Brain, Behavior, and Immunity 59 (2017) 62–66

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Short Communication

The application of protein microarray assays in psychoneuroimmunology

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ARTICLE INFO

Article history:
Received 29 June 2016
Received in revised form 2 September 2016
Accepted 13 September 2016
Available online 14 September 2016

Keywords:
Microarray
ELISA
Psychoneuroimmunology
Sera
Multiplex
Assay

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.

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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 sample, antigen, reagents, and time required to perform the assay (Leng et al., 2008). Additional advantages of protein microarrays include: a wider signal detection range (0–65,535 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

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http://dx.doi.org/10.1016/j.bbi.2016.09.013
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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 Section 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 μm 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 Section 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 (8 ml) 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 2000*g* for 10 min 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 50 μg/ml in printing buffer (PBS-Trehalose-Tween) and added to a 384-well plate (Genetix). Alongside this, 19 twofold serial human IgG dilutions (range 100 μg/ml–0.2 ng/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 h with 100 μl of 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS). Wells were then aspirated and washed for five cycles of 1 min with 150 μl 0.1% Tween-20 solution in PBS. Sera were diluted at 1:8000 in a two-step process with the final dilution being made into antibody diluent (Dako). 100 μl of diluted sera was added to each well for 1 h. Wells were then aspirated and washed again (for 3 min per cycle) before 100 μl biotinylated anti-human IgG (Vector Labs) diluted to 1:20,000 in 5% BSA in PBS was added for 1 h. Wells were then aspirated and washed again (1 min washes), before a final incubation with 100 μl Streptavidin

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