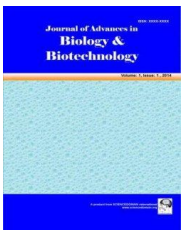

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Biomarker Analysis with Grating Coupled Surface Plasmon Coupled Fluorescence

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Authors' contributions

This work was carried out in collaboration between all authors. Author AM performed all of the analyses and wrote the first draft of the manuscript. Author JAD made and provided all of the peptides. Author TZ collected bloods from children with autism. Author DAL designed the study, wrote the protocol and assisted with the writing of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

One of the greatest hurdles in protein microarray technology has been the limited sensitivity. However, the strategy of amplifying the SPR signal with fluorescence improves sensitivity. Herein, we report a simple and efficient method, Grating Coupled Surface Plasmon Coupled Fluorescence (GCSPCF) with a peptide microarray to screen sera of human subjects and a mouse strain with autistic-like behavior for quantification of the epitope specificities of their circulating antibodies. The GCSPCF peptide microarray is a novel assay that facilitates high-throughput screening of antibody epitope specificities or binding of interacting analytes with a small quantity (<50 µl) of sample. This study utilized over 600 peptides with the eventual goal of being able to identify antibodies that could be used as biomarkers of particular disorders. The GCSPCF technology was able to detect antibodies to the tripeptide glutathione; it was also capable of sequential screening antibodies of different immunoglobulin isotypes (IgG1, IgG4 and IgE) to the same peptide. The GCSPCF broad-based screening approach was able to begin the identification of

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epitope-specific antibody binding patterns in an effort to develop a predictive signature that could be further developed for clinical trials. In addition to evaluation of antibody specificities with hundreds of spotted peptides at multiple regions of interest (ROI), the addition of spotted antibodies could provide an even more complete biomarker profile with capture of analytes, such as stress proteins and cytokines. Differences between a mouse strain with normal behaviors (C57BL/6J) and a strain with autistic-like behaviors (BTBR *T*$^{+}$*tf*/J) as well as healthy and autistic participants are used for comparative analyses.

Keywords: SPR; grating-coupled surface plasmon coupled fluorescence; synthetic peptides; epitopes; circulating antibodies; autism.

ABBREVIATIONS

Surface Plasmon Resonance (SPR); Grating-Coupled Surface Plasmon Coupled Fluorescence (GCSPCF); Mean Fluorescence Intensity (MFI); Regions of Interest (ROI); Autism Spectrum Disorders (ASD).

1.INTRODUCTION

Surface plasmon resonance (SPR) technologies have often been used to measure dielectric functions of chemical and/or biochemical properties as well as thickness of thin film materials [1]. Recently, the SPR spectroscopic technology has developed into an analytical technique for the characterization of thin metal films (i.e. silver and gold) at interfaces as well as a sensitive detection tool for monitoring real-time kinetic processes of interfacial binding reactions [2]. The combination of a sensor chip (i.e., glass slide/dielectric grating/metal layer) with SPR (Fig. 1A) is termed grating coupled surface plasmon resonance (GCSPR), which allows detection of analytes by changes in the refractive index (RI) [3]. This configuration can best be used as a biosensor since a biological component provides a sensing mechanism for detecting small changes in the refractive index resulting in a shift in the SPR angle (Fig. 1B) that occurs from analyte binding at or near the surface of the metal surface, which are capable of supporting surface plasmons [4]. The current sensitivity of the RI change for GCSPR is reported to be on the order of 3×10^{-7} refractive index units (RIU) [5]. This biosensor platform is for receptor-ligand binding reactions involving multiple soluble and particulate analytes in complex biological samples such as proteins, antibodies, antigens, cytokines, and mammalian cells and their secreted products for analytical applications. Thus, the use of GCSPR spectroscopy for detection and quantification of a particular bioanalyte is accomplished through signal transduction associated with antigen-antibody pairs that requires a suitable printing of a capture reagent, like antibodies, on a biosensor chip before the flow of ligands, like antigens, over the chip.

Recently, there have been an increasing number of studies involving the maternal immune system and how it develops and transfers antibodies to the fetal brain [6]. Antibodies in the blood of mothers with autistic children had significantly higher reactivity to fetal brain proteins in comparison to mothers without autistic children [7]. However, autoantibody development and specificity to brain antigens in people with autism spectrum disorders (ASD)has yet to be fully defined, which is important in discovering mechanistic pathways that are responsible for the development of ASD and thereafter developing new therapies to improve the behaviors associated with ASD. This preliminary study reports the use of a peptide microarray method based on Grating-Coupled Surface Plasmon Coupled Fluorescence

(GCSPCF) imaging for detecting circulating antibodies in sera to assess antibody specificities based on epitope recognition. Thus, creating an epitope signature profile that could be important in finding antibodies that interfere with normal brain development or be used to predict patterns of neurodevelopmental disorders. GCSPCF is closely related to GCSPR in that excitation of surface plasmons near the metal: dielectric interface greatly increases sensitivity of the fluorescence signal through the enhanced excitation rate of fluorophores [8]. This feature takes advantage of the electromagnetic field enhanced by the resonant excitation of surface plasmons allowing the detection of small molecules and/or analytes at low concentrations since a higher fluorescence signal is achieved [8]. Furthermore, compared to conventional fluorescence techniques, the excitation of fluorophores near the metal surface suppresses background fluorescence emission, which allows increased sensitivity and efficiency in collecting fluorescence signals [9].

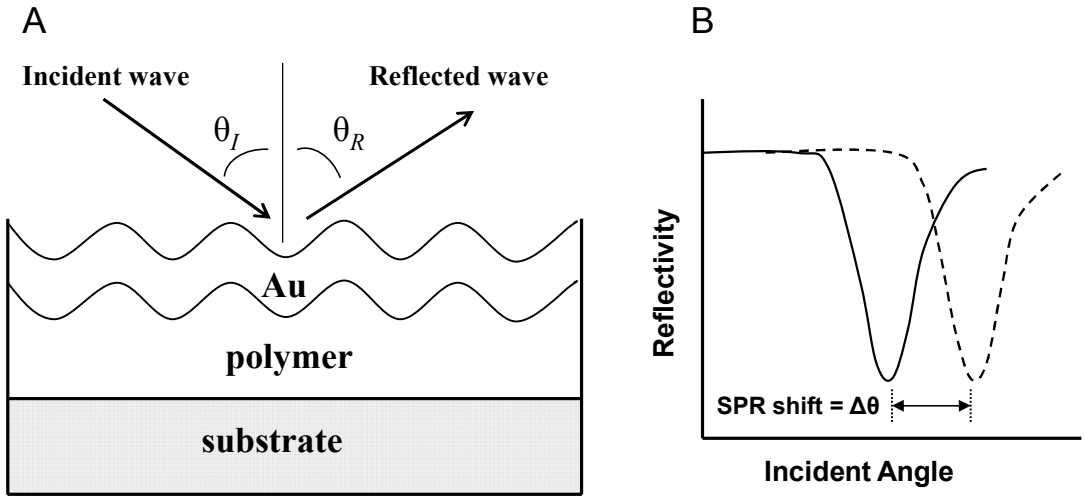

Fig. 1. A) Schematic representation of a grating coupled surface plasmon resonance (GCSPR) sensor chip. B) Schematic representation of SPR spectra where there is a shift in angle because of change in refractive index. A biological (proteins, cells or other molecules) component provides a sensing mechanism for detecting small changes in the refractive index resulting in a shift in the SPR angle that occurs from binding at the regions of interest (ROIs) near the surface of the metal surface, which are capable of supporting surface plasmons

Peptides are cost effective, easy to synthesize, and provide an ideal target as a diagnostic biomarker for the early diagnosis of infectious diseases [10], allergies [11], cancers [12] and autoimmune diseases [13,14]. Peptides are generally synthesized in short amino acid sequences and are used as substitutes for proteins in capturing antigen-specific antibodies [15]. Unlike proteins that could lose their biological function because of their length and complex structures under different conditions, peptides are relatively stable [16], and can be synthesized to mimic the epitope-specific recognition sites of antibodies [17]. In this study, the aim was to determine the usefulness of peptides with the GCSPCF technology and then to identify a pattern of epitope-specific antibody signatures associated with ASD by initially optimizing conditions for screening sera with GCSPCF and peptides followed by assaying antibody specificity using sera from BTBR-*T*$^{+}$*tf*/J (BTBR) and C57BL/6J (B6) mice and humans. BTBR mice are an inbred strain of mice commonly used in research because of

their behavioral phenotypes, which mimic many of the behavioral features that define ASD [18]. These features include repetitive self-grooming, unusual patterns of ultrasonic vocalization, and deficits in social interactions and social approach [18,19]. B6 mice, on the other hand, are an inbred strain of mice used as a control strain because of their high social and low self-grooming behavior [19].

