The Application of Protein Microarray Assays in Psychoneuroimmunology

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Abstract

Protein microarrays are miniaturized multiplex assays that exhibit many advantages over the commonly used enzyme-linked immunosorbent assay (ELISA). This article aims to introduce protein microarrays to readers of *Brain, Behavior, and Immunity* and demonstrate its utility and validity for use in psychoneuroimmunological research. As part of an ongoing investigation of psychological and behavioral influences on influenza vaccination responses, we optimized a novel protein microarray to quantify influenza-specific antibody levels in human sera. Reproducibility was assessed by calculating intra- and inter-assay coefficients of variance on serially diluted human IgG concentrations. A random selection of samples was analyzed by microarray and ELISA to establish validity of the assay. For IgG concentrations, intra-assay and inter-assay precision profiles demonstrated a mean coefficient of variance of 6.7% and 11.5% respectively. Significant correlations were observed between microarray and ELISA for all antigens, demonstrating the microarray is a valid alternative to ELISA. Protein microarrays are a highly robust, novel assay method that could be of significant benefit for researchers working in psychoneuroimmunology. They offer high throughput, fewer resources per analyte and can examine concurrent neuro-immune-endocrine mechanisms.

Keywords: Microarray, ELISA, Psychoneuroimmunology, Sera, Multiplex, Assay

1. Introduction

Researchers interested in brain, behavior, and immune links are frequently required to quantify levels of proteins in liquid samples. Common scenarios include the measurement of antibodies, antigens, cytokines and/or chemokines in sera or saliva. The enzyme-linked immunosorbent assay (ELISA) is the traditional method for such scenarios and is widely used for a variety of purposes in psychoneuroimmunology (e.g., Kohut et al., 2005; Shimizu et al., 2007). There are, however, practical limitations of ELISA: it has a fairly small dynamic range (values showing a linear relationship between absorbance and protein quantity) meaning that samples often require considerable dilution to fall within this range. Even following dilution, samples with particularly high or low protein levels may fall outside this range and therefore require re-analysis at a different dilution, potentially exaggerating differences (Leng et al., 2008). Further, ELISA is a monoplex assay (i.e., only one protein of interest can be measured per well), thus requiring considerable quantities of reagents and samples, especially if multiple proteins are of interest. This limits the suitability and practicality of ELISA when a large number of samples require processing; when more than one protein is of interest, and/or where many replicates are desired to improve the assay's robustness.

Recently, the development of protein microarrays has provided an alternative approach to quantifying proteins in liquid samples, avoiding the aforementioned limitations of ELISA. Protein microarrays are, in essence, miniaturized versions of the ELISA assay. Like ELISA, protein microarrays are highly flexible and can be adapted to measure almost any protein that can be examined via ELISA. However, unlike ELISA, protein microarrays are multiplex, meaning that many types of proteins can be detected within a single processing of a sample (Negm et al., 2015). This vastly reduces the quantities of sera, antigen, reagents, and time required to perform the assay (Leng et al., 2008). Additional advantages of protein

microarrays include: a wider signal detection range (0-65535 arbitrary units) based on fluorescence rather than colorimetric detection (typically 0-3 optical density units for ELISA), a greater capacity for large standard curves with many reference points, and lower dilution requirements for samples. Large parts of the microarray process can be automated, producing a highly specific continuous outcome with a larger dynamic range than ELISA (Rampal, 2007). Further, protein microarrays are highly robust as large number of replicates can be performed simultaneously and they can be adapted to include multiple internal quality control measures (Negm et al., 2015).

To date, microarray use has been relatively uncommon in psychoneuroimmunological research, most likely due to its relatively recent development and a lack of familiarity with the technique. The notable exception to this is research on gene expression (e.g., Carroll et al., 2016; Cole et al., 2007; Miller et al., 2014; Vedhara et al., 2015) where the multiplex abilities of DNA microarrays (which were the pre-cursor to protein microarrays) have long been established as a valuable technique, permitting the analysis of thousands of gene sequences simultaneously.

More recently we have seen the application of protein microarrays to rapidly quantify clinically relevant proteins such as antibodies to *Clostridium difficile* (e.g., Negm et al., 2015) and a variety of human cytokines simultaneously in a single sample (e.g., Selvarajah et al., 2014). Both applications indicate this method could be of considerable utility in psychoneuroimmunology.

