Please Note:
The research described below represents a small portion of an on-going technical development project. Any and/or all of the methods used for this project may or may not be implemented in the final NIDA Neuroproteomics Center microarray protocols.

TESTING AND ESTABILISHING OPTIMAL PROTEIN ARRAY CONDITIONS TO BE APPLIED TOWARDS THE DARPP-32 PHOSPHOPROTEIN MODEL SYSTEM

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ABSTRACT

The ability to quantitate multiple proteins simultaneously has a wide array of applications including basic biological research, identification of therapeutic markers and targets, molecular classification and disease diagnosis. The challenge for microarray fabrication becomes one of choosing and optimizing conditions for printing and using the arrays that are robust enough to be useful for many diverse proteins. The major parameters that will be addressed include surface chemistry for printing; spotting method; protein concentration for printing; blocking and conjugation method; and signal generating molecule and labeling. The data obtained will be used to formulate standard operating protocols for the DARPP-32 array.

Abbreviations:

SE: SuperEpoxy; PLL: Poly-L-lysine; DARPP-32: dopamine and cAMP-regulated phosphoprotein, M_r = 32,000; GST: glutathione s-transferase; IgG: Immunoglobulin G; Ab: Antibody; SMP: Stealth Microspotting Pin; BSA: Bovine serum albumin

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INTRODUCTION

Classical approaches to biological research have involved the study of one or a few genes at a time. Gene expression analyses at the mRNA level were completed using techniques such as Northern blotting, differential display, serial analysis of gene expression (SAGE), and dotblot analysis (van Hal et al, 2000). For the past decade, emphasis has been placed on generating DNA sequence information as a key to solve various biological problems and to gain a better understanding of the biological system.

The abundance of biological data is due to the rapid technology development for the analysis of biological systems. The development of high-throughput instruments for automated DNA sequencing have accelerated the pace of gene discovery and led to the completion of the human genome and the genomes of other organisms (Zhu, 2001; Shaw et al, 2003). The exponential increase in DNA sequence data requires the development of new technologies and methods to use this vast amount of information to answer the complex biological questions that lie ahead. The challenge of understanding the function of each gene in the genome has led to the development of high-throughput experimental techniques referred to as functional genomics. Functional genomic studies include systematic disruption of predicted genes, mRNA expression profiling based on DNA microarray technology, protein expression profiling using two-dimensional electrophoresis (2D-GE) and mass spectrometry and large scale mapping of protein-protein interactions (Palzkill, 2002).

The integration and advancement of prior gene expression analysis technology, along with DNA sequencing technology, has evolved into microarray technology. DNA microarray technology allows simultaneous assays of thousands of genomic features in a single experiment, based on the biochemical process of hybridization reactions between single-stranded fluorescent molecules and single-stranded sequences immobilized on a platform, e.g. glass microscope slide (Schena et al, 1999).

DNA microarrays have been used to genotype viruses, identify single nucleotide polymorphisms, identify targets of transcription factors and monitor gene expression in a variety of biological samples (Sreekumar, 2001). They allow for high throughput analysis of nucleic acids at their transcriptional level. At the mRNA level of gene expression, DNA microarrays enable semi-quantitative assessment of changes in the expression levels of all the genes in the genome with a single experiment (Shaw et al, 2003). The gene expression data provides only information obtained on steady-state mRNA levels. The mRNA levels do not always correlate with protein levels due to the variable rates in protein translation, modification and degradation (Zhu, 2001). They cannot provide insight into the function of individual genes since their transcriptional activity does not reflect the production or activity of the translated protein (Gygi et al, 1999).

It is estimated that the human genome has around 30,000 genes, but there may be as many as 1 million protein-based structures in the human proteome. The proteome is more complex than the genome because many genes encode multiple variants of a protein; many proteins are post-transitionally modified in a combination of different ways; the proteome is dynamic, with the proteomics phenotype evolving during onset or progression of disease (Mann et al, 2004).

For the past few years, more attention has been given to proteomics, the study of proteins and their activities expressed by a given genome. Protein properties such as expression levels, post-translational modifications, such as phosphorylation and glycosylation, and interactions with other molecules are necessary to study to obtain a global view of cellular processes at the protein level. It is necessary to study proteins rather than just genes because protein levels and their activities can differ significantly from mRNA levels; many functions of genes can only be studied using biochemical techniques; and much regulation of gene activity occurs at the protein level, including protein stability, modification, and localization (Snyder et al, 2002; Sreekumar, 2001).

Microarrays have been used to generate expression profiles of cDNA populations. Groups of genes within known pathways that are differentially regulated in disease have been published, but there is a high degree of false positives within the results. The false positives are due to repetitive DNA sequences and cross-hybridization. The large number of false positive results conceals the genes that are differentially expressed. It is not possible to confirm all genes using techniques such as real time PCR due to time and cost restraints. A substantial number of cDNAs spotted on a microarray come from expressed sequence tags rather than characterized genes, hindering functional analysis. The correlation between DNA and protein levels is 25 to 50%; few genes can be shown to be up or down regulated at the protein level. An alternate and more direct approach is to study protein levels directly (Shoemaker and Linsley, 2002).

The conventional methods for protein analysis including mass spectrometry, crystallography, gel electrophoresis, column chromatography generate intricate details of a single protein but these approaches also require expensive equipment, are time consuming and difficult to relate to the whole proteome (Sreekumar, 2001). As the

complexity of an organism increases, the extent of proteomics coverage decreases dramatically from the 73% observed and the 32% characterized in *Mycoplasma genitalium* (Wasinger et al, 2000). Sample processing time increases at the expense of throughput, peptide coverage and analytical reproducibility as protein abundance decreases. The majority of proteins, including ones with 'housekeeping' functions occur at very low intracellular abundance. 90% of the protein content in body fluids such as serum contains albumin, transferrin, haptoglobulin, and immunoglobulin. The low abundance proteins are beyond the resolution and analytical capacity of traditional proteomics (Washburn et al, 2001).

The growing importance of molecular studies that characterize physiology, development, and disease has brought on the need for technology that will allow for high throughput quantitation of specific proteins (Sreekumar, 2001). The ability to quantitate multiple proteins simultaneously has a wide array of applications including basic biological research, molecular classification and disease diagnosis. Currently available assays that meet the need for high throughput analyses such as microtiter plates and two-dimensional gels, but sample consumption can be problematic in large-scale studies. Protein array technology resolves the latter drawbacks and allows for measurement of thousands of proteins simultaneously using small amounts of sample input (Haab, 2001). Protein array studies hold the promise of adding a further dimension to the knowledge base of cellular and molecular biologists by providing a better, more conclusive insight on gene expression because they convey the true cellular manifestation of gene activity (Wildt et al, 2000).