Antibodies and other capture analytes were also utilized in this study. A recent study reported enhanced lipid peroxidation and decreased levels of glutathione peroxidase resulting from increased oxidative stress in children with ASD [20]. Since oxidative stress has been implicated in various disorders [21] including ASD [22] and since the brain is vulnerable to oxidative damage [23], we assayed sera for autoantibodies against stress proteins and determined if the technology might be able to quantify glutathione, a major anti-oxidant. Autoantibodies to stress proteins in autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) have previously been reported [24] as well as circulating immune complexes (CICs) in these diseases [25]. However, no clear correlation has been made between the increased auto antibodies to the stress proteins and the pathogenesis of the disorders.

2. MATERIALS AND METHODS

2.1 Materials

Gold-coated GCSPR biosensor microchips, 1 cm^2, were purchased from Ciencia, Inc. (East Hartford, CT). Antibodies were obtained from a number of sources. Alexa Fluor® 647 goat anti-mouse IgG (H+L) and Alexa Fluor® 647 Streptavidin were purchased from Invitrogen (Carlsbad, CA). Biotin labeled goat anti-human IgE was purchased from KPL (Gaithersburg, MD). Human AB serum was purchased from Sigma-Aldrich (St. Louis, MO, USA). Goat anti-human (h) IgA, IgE, IgG, and IgM were purchased from Caltag Laboratories (Burlingame, CA), while anti-mouse (m) IgG antibodies were purchased from The Binding Site (Birmigham, UK). The biotinylated anti-human IgG4 and IgG1 were purchased from Alpha Diagnostic Intl. Inc. (Texas, USA). The GCSPR/GCSPCF instrument, a second generation dual-mode instrument, was designed, built and purchased from Ciencia, Inc. (East Hartford, CT).

2.2 Mouse Strains

BTBR *T*$^+$*tf*/J (BTBR) and C57BL/6J (B6) mice were originally obtained from Jackson Laboratory (Bar Harbor, ME), and were bred and housed in a specified pathogen-free environment with food and water *ad libitum* at The Wadsworth Center. All mice were maintained on a 12-hr light/dark cycle with lights on from 7 AM to 7 PM. BTBR (n=6) and B6 mice (n=6) were bled and blood collected into EDTA-containing tubes following approved procedures by the Institutional Animal Care and Usage Committee (IACUC) of The Wadsworth Center, NY State Department of Health.

2.3 Human Sera Samples

All patient sera were obtained with IRB approval from New York State Department of Health (protocol 99-412) and/or Center for Disability Services (protocol CFDS# 10-01). All participating families signed the approved consent form.

2.4 Peptide Synthesis

The peptide microarray consisted of over 800 synthesized peptides ranging in length from 6 to 55 amino acid sequences. In brief, the peptides were synthesized by F-MOC chemistry using an Applied Biosystems 431A peptide synthesizer (Foster City, California). The crude peptides were purified by chromatography on a reverse phase HPLC (C-18 Delta PACK, 19 x 300 cm) in which a single peak was obtained and lyophilized to a powder. The lyophilized powder was stored in a desiccator until used and further characterization for molecular weight was performed by Ion trap-micro electrospray mass spectrometry using a Thermo Finnigan LCQ Deca MS instrument. Peptides were then resuspended in dry dimethyl sulfoxide (DMSO) at concentrations ranging from 2 to 10 mg/mL, diluted to final a concentration of 1 mg/mL in carbonate buffer (pH 8-9), transferred to a 384-well microtiter plate (Thermofisher, IL), and immediately pin spotted onto the biosensor microchip as described below.

2.5 GSNEM Synthesis

Stock glutathione-*N*-ethylmaleimide (GS-NEM) solutions were prepared by slow addition of 7.68 mg (0.025 mmole) of reduced GSH (Sigma) dissolved in 500 µL of PBS to 3.13 mg (0.025 mmole) *N*-ethylmaleimide (NEM; Sigma) in 500 µL PBS at room temperature. The reaction was followed by UV-visible spectroscopy (λ 304-315 nm, NEM) and thin layer chromatography (TLC) until complete. Serial dilutions from the standard stock GS-NEM (10.8 mg/mL, 2.28 x 10^{-2} M) solution were prepared ranging from 10 mg/mL to 0.1 mg/mL, pin spotted, and developed by using 8.1-GSH, a mouse monoclonal IgG1 antibody against GSH adduct with NEM (GS-NEM) [26], conjugated to Alexa Fluor 647 as described below.

2.6 GCSPR Biosensor Microchips and Peptide Printing

The gold coated GCSPR biosensor microchips were initially rinsed with 70% ethanol, distilled water and dried under stream of filtered air. The chip was then placed in a 35 x 10 mm style petri dish (Becton Dickinson, Franklin Lakes, NJ) and cross-linker reagent dithiobis (succinimidyl propionate) (DSP) (Pierce, Rockford, IL) was added at a concentration of 4 mg/mL in dry dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO). The mixture was allowed to incubate for 30 minutes at room temperature, the chip was then rinsed with DMSO, water and dried under a stream of filtered air. The chip was immediately placed in an *Arrayit* robotic microarray spotter, SpotBot II (Arrayit, CA) configured to use one 946MP4 contact pin that has a delivery volume of 1.1 nL and a diameter of 135 µm per spot, and spotted with peptides, bovine serum albumin (essentially globulin-free BSA) (Sigma-Aldrich, St.Louis, MO), which was used as a reference, and goat anti-mouse IgG (or goat anti-human IgG for human samples) prepared at a concentration of 1 mg/mL. A total of 105 different peptides including controls were pin spotted on each 1 cm^2 microchip in triplicate (n = 3 ROIs per sample) at room temperature with a relative humidity of 80-90%. The number of samples was limited to 105 (315 ROI) simply because this was the maximum number of samples that could be printed with proper spacing between each ROI in order to prevent overlapping. After being spotted, the microchip was incubated for 1 hr at room temperature with a relative humidity of 80-90% and stored at 4ºC in a desiccator until used.

