild Antigen Stripping solution 10X (Millipore/Chemicon)
3. Primary antibody of choice
4. Biotinylated secondary antibody, species matched to primary antibody
5. Dako CSA kit (Dako)
6. Biotin blocking system (Dako)

7. Antibody diluent with background reducing components (Dako)
8. Tris Buffered Saline with Tween (TBST, Dako)
9. DAB+, Liquid Substrate Chromogen System (Carcinogenic, contact hazard: wear gloves while handling) (Dako)

2.5. Image Acquisition

1. High resolution flat-bed scanner
2. Laser scanner or CCD scanner (excitation 280nm, emission 618nm)

2.6. Sypro Ruby Protein Blot stain

1. Sypro Ruby Protein Fixative Solution (7% v/v Acetic Acid and 10% v/v Methanol in deionized water). Close tightly and store at room temperature. Solution is stable for 2 months.
2. Sypro Ruby Protein Blot Stain (Invitrogen).

3.0. Methods

Tissue procurement

Based on current best practices for protein preservation, the tissue sample should be frozen as soon as possible after procurement to minimize phosphoprotein fluctuations (1). While there appears to be great variation in the fluctuation times between tissue types due to intrinsic kinases, nucleases, proteases and phosphatases, prompt freezing of the tissue limits these potential molecular changes. Freezing the tissue sample in an embedding media such as Sakura Finetek's O.C.T. compound prevents the formation of water crystals that can disrupt a tissue's cellular structure. In addition, this aqueous polyvinyl alcohol compound provides support to the tissue and aids in the cryo-sectioning process.

3.1. Tissue lysis and protein extraction

1. The desired maximum final total protein concentration for a whole cell lysate is 0.5µg/µL total protein. Snap frozen tissue (without cryopreservative): Weigh frozen tissue sample on an analytical balance. Pulverize frozen tissue. Place the frozen tissue in a microcentrifuge tube. Add 1000 µL tissue lysis buffer for each 200mg of tissue. Fresh tissue: Weigh tissue sample on an analytical balance. Place the fresh tissue in a microcentrifuge tube. Add 1000 µL tissue lysis buffer for each 200mg of tissue. Immediately homogenize tissue (*see Note 2*).
2. Briefly vortex the microcentrifuge tube containing the whole cell lysate.
3. Immediately heat the lysate at 100°C for 5-8 minutes. After tissue lysis, the lysates should be printed on the microarray as soon as possible. If a delay in printing is anticipated, the lysates may be stored at -80°C (*see Note 8*).

3.2. O.C.T. embedded frozen tissue for frozen section preparation

1. Prepare all supplies prior to the biopsy procedure to avoid delay once the specimen has been obtained.
2. Label the handle and the front surface of the cryomold with the sample identifying information.
3. Cover the bottom of the cryomold with O.C.T. to a depth of 2-4mm.

4. Orient the specimen on top of the O.C.T. in the cryomold in the desired position, keeping in mind that the side facing down will be the first tissue surface cut.
5. Completely cover the tissue with O.C.T. and place immediately, in a horizontal position, in a container of dry ice. Covering the container of dry ice will speed freezing.
6. O.C.T. will appear white once it has been frozen. The embedded tissue may or may not be visible within the O.C.T. To add further protection, the cryomold can be wrapped in aluminum foil, placed inside a 2×4cm Bitran plastic bag, or placed in a 50ml conical Falcon tube.
7. The frozen tissue should be stored at -70° to -80°C .