Method	Application	Features
2D-GE/MS and LC/CE/MS	Protein abundance/abundance/identification/ posttranslational modifications	Very sensitive
Protein Localization	Subcellular localization of proteins	Defines subcellular compartment Data helps increase confidence of protein interaction data Proposes function solely from localization
Two-Hybrid	Protein-protein interactions	Mostly binary interactions Able to detect transient interactions Very automatable High false-positives/false-negatives
MS Identification of Copurifying Proteins	Protein-protein interactions	Able to detect components in small-to-large protein complexes Proteins are in their native environment Most interactions not binary High false-positives
Solution Screening Biochemical Activities	Demonstrate biochemical activities for proteins of known/unknown function	Ability to vary assay conditions Bias for well expressed proteins
Protein Microarrays	protein-protein interactions protein-RNA interactions protein-DNA interactions protein-sugar interactions protein-lipid interactions protein-small-molecule interactions interaction profiling posttranslational modifications enzymatic assays	Very versatile for detecting molecular interactions, including, but not limited to, protein-protein interactions Able to control experimental parameters Very high-throughput Ability to prove binary interactions Proper folding/function for large numbers of immobilized proteins unknown

Table 1. Proteome Investigative Methods
(From: Snyder et al, 2002)

The different protein interactions that can be generated on a microarray platform range from antibody/antigen, enzyme/substrate, ligand/target, protein/nucleic acid to name a few. Antibody arrays are well suited for profiling protein expression and the antigen-antibody interactions are highly specific. Antibody arrays can be used to analyze the expression of one or more proteins in a mixture such as cell extract without the need to work on the individual components to separate, analyze the differences in patterns and then to identify each protein. Antibody arrays have the ability to profile protein expression concentrations and also posttranslational modifications (Wildt, 2000).

Protein-detecting microarrays employ current immunoassay techniques such as sandwich, antigen capture and direct immunoassays. In sandwich assays, antibodies are captured on the glass substrate and detected using secondary labeled antibodies. The sandwich assays are analogous to ELISA assays. Sandwich assays are highly specific and allow for minimal cross reactivity. One disadvantage with this method is the need to use two non-competing affinity reagents for each protein.

Antigen capture is an alternative approach to sandwich assays without using a secondary antibody. Either a label-free detection method, such as mass spectrometry or surface plasma resonance, or chemical labeling of proteins in the sample is used. The two-color approach is not as sensitive since signal amplification is unattainable beyond that provided by the fluorophores. The two-color approach, does however, allow for a more accurate quantification of the relative abundance of the proteins. Antigen capture is limited by the specificity of the affinity reagents. Labeling proteins creates problems because some proteins may label on their antigenic epitopes, losing their ability to be captured by the affinity reagents.

In direct immunoassays, the samples are immobilized onto the glass substrate and detected and quantified by probing with labeled antibodies. Direct immunoassays permit signal amplification, however, proteins that are in low abundance must compete with more abundant proteins for immobilization on the glass substrates.

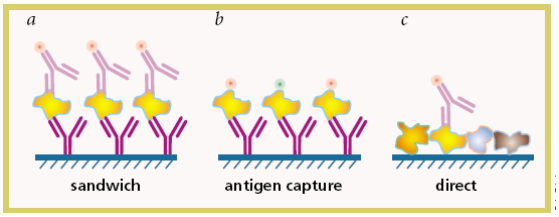


Figure 1. Strategies used for protein detection using microarrays
(From: MacBeath, 2002)

Many of the approaches used in generating protein microarray technology have replicated DNA microarray technology in many ways. However, there are some fundamental differences between proteins and DNA that are reflected in the complexity of the

challenges faced in attempting to produce robustly functioning protein arrays (Sreekumar, 2001).

The shift from conventional methods towards high throughput methods such as microarrays demands focusing on larger sets of proteins instead of one protein at a time. Proteins are very diverse in nature. Nucleic acids are composed of four nucleotide bases and have a hydrophilic, negatively charged backbone. In contrast, the components of proteins are 20 amino acids that can be hydrophilic or hydrophobic, acidic or basic in nature. Proteins, therefore, have a broader diversity than nucleic acids in their solubilities, tertiary structures and active sites. These are precisely the properties that give each protein its individual functionality (Mann et al, 2004) and that create the challenges when making protein microarrays.

Proteins are physically and chemically heterogeneous; they have a three-dimensional structure that is critical to their function. No amplification technique, analogous to PCR for nucleic acids, is available to boost cellular levels of low abundance protein when compared to DNA techniques; instead analyte enrichment and/or signal amplification strategies must be implemented. Protein-protein interactions and conjugations depend on the folded three dimensional protein structures, unlike hybridization of DNA microarrays, which involve interactions between linear DNA or RNA sequences (Sreekumar, 2001). Many proteins are also known to lose activity when they are bound to a solid surface and many absorb nonspecifically to each other, to substrate materials, and to other proteins (Metzger, 2002). Because of their level of heterogeneity, the challenge for microarray fabrication becomes one of choosing and optimizing conditions for printing and using the arrays that are robust enough to be useful for many diverse proteins. The major parameters that must be addressed include, but are not limited to:

1. Surface chemistry for printing
2. Spotting method
3. Protein concentration for printing
4. Blocking method
5. Conjugation method
6. Signal generating molecule and labeling
7. Slide storage and shelf-life

We will attempt to begin to address issues 1-6 as they pertain to developing and optimizing techniques for printing a neuroproteomic-signaling array using DARP32 as a model protein.

Surface Chemistry

In the production of protein microarrays, the selection of the support material is a vital part of the initial optimization process. Protein arrays were initially produced on nitrocellulose and PVDF (polyvinylidene fluoride) membranes or on glass slides designed for DNA arrays. A gradual introduction of alternate surfaces have been characterized and studied (Agenendt et al, 2002). The ideal support material should robustly attach all the chosen proteins in an appropriate orientation without altering their

conformation. Since proteins have a variety of polarities, hydrophobicities, charges, sizes and structures, it is a challenge to attach the different types of proteins while repelling non-specific binding. It is difficult to achieve both low nonspecific binding and high protein attachment.

The main protein attachment strategies include adsorption, affinity binding, and covalent binding. Adsorption allows for attachment via electrostatic or hydrophobic forces. Substrates including PVDF, nitrocellulose, polystyrene, or poly-L-lysine adsorb well. However, non-specific binding is difficult to maintain since the surface binds all proteins. The use of various blocking agents, such as milk proteins, to saturate the free binding sites on the substrates is an option in reducing non-specific binding (Agenendt, 2002). Not all proteins are able to bind to the substrate via adsorptive attachment, depending on the protein's chemistry. A weakly bound protein may not stay attached during washing of the slide surface if stringent washes with high salt or high detergent concentrations are used.

Affinity binding uses specific biological interactions such as biotin and avidin or between protein A and IgG to increase protein attachment. An example is of a polyethylene glycol (PEG)-coated surface that is functionalized with biotin and streptavidin. Spotted antibodies containing biotin are attached to the surface and nonspecific background proteins are repelled by the PEG (Ruiz-Taylor et al, 2001). The surface and the protein to be attached may need to be altered with the components of the interaction. The proteins do have to be modified prior to spotting, which has the potential of altering the protein structure.

