Reverse phase protein microarray technology in traumatic brain injury

Andrea B. Gyorgy *, John Walker, Dan Wingo, Ofer Eidelman, Harvey B. Pollard, Andras Molnar, Denes V. Agoston

Department of Anatomy, Physiology and Genetics, School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA

ARTICLE INFO

ABSTRACT

Antibody based, high throughput proteomics technology represents an exciting new approach in understanding the pathobiologies of complex disorders such as cancer, stroke and traumatic brain injury. Reverse phase protein microarray (RPPA) can complement the classical methods based on mass spectrometry as a high throughput validation and quantification method. RPPA technology can address problematic issues, such as sample complexity, sensitivity, quantification, reproducibility and throughput, which are currently associated with mass spectrometry-based approaches. However, there are technical challenges, predominantly associated with the selection and use of antibodies, preparation and representation of samples and with analyzing and quantifying primary RPPA data. Here we present ways to identify and overcome some of the current issues associated with RPPA. We believe that using stringent quality controls, improved bioinformatics analysis and interpretation of primary RPPA data, this method will significantly contribute in generating new level of understanding about complex disorders at the level of systems biology.

Published by Elsevier B.V.

1. Introduction

Reverse phase protein microarray (RPPA) technology is a sensitive and high throughput antibody-based assay (Kricka and Master, 2008; MacBeath, 2002; Madoz-Gurpide et al., 2001; Schweitzer and Kingsmore, 2002; Spurrier et al., 2008; Zong et al., 2007). The technology has the potential to become a widely used tool in studying complex physiological conditions and diseases (Belluco et al., 2005; Chan et al., 2004; Espina et al., 2009a; Espina et al., 2003; Kumble, 2003; Pollard et al., 2007; Ramaswamy et al., 2005; Spurrier et al., 2008). RPPA is especially the method of choice when large numbers of samples in very small quantities need to be analyzed (Espina et al., 2007; Spurrier et al., 2008; VanMeter et al., 2007). Importantly, the technology can provide high-resolution proteomics data in a quantitative and affordable manner (Agoston et al., 2009; Liotta et al., 2003a,b; Liotta and Petricoin, 2003; Pollard et al., 2007).

RPPA is a large-scale version of the original dot blot assays in a microdot format (Paweletz et al., 2001; Spurrier et al., 2008). Impor-

tantly, as in the dot blot assay, proteins are not separated, thus the quality of antibodies (Bordeaux et al., 2010) is essential to the technology. The intactness and accessibility of epitopes is another key determinant of successful application of the method. As the technology is becoming increasingly used (Anderson et al., 2009; Espina et al., 2009a,b), we feel that it is important to share technical issues we have encountered and also solutions allowing the technology to be used to its potential.

We have used RPPA for quantifying changes in the levels of proteins associated with cellular damage in brain tissue extracts, cerebrospinal fluid (CSF), and serum after traumatic brain injury (TBI) (Agoston et al., 2009; Bauman et al., 2009). We needed to develop protocols for assessing antibody specificity so they can be reliably used in quantitative analysis by RPPA. We have found that generating individual dilution curves for every sample substantially improves accurate quantification and increases dynamic range. We provide a novel method for quantification enabling comparing data in a reliable and reproducible way.

2. Materials and methods

2.1. Biosamples

Serum, CSF, and brain tissues were obtained from a swine model of explosive blast induced TBI. Experiments were carried out and biosamples, blood, CSF and brain were collected, handled and stored as described earlier (Bauman et al., 2009).

2.2. Preparation of samples

Brain tissues were pulverized in liquid nitrogen using a mortar and a pestle. 200 mg of the powder was transferred into 1 ml of T-per lysis buffer (#78510 Thermo Fisher, Waltham, MA) with ethylene diamine tetraacetic acid (EDTA)-free Halt protease inhibitor cocktail (#78441 Thermo Fisher) in 1.5 ml tubes on ice. The suspensions were kept on ice and sonicated using a Misonix S-4000 automated sonicator (Misonix, Farmingdale, NY) using 12 repeats of 10 s bursts at an amplitude of 20, with 50 s cooling breaks between bursts. Samples were then centrifuged at 4 °C at 15,000 g for 15 min. Supernatants were aliquoted, and stored at −80 °C. Serum samples were diluted 1:10 volume:volume with Tissue Solution, aliquoted and kept frozen at −80 °C until use. CSF samples were diluted 1:3 volume:volume with Tissue Solution, aliquoted and frozen at −80 °C until use. Protein concentrations were measured using the bicinchoninic acid (BCA) assay (Thermo Fisher, Waltham, MA).

Tissue samples were diluted in print buffer (10% glycerol, 0.05% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol (DTT) in 1× tris-buffered saline (TBS) to a final protein concentration of 1 mg/ml; serum samples were printed with an initial dilution of 1:10, while CSF samples were diluted 1:1 with print buffer. Then samples were transferred into a JANUS Varispan Integrator and Expanded Platform Workstation (PerkinElmer, Waltham, MA). The JANUS workstation was programmed to perform the standard 11-point serial 1:2 dilution of each sample which was followed by a buffer-only control in the 12th row in 96-well plates; then each dilution was transferred to Genetix 384-well plates (X7022, Fisher Scientific, Pittsburg, PA). Plates were centrifuged at 4 °C at 1500 g for 5 min and transferred either into a Q-Array Mini microarray printer (Genetix, Boston, MA) or into an Aushon 2470 Arrayer (Aushon Biosytems, Billerica, MA) for printing. The following single-pad nitrocellulose coated glass slides were tried: Nexterion white (NC-W) and dark NC-D (SCHOTT, Elmsford, NY), PATH (Thermo Fisher Scientific, Waltham, MA), Whatman FAST (Fisher Scientific, Pittsburg, PA), ONCYTE Avid and ONCYTE Nova slides (Grace Bio-Labs, Bend, OR); the results shown were obtained using slides from Grace Bio-Labs: Avid for CSF and tissue samples and Nova for serum.