3.2.1. Frozen Section Preparation

1. Label glass microscope slides with a pencil. Place slides face-up on top of the cryostat.
2. Remove the cryomold containing tissue from the freezer and place it in a box with dry ice. Peel the cryomold from the O.C.T. tissue block. Place the tissue block either in the dry ice or in the cryostat to keep it frozen.
3. Place a small amount of O.C.T. on a room temperature chuck. Place the O.C.T. embedded tissue block directly on the room temperature O.C.T. on the chuck. Immediately place the chuck in the cryostat.
4. Allow the O.C.T. to freeze, forming a bond between the tissue block and the chuck.
5. Place a blade in the knife holder.
6. Place the chuck containing the tissue block in the chuck holder and tighten the holder.
7. Align the tissue face parallel with the blade.
8. Set the micrometer setting to the desired thickness (5-8 μm is optimal for laser capture microdissection).
9. Cut sections until a full tissue thickness is obtained. The micrometer may be adjusted to cut thicker sections until the tissue face is reached.
10. Place the tissue section on a room temperature glass slide. Hold the slide so the tissue will adhere to the clean, front surface of the slide (*see Note 9*).
11. Do not allow the frozen section slides to thaw on the slide or air dry at room temp. Keep the slides frozen by placing them in the cryostat box or directly into a pre-chilled slide box kept on dry ice.
12. After all the frozen section slides for one sample have been cut, remove the blade from the knife holder, and discard the blade in a sharps container or blade holder.
13. Loosen the chuck from the chuck holder and remove the chuck.
14. Squirt a small amount of O.C.T. on top of the tissue block to cover the tissue.
15. Immediately place the tissue in the Pelletier to rapidly freeze the O.C.T. Allow to freeze for 2-4 minutes. Alternatively, a piece of pre-chilled smooth metal may be placed on top of the O.C.T. to freeze the O.C.T.
16. Remove the tissue block from the Pelletier.
17. Use a room temperature putty knife to pry the O.C.T. block away from the chuck.

18. Put the cryomold and tissue block in a Bitran bag, or wrap the cryomold and tissue block in aluminum foil. Label the bag/aluminum foil.
19. Store the tissue block and frozen section slides at -80°C .

3.3. Reverse phase protein microarray construction

Reverse phase protein microarrays are a multiplexed proteomic platform used to evaluate cell signaling protein levels or phosphoprotein profiles in many samples printed on one array for one specific endpoint per array (18-23). Over 100 array slides can be printed with 40 μL of protein lysate and each array is probed with a single antibody. In addition to printing sample lysates, it is also essential to print control lysates such as commercial cell lysates, recombinant peptides, or peptide mixtures that are known to contain the antigens being investigated. All samples are printed in a dilution curve, which permits the selection of the optimal sample protein concentration for individual antibodies that have varying affinities.

The Aushon 2470 arrayer utilizes a solid pin format for the application of cell lysates or other protein containing fluids onto a matrix of nitrocellulose mounted on a glass microscope slide (*see Note 10*). Prior to printing cell lysates on a RPMA, the number of cells required should be optimized preceding the final array construction (*see Note 11*) (Figure 3). The arrays are subsequently stained using a Dako CSA (Catalyzed Signal Amplification) System that includes blocking and signal amplification reagents that are compatible with chromogenic (DAB), chemiluminescent, or fluorescent (Li-Cor® IRDye680) detection reagents.

1. Fill the Aushon 2470 arrayer water wash container with deionized water and empty the waste container. Care should be taken to verify that the tubing is sufficiently inserted into each container.
2. Fill the humidifier with deionized water (*see Note 12*).
3. Load nitrocellulose coated slides onto slide platens with the frosted edge of the slide on the right. Insure the slides are securely held by the platen clips. The array slide printing order is top left to bottom left of the platen, followed by top right to bottom right. Place the slide platens into the Aushon 2470 arrayer.
4. If the lysates have been stored frozen, thaw the lysates and heat the lysates (do not heat commercial cell lysates samples) in a dry heat block or boiling water bath for 7 minutes at 100°C . Cool to room temperature.
5. Load samples into a 384 well plate, creating a 4 point, 2-fold dilution curve. Refer to Figure 4 for an example plate map based on a 20-pin print head configuration (*see Note 13*).
6. Place the lid on top of the 384 well plates (*see Note 14*).
7. With the 2 metal clips on the plate holder open and A1 in the lower left hand corner, slide the 384-well plate to the back of the holder. Flip the metal clips to the closed position.
8. Place the plate holder in the elevator with A1 facing the outside of the instrument.
9. Turn on the power to the Aushon 2470 arrayer. Start the arrayer software by double-clicking on the “Aushon 2470” icon. Enter the username and password.
10. The array program window will be displayed. Define the number of microtiter plates to be used for printing and the location of samples in each microtiter plate.