Covalent binding allows for strong attachment of proteins to surfaces. It allows for high-stringent binding conditions and washes away weakly bound proteins. The reaction of amine groups on proteins with the immobilized functional groups such as aldehydes or succinimides is the most common method (Haab, 2003).

Others have used several types of printing substrates in the design of protein arrays including glass slides, porous gel pad slides and microwells. Glass slides have an advantage over the other platforms since they can be used with standard microarrayers and scanners used for DNA microarrays (Zhu, 2001). Glass slides also offer higher density arrays and lower sample volumes. The two major classes of microarray slides include gel-coated surfaces such as polyacrylamide or agarose and non-gel-coated modified glass slides such as aldehyde, poly-L-lysine or nickel-coated slides. Non-gel coated modified glass slides are chemically modified to bind proteins and antibodies via electrostatic and covalent interactions. The slides of interest in this study are outlined below.

<u>SuperEpoxy Slides (TeleChem)</u>
The slides are derivatized with epoxysilane. Proteins are covalently attached through an epoxide ring-opening reaction primarily via their surface amino, hydroxyl and thiol groups, giving higher binding affinity than amine-coated slides.

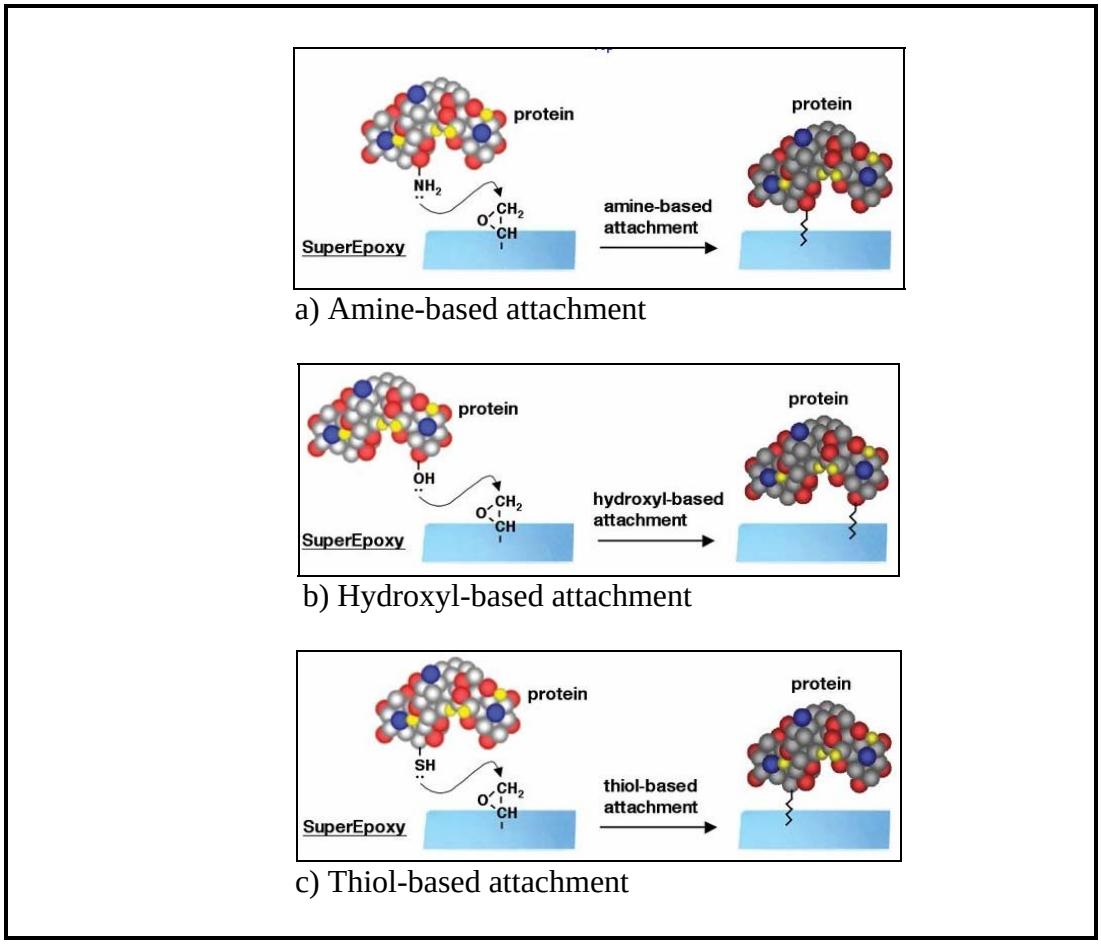


Figure 2. SuperEpoxy surface chemistry for attachment
(From: TeleChem Inc, www.arrayit.com)

Hydrogel Slides (Perkin Elmer)

Hydrogel slides have a porous layer of gel matrix 20-30µm in thickness onto which the proteins are immobilized. The hydrophilic environment created by the matrix aids in the reduction of evaporation and minimizes protein denaturation. The slides have demonstrated lower detection limits but with greater intra- and interfiled variation (Agenendt et al, 2002).

Fast Slides (Schleicher & Schuell)

The slides are coated with 15µm layer of nitrocellulose. The slides allow for higher sample loading and are able to retain more protein during slide processing. They require at least 8 hours of incubation for proteins to immobilize in the matrix. The nitrocellulose matrix does contribute to higher intrinsic background level due to light scattering.

Poly-L-Lysine Slides (coated using Patrick Brown's protocol)
Proteins are bound to the positively charged slide through electrostatic interactions to form salt bridges. The slides are easy to prepare manually, with good signal uniformity and reproducibility.

Spotting Methods
An arraying robot is essential in the production of protein microarrays. The arrayer has three basic components: a source plate containing the samples to be arrayed; the pins used for spotting the samples; and the substrates to contain the samples. It is responsible for the fabrication of quality arrays with good spot morphology. There are two distinct types of arraying robots, ones that use pin or contact printing and ones that use Piezo-electric non-contact printing. There are contradicting reports on which method is better suited for printing proteins. It has been argued that standard capillary-based spotters (contact printing method) are unsuitable due to the fragile nature of proteins. Also, that contact printing is destructive to the slide surface (Nock et al., 2003). Non-contact printing is faster at multi-dispensing a single sample, but slower at changing between samples as compared to contact printing. The available Piezo instruments have a tendency to smear spots or produce uneven intensity across the spot. Contact printing pins can either be solid tip pins or split pins. The solid pins need to be dipped into the sample between each spotting, whereas the split pins do not need to be re-loaded with sample as frequently. The solid pins may have an advantage over the split pins because they are cheaper, more robust, and not prone to clogging. The split pins hold a greater volume of sample by capillary action and dispense a defined sample volume onto the slide with each contact, resulting in less sample usage and faster printing of samples (Mann et al., 2004). Split pins provided by TeleChem are preferred for the fabrication of protein arrays. TeleChem's stealth microspotting pins have flat tips and defined uptake channels. A 25µm layer of sample is formed at the end of the pin and printing is performed by light contact with the slide.