2.7 GCSPR Biosensor Microchips and antibody printing

The GCSPR microchips were prepared as described above and antibodies to the following proteins (StressMarq Biosciences Inc., Canada) were printed directly onto the microchip: Cavbeta1, KIR2.3, KChIP3, KvBeta1.1, Nav1.8, KV3.1, CAV1.3, CAV1.2, KV3.4, CASPR,KCNQ4,Pan-KvBeta, BK Beta3a, DNMT3L, TrpM7, Pan-SAPAP, Kv1.1 Extracellular, KCNQ2, S26A-23,GABA(A)R Delta, PSD-95, TrpC7, TrpV3, Kv2.1, Slo3, HCN4, Kv2.2, Pan-MAGUK,GABA(A)R, Beta1, NR2B, SHANK1,HCN2, CAV1.3, HCN3, Slo2.2 (SLACK), Nav1.1, Kv1.5, Kv1.4,TrpC5,Kv2.1 Extracellular, Kv1.1, Kv1.2,CavBeta2, Nav1.2, Pan-KCHIP,Slo2.1 (SLICK), PAN-SHANK, TrpC4,SHANK3, KIR2.2, BK Beta2, Kv1.6, CASPR2, SAP97,PICK1,Co-Rest/RCOR1,Kv4.3, KCNQ1, NR2B, GABA(A)R- Beta3, GABA(A)R- Alpha1, Kir2.1, Kv2.1, NAV1.7, KChIP2, HCN1,CAV3.2, Nav1.6,Pancortin, IP3 Receptor, Contactin/F3, GluR2,CHAPSYN-110 (PSD93), TrpV3,Sodium-Iodide symporter, Slo1, Thorase/Atad1, Kv1.3, CASK, KChIP1, Sodium-Iodide symporter, Kv4.2,CNGA1/3, KvBeta1.2,Zinedin (Striatin 4), KvBeta2, SHANK2, Cavbeta4, WAVE1/SCAR,SH3GL1,BK Beta4, HIF2 alpha- EP190b, p38 alpha MAPKinase, HSF-2,Hsp90, SAP102, Hsc70 (Hsp73), DMPO- N1664A, SNAP-25- SP-12, TSP23, Hsp70,and p23.

2.8 GCSPR Analysis

The printed microchips were assembled at room temperature using a double sided adhesive gasket (0.50 mm) and a glass window (5 mm) to create a flow cell, 50 µL, and placed in the GCSPR instrument for real-time kinetic binding and fluorescence analysis. At this point, all experiments performed in the instrument were at 32ºC and all buffer solutions were degassed under vacuum, 25 psi, for 1 hr before use. The chip was initially washed with freshly degassed HEPES buffer (10 mM HEPES, 150 mM NaCl, 3.5 mM EDTA and 0.05% Tween 20) for 10 min at a flow rate of 200 µL/min, blocked with 5% L-lysine dissolved in HEPES buffer for 60 min at a flow rate of 200 µL/min, washed for 10 min and 1.4 mL of diluted (1:70 (v/v)) mice serum in HEPES buffer was flowed and recirculated over the chip for 20 min at the flow rate of 200 µL/min. The chip was then washed for 10 min and anti-mIgG (H+L) AlexaFluor 647 diluted to 2 µg/mL in HEPES buffer was flowed over the chip at a flow rate of 200 µL/min for 20 min. The chip was washed with HEPES buffer for a final time for 10 min and imaged for fluorescence analysis.

The human AB serum experiments were performed in a similar manner as described above in which peptides #386 - #504 were printed on two separate chips on different dates along with BSA, goat anti-hIgG and anti-hIgE. The chip was washed for 10 min, blocked with 5% L-lysine for 60 min, washed, and human AB serum diluted 1:7(v/v) in HEPES buffer was flowed over the chip for 30 min. The chip was washed again, and biotin labeled anti-human IgE at a concentration of 2 µg/mL in HEPES buffer was flowed over the chip for 30 min. Afterwards, the chip was washed, and Streptavidin Alexa Fluor 647 at a concentration of 400 ng/mL was flowed over the chip for a period of 45 minutes followed by a final 10 min wash before fluorescence imaging analysis. The same procedure was repeated sequentially with biotin labeled anti-hIgG4 and then anti-hIgG1.

Experiments involving stress proteins were initially setup as described above. In brief, the microchips were assembled, initially washed with freshly degassed PBS buffer (pH 7.4) containing 0.05% Tween 20 for 10 min at a rate of 200 µL/min, blocked with 2% BSA dissolved in PBS buffer for 60 minutes at a flow rate of 200 µL/min, washed for 10 min, and human sera diluted 1:70 (v/v) in PBS from healthy control or autistic subjects was flowed

over the microchip at a rate of 150 µL/min for 30 minutes. The microchip was then washed for 10 minutes, and biotin conjugated anti-hIgG (Sigma-Aldrich, USA) at a concentration of 2 µg/mL was flowed over the microchip for 30 min at a flow rate of 200 µL/min. The chip was washed for 10 min, and Streptavidin AlexaFluor 647 at a concentration of 400 ng/mL was flowed over the chip for a period of 45 min followed by a final 10 min wash before fluorescence imaging analysis.

2.9 GCSPR Imaging Analysis

In either GCSPR or GCSPEF mode, an output folder for each experiment was created in which files of output data, experimental description and annotations, instrument configurations, ROI templates, and experimental protocols were stored. All the configurations and template files, once created, can be reloaded to repeat an experiment or as components of a new experiment. Images of the surface of the peptide microarray were captured electronically with a CCD camera and stored. The camera sensor in the SPR instrument is 1392 pixels wide by 1040 pixels high in which each pixel is 6.45 µm square. Pixels in the sensor are arranged in a rectangular array and are numbered from the upper left-hand corner (i.e. rows are numbered left to right and columns from top to bottom). Regions of interest (ROIs) were defined on the image that isolates each spot along with reference regions. ROIs are defined by recording the column and row pixel numbers of the upper left-hand corner and the lower right-hand corner of each ROI. In GCSPR mode, the angle of a beam of illuminating light is varied and the SPR resonant angle determined for each ROI. The primary output of the SPR mode is the resonant angles for each ROI as a function of time. The resonant angle shift is proportional to the mass of the material captured at each ROI. In GCSPCF fluorescence mode, the primary output is the fluorescence intensity of each ROI. Fluorescence measurements are made as a function of time in which the sum of the intensity of each pixel is divided by the number of pixels in the ROI [27].

2.10. GCSPR Data Processing

The SPR instrument operating software is an executable program written in LabView (National Instruments, Austin, TX). This operating software controls motion, data acquisition and image processing. Furthermore, the software also converts camera units into refractive index units, performs background referencing and allows for measuring binding signal responses at various time points. Calculated GCSPCF fluorescence intensities represent average values of 3 ROIs per sample ± standard deviation performed on Microsoft Excel in which BSA was used as a reference/negative control. Expression profiles were plotted as heat maps using JMP9 Heat Map Software.

3. RESULTS

Our results are based on GCSPR or GCSPCF analysis. Fluorescence analysis of antibodies bound to printed peptides was used to assess the presence of circulating antibody specificities of IgG antibodies in the sera of both humans (healthy control and ASD participants) and mice (BTBR and B6). The GCSPCF peptide microarray consisted of over 600 synthesized peptides ranging in length from 6 to 55 amino acids. To determine if a molecule as small as a tripeptide (γ-glutamyl-cysteinylglycine) could be attached to the microchip and detected by an antibody, we optimized crosslinking of GS-NEM at concentrations ranging from 10 to 0.1 mg/ml, as described in methods. There were no significant differences with the 10, 5, and 2 mg/ml amounts of spotted GS-NEM (Fig. 2). The

spotted GS-NEM was detected with AlexaFluor647-monoclonal antibody 8.1GSH [26]. On the same microchip, mouse (m) IgG at concentrations ranging from 5 to 0.1 mg/mL were spotted at separate ROI, and detected with AlexaFluor647-goat anti-mIgG (Fig.2). This initial analysis determined that a peptide and protein could be detected above the back ground mean fluorescence intensity (MFI) with GS-NEM at 0.5 mg/ml. Since each spot is made with 1.1 nl, the GCSPCF system can detect approximately 0.5 ng of a tripeptide/ROI. With mIgG on the same microchip at separate ROI and subsequent analysis of antibody (goat anti-mIgG) binding to its antigen, it was determined that polyclonal antibody binding toa protein antigen could be detected with less analyte/ROI, in that the amount needed to achieve detectable antibody binding above the background was spotting 0.1 mg/ml or 0.1 ng/ROI (Fig. 2). Additionally, with sequential analysis of detection, the proportional MFI values for the diluted spotted peptide and protein on the same microchip indicated no substantial interference or modification of previous MFI values at each ROI.