11. Double-click on a microtiter plate listed in the source well plate library. This automatically places a microtiter plate in the well plate hotel. Repeat this step for each microtiter plate that will be used in the print run.
12. Click on the first microtiter plate listed in the well plate hotel. The highlight color will change from green to blue.
13. Click on “overlay extractions” beneath the selected well plate image. This allows you to visualize which microtiter plate wells will be used for printing for a given plate. An extraction is equivalent to one dip of the print head into a set of wells. For a 20 pin format with 350 μ m pins, one extraction corresponds to wells A1, A2, A3, A4, A5, B1, B2, B3, B4, B5, C1, C2, C3, C4, C5 and D1, D2, D3 D4, D5. To program the arrayer to print 80 samples, from 4 microtiter plates, in rows A-P, use the following format: Select 4 unique extractions, start at 1 for plate #1 Select 4 unique extractions, start at 5 for plate #2 Select 4 unique extractions, start at 9 for plate #3 Select 4 unique extractions, start at 13 for plate #4
14. Designate the left offset and feature to feature spacing of the x and y axis (Table 1) (*see Note 15*).
15. Select 3 depositions per feature, which will print approximately 30 nL lysate per spot (*see Note 16*).
16. Select the number of super arrays per substrate: 1 if using microtiter wells A-H; 2 if using microtiter plate wells I-P in addition to rows A-H. Replicate positioning: Linear (vertical) Number of replicates: 1 for duplicates, 2 for triplicates.
17. Click “Next” to set the wash parameters. Submerged dwell time: 4 seconds (*see Note 17*).
18. Click “Next” to select the number of slides to be printed. One to ten slides may be printed on any platen.
19. If the humidity is less than the 50%, set the humidity control to 50%.
20. Verify that all instrument preparation steps and programming steps have been completed.
21. Click the green “Start Deposition” icon. The microtiter plate door and the slide platen door will automatically lock. The system will begin initialization by homing all components and taking inventory of the microtiter plates and slide platens.
22. Deposition is complete when the arrayer software displays the message “Quit or Continue”. Select “quit” to terminate printing. Select “continue” to unload the printed array slides and load additional slides for printing (*see Note 18*).
23. To thoroughly clean the pins after each print run, load 20 μ L of 70% Ethanol into wells A1-D20 of a 384 well microtiter plate. Load one nitrocellulose coated slide into the arrayer. Program the arrayer as outline above to print 3 depositions/feature on one slide. Start the deposition. At the end of the deposition process, select “quit” to terminate printing. Remove and discard the nitrocellulose slide and the microtiter plate.
24. Turn the Aushon 2470 arrayer power off, and place the printed array slides in a slide box. Store the slide box in a plastic storage bag with desiccant at -20°C (*see Note 19*).

3.4. Reverse Phase Protein Microarray Immunostaining

3.4.1. Array slide pretreatment—RPMA technology allows the simultaneous detection and analysis of signal intensity among a group of samples. This method requires a single antibody-epitope interaction with the protein of interest. All RPMA slides, with the exception of the one probed with the fluorescent Sypro Ruby staining solution, should be blocked prior to the staining procedure.

1. Allow frozen RPMA slides to warm at room temperature for approximately 5-10 minutes. Leave the slides in the box with dessicant during this time.
2. Prepare a 1X solution of Mild Re-Blot (stock is 10X) in deionized water.
3. Incubate the microarray slides that are to be stained with antibodies in 1X Mild Re-Blot™ solution for 15 minutes on a rocker/shaker (*see Notes 20 and 21*). Do not use Re-Blot™ for arrays printed with serum or low molecular weight (LMW) serum fractions, or for arrays to be stained with Sypro Ruby Total Protein Blot stain. For serum/LMW serum fraction arrays, place the slides directly in I-Block solution.
4. Remove the Re-Blot™ solution and wash the microarray slides with 1X PBS (calcium and magnesium free) twice for 5 minutes each.
5. Decant the last PBS wash and immediately place the slide in blocking solution (I-Block solution). Incubate in I-Block at room temperature with constant rocking for a minimum of 60 minutes (*see Note 22*).