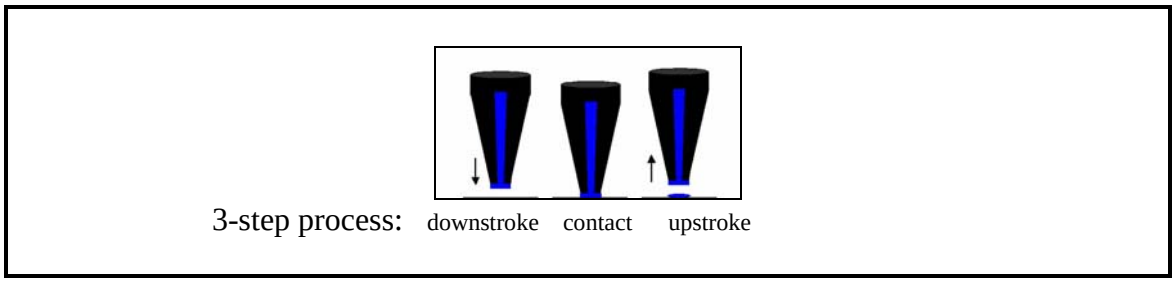


Figure 3. Printing mechanism of Stealth Microspotting Pins
(From: TeleChem - www.arrayit.com)

The pins are available in a range of sizes from 62.5µm to 600µm in diameter. SMP3, SMP4, and SMP9 pins each uptake the same amount of sample volume, but the amount delivered on the glass substrates varies greatly and therefore the spot diameter produced by each pin is distinct. SMP3 creates the smallest spot diameter in the group of 100µm and SMP9 creates the largest spot diameter of 300µm. Each pin has its advantages and disadvantages. Although a greater spot diameter is generated with a SMP9 pin, the array density is low. The maximum number of spots that can fit on a 18mm x 72mm slide with

64 pins is only 9,216 spots compared to 82,944 spots from SMP3 pins. Even though TeleChem provides the number of spots each pin can print with a single sample uptake, it is necessary to test this with each different sample due to heterogeneity between buffers used.

Pin Type	Spot diameter (µm)	Uptake volume (µl)	Delivery volume (nl)	Number of spots per loading	Minimum spot spacing (µm)	Maximum subgrid per pin (spots)	Density (spots/ cm^2) per 4 pins	Maximum number of spots (18 mm x 72 mm) per 64 pins
SMP3	100	0.25	0.7	200	120	36 x 36	5,184	82,944
SMP4	135	0.25	1.1	185	160	28 x 28	3,136	50,176
SMP9	300	0.25	3.3	110	360	12 x 12	576	9,216

Table 2. Manufacturer's Comparison of the three TeleChem pin types
General physical parameters of spots created with SMP3, 4 and 9 pins with regard to spot diameter, delivery volume, number of spots printed per one sample loading and number of spots per standard subarray.
(From: TeleChem - www.arrayit.com)

Arraying environment

Arraying conditions should be optimized to achieve a non-denaturing environment, minimize non-specific interactions, and to provide good spot morphology, homogeneity and spot-to-spot reproducibility. The Microarray Resource print rooms are 100 class cleanrooms at 72ºF, 55% relative humidity.

A printing buffer of low or physiological salt content at neutral pH is preferred. The addition of a mild detergent at low concentration, such as 0.01% (v/v) Tween 20 (polyoxyethylenesorbitan monolaurate) will aid in the reduction of non-specific interactions and also improve spot morphology. The detergent has the capability of decreasing surface tension between the buffer and the slide surface. Higher concentrations of detergent may cause the spots to spread on the two-dimensional surface (Mann et al., 2004).

DARPP-32 Background

DARPP-32, dopamine and cAMP-regulated phosphoprotein, M_r = 32,000 (as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis), is a cytosolic protein that is expressed in very high concentrations (~50µM) in neostratial medium spiny neurons (Ouimet et al, 1984; Walaas and Greenhard, 1984). DARPP-32, when phosphorylated by cAMP-dependent protein kinase (PKA) on a single threonine residue, Thr34, converts into a potent inhibitor of protein phosphatase-1 (PP-1) (Hemmings et al, 1984). Dopamine and numerous other neurotransmitters have been shown to regulate the phosphorylation/dephosphorylation of DARPP-32 at Thr34 in neostratium, thereby altering the activity of PP-1 and regulating the phosphorylation state and activity of many downstream physiological effectors including various neurotransmitter receptors and voltage-gated ion channels (Greenhard et al, 1999).

In addition to phosphorylation at Thr34, DARPP-32 is phosphorylated at Thr75 by cdk5, a neuronal member of the family of cyclin dependent protein kinases (Bibb et al, 1999). Phosphorylation of Thr75 converts DARPP-32 into an inhibitor of PKA. Under conditions of reduction of phosphor-Thr75 by a cdk5 inhibitor or where the cdk5 activator, p35, is deleted, the results is increased phosphorylation of PKA substrates. This has revealed a role for regulation of cdk5 in the actions of psychostimulants (Bibb et al, 2000). Cdk5 levels and the phosphorylation of Thr75 in DARPP-32 are upregulated following chronic treatment with cocaine, in turn diminishing the effectiveness of dopamine/cyclic AMP/ protein kinase A/ DARPP-32/ protein phosphatase-1 signaling (Robinson and Kolb, 1999).

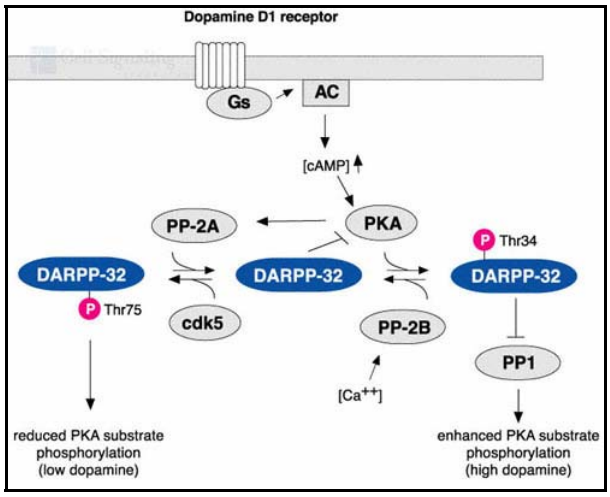


Figure 4. DARPP-32 signaling pathway
(From: Cell Signaling Technologies, http://www.cellsignal.com/)

It will be a great benefit to studies of drugs of abuse to routinely analyze the phosphorylation of DARPP-32 at Thr34 by PKA or at Thr75 by cdk5. Dr. Angus Nairn has spent years of research preparing highly specific phosphor-specific antibodies to various sites in DARPP-32. It is a constant challenge to be able to prepare antibodies that not only recognize the phosphor-form of a particular phosphorylation site, but that are also specific enough to prevent cross-reactivity to other proteins. In many cases this has proven difficult given the conservation in primary amino acid sequences in consensus phosphorylation sites for multi-functional kinases like PKA or cdk5. As an example for the latter, a mouse monoclonal antibody recognizing phosphor-Thr34 of DARPP-32 (mAB-23) also binds non-specifically to proteins present in brain regions where DARPP-32 is not highly expressed or even to brain tissues from a DARPP-32 knockout mouse (Bibb et al, 2000). MAB-23 antibody referred to above was prepared using ~10 amino acid peptides as antigens with the respective phosphor-Thr residue situated in the middle of the peptide. Dr. Nairn and colleagues believed that longer peptides would expose epitopes surrounding the amino acid side chains other than the phosphor-amino acid and reduce the probability of generating a predominant reaction to the phosphor-epitope. The

new antibodies prepared using ~25 amino acid peptides as antigens have demonstrated by Dr. Nairn's group as showing a high degree of specificity. These highly specific antibodies, along with peptide epitopes from DARPP-32 knock-out and transgenic tissues will be useful in not only establishing protocols for protein arrays, but serving as a foundation for obtaining working knowledge of proteins and their interactions on glass substrates.