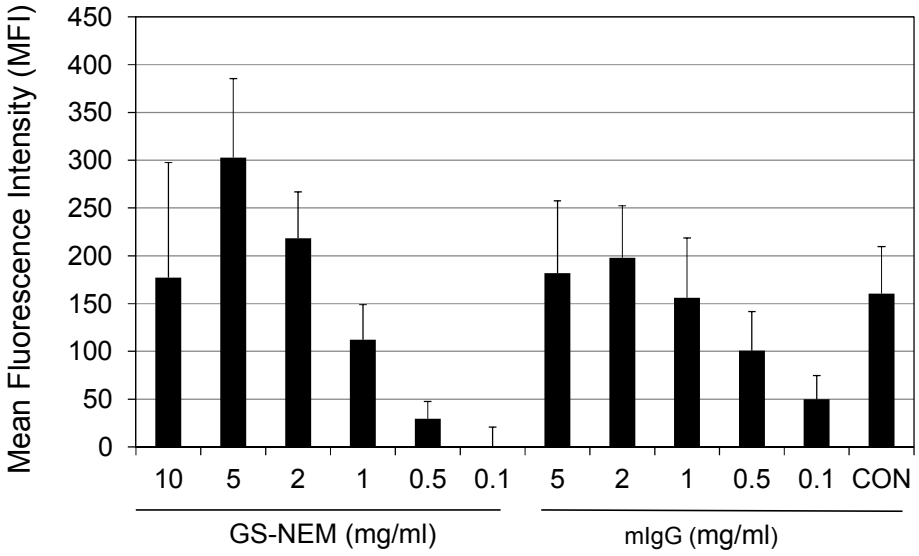

Fig. 2. GCSPCF fluorescence results of GS-NEM spotted and cross-linked onto a microchip at various concentrations along with mIgG and a control (Alexa Fluor 647 mouse IgG, 0.5 mg/mL) spotted at separate ROI. GS-NEM was detected by flowing 8.1-GSH, a mouse monoclonal IgG1 antibody against GSH adduct with N-ethylmaleimide (GS-NEM) conjugated to Alexa Fluor 647, over the microchip for 60 min. While mIgG was developed by flowing Alexa Fluor 647 anti-mouse IgG (H+L) (2 µg/ml) over the microchip for 60 min

The inverse of the spotting of antigen and capture of antibody is shown for use of spotted goat-anti-hIgG and the capture of antigen (IgG) from human serum. The antibody was spotted at 1 mg/mL and dilutions of human AB serum were sequentially flowed over the microchip with GCSPCF analysis after each dilution. With 3 µL of serum, which is equivalent to about 36 µg IgG, in 1.4 mL flowed over the microchip, the MFI value was significantly above the background MFI. Although the 1:466 dilution of human serum gave a MFI signal above background, sera diluted 1:7 or 1:70 were considered more ideal for further analysis of specimens (Fig. 3).

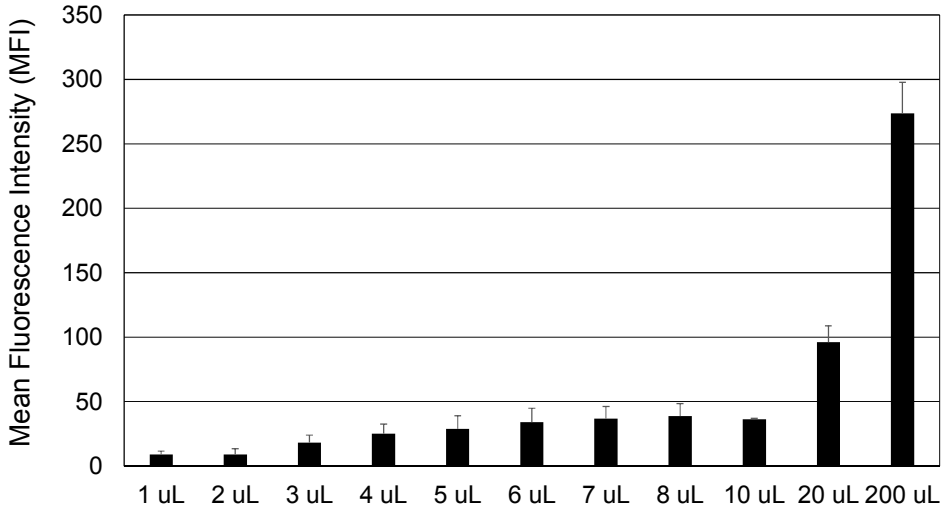

Fig. 3. GCSPCF analysis of IgG in human AB serum. Serial dilutions of human AB serum starting at 1 µL diluted to 1400 µL (dilution of 1:1400 v/v) and ending at 200 µL (dilution of 1:7 v/v) were screened. The SPR microchip was spotted with anti-human IgG (H+L) at 1 mg/mL, and the diluted serum was flowed over the microchip for 30 min. ROI were analyzed for binding of IgG by flowing biotinylated goat anti-human IgG (2 µg/mL) over the microchip for 30 min, and developed by flowing AlexFluor647-Streptavidin (400 ng/mL) over the microchip for 45 min followed by MFI analysis

Reproducibility of the GCSPCF peptide microarray was determined with use of human AB serum in replicate microarrays. The results were assessed with Pearson correlation analysis. A serum was sequentially assayed for IgE, IgG4 and IgG1antibodies to the various spotted peptides, which were spotted on two separate SPR microchips. The microchips were spotted and utilized approximately a month apart. IgE (Fig. 4A), IgG4 (Fig. 4B) and IgG1 (Fig. 4C) antibodies appeared to have comparable reproducibility with correlation coefficients of $r = 0.94$, $r = 0.94$ and $r = 0.91$, respectively, with $P<0.01$.This analysis also suggests that all three isotypes can be quantified on the same microchip as long as the ROI does not become saturated, which is why one must start with the ligand least likely to saturate the ROI. In the case shown, this would be IgE followed by IgG4 and then IgG1. IgE antibodies would be expected to bind the least since the approximate IgG1, IgG4, and IgE ranges are 420-1292 mg/dl, 1-291 mg/dl, and 0.24-48 µg/dl, respectively. Thus, as shown (Fig. 5), the technology might be useful for the screening of an allergic serum for the amount of different antibody isotypes binding to the epitope of an allergen. Reproducibility of the GCSPCF microchip spotting and analysis was also assessed with separate microchips (n=3) spotted and used at monthly intervals with antibodies to stress proteins and analysis of a human serum. The separate analyses gave a %CV = 22.98(data not shown).

Antibody specificities with the peptide microarray were assessed with use of a serum pool from BTBR (n=6) and B6 (n=6) mice. An example of a GCSPR image of the ROI (18 x 18)matrix (Fig. 6A) and a fluorescence image of the GCSPCF peptide microarray after being treated with diluted 1:70 (v/v) mouse sera and developed with AlexaFluor647 goat anti-mIgG is illustrated (Fig. 6B). Analysis of the fluorescence clearly demonstrated binding above background, which indicates circulating antibodies in the sera of the mice show specificity towards certain peptides; the fluorescence image before the addition of sera is shown for

comparison (Fig. 6C). Furthermore, since the intensity signal varied from peptide to peptide of various lengths, it's likely this was a result of affinity differences and/or amounts of the antibodies toward the peptides; additionally, it is possible for longer peptides that antibodies with different specificities are binding to separate epitopes of a single peptide. The heat map, which shows 480 of the peptides used, further summarizes the distinct antibody-peptide binding interaction of the peptides used in the GCSPCF peptide microarray (Fig. 7). Most peptides bound no or low amounts of IgG implying that there were minimal antibody amounts or affinities to those peptides. Some peptides (e.g., peptide #25 of column 1) bound equivalent amounts of circulating antibodies from both BTBR and B6 sera. Although the various peptides displayed different amounts of bound IgG from B6 and BTBR mice, both strains displayed different MFI values (positive and negative) to the majority of the same peptides. There were only 24 peptides that bound more BTBR antibody than B6 antibody.