3.4.2. Microarray Immunostaining—The Dako autostainer allows simultaneous staining of 48 slides (*see Note 23*). The number of slides to be stained is chosen in relation to the number of endpoints of interest and the number of species used to generate the primary antibodies. Antibodies from different animal species can be used during the same Autostainer run. However, to quantify the non-specific background signal generated from the interaction between the secondary antibody and samples, it is essential to include in each staining run one slide that is probed with secondary antibody only for each species of secondary antibody used. The secondary antibody control slides must be matched to the primary antibody species. For example, if the primary antibodies selected consist of mouse and rabbit antibodies, then two secondary antibody control slides are required, one for the rabbit antibodies and one for the mouse antibodies. The signal intensity of the slide probed with secondary antibody only is subtracted from the signal intensity of the primary +secondary antibody stained slide.

1. Select unconjugated primary antibodies of interest (*see Note 24*).
2. Select biotinylated secondary antibodies corresponding to the species of the primary antibodies.
3. Program the Dako Autostainer (Table 2).
4. Prepare CSA solutions according to the manufacturer's directions.
5. Fill the buffer reservoir with 1X TBST and the water carboy with deionized water. Empty the waste container if necessary.
6. Load the reagents and slides on the Autostainer. Prevent the nitrocellulose from drying during slide loading. If necessary, rinse the slides with 1X TBST buffer during the slide loading process (*see Note 25*).
7. Prime the water first and then the buffer before starting the run.

8. At the end of the Autostainer run, remove the slides, rinse them with deionized water, and allow them to air dry.
9. Label the microarray slides specifying the date, study, and antibody that has been used in the staining procedure.

3.5. Colorimetric system image acquisition and data analysis

Any high-resolution scanner, provided with grayscale option, can be employed for image acquisition of diaminobenzidine (DAB) stained microarrays, providing it generates 14 or 16-bit scanned images.

1. Adjust the image appearance (inverted/not inverted) as required by the image analysis software. Save the adjusted image as a TIFF file (*see Note 26*). Tiff images can be imported to a variety of data analysis software programs.
2. The pixel intensity of each spot is proportional to the amount of measured analyte per spot. Final intensity values for the RPMAs are obtained after subtraction of the negative control intensity value/spot (secondary antibody alone) and normalization to the total protein value/spot.

3.6. Sypro Ruby Total Protein stain

The concentrations of total, phosphorylated, and cleaved proteins present in different samples can be determined by RPMA. The signal intensity normalization process can be based on total protein values, allowing the comparison of samples with different protein concentrations (*see Note 27*). Sypro Ruby Protein Blot Stain is a reversible fluorescent dye that binds to primary amino groups on proteins in an acidic environment. The dye has two excitation maxima at ~280 nm and at ~450 nm and an emission maximum near 618 nm (24, 25). Sypro Ruby Blot stain has a sensitivity of 1.0 ng to 1.0 µg protein per microliter of sample. Images of Sypro Ruby stained slides can be acquired with a laser scanner or a CCD camera (*see Note 28*).

1. If the array slides were stored frozen (-20°C), allow the selected slide(s) to room temperature.
2. Wash array slide(s) in deionized water for 5 minutes with constant rocking/shaking.
3. Incubate array slides in Sypro Ruby Protein Blot fixative solution at room temperature for 15 minutes with constant shaking.
4. Discard Sypro Ruby Protein Blot fixative solution and wash slides with deionized water 4 times for 5 minutes each.
5. Incubate slides with Sypro Ruby blot stain for a minimum of 30 minutes. Sypro Ruby is a photo-sensitive dye; therefore, protect the array slides from light by covering the container with aluminum foil.
6. Discard Sypro Ruby Blot stain. Rinse slides with deionized water 4x for one minute each. Protect from light.
7. Allow slides to air dry. Protect the stained microarray slides from light.
8. Acquire slide images with a laser scanner, such as Revolution® 4550 Scanner (VIDAR) or a CCD camera such as the NovaRay (Alpha Innotech).