Immunoblot analysis and immunocytochemical analysis has already been performed by Dr. Nairn's group using various total and phosphor-specific DARPP-32 antibodies. As a member of a core facility, whose focus is nucleic acid arrays, it is a challenging task to transition into protein arrays, due to proteins structural and functional complexity. Once we have demonstrated array printing and assay conditions the selected DARPP-32 antibodies that result in equivalence of array data to that obtained from the immunoblot and immunocytochemical analysis, this will be an extremely beneficial platform for immobilizing antibodies on glass substrates and testing numerous other DARPP-32 and neuroproteome signaling antibodies. Once all the major technical issues have been addressed, the focus can be turned to incorporating numerous antibodies onto an array. However, it is beneficial to initially focus on only a few antibodies in order to generate a good platform for fabricating protein arrays.

Commercially available protein arrays are expensive and are limited in their diversity. Advances in academia would benefit from a robust, cost effective platform, with versatility to display custom proteins for multiple systems and applications. The complexity of proteins and the challenges posed by protein array fabrication and usage are miniscule compared to the potential abundance of information obtainable through their usage.

It was originally planned to print DARPP-32 protein array to test and establish a set of protocols for printing and processing protein arrays in an academic core facility setting. Unfortunately, we were not able to print DARPP-32 because underlying technical issues needed to be addressed and more time had to be spent optimizing basic conditions.

MATERIALS AND METHODS

Unless otherwise noted, each slide is printed on an Omnigrid-100 (Genomic Solutions) with 1 SMP3, 2 SMP4 and 1 SMP9 TeleChem split pins. Both poly-L-lysine and SuperEpoxy slides were used for each print. All slides are spun dry in Corning 50 ml plastic screw-cap tubes at 1000 rpm for 5mins. The slides are scanned using an Axon 4000A laser scanner with 650 PMT setting at 532nm and 635nm wavelengths.

Spot morphology and Quality Control

Slide staining using a hand spotted BSA slide

To perform a quality control stain to visualize spots on the printed arrays prior to using a batch of arrays for conjugation, Sypro Red protein gel stain at 5000X concentration in DMSO (Molecular Probes, S-6654) was diluted 1:1000 and 1:500 in dH_2O. 1µg/ml of BSA was hand spotted onto a poly-L-lysine coated slide. The slide was incubated in the

dark with Sypro Red at room temperature for 15mins. The slide was washed three times in dH_2O for 5mins each. The slide was air dried and scanned.

Slide staining using a printed BSA slide
Test slides with four different BSA concentrations (1µg/ml, 0.75µg/ml, 0.50µg/ml, 0.25µg/ml) and 1X PBS were printed on poly-L-lysine coated glass slide using a GeneMachines Omnigrid (Genomics Solutions). Four TeleChem split pins, 1 SMP3, 2 SMP4, 1 SMP9, were used to print 3 replicate arrays on each slide with three replicate spots per sample. Sypro Red dilutions of 1:500, 1:250, 1:100, and 1:50 in dH_2O were tested on each array. The slide was incubated with Sypro Red for 15mins in the dark at room temperature. The slides were washed in dH_2O three times for 5mins. The slides were spun dry at 1000 rpm for 5mins and scanned as described above.

Carryover Test
The cleaning cycle between each sample uptake was optimized to ensure that each pin was washed thoroughly and did not contain residual sample. The cleaning cycle containing a loop of three long washes in dH_2O and three short dries and one short wash and a long dry, used originally with cDNA and oligonucleotides, was used as the starting point for testing pin cleaning for protein printing. Cyanine 3 labeled anti-mouse and Cyanine 5 labeled anti-rabbit (given by Dr. Pazhani Sundaram, Recombinant Technologies LLC), along with 1X PBS were printed to test for sample carryover. The labeled anti-rabbit and anti-mouse were diluted 1:50 in 1X PBS. In a 384 v-bottom print plate (Genetix), labeled anti-mouse and anti-rabbit were placed in the first index of the plate (A1-C2), followed by an index of 1X PBS (A3-C4), then a blank index (A5-C6), and then labeled anti-mouse and anti-rabbit in the last index (A7-C8). The sample sequence was printed twice on poly-L-lysine slide with three replicate spots per sample. The sample sequence on the poly-L-lysine slide was three spots of Cyanine labeled sample, three spots of 1X PBS, three blank spots, three spots of Cyanine labeled sample. The slide was scanned immediately afterward printing. The above method was repeated with changes to the cleaning cycle until no traces of Cyanine labeled anti-rabbit and mouse were detected where the blank spots were printed.

Pin comparison
Cyanine labeled anti-mouse and anti-rabbit antibodies (Ab) were used to estimate the optimum number of Ab spots that could be printed with each of the SMP3, SMP4 and SMP9 pins with a single uptake of sample. A 384 v-bottom plate with 5µl of the cyanine labeled sample was used for the test. The four pins were programmed to print 300 spots from a single sample uptake with 300µm center-to-center spot diameter. The slides were immediately scanned. The number of spots printed by each pin, the spot morphology, the signal intensity were noted and graphed.

Printing buffer trials

Antibody test print in 1X PBS

Antibody test slides containing: glutathione s-transferase (GST) protein (Recombinant Technologies LLC, Product no: 200101), anti-Mouse IgG (QED Bioscience Inc., Catalog no: 80000), anti-Rabbit IgG (QED Bioscience Inc., Catalog no: 81000), anti-GST (QED Bioscience Inc., Catalog no: 18803), bovine serum albumin, R911-polyclonal rabbit Ab (gift, Dr. Nairn), R500-polyclonal rabbit Ab (gift, Dr. Nairn), M6-mouse monoclonal Ab (gift, Dr. Nairn) were diluted in 1X PBS at 1µg/ml, 0.75µg/ml, 0.50µg/ml, and 0.25µg/ml.