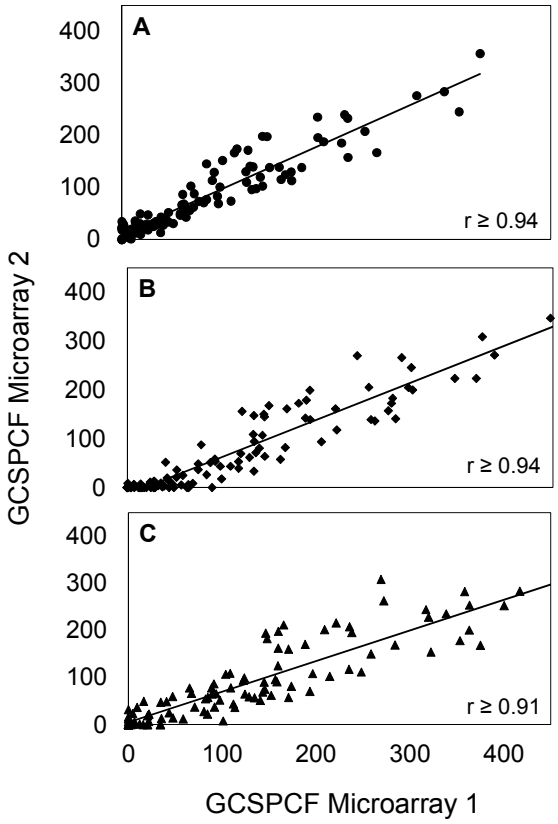

Fig. 4. Reproducibility of GCSPCF analysis of IgE (A), IgG4 (B) and IgG1 (C) from human sera in replicate arrays assessed with Pearson coefficient analysis. The serum was analyzed with peptides (1 mg/mL) cross-linked onto two separate microchips, followed by flowing the serum (200 µL, 1:7 dilution) over the microchips for 30 min. Binding of IgE, IgG4, and IgG1to each peptide/ROI was sequentially assessed by flowing biotinylated goat anti-human IgE (2 µg/mL) over the microchip for 30 min, and developed by flowing AlexaFluor647-Streptavidin (400 ng/mL) over the microchip for 45 min followed by MFI analysis. Subsequently, each ROI was similarly assayed with biotinylated goat anti-human IgG4 (2 µg/mL) and then with biotinylated anti-human IgG1 (2 µg/mL)

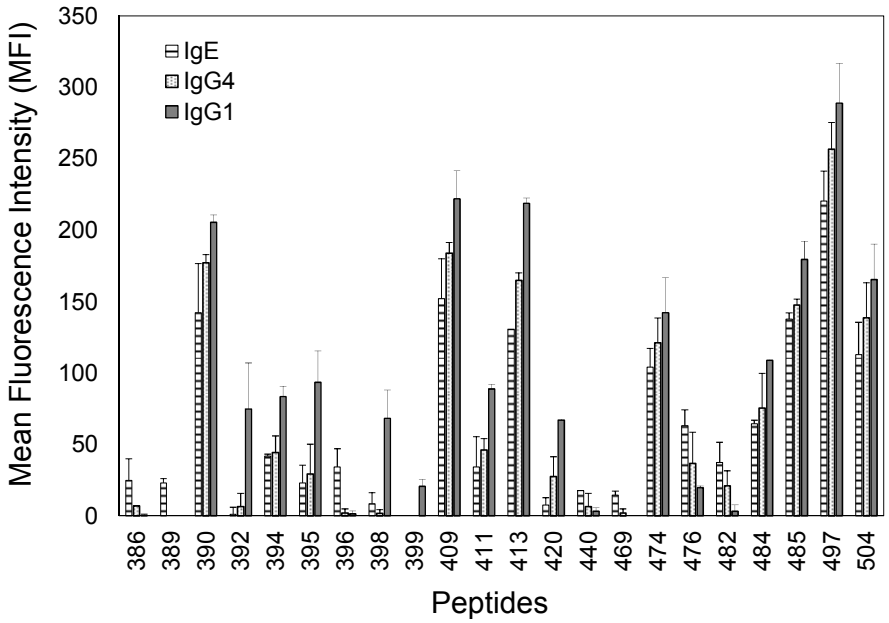

Fig. 5. GCSPCF sequential analysis of IgE, IgG4 and IgG1 from human sera. Sera was analyzed by crosslinking peptides (1 mg/mL) onto a microchip, followed by flowing diluted (1:7 v/v) human sera over the microchip for 30 min and further analysis as described for Fig. 4

In comparison, Fig. 8 shows GCSPCF binding results of IgG antibodies to hundreds of peptides using diluted 1:70 (v/v)human sera from healthy controls(n=3) and subjects with ASD(n=3).The MFI values with the control and ASD sera indicate whether the peptide array could differentiate differences between control and ASD sera (Fig. 8). The Y-axis values above 0 indicate that the IgG of ASD sera bound to a peptide to a greater extent than that of the control sera (ASD MFI minus Control MFI). The X-axis values indicate the extent of IgG binding in both ASD and control sera (Total MFI = control MFI + ASD MFI). For differentiation of the IgG-peptide interactions, the peptides were arbitrarily grouped into four separate categories. There were peptides bound by IgG from control and ASD subjects (MFI>150), but IgG from ASD subjects bound better with an MFI difference >100 (Table 1). Some peptides bound a negligible amount of IgG from the controls (MFI <12) but had MFI values >50 with IgG from ASD subjects (Table 2). The majority of the peptides bound negligible amounts of IgG (total MFI <50) from control and ASD subjects with low differences in binding (MFI <50) with control and ASD sera (Group 3). There also were some peptides that bound more IgG from control sera than ASD sera (Group 4). Peptides that bound more IgG from ASD subjects than from controls and that were not listed in Tables 1 or 2 are listed in a Supplemental Table. Most peptide sequences used were from known proteins; however, some were from random amino acid sequences. Occasionally, peptides of the same sequence made with a separate synthesis were spotted and compared; they gave similar results and their MFI values were averaged. Some peptides with equivalent portions of the same sequence (e.g., peptide 227 and 234 in Table 1) also were spotted, and they gave equivalent MFI values. With the hundreds of peptides spotted, 65 peptides showed binding of IgG from ASD subjects better than (MFI >50) IgG from the controls. For the most part, antibody specificity from human sera showed similar, but not identical binding patterns that varied from person to person. In contrast to the human IgG binding characteristics, serum

IgG from BTBR and B6 mice showed binding differences with only 35 peptides, and of these peptides only 24 were bound to a greater extent with antibodies from BTBR mice (Table 3).