4.0. Notes

1. The tissue should be cut to a size no greater than one half the area of the cryomold so that it will fit into the cryomold without touching the sides of the mold. For the

standard cryomold, specimen samples should not exceed 1cm in height or width, or a thickness of more than 0.5 cm.

2. Any type of manual or automated tissue disruptor may be used that is compatible with tissue lysis buffer. Automated tissue disruptors may also be used such as pressure cyclers (Barocycler®, Pressure BioSciences Inc), instruments containing a lysing matrix such as glass beads (Fast Prep®, MP Bio), or combination pressure/ultrasound disruption instruments (Adaptive Focused Acoustics® series, Corvaris, Inc.).
3. Each Aushon 2470 arrayer is supplied with microtiter plate holders (referred to as source plates) that are designed to hold a specific vendor's microtiter plate. An example microtiter plate compatible with the Aushon 2470 arrays is the Genetix polystyrene 384 well plate, catalog number X6003. Microtiter plate lids are required for compatibility with the Aushon 2470 arrayers. The arrayer is equipped with a suction cup to remove the lid of the microtiter plate.
4. Each lot number of nitrocellulose coated slides should be thoroughly examined prior to use including visual macroscopic examination of the membrane surface. Examine the nitrocellulose for defects such as scratches, holes, and alignment of the pad on the glass surface. Nitrocellulose coated slides are available in a variety of formats including single pad and multi-pad configurations.
5. Every printed array slide should include lysates of known total protein concentration and performance with the detection system, such as commercial cell lysates, homebrew cell lysates, and/or peptides or phosphopeptides. These samples are for process control, indicating adequate deposition of protein and recognition by the primary antibody.
6. The lysate volume determines the number of arrays that can be printed. 20µL of lysate is sufficient to construct 30-40 arrays in serial two-fold dilutions. To prepare whole slide lysates for RPMA, add 10-20 µL of tissue lysis buffer per tissue section based on the area of the section. For microdissected tissue, add 1-3 µL of tissue lysis buffer per 1000 cells.
7. Avoid boiling the I-Block solution. Heating the solution at low/mid heat levels for 10-15 minutes is usually adequate to completely solubilize the I-Block powder. I-Block is a casein based protein solution. Boiling will cause protein degradation and potential alterations in blocking efficiency.
8. Whole cell protein lysates that have been stored frozen should be heated at 100°C for 5—8 minutes prior to preparing the lysate for microarray printing.
9. The tissue should be in the center of the slide. This is of particular importance for cells that will be procured by laser capture microdissection, as tissue that is too close to the end of the slide, or the sides, can not be microdissected (26).
10. The Aushon 2470 arrayer employs a proprietary pin technology for printing samples. The pin design/manufacturing process limits fluid from adhering to the shaft of the pin. The pins are positioned in a print head which moves in the z-axis only, while the microtiter plate and slides move in the x and y axis.
11. For arrays prepared from LCM procured tissue cells, a minimum of 15,000 cells are needed for printing multiple arrays (up to 50 arrays). LCM procured cells are lysed using tissue lysis buffer. Add 1-3 µL of tissue lysis buffer per 1000 cells. The resulting lysates are diluted into two-fold dilution curves in a 384-well microtiter plate (26).