In this article, we provide a brief conceptual overview of protein microarrays using the example of a novel microarray assay we developed to quantify influenza IgG antibodies in

serum samples taken as part of a psychoneuroimmunological study investigating the influence of psycho-behavioural influences on responses to influenza vaccination (a complete protocol for the assay is presented in 2.3.1). We then present data that demonstrates the assay is reliable and correlates well with sera analyzed by traditional ELISA.

1.1 Conceptual Overview of Protein Microarray

The central feature of protein microarrays is that tiny quantities (less than 200 microns in diameter) of assay-specific capture proteins are 'printed' by a robotic arrayer onto a reactive slide surface (typically a treated glass slide). These proteins become bound as small discrete 'spots' on the slide surface, before the remainder of the slide is blocked to prevent further binding. This process is analogous to the coating and blocking stages of a traditional ELISA, and can be accomplished using the same antigens as would be used for ELISA. To give a more concrete example, we printed multiple replicates of the three antigens contained in the 2014/15 influenza vaccine (formalin inactivated, partially purified H1N1, H3N2 and B viruses) alongside a calibration curve of human IgG onto commercially available aminosilane coated glass slides (for methodology see 2.3.1).

Diluted samples are then added to the slide surface, at which point proteins of interest in the sample (in our case antigen-specific antibodies in human sera) bind to the printed proteins. The remaining unbound sample is then washed away. At this point the remaining steps can vary depending on the protein of interest and is much like ELISA, in that protein microarrays can accommodate assay formats analogous to all variations of ELISA including direct, indirect and capture assays. In our example, the next steps of our microarray assay are analogous to that of an indirect ELISA. A secondary antibody (in our case anti-human IgG) that binds specifically to the bound protein is added. This secondary antibody is 'labelled'

with biotin, a small molecule that can be readily bound to a number of commonly used fluorescent labelling dyes. Finally, a fluorescent dye that binds to biotin is added (in this case streptavidin cyanin 5), which in turn can be detected by laser scanning. The fluorescence of a given sample (measured in arbitrary fluorescence units, AFU) is proportional to the number of antibodies in the serum sample that bound to the printed antigen, with greater fluorescence indicating more antibodies. Like ELISA, signals can be interpolated against a calibration, or standard, curve printed alongside proteins of interest if required.

2. Methods

2.1 Human Serum Samples

Venous blood samples (8ml) were obtained as part of an ongoing study to assess psycho-behavioral influences on influenza vaccination response in older adults (65-85 years). Samples were collected via venipuncture by trained phlebotomists using BD Vacutainer® tubes containing clot activator and gel for separating serum. After clotting at room temperature, samples were centrifuged at 2000g for 10 minutes after which sera were separated and aliquoted into Eppendorf tubes. Samples were stored at -80°C until analysis. For the ongoing study, over 400 samples were analyzed via microarray comprising sera collected at baseline, 4 weeks and 16 weeks post-vaccination. The validation and comparative analyses against ELISA presented in this article involved random selections of these samples.

2.2 Procedure

To assess the reproducibility of the microarray assay, serial dilutions of human IgG standard (R&D systems) were processed on two separate occasions using a random selection of 15

serum samples (note that these samples where only included for practical reasons, they do not influence the signals of the calibration curve). Coefficients of variation (CV) were calculated for the IgG dilutions in accordance with established guidelines (U.S. Department of Health and Human Services, 2013) to assess both intra-assay reliability (referring to variability in signals between replicates within a block across a single slide) and inter-assay reliability (in this case referring to variability in signals in calibration curves performed on two separate occasions). By convention, intra- and inter-assay CVs are considered acceptable if less than 15% and 20%, respectively. To compare the microarray assay with ELISA, another random selection of samples (n=40) were analyzed by both microarray and ELISA with results compared using the non-parametric Spearman's rank correlation coefficient (rho) as IgG levels were not normally distributed (positive skew).