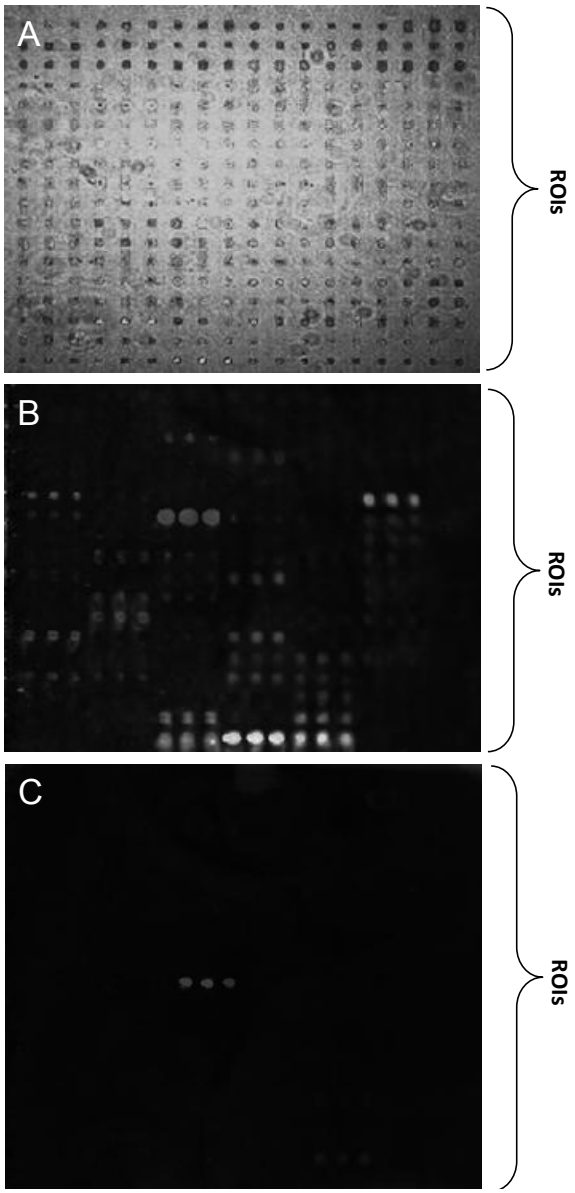

Fig. 6. SPR sensor microchip with spotted peptides (1 mg/mL) cross-linked to the gold (Au) surface and imaged by GCSPR (A) or by GCSPCF imaging (B) after diluted (1:70) mouse serum was flowed over the microchip for 30 min and developed by flowing AlexaFluor647-anti-mouse IgG (H+L) (2 µg/mL) over the microchip for 30 min. GCSPCF image of the peptide microarray taken before the start of the assay (C) shows only the fluorescent internal ROI markers spotted along with the peptides/antibodies in order to properly align ROI matrix

Table 1. Peptides binding greater amounts of IgG (MFI >100) with high (>100) MFI difference

Peptide #	Amino acid sequence	Total MFI	MFI difference
125	ANERADLIAYLAQATK	141	122
131	HAIIQGHWPECVSFGSTDRFVLAKEHRSAHSEFSSK	569	124
132	HKVNSQVE	247	143
219	ISQAVHAAHAEAPV	170	122
221	RKRRKMSRGL	271	229
227	EMISQAVHAAHAEA	224	120
234	HQEMISQAVHAAHA	198	104
443*	VKCFNCGKEGHIARNCRA	660	184
502	PSKKGSRPQRQRRGA-MAPS 4	574	109
525	CYFDDNSNVICKKYRS	183	104
641	Human neuropeptide Y	292	172
644	GVAMLNGLIYVIGGV-OH	196	149

**average MFI values for 3 peptides with same sequence*

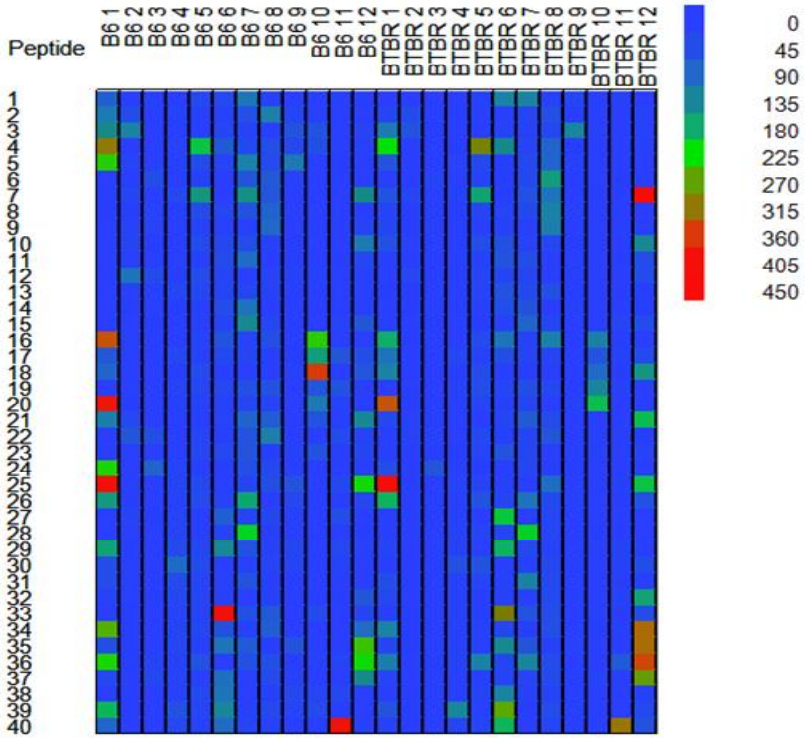

Fig. 7. Heat map of GCSPCF analysis of BTBR and B6 serum IgG. The SPR microchip was spotted with hundreds of peptides and sera (1:7 v/v dilution) from mice was flowed over the microchip for 30 min. ROIs were analyzed for binding of IgG by flowing AlexFluor647-goat anti-mouse IgG (H+L) (2 µg/mL) over the microchip for 30 min. Each peptide was designated a different number, and each microchip was assayed with a serum pool of BTBR (n=6) and B6 (n=6) sera. The means of the sera were used to calculate the fluorescence values for each peptide. Each B6 or BTBR column (1-12) hadthe same set of 40 peptides, thus heat map shows IgG binding to 480 different peptides

Table 2. Peptides bound preferentially by IgG from ASD participants

Petide #	Peptide sequence	Total MFI value	ASD MFI value	Control MFI value
2	RVIEVVQGACRAIRHIPRR	80	80	0
72	MRKMKLGLVK	81	81	0
91	FIVESNSSSSTRSAVDMAC	55	53	2
110	RNASVLKSSKNAKRYLRCNLKA	86	84	2
119	VERVGCAGLSMVECDM	60	60	0
127	GKKVITAFAEGLK	88	88	0
151	LENLKKLRARSTYNLKKLPTLEKLVALMEASLTY	90	78	12
163	D-Phe PRGPRGENGDFEEIPEEYL	71	71	0
177	SDIAKSAN	53	53	0
205	SRSESSIENFLCRSA	91	91	0
210	HQEMSTATNSDVPVQ	154	154	0
249	MLATRVFSLIGRR	89	85	4
296	CGIKVAV	60	60	0
361	VTKAWSICTA	68	59	9

Table 3. Peptides binding greater amounts of IgG from BTBR mice compared to B6 mice

Peptide #	Peptide Sequence	MFI difference value	BTBR MFI value	B6 MFI value
522	CYFDDSSKVICKKYRS	270	420	150
554	NTVGGHQAAMQMLKE	238	332	94
285	SYSMEHFRWGKPVLYSFKPL (D-Ala)R	134	266	132
269	CYFDDSSNVICKKYRGSGC-(C-dimer)	122	204	82
562	RRQISELHPICNKSILRQEVDYMTQARGQR SS	110	319	209
551	QMVHQAISPRTLNAW	98	174	76
286	YSFKPMPL(D-Ala)R	96	190	94
534	GHCASLSRSTAFLRWKDYNCNV (cyclized)	90	162	72
344	ACNTATCVTHRLAGLLSRSGGVVKSNFVP TNVGSKAF-NH2	90	168	78
239	4x(LNKEKTYLRDQHFLEQHPLL)-3K-BA-MAP4	88	124	36
334	CYFNGKEQIICGKIPS	72	127	55
444	VKCFNCGKEGHIARNCRA	72	164	92
180	4x(CSGDEILTMELMIMKALKWR)-3K-BA-MAP 4	70	134	64
357	CHHRICHCSNRVFLC	69	122	53
243	GTLSVFGMQARYSLRDEFPLLTTKRV	69	147	78
385	LKGTNDSLMRQMREL-MAPS 4	64	130	66
7	MGDHDVALCHVSRYNC	62	62	0
231	4x[EIDRDTKKCAFRTHTG]-3K-BA- MAP-4	59	120	61
10	RRQISELHPICNKSILRQEVDYMTQARGQR SS-QYIKANSKFIGITEL-MAP 4	58	58	0
506	KPDAFNPCEDIMGYNILR	57	78	21
537	QRWTTASSSVIRC	57	196	139
567	FQDAYNAAGGHNAVF	56	64	8
329	4x(MPRERRERDAKERDT)-3K-BA-MAPS4	56	122	66
271	MFRDLKVQCGWVELENRLTK	50	187	137