12. Use ddH₂O in the humidifier to prevent mold/bacterial growth. Every 2 weeks, empty the humidifier's water chamber and clean the water chamber by rinsing with 70% ethanol, followed by several water rinses, and air-dry the water chamber.
13. Samples that fill an entire array (640 spots maximum from 350µm pins) can be loaded into 4 individual 384 well plates to prevent significant evaporation during the pipetting and printing process. Samples in rows A-D can be loaded in plate 1, rows E-H in plate 2, rows I-L in plate 3, and rows M-P in plate 4.
14. The Aushon 2470 arrayer is equipped with suction cups to remove the lid from the microtiter plates. A lid **MUST** be placed on every microtiter plate that is loaded in the arrayer. The lid should be clean and dry, free of dust, adhesive and liquid.
15. Top offset settings may vary slightly with each arrayer and/or slide manufacturer. Food dyes, used for baking or egg coloring, can be diluted in PBS or water to substitute as "samples" for evaluating spot placement by the robotic arraying device. Clean the arrayer pins thoroughly following printing of food dyes. 70% (v/v) ethanol dispensed into a microtiter plate can be used to effectively clean the pins. Dispense 20 µL of 70% ethanol into wells 1-20 in rows A, B, C and D of a 384 well microtiter plate. Load one nitrocellulose coated slide into the arrayer. Program and execute a print run for one slide, from one microtiter plate with 4 unique extractions, at 3 depositions/feature.
16. Samples with protein concentrations less than 0.5 µg/µL can be effectively concentrated on an array by printing more depositions per feature. If more than 5 depositions/spot are necessary, first print at 5 depositions/spot, allow the spots to dry for 10 minutes and then print additional depositions/spot. Nitrocellulose has a finite protein binding capacity based on its porosity and depth (Figure 3) (27, 28). Therefore, printing more than 5 depositions/spot is generally not recommended as the nitrocellulose becomes saturated.
17. Carryover experiments should be conducted with each instrument to determine the optimal pin washing time for various sample matrices.
18. A gal file is generated with each print run and is saved in C:\Documents and Settings\user\My Documents\user\Array Data Files. It can be uploaded into software analysis packages for array layout spot identification.
19. Proteins immobilized on nitrocellulose slides are stable at -20°C for up to 3 years (personal experience) if stored in dry (with dessicant) conditions.
20. Do not use Re-Blot™ for arrays composed of serum or low molecular weight fractionated serum samples. Re-Blot™ causes diffusion of the serum sample and/or buffer components beyond the printed spot resulting in a blurry, poorly defined spot.
21. The Re-Blot™ solution further denatures the protein immobilized onto the nitrocellulose slides thus improving the antibody-epitope recognition. Do not exceed the suggested incubation time (15 minutes). Re-Blot™ is a very basic solution (pH 14). Over-exposure to Re-Blot™ solutions may cause nitrocellulose alterations or nitrocellulose detachment (delamination) from the glass slide.
22. I-Block™ solution is a protein based blocking reagent that is useful for blocking the nitrocellulose prior to immunostaining the array. A minimum blocking time of 1 hour at room temp, with gently rocking, is recommended while longer blocking times are not detrimental. If blocking must be performed overnight, block the slides at 4°C.

23. Although the Dako Autostainer has a maximum capacity of 48 slides, we have found it best to stain a maximum of 36 slides per staining run. The nitrocellulose slides have a tendency to dry out during extended staining runs. Paper towels soaked in water may be placed inside the sink area of the Autostainer to maintain humidity during the staining run. Alternatively, a shallow dish of water may be placed inside the left side of the Autostainer chamber.
24. Each primary antibody must be validated by Western blotting to confirm specific interaction between the protein of interest and the antibody, using complex samples similar to those which will be used on the array.
25. TBST contains a high concentration of salt. If the TBST is not rinsed from the stained nitrocellulose arrays, salt crystals may form on the nitrocellulose surface. Consequently, the autostainer program includes a water rinse after DAB deposition. Moreover, it is possible to add a further water rinse (auxiliary step) after the final water rinse in order to pause the instrument for 840 minutes. By doing this, the autostainer is programmed to be in an idle status for 14 hours, at which time the slides will be rinsed again with deionized water (Table 2).
26. Image adjustments should only include those adjustments that change all pixel intensities in an image in a linear, consistent manner. All image adjustments must be performed prior to spot analysis and must be consistent for all array slides. It is important to note that some image manipulation programs are capable of changing the actual pixels in the image.
27. The total protein concentration in a large set of printed array slides may vary from the initial slides printed to the last slides printed due in part to sample evaporation and the volume of fluid held by the pin. It is recommended to stain 1 of every 25 slides with Sypro Ruby Protein Blot stain. For example, if 120 microarrays have been printed it is suggested to use slides 25, 50, 75 and 100 for total protein quantification.
28. A UV transilluminator (~300nm), a blue-light transilluminator, or a laser scanner that emits at 450, 473, 488 or 532 nm is appropriate for imaging a Sypro Ruby stained array. Example imaging systems are: Kodak 4000 MM imager; Alpha Innotech NovaRay; Tecan Reloaded LS; Perkin Elmer ProScanArray HT; Molecular Devices GenePix 4000B.