Array Layout:

1ug/ml	**0.75ug/ml**	**0.50ug/ml**	**0.25ug/ml**
M6	M6	M6	M6
R500	R500	R500	R500
R911	R911	R911	R911
GST	GST	GST	GST
BSA	BSA	BSA	BSA
Anti-Mouse	Anti-Mouse	Anti-Mouse	Anti-Mouse
Anti-Rabbit	Anti-Rabbit	Anti-Rabbit	Anti-Rabbit
Anti-GST	Anti-GST	Anti-GST	Anti-GST
Empty	Empty	Empty	Empty
PBS	PBS	PBS	PBS

Slide Printed: AB-PLL 01-09; 19-27
AB-SE 10-18; 28-36

Three replicate spots were printed for each sample on three replicate arrays. Each array contained four 10x12 subarrays, printed with 1 SMP3, 2 SMP4, 1 SMP9 pins, respectively. Eighteen poly-L-lysine slides (made in house) and 18 SuperEpoxy slides (TeleChem) were printed. After every 6 slides, the pins were replenished with sample, as so-called re-dip, allowing for 58 spots per sample from a single sample uptake. After each sample uptake, the pins blotted four times on a blot pad to remove excess sample before printing on the glass slides.

Antibody test print in 1X PBS and 0.05%, 0.10%, 0.25% Triton-100X

Antibody test slides containing: mouse IgG (Sigma-Aldrich, product no: I8765) and rabbit IgG (Sigma-Aldrich, product no: I8140) were diluted in 1X PBS, 1X PBS/0.05% Triton, 1X PBS/0.10% Triton, 1X PBS/0.25% Triton at 1µg/ml, 0.75µg/ml, 0.50µg/ml, and 0.25µg/ml.

Array Layout:

1ug/ml	**0.75ug/ml**	**0.50ug/ml**	**0.25ug/ml**	
Mouse IgG	Mouse IgG	Mouse IgG	Mouse IgG	**1X PBS**
Mouse IgG	Mouse IgG	Mouse IgG	Mouse IgG	**0.05% Triton**
Mouse IgG	Mouse IgG	Mouse IgG	Mouse IgG	**0.10% Triton**
Mouse IgG	Mouse IgG	Mouse IgG	Mouse IgG	**0.25% Triton**
Rabbit IgG	Rabbit IgG	Rabbit IgG	Rabbit IgG	**1X PBS**
Rabbit IgG	Rabbit IgG	Rabbit IgG	Rabbit IgG	**0.05% Triton**
Rabbit IgG	Rabbit IgG	Rabbit IgG	Rabbit IgG	**0.10% Triton**
Rabbit IgG	Rabbit IgG	Rabbit IgG	Rabbit IgG	**0.25% Triton**
PBS	PBS/0.05% Triton	PBS/0.10% Triton	PBS/0.25% Triton	
Mouse/Rb IgG	Mouse/Rb IgG	Mouse/Rb IgG	Mouse/Rb IgG	

Slides printed: Triton-PLL 05-07
Triton-SE 08-10

Three replicate spots were printed for each sample on three replicate arrays. Each array contained four 12x10 subarrays, printed with 1 SMP3, 2 SMP4, 1 SMP9 pins, respectively. 3 poly-L-lysine slides and 3 SuperEpoxy slides (TeleChem) were printed. After each sample uptake, the pins blotted four times on a blot pad to remove excess sample before printing on the glass slides.

Antibody test print in 0.05%, 0.01%, 0.001% Triton-100X

Antibody test slides containing mouse IgG (Sigma Aldrich) and rabbit IgG (Sigma Aldrich) were diluted in 1X PBS/0.05% Triton, 1X PBS/0.01% Triton, 1X PBS/0.001% Triton at 1µg/ml. The z-axis of the arrayer was re-calibrated to allow for light contact of the pins with the slide surface, decreasing the depth of the pins.

Array Layout:

Mouse IgG	buffer	**0.05% Triton**
Mouse IgG	buffer	**0.01% Triton**
Mouse IgG	buffer	**0.001% Triton**
Rabbit IgG	buffer	**0.05% Triton**
Rabbit IgG	buffer	**0.01% Triton**
Rabbit IgG	buffer	**0.001% Triton**
Mouse/Rabbit IgG	buffer	**0.05% Triton**
Mouse/Rabbit IgG	buffer	**0.01% Triton**
Mouse/Rabbit IgG	buffer	**0.001% Triton**

Slides printed: Triton-PLL 11-14
Triton-SE 15-18

Three replicate spots were printed for each sample, along with three replicate spots of buffer on three replicate arrays. Each array contained four 6x9 subarrays, printed with 1 SMP3, 2 SMP4, 1 SMP9 pins, respectively. 4 poly-L-lysine slides and 4 SuperEpoxy slides (TeleChem) were printed. After each sample uptake, the pins blotted four times on a blot pad to remove excess sample before printing on the glass slides.

The antibody test print with Triton-100X was repeated.

Array Layout:

Mouse IgG	buffer	**0.05% Triton**
Mouse IgG	buffer	**0.01% Triton**
Mouse IgG	buffer	**0.001% Triton**
Rabbit IgG	buffer	**0.05% Triton**
Rabbit IgG	buffer	**0.01% Triton**
Rabbit IgG	buffer	**0.001% Triton**
Mouse/Rabbit IgG	buffer	**0.05% Triton**
Mouse/Rabbit IgG	buffer	**0.01% Triton**
Mouse/Rabbit IgG	buffer	**0.001% Triton**

Slides printed: Triton-PLL 19-27
Triton-SE 28-36

Three replicate spots were printed for each sample, along with three replicate spots of buffer on three replicate arrays. Each array contained four 6x9 subarrays, printed with 1 SMP3, 2 SMP4, 1 SMP9 pins, respectively. 9 poly-L-lysine slides and 9 SuperEpoxy slides (TeleChem) were printed. After every 6 slides, the pins were replenished with sample allowing for 58 spots per sample from a single sample uptake. After each sample uptake, the pins blotted four times on a blot pad to remove excess sample before printing on the glass slides.

Protein and antibody labeling

Using an antibody labeling kit

Labeling of GST, Anti-GST, Rabbit antibody

Alexa 647 and Alexa 532 antibody labeling kit from Molecular Probes were used (catalog no: A20186, A20182). The labeling protocol was used with 50µl of protein volume instead of the recommended 100µl. The labeling kit is designed to label 1mg/ml of protein successfully. Anti-GST Ab, at 1mg/ml, was labeled with Alexa 647. GST protein at 0.8mg/ml was labeled with Alexa 532. Rabbit antibody at a measured concentration of 2.84mg/ml, obtained by the absorbance readings at 280nm and 260nm, was labeled with Alexa 532. For each labeling reaction, $1/10^{th}$ volume 1M sodium

bicarbonate was added to each 50µl of protein sample. Each sample was added to a vial of dye and incubated in the dark for 3 hrs. Three columns for removal of unconjugated dye were prepared using the purification resin supplied in the labeling kit. The dye mixture was placed onto the column and allowed to absorb for 5mins. The labeled protein was eluted by centrifugation at 1000 rpm for 5mins two times.