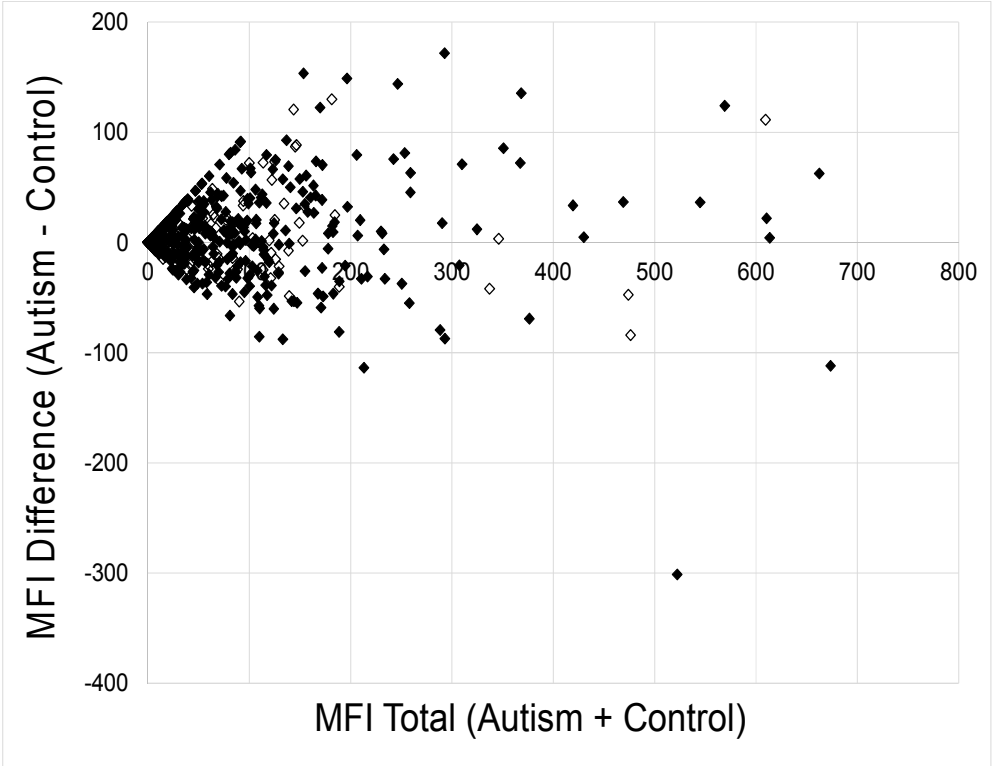

Fig. 8. GCSPCF analysis of peptide specificities of IgG in sera from healthy control and ASD participants. Microchips were spotted with hundreds of peptides and ROIs were analyzed for binding of IgG after serum was flowed over the microchip for 30 min. Analysis was performed by flowing biotinylated goat anti-human IgG (2 µg/mL) for 30 min followed by flowing AlexFluor647-Streptavidin (400 ng/mL) for 45 min. Each peptide was designated a different number on each microchip and assayed with healthy control (n=3) and ASD (n=3) sera. The means from the ASD and Control sera were used to calculate the difference between their fluorescence values for each peptide (Y axis) and their additive values for each peptide (X axis). The unfilled diamonds represent the average MFI values with peptides of the same sequence synthesized at different times

The control and ASD sera also were assayed with antibodies to stress proteins that were spotted directly on the microchips. We found NMDA receptor 2B (NR2B), Synaptosomal-associated protein 25 (SNAP-25), Zinedin (Striatin 4), transient receptor potential channel 5 (TrpC5), postsynaptic density protein 95 (PSD-95) and CA_V1.3immune complexes to be expressed at a higher MFI with sera from ASD subjects compared to that with sera from healthy control subjects (Fig. 9). Interestingly, these analytes were quantified with biotin-goat anti-hIgG and AlexaFluor647-streptavidin, which indicates that the captured analytes were in association with IgG antibodies.

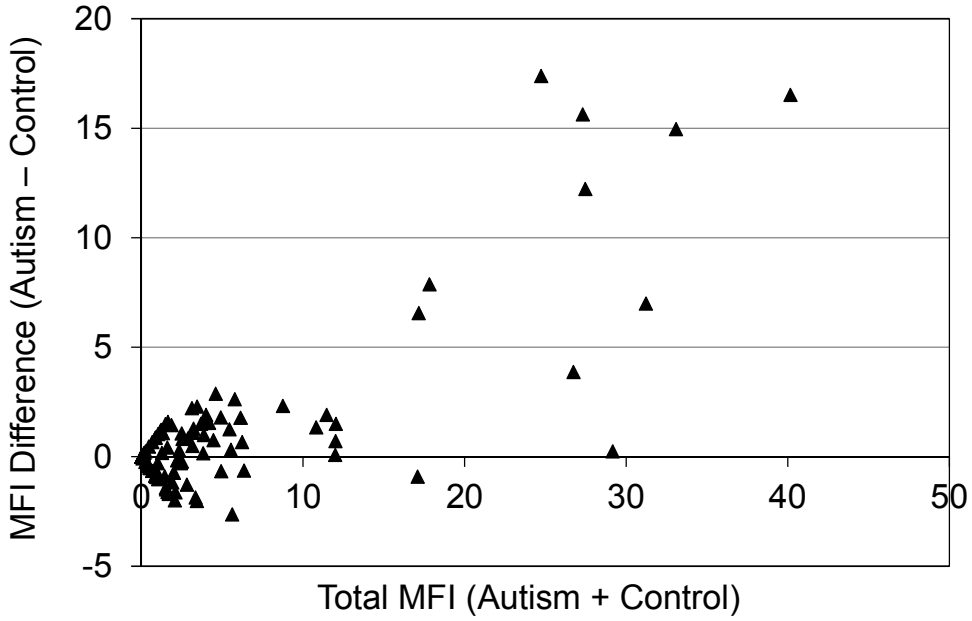

Fig. 9. GCSPCF analysis of stress proteins in the sera of healthy control and ASD participants. Microchips were spotted with hundreds (101 per microchip plus controls) of stress protein antibodies, and ROIs were analyzed for binding of proteins with biotinylated goat anti-human IgG (2 µg/mL) whole molecule after a serum sample was flowed over the microchip for 30 min. The microchip was developed by flowing AlexFluor647-Streptavidin (400 ng/mL) for 45 min. Each capture antibody was designated a different number on each microchip and assayed with ASD (n=3) and control (n=3) sera. The means from the ASD and control sera were used to calculate the difference between their fluorescence values for each stress protein-antibody complex (Y axis) and their additive values (X axis)

4. DISCUSSION

The results obtained for this study are from a relatively broad GCSPCF microarray in which various experiments involving peptides, Ig isotypes and stress proteins were performed using human and/or mouse sera. As previously described [28,29], the dual-mode GCSPR technology is unique in that it is capable of detecting real-time binding kinetics in GCSPR mode and can be switched over to GCSPCF (fluorescence) mode to increase sensitivity. The detection range in GCSPR mode is ng-mg/ml; however, sensitivity increases to pg-ng/ml in the GCSPCF mode. Thus, low concentrations of antibodies and/or analytes can be detected.