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Figure 1. Stoichiometry between protein kinases and protein phosphatases

Phosphorylated cell signaling cascades are regulated by a series of kinases and phosphatases which act in concert to activate/deactivate a protein. Phosphatase inhibitors, in the absence of kinase inhibitors, will cause a false elevation of phosphorylated proteins if the kinase remains functional and active.

Figure 2. Reverse phase protein microarray format

Binding capacity of the nitrocellulose membrane can be determined empirically by varying the amount of sample deposited on the nitrocellulose. A. Whole cell lysates were printed in the vertical direction in two-fold serial dilutions. In the horizontal direction, the lysate was printed at 2, 3, 4, or 5 depositions/feature (hits/spot). Spot morphology for each set of depositions was assessed using MicroVigene spot analysis software (Vigene Tech). B. Typical reverse phase protein microarray constructed with whole cell lysates. Each sample, control and standard was printed in duplicate, serial two-fold dilutions using an Aushon Biosystemes 2470 arrayer equipped with 350 μm pins. The dilutions were printed in the horizontal direction.

Figure 3.
Overview of tissue phosphoprotein preservation variability.

A1	A6	A11	A16	A2	A7	A12	A17	A3	A8	A13	A18	A4	A9	A14	A19	A5	A10	A15	A20	F1	F6	F11	F16	F2	F7	F12	F17	F3	F8	F13	F18	F4	F9	F14
A1	A6	A11	A16	A2	A7	A12	A17	A3	A8	A13	A18	A4	A9	A14	A19	A5	A10	A15	A20	F1	F6	F11	F16	F2	F7	F12	F17	F3	F8	F13	F18	F4	F9	F14
E1	E6	E11	E16	E2	E7	E12	E17	E3	E8	E13	E18	E4	E9	E14	E19	E5	E10	E15	E20	M1	M6	M11	M16	M2	M7	M12	M17	M3	M8	M13	M18	M4	M5	M14
E1	E6	E11	E16	E2	E7	E12	E17	E3	E8	E13	E18	E4	E9	E14	E19	E5	E10	E15	E20	M1	M6	M11	M16	M2	M7	M12	M17	M3	M8	M13	M18	M4	M5	M14
B1	B6	B11	B16	B2	B7	B12	B17	B3	B8	B13	B18	B4	B9	B14	B19	B5	B10	B15	B20	J1	J6	J11	J16	J2	J7	J12	J17	J3	J8	J13	J18	J4	J5	J14
B1	B6	B11	B16	B2	B7	B12	B17	B3	B8	B13	B18	B4	B9	B14	B19	B5	B10	B15	B20	J1	J6	J11	J16	J2	J7	J12	J17	J3	J8	J13	J18	J4	J5	J14
F1	F6	F11	F16	F2	F7	F12	F17	F3	F8	F13	F18	F4	F9	F14	F19	F5	F10	F15	F20	N1	N6	N11	N16	N2	N7	N12	N17	N3	N8	N13	N18	N4	N5	N14
F1	F6	F11	F16	F2	F7	F12	F17	F3	F8	F13	F18	F4	F9	F14	F19	F5	F10	F15	F20	N1	N6	N11	N16	N2	N7	N12	N17	N3	N8	N13	N18	N4	N5	N14
C1	C6	C11	C16	C2	C7	C12	C17	C3	C8	C13	C18	C4	C9	C14	C19	C5	C10	C15	C20	K1	K6	K11	K16	K2	K7	K12	K17	K3	K8	K13	K18	K4	K5	K14
C1	C6	C11	C16	C2	C7	C12	C17	C3	C8	C13	C18	C4	C9	C14	C19	C5	C10	C15	C20	K1	K6	K11	K16	K2	K7	K12	K17	K3	K8	K13	K18	K4	K5	K14
D1	D6	D11	D16	D2	D7	D12	D17	D3	D8	D13	D18	D4	D9	D14	D19	D5	D10	D15	D20	O1	O6	O11	O16	O2	O7	O12	O17	O3	O8	O13	O18	O4	O5	O14
D1	D6	D11	D16	D2	D7	D12	D17	D3	D8	D13	D18	D4	D9	D14	D19	D5	D10	D15	D20	O1	O6	O11	O16	O2	O7	O12	O17	O3	O8	O13	O18	O4	O5	O14
D1	D6	D11	D16	D2	D7	D12	D17	D3	D8	D13	D18	D4	D9	D14	D19	D5	D10	D15	D20	L1	L6	L11	L16	L2	L7	L12	L17	L3	L8	L13	L18	L4	L5	L14
D1	D6	D11	D16	D2	D7	D12	D17	D3	D8	D13	D18	D4	D9	D14	D19	D5	D10	D15	D20	L1	L6	L11	L16	L2	L7	L12	L17	L3	L8	L13	L18	L4	L5	L14
H1	H6	H11	H16	H2	H7	H12	H17	H3	H8	H13	H18	H4	H9	H14	H19	H5	H10	H15	H20	P1	P6	P11	P16	P2	P7	P12	P17	P3	P8	P13	P18	P4	P5	P14
H1	H6	H11	H16	H2	H7	H12	H17	H3	H8	H13	H18	H4	H9	H14	H19	H5	H10	H15	H20	P1	P6	P11	P16	P2	P7	P12	P17	P3	P8	P13	P18	P4	P5	P14