The purified conjugate was diluted in PBS and the absorbance measured in a cuvette with a 5mm pathlength at 280nm and 530nm.
The concentration of the sample labeled with Alexa 532 was computed using the formula:

$[[A_{280} - (A_{530}*.09)] * \text{dilution factor}]/203{,}000$

where: 0.09 is the correction factor for the fluorophore's contribution to the absorbance at 280nm, and
203,000 is the molar extinction coefficient in $cm^{-1}M^{-1}$ of a typical IgG at 280nm.

The labeling efficiency of Alexa 532 was computed using:

$(A_{530} * \text{dilution factor})/(81{,}000 * \text{protein concentration})$

where: $81{,}000 cm^{-1}M^{-1}$ is the molar coefficient of the Alexa Fluor 532 dye at 530nm.

A successful Alexa 532 labeling reaction should result in 3-7 moles of Alexa 532 dye per mole antibody.

GST protein, labeled with Alexa 532, had a protein concentration of 1.13×10^{-5}M and a labeling efficiency of 2.06 moles dye/mole protein.
Rabbit antibody, labeled with Alexa 532, had a protein concentration of 0.85×10^{-5}M and a labeling efficiency of 2.71 moles dye/mole protein.

The purified Alexa 647 conjugate was diluted in PBS and the absorbance measured in a cuvette with a 5mm pathlength at 280nm and 650nm.
The concentration of the sample labeled with Alexa 647 was computed using:

$[[A_{280} - (A_{650}*.03)] * \text{dilution factor}]/203{,}000$

where: 0.03 is the correction factor for the fluorophore's contribution to the absorbance at 280nm and
203,000 is the molar extinction coefficient in $cm^{-1}M^{-1}$ of a typical IgG at 280nm.

The labeling efficiency of Alexa 647 was computed using:

$(A_{650} * \text{dilution factor})/(239{,}000 * \text{protein concentration})$

where: $239{,}000 cm^{-1}M^{-1}$ is the molar coefficient of the Alexa Fluor 647 dye at 650nm.

A successful Alexa 647 labeling reaction should result with 3-7 moles of Alexa 647 dye per mole antibody.

Anti-GST Ab, labeled with Alexa 647, had a protein concentration of 0.76×10^{-5}M and a labeling efficiency of 3.18 moles dye/mole protein.

Using an antibody labeling kit with microcon columns

Labeling of GST, Rabbit antibody, Mouse antibody

Alexa 647 and Alexa 532 antibody labeling kit from Molecular Probes were used as described earlier with a few minor changes. The labeling kit was used with 100µl of sample volume. Rabbit antibody (Sigma Aldrich) at 11.2mg/ml was labeled with Alexa 532. Mouse antibody (Sigma Aldrich) at 11.4mg/ml was labeled with Alexa 647. GST protein (Recombinant Technologies) at 0.8mg/ml was labeled with Alexa 532. Each protein sample was diluted to 1mg/ml in 1X PBS. $1/10^{th}$ volume of 1M sodium bicarbonate was added to each sample to achieve 100µl of total volume. Each protein sample was added to the respective dye vial and incubated at room temperature for 60mins in the dark. Microcon columns (YM-30) were used instead of the resin provided in the labeling kit. 400µl of 1X PBS was added to the dry column and then 100µl of the labeled probe was added. The column was placed in a collection tube and spun at 13,000 for ~10mins. The column was checked regularly to ensure it did not dry out. The flow thru was discarded and repeated again with the addition of 400ul of 1X PBS. The column was inverted into a new collection tube to collect the probe. The column was spun at 10,000 with 3 bursts of high speed. The volume was adjusted with the addition of 1X PBS to achieve a final volume of 100µl. If a substantial amount of dye remained stuck on the column bed, 30µl of 1X PBS was added to the column bed and let stand for a few minutes. The column was inverted on the collection tube and the sample collected.

The concentration of the sample labeled with Alexa 532 was computed using:
$[[A_{280} - (A_{530}*.09)] * \text{dilution factor}]/203{,}000$
GST protein, labeled with Alexa 532, had a protein concentration of $0.37M \times 10^{-5}$ and a labeling efficiency of 4.51 moles dye/mole protein.

The concentration of the sample labeled with Alexa 647 was computed using:
$[[A_{280} - (A_{650}*.03)] * \text{dilution factor}]/203{,}000$
Mouse antibody, labeled with Alexa 647, had a protein concentration of $0.116M \times 10^{-4}$ and a labeling efficiency of 1.45 moles dye/mole protein.

The concentration of the sample labeled with Alexa 532 was computed using:
$[[A_{280} - (A_{530}*.09)] * \text{dilution factor}]/203{,}000$
Rabbit antibody, labeled with Alexa 532, had a protein concentration of $0.59M \times 10^{-5}$ and a labeling efficiency of 0.99 moles dye/mole protein.

Mouse and rabbit antibody was re-labeled using the steps outlined above. Mouse antibody, re-labeled with Alexa 647, had a protein concentration of $0.63M \times 10^{-5}$ and a labeling efficiency of 6.41 moles dye/mole protein.

Rabbit antibody, re-labeled with Alexa 532, had a protein concentration of $0.31M \times 10^{-5}$ and a labeling efficiency of 6.98 moles dye/mole protein.

Slide conditions and blocking methods tested

The unprocessed slides were stored at 4ºC. The slides to be used for detection were labeled and the placement was each array was delineated using a diamond scriber on the opposite side of the array.

BSA blocking

Prior to use, the slides were washed in 1X PBS. The slides were blocked in 1X PBS/1% BSA for 60mins at room temperature. The slides were washed in 1X PBS for 2mins, 3 times. Excess liquid was removed and a separation between each array was made using PapPen (creates a hydrophobic barrier). The labeled probe was diluted in 1X PBS/0.5% BSA. 12µl of the labeled probe was placed on a $18mm^2$ coverslip and inverted onto the array. The slides were placed in a GeneMachines hybridization chamber with 1X PBS at the bottom for adequate humidification. The chamber was placed in the dark at room temperature for 60mins. The slides were washed in 1X PBS for 5mins, 3 times. The slides were spun dry and scanned.

Nonfat Milk blocking

The slides were rinsed in 3% nonfat milk/PBS/0.1% Tween-20. The slides were transferred to 3% nonfat milk/PBS/0.02% sodium azide blocking solution and placed at 4ºC for overnight incubation. The slides were washed in 1X PBS for 1min, 3 times. Excess liquid was removed and a separation between each array was made using PapPen. The labeled probe was diluted in 1X PBS/0.5% BSA. 12µl of the labeled probe was placed on a $18mm^2$ coverslip and inverted onto the array. The slides were placed in a GeneMachines hybridization chamber with 1X PBS at the bottom for adequate humidification. The chamber was placed in the dark at 4ºC for two hours. The coverslip was removed in 1X PBS. The slides were washed in 1X PBS/0.1% Tween-20 for 20mins; 1X PBS for 10mins twice; dH_2O for 10mins twice. The slides were spun dry and scanned.