2.3 Preparation and Processing of Samples

2.3.1 Microarray Protocol

Antigens for the 3 strains contained within the 2014/15 influenza northern hemisphere vaccine (H1N1 A/California/7/2009; H3N2 A/Texas/50/2012; B/Massachusetts/2/2012; National Institute of Biological Standards and Control) were diluted to 50µg/ml in printing buffer (PBS-Trehalose-Tween) and added to a 384-well plate (Genetix). Alongside this, 19 two-fold serial human IgG dilutions (range 100µg/ml - 0.2ng/ml) were added to the plate to form a calibration curve. Antigens and human IgG dilutions were spotted in 4 replicates on aminosilane-coated glass slides (Schott) in a 16 block format using a Biorobotics MicroGridII arrayer (Microgrid 610, Digilab).

Spotted slides were loaded into 16-well slide holders with hydrophobic barriers to separate wells. Wells were blocked for 1 hour with 100µl of 5% bovine serum albumin (BSA) in

phosphate buffered saline (PBS). Wells were then aspirated and washed for five cycles of 1 minute with 150µl 0.1% Tween-20 solution in PBS. Sera were diluted at 1:8,000 in a two-step process with the final dilution being made into antibody diluent (Dako). 100µl of diluted sera was added to each well for 1 hour. Wells were then aspirated and washed again (for 3 minutes per cycle) before 100µl biotinylated anti-human IgG (Vector Labs) diluted to 1:20,000 in 5% BSA in PBS was added for 1 hour. Wells were then aspirated and washed again (1 minute washes), before a final incubation with 100µl Streptavidin Cyanin-5 dye (eBioscience) diluted to 1:1,000 in antibody diluent for 15 minutes (covered in foil). Wells were then aspirated and washed (as above), before a final wash of 3 minutes with PBS only. All washes and incubations were conducted at room temperature with gentle agitation on a microplate shaker. Slides were then removed from holders and rinsed with distilled water before being dried by centrifugation at 1,200*g* for up to 10 minutes. Slides were scanned using a GenePix 4200AL scanner with a wavelength of 635nm, and 100% power. Photomultiplier gain (PMT) was set to 400 for each scan. Produced TIFF images were processed for fluorescence data via Axon Genepix Pro-6 Microarray Image Analysis software (Molecular Services Inc.). To improve reliability of microarray data, spots were robustly filtered such that those with circularity values <50, diameter <70 microns, and with less than 50% of spot pixels at least 2 standard deviations above local background were removed prior to analysis. The outcome variable of interest was spot median fluorescence minus local background.

2.3.2 ELISA Protocol

Wells of reactive ELISA plates (Fisher Scientific) were coated, in triplicate, with 50ul of antigens (see 2.3.1) diluted to 1µg/ml in carbonate-bicarbonate buffer (Sigma-Aldrich), alongside a calibration human IgG standard curve (25µg/ml to 0.2µg/ml) and refrigerated at

4°C overnight. The following morning, wells were aspirated and washed 3 times with 0.05% Tween-20 in PBS using an automated plate washer (BioTek) and blocked for 1 hour with 100µl of 3% BSA. Plates were then aspirated and washed again (as above) before 50µl of serum diluted at 1:8,000 in PBS was added and incubated for 1 hour. After a repeated aspiration and washing, 50µl of biotinylated anti-human IgG diluted to 1:20,000 in PBS was added for 1 hour. Plates were then aspirated and washed again, before 50µl of Streptavidin-HRP (R&D systems) at 1:40 in PBS was added to each well and incubated for 15 minutes. After a final aspiration and wash, 50µl of tetramethylbenzidine substrate solution (Sigma-Aldrich) was added to each well for 10 minutes, before the reaction was stopped with 1N solution of H_2SO_4 (sulphuric acid). Plates were then scanned at 450nm on a FLUOstar Omega plate reader (BMG Labtech).

3. Results

3.1 Microarray Reliability

Intra and inter-assay reliability statistics are presented for IgG concentrations ranging from 100-0.78µg/ml in Table 1, as signals for lower concentrations were often not above background levels. Mean intra-assay reliability was found to be excellent, with CVs for all concentrations ranging from 4.3%-12.3%, with a mean of 6.7%. Inter-assay reliability CVs also met conventional thresholds, ranging from 2.1% to 19.4%, with a mean of 11.5%.