Super Array 1

Super Array 2

Figure 4. Example 384 well platemap

The figure represents 1) which microtiter wells contain samples to construct a duplicate 4 point, two-fold dilution series, and 2) the location of the spots on the RPMA from those wells. The microtiter plate well designations, such as A1, B1, C1 etc. represent which well contains a given sample. The position of the sample on the printed RPMA is depicted by the location of the well in the figure. For example, the sample in well A1 will be printed in the top left corner of the array, while the sample in A6 will be printed adjacent to A1. Samples in consecutive microtiter wells are not printed next to each other due to the pin head configuration, pin diameter, and microtiter plate well spacing.

Table 1

Example spot parameters for the Aushon 2470 arrayer

Parameter	350µm Pins	185µm Pins
Top Offset (Arrayer Specific)	4.5	4.5
Left Offset	5.0	5.0
Deposited Spot Diameter	650µm	250µm
Feature-to-feature spacing x-axis	1125	500
Feature-to-feature spacing y-axis	1125	562.5
Number of depositions per feature	3	3
Max Feature in Y-Dir	4	8
Max Feature in X-Dir	4	9

Table 2

Dako Autostainer programming grid for reverse phase protein microarrays using a Catalyzed Signal Amplification (CSA) method

Reagent	Time (minutes)	Reagent Category
Buffer		Rinse
Hydrogen peroxide block	5	Endogenous Enzyme Block
Buffer		Rinse
Avidin Block	10	Auxiliary
Buffer		Rinse
Biotin Block	10	Auxiliary
Buffer		Rinse
Protein Block	5	Protein Block
Blow Air		Rinse
Primary Antibody	30	Primary Antibody
Buffer		Rinse
Buffer	3	Auxiliary
Buffer		Rinse
Secondary Antibody	30	Secondary Antibody
Buffer		Rinse
Buffer	3	Auxiliary
Buffer		Rinse
Streptavidin Biotin Complex	15	Secondary reagent
Buffer		Rinse
Buffer	3	Auxiliary
Buffer		Rinse
Amplification (Biotinyl tyramide)	15	Secondary reagent
Buffer		Rinse
Buffer	3	Auxiliary
Buffer		Rinse
Streptavidin-HRP	15	Tertiary reagent
Buffer		Rinse
Buffer	3	Auxiliary
Buffer		Rinse
Switch to toxic waste		
DAB	5	Chromagen
Water		Rinse
Overnight Water *	840	Auxiliary

* Optional step if operating the Autostainer overnight