Proteins and antibodies were spotted directly on the gold (Au) coated microchip without a cross-linker. Proteins and antibodies adsorb onto Au surfaces through nonspecific electrostatic and hydrophobic interactions [30]; thus, a cross-linker was not necessary. Peptides were immobilized on the microchip with use of the cross-linker, dithiobis (succinimidyl propionate) or DSP [31], which is an amine reactive cross-linker that forms a stable bond between the gold surface of the microchip and the peptide. Cross-linking peptides with DSP, which has a spacer arm of 12 Å, is an ideal technique for maintaining the

peptides biological activity, site specificity, and linkage stability [32]. However, depending on the peptide length and the amino acid composition, the utilization of this method might lead to peptide conformational changes from multiple conjugations at different sites of the peptide, but the changes don't necessarily result in the inactivation or deactivation of the peptide [33]. In our studies, peptides consisting of 15 amino acids or less were considered to be linear epitopes, and if conformational changes did occur, they reflect different binding regions that form a composite epitope [34], which can be recognized by antibodies. As was demonstrated with peptide # 227 (EMISQAVHAAHAEA) and peptide # 234 (HQEMISQAVHAAHA), which bound equivalent amounts of IgG, the epitope within a sequence seems to remain intact; however, new epitopes definitely could be created dependent on the coupling to the microchip. Even intact proteins may take on a new epitope or lose an epitope when adhered to the gold surface. Thus, once the antibody specificity profiles are defined for a particular disorder, the specificities of the biomarker signature would need to be assayed by Western blot analysis, immunoprecipitation, or immunohistochemistry to delineate the appropriate autoantigens. However, the peptide screening will identify the number of specificities that need to be further considered for protein evaluations and the relevant epitope would hypothetically already be known.

Our current data suggests a significant variation between different individuals. These different binding patterns could reflect different genetic and environmental influences on the production of the antibodies to self-epitopes or environmentally altered self-epitopes. Individuals with autoimmune thyroiditis as well as healthy controls have antibodies to epitopes of thyroglobulin, but the amounts and epitope specificities of the antibodies are greater in those with thyroiditis [35,36]. It has also been reported that greater iodine ingestion increases anti-thyroglobulin levels and specificities [37,38]. It is not surprising that sera from healthy humans and mice have antibodies to as many different peptides as those from humans and mice with a disorder such as Parkinson's disease [39] or lupus [40], which is why a differential profile (disorder-related antibody signature) needs to be defined.

Since the initial evaluation of the GCSPCF technology included sera from humans and mice with central nervous system (CNS) disorders, neuroendocrine differences also might affect antibody amounts and specificities [41]. Serum antibodies from an ASD subject has been reported to possess higher binding reactivity to cerebellum antigens with use of rat brain sections [42]. The prevalence of autoimmune diseases is known to be greater in families with an ASD person [43] and autoantibodies are known to be present in the sera of mothers of autistic children and the ASD persons, themselves [44]. It also is well known that people with ASD suffer from multiple abnormalities including the gastrointestinal (GI) tract [45], food allergies [46] and immune system [47]. Additionally, a previous study reported that serum antibodies from children with ASD showed reactivity to proteins such as myelin basic protein (MBP), glial acidic fibrillary protein (GFAP), and brain derived neurotropic factor [48]. Thus, our results could reflect multiple factors. It is also possible that the differences amongst the ASD participants is due to antibodies to the same proteins but different epitopes of the proteins which could affect the degree to which the antibodies affect the biological activity of the protein.

The BTBR mice are unique in that their immune response differs from B6 mice as previously reported [49]. Furthermore, there are multiple differences between BTBR mice compared to B6 mice, including metabolism [50], increased corticosterone (stress hormone) levels [51], increased pro-inflammatory cytokines levels [52], and higher serum IgG and IgE as well as IgG anti-brain antibodies [52]. Thus, the physiological and neurological features of BTBR mice could potentially be used to develop reliable biomarkers for ASD.

In the evaluation of the human (control/ASD) and mouse (B6/BTBR) serum antibodies, we found five peptides (CVAGSLPRRPLPPA, RRQISELHPICNKSILRQEVDYMTQARGQRSS-QYIKANSKFIGITEL-MAPS4, LSEDKLLACGEGAADIIIGHLCIRHEMTPVNPGV, VITSHQETSTATNSD and DPAGSGING) that were bound by serum antibodies from only BTBR and ASD sera. However, the initial results suggest more of a difference than similarity since a high variability was observed between human ASD subjects. Thus, a larger number of human ASD serum samples are necessary to make a possible biological relationship between ASD subjects and the BTBR mouse model, which might be a preclinical animal model for ASD. Our results do suggest BTBR and ASD serum antibodies appear to have a low and/or high affinity towards these five peptides; however, no distinction was made between male and female mice and a pooled mouse sera sample was used.

We also found NR2B, SNAP-25 (SP-12), Zinedin (Striatin 4), TrpC5, PSD-95 and CA_V1.3proteins to have a higher expression in serums from ASD subjects compared to healthy control subjects. Proteins such as SNAP-25 and Zinedin (or Striatin 4) are involved in neuron signaling pathways whereas Ca_v1.3 and TrpC5 play an important role in calcium, Ca^{2+}, channel signaling [53,54,55,56]. PSD-95 is most notably known for its vital role in neuronal synaptic plasticity [57], while NR2B is involved in neurotransmission of glutamate [58].Even though various research associated with autism has been done in areas involving misfolded proteins [59] and oxidative stress [22], these proteins could potentially be related to the etiology of ASD. For example, elevated levels of SNAP-25 have been found in patients with bipolar disorder and schizophrenia [56], whereas TrpC5 has been implicated in neurological behavior [60]. Additionally, NR2B was found to be over expressed in a rat model following exposure to valproic acid (VPA), which is a teratogen causing core behavioral changes [61]. These proteins could play multiple roles and trigger downstream cascades that control survival signaling or lead to neurodegeneration.

The use of GCSPCF analysis of epitope recognition to assess allergies in human subjects also could become an important new screening assay. The GCSPCF peptide microarray was used to look at IgE, IgG4 and IgG1 specificities with encouraging results; however, further studies are needed to optimize and validate this aspect of microarray isotype screening. Assessment of antibody isotypes to peptides may aid differential evaluation of disorders in addition to allergies. For example, IgG 4 has been associated with sclerosing disease and other diseases [62]. Ig isotypes have different biological activities, including their differential binding to Fcgamma receptors (FcγR), activation of complement activities, and translocation through the placenta [63,64]. The biological complexities of the immunoglobulin isotypes have been evaluated as influences affecting different disorders [65]. Since the GCSPCF technology requires only small volumes and Ig isotypes have been quantified from punches of newborn dried blood spots [66], the assay might be able to provide early analysis of babies for improved early interventions.

5. CONCLUSION

The GCSPCF peptide microarray presented above provides a versatile and inexpensive immunoassay for screening sera with synthetic peptides and various antibodies. The SPR instrument is both flexible and highly automated. Our results suggest a small number of circulating antibodies from sera were affinity captured by peptides having the specific epitope, which makes capturing epitope-specific antibodies from sera possible. Thus, the unique dataset created from a large number of peptides, allows the creation of a peptide biomarker profile in which peptides can be further developed to predict antibody epitopes or

target specific proteins. Even though our interests were focused on screening of circulating antibodies specific to peptides, screening for other analytes could have been performed simultaneously on the same microchip. In addition, considering the GCSPCF technology is capable of detecting antibody concentrations in the range of pg to ng/mL, this technology is highly important in detecting relatively low concentrations of antibodies with small amounts of serum, which has clinical significance. Our goal is to utilize this technology to create epitope-specific signatures for autoantigens associated with autoimmune diseases including autism, rheumatoid arthritis, type-1 diabetes and systemic lupus erythematosus using serum from human subjects, which is the current focus of our research.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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