[INSERT TABLE 1 HERE]

3.2 Comparison with ELISA

For the ELISA standard curve, the top two human IgG concentrations exceeded the detection limits of the assay, leaving a remaining range of 6.25μg/ml to 0.2μg/ml for which reliability could be assessed. Intra-assay CV's for ELISA standard curve concentrations ranged from 2.0%-22.8%, with a mean of 11.7%. Inter-assay reliability CV's for ELISA standard curves ranged from 9.6%-30.1%, with a mean of 18.2% indicating marginally poorer reliability compared to the microarray assay. Correlations between signals obtained for samples using microarray and ELISA were statistically significant and moderate to high in magnitude (see Figure 1), indicating the microarray assay is an acceptable alternative to ELISA (H1N1: rho(39) = .757, p<.001; H3N2: rho(39) = .661, p<.001; B: rho(37) = .568, p<.001).

[INSERT FIGURE 1 HERE]

4. Conclusion

In this article we have introduced protein microarrays and provided an example of their application in a common psychoneuroimmunology context: measuring antibodies in human sera. Our results demonstrate that the protein microarray assay had good intra- and inter-reliability and demonstrated significant correlations with the more established ELISA. Furthermore, the assay allowed us to analyze a large number of sera samples in a comparatively short time-frame. It is noteworthy that the dilution level chosen for comparisons between protein microarray and ELISA (1:8,000) was used to ensure samples optimally fell within the limited dynamic range of the ELISA. A considerably lower dilution (e.g., 1:1,000) could have been used for the protein microarray assay, which would potentially further improve its reliability profiles. While signals obtained from protein microarray and ELISA do correlate, it is important to emphasize that researchers would be

advised to be consistent in their assay approach across studies if they wish to draw direct comparisons between data-sets (Richens et al., 2010).

For researchers examining behavioral, neural, endocrine and immune system interactions, protein microarrays offer a multitude of potential uses beyond the specific example of illustrated in this article. For example, many recent articles featured in *Brain, Behavior, and Immunity* report the use of immunoassays that could alternatively be performed by protein microarray. These include measuring high-sensitivity C-reactive protein in obese humans (Lasselin et al., 2016), plasma corticosterone levels in mice with *Trypanosoma Cruzi* infection (Roggero et al., 2016), and protein expression in sonicated spinal cord tissue from rats exposed to morphine (Ellis et al., 2016). Furthermore, in a single blood sample (human or animal), protein microarrays can be adapted to rapidly quantify a host of endocrine and immune components concurrently. For example, immunoglobulin isotypes and sub-types (antigen specific or generic), endocrine factors including adrenocorticotropic hormone and glucocorticoids and a panel of inflammatory cytokines can be measured in serum. Similarly, protein abundance in red blood cell lysates and cell surface antigen presentation can also be measured in PBMC's (Kopf, Shnitzer, & Zharhary, 2005; Wolf-Yadlin, Sevecka, & MacBeath, 2009). While other assay methods can be used for each of these factors individually, protein microarrays can measure these factors in minute quantities of sample, often simultaneously. This opens up new and greater possibilities for researchers to examine concurrent changes in hormonal, inflammatory and immune processes and to better understand the interactions between these factors.

On a practical level, our experience of protein microarrays is, that after an initial optimization stage (which, in a laboratory experienced with protein microarrays can be feasibly completed

within a week), a large number of samples can be processed much more quickly than would be possible using ELISA. This high throughput is an especially attractive feature, as the resources (time and money) required to perform more traditional immunological assays is considerable and can be prohibitive to analyzing larger numbers of participant samples. This is particularly salient in psychoneuroimmunology, which has historically received criticism for an over-reliance on small, often under-powered, studies (Segerstrom & Smith, 2012). Further, the increased numbers of replicates that can be performed for each sample, permit us to be more confident of the assay's robustness and precision. Crucially, readers should note that the procedure could be easily adapted to assess a wide variety of other more or less abundant proteins in most clinically relevant liquid samples. A minor limitation of this method is, however, that the commercially produced reactive slides suitable for protein microarrays have a relatively short shelf-life, compared to ELISA plates and therefore must be purchased close to the time of analysis.

Conflicts of interest

All authors have no conflict of interest to declare.

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