2.3. Printing parameters

The Q-Array Mini, equipped with quill pins, was programmed to perform 3 stampings per ink (samples) and 3 stampings per spot. The stamping time and inking time were both set at 500 ms. The pins were washed between dipping into a new sample for 3000 ms in water, with a 500 ms break, then in ethanol for 1000 ms followed by 500 ms air-drying time. The final wash was in ethanol for 5000 ms, followed by a 4000 ms air dry. Once printing was completed, slides were placed into a desiccator at 4 °C to dry overnight.

The Aushon Arrayer was programmed to use 16 pins. Each sample was printed in 12 dilutions (12 rows), in triplicate (3 columns), resulting in a block of 3 × 12 dots. The spot diameter was set to 250 nm with a spacing of 500 nm between dots on the x-axis and 375 nm on the y-axis. Wash time was set at 1.2 s without delays. For printing serum and tissue extracts, the arrayer was programmed for a single deposition per dot. For CSF samples, the arrayer was programmed to deliver 3 depositions per dot.

2.4. Immunochemical detection

The slides were blocked with 5% dried milk in 1× TBS with 0.1% Tween-20 (TBST). Unblocked slides were treated with TBST without dried milk for 1 h at room temperature. Primary antibodies were diluted to 10× of the concentration found optimal by Western analysis in antibody incubation buffer (0.1% BSA, protease inhibitors (EDTA-free Halt protease and phosphatase inhibitor cocktail, Thermo Fisher, Waltham, MA), 1× TBS, 0.5% Tween-20). Primary antibodies were used in the following dilutions for RPPA: Caspase-7/MCH-3 (BD labs, Cat# 610812) 1:100; brain-derived neurotrophic factor (BDNF) (Santa Cruz, Cat#sc-546) 1:20; and N-Cadherin (Santa Cruz, Cat#sc-31031) 1:50. Two hundred microliters of the primary antibody solution was then distributed over the entire nitrocellulose surface of the slide, covered with a cover slip (Nunc* mSeries LifterSlips, Fisher Scientific, Pittsburg, PA); and incubated in a humidity chamber at 4 °C overnight, while gently rotated. The next day, slides were washed three times in 0.1% TBST for 5 min each, and then incubated with an Alexa Fluor® 635 goat anti-mouse (Cat# A-31574) or goat anti-rabbit (Cat# A-31576) or rabbit anti-goat IgG (H + L) (Cat# A-21086) secondary antibody from Invitrogen at 1:6000 dilution in antibody incubation buffer and gently rotated for 1 h at room temperature. Slides were washed three times in 0.1% TBST 5 min each, and then three times again in 1× TBS. Slides were placed vertically into 50 ml conical tubes, and centrifuged at 1500 g for 5 min to remove excess liquid and facilitate drying. Slides then were allowed to fully air dry in the dark. The fluorescent signals were measured by scanning the slides with a 633 wavelength laser using a 647 nm filter in a Scan Array Express microarray scanner (PerkinElmer, Waltham, MA). Data were imported into a Microsoft Excel-based bioinformatics program developed in house for analysis.

All antibodies were pre-tested for specificity by Western blot analysis using tissue extracts (or serum) along with a specific positive control. When possible, an identical blot was made and incubated with the primary antibody pre-absorbed with the specific blocking peptide. The following reagents were used: S100b (ab41548-antibody, ab41547-blocking peptide, Abcam, Cambridge, MA); glucocorticoid receptor (ab3578-antibody; ab5018-blocking peptide, Abcam, Cambridge, MA); Caspase 7 (BD labs, Cat# 610812).

2.5. Data analysis and bioinformatics

A Microsoft Excel-based analysis tool was developed in house to analyze the primary data. To plug in the data from the scanned image, copy then paste the data from the appropriate slides to the analysis tool. See the detailed plug-in directions on the first sheet ("Instructions"). The tool imports intensity data from the scanner output and calculates the total net intensity after local background subtraction for each spot. The background subtracted intensity data from the dilution series of each sample are then plotted against dilution on a log–log graph. Regression (linear regression of the log–log data) of the data is done after removal of flagged data. Flagged data include spot intensities in the saturation range or noise range, signal to noise ratio less than 2, or high variability between duplicate spots (>10–15%). The total amount of the antigen is determined by the Y-axis intercept i.e., extrapolating the regression to zero (the undiluted sample).

3. Results and discussion

We have successfully utilized RPPA technology for quantitative analysis of biomarkers associated with traumatic brain injury (Agoston et al., 2009; Bauman et al., 2009; Gyorgy et al., in preparation) thus we feel this method provides a reliable and reproducible means to determine TBI induced changes.

RPPA is a highly sensitive method of protein quantification, able to detect 10^{-20} mol of protein in a sample, depending on the individual antigen–antibody being used (Paweletz et al., 2001), while commercial ELISAs detection limit is typically in the several pg