TeleChem 1X BlockIt buffer

Prior to use, the slides were washed in 1X PBS. The slides were blocked in 1X BlockIt buffer for 60mins at room temperature. The slides were washed in 1X PBS for 5mins, 3 times. The labeled probe was diluted in 1X BlockIt buffer. Excess liquid was removed and a separation between each array was made using PapPen. 12µl of the labeled probe was placed on a $18mm^2$ coverslip and inverted onto the array. The slides were placed in a GeneMachines hybridization chamber with 1X PBS at the bottom for adequate humidification. The chamber was placed in the dark at room temperature for 60mins. The slides were washed in 1X PBS for 10mins, 3 times. The slides were spun dry and scanned.

Testing the effects of blocking buffer and labeled probe concentrations on signal intensity

Conjugations using antibody test slides with 1X PBS printing buffer and 1:1000 to 1:100 labeled probe

The blocking conditions outlined above were tested on poly-L-lysine and Super Epoxy slides. Each blocking condition and probe concentration was done with three technical

repeats. Anti-GST, GST, Rabbit Ab, and Mouse Ab were labeled using an antibody labeling kit.

Slide Name	**Blocking Conditions**	**Conjugation Probe**		
		Array 1	**Array 2**	**Array 3**
AB_PLL 01, 02*, 03 AB_SE 10, 11, 12	BSA Blocking	Anti-GST/Rabbit Ab 1:1000 (Alexa 647/Alexa 532)	GST 1:1000 (Alexa 532)	1X PBS/0.5% BSA
AB_PLL 04, 05, 06* AB_SE 13, 14, 15	Nonfat Milk blocking	Anti-GST/ Rabbit Ab 1:1000 (Alexa 647/ Alexa 532)	GST 1:1000 (Alexa 532)	1X PBS/ 0.5% BSA
AB_PLL 07*, 08 AB_SE 17, 18	BlockIt buffer	Anti-GST 1:1000 (Alexa 647)	GST 1:1000 (Alexa 532)	1X BlockIt buffer
AB_PLL 09	Nonfat Milk blocking	GST 1:500 (Alexa 532)	GST 1:100 (Alexa 532)	GST 1:50 (Alexa 532)
AB_PLL 19*	Nonfat Milk blocking	Anti-GST 1: 500 (Alexa 532)	Anti-GST 1: 100 (Alexa 532)	Anti-GST 1:50 (Alexa 532)
AB_PLL 20	Nonfat Milk blocking	Rabbit Ab 1:500 (Alexa 532)	Mouse Ab 1:500 (Alexa 647)	Rabbit Ab/Mouse Ab 1:500 (Alexa 647/Alexa 532)
AB_PLL 21	Nonfat Milk blocking	GST 1:500 (Alexa 532)	Anti-GST 1:500 (Alexa 647)	GST/Anti-GST 1:500 (Alexa 647/Alexa 532)
AB_PLL 22	BSA Blocking++	Rabbit Ab 1:500 (Alexa 532)	Mouse Ab 1:500 (Alexa 647)	Rabbit Ab/ Mouse Ab 1:500 (Alexa 647/Alexa 532)
AB_PLL 23	BSA Blocking++	GST 1:500 (Alexa 532)	Anti-GST 1:500 (Alexa 647)	GST/Anti-GST 1:500 (Alexa 647/Alexa 532)

Table 3. Blocking and conjugations conditions for each slide.
***** slides are discussed in depth in the discussion section
BSA blocking++: The steps outlined previously were followed with an increase in the first wash after blocking to 3 times for 5mins each in 1X PBS and the second wash after conjugation to 3 times for 10mins each in 1X PBS.

Conjugations using antibody test slides with 1X PBS and 0.05%, 0.10%, 0.25% Triton-100X and 1:100 to 1:50 labeled probe

The blocking conditions outlined above were tested on poly-L-lysine and Super Epoxy slides. The altered BSA blocking method was implemented, with an increase in the first wash after blocking to 5mins in 1X PBS, 3 times and the second wash after conjugation to 10mins in 1X PBS, 3 times.

Labeled anti-rabbit Cy3 and anti-mouse Cy5 (Amersham Bioscience, product no: PA43004, PA45002) at 1mg/vial was reconstituted in 1.0ml of dH_2O and diluted accordingly.

Slide Name	Blocking Conditions	Conjugation Probe		
		Array 1	**Array 2**	**Array 3**
Triton 06* PLL	BSA blocking	Anti-mouse 1:100 (Cy5)	Anti-rabbit 1:100 (Cy3)	Anti-mouse/anti-rabbit 1:100 (Cy5/Cy3)
Triton 09* SE	BSA blocking	Anti-mouse 1:50 (Cy5)	Anti-rabbit 1:50 (Cy3)	Anti-mouse/anti-rabbit 1:50 (Cy5/Cy3)
Triton 07* PLL	BlockIt buffer	Anti-mouse 1:100 (Cy5)	Anti-rabbit 1:100 (Cy3)	Anti-mouse/anti-rabbit 1:100 (Cy5/Cy3)
Triton 10 SE	BlockIt buffer	Anti-mouse 1:50 (Cy5)	Anti-rabbit 1:50 (Cy3)	Anti-mouse/anti-rabbit 1:50 (Cy5/Cy3)
Triton 05 PLL	Nonfat Milk blocking	Anti-mouse 1:100 (Cy5)	Anti-rabbit 1:100 (Cy3)	Anti-mouse/anti-rabbit 1:100 (Cy5/Cy3)
Triton 08 SE	Nonfat Milk blocking	Anti-mouse 1:50 (Cy5)	Anti-rabbit 1:50 (Cy3)	Anti-mouse/anti-rabbit 1:50 (Cy5/Cy3)

Table 4. Blocking and conjugations conditions for each slide.
*****slides are discussed in depth in the discussion section

Conjugations using antibody test slides in 1X PBS and 0.05%, 0.10%, 0.25% Triton-100X and 1:50 labeled probe

The altered BSA blocking method was implemented, with an increase in the first wash after blocking to 5mins in 1X PBS, 3 times and the second wash after conjugation to 10mins in 1X PBS, 3 times.

1X PBS/1.0% BSA blocking solution was filtered using a .22µm filter. Three slide conditions were used: 1) PapPen was placed around each array before the slide was blocked; 2) A mask was made using label tape around each array before the slide was blocked; 3) PapPen was placed around each array after the slide was blocked.

Slide Name	Blocking Conditions	Conjugation Probe		
		Array 1	**Array 2**	**Array 3**
Triton 11 PLL (PapPen before)	BSA Blocking	Anti-mouse 1:50 (Cy5)	Anti-rabbit 1:50 (Cy3)	1X PBS/0.5%BSA
Triton 12 PLL (Label mask)	BSA Blocking	Anti-mouse 1:50 (Cy5)	Anti-rabbit 1:50 (Cy3)	1X PBS/0.5%BSA
Triton 15* SE (PapPen before)	BSA Blocking	Anti-mouse 1:50 (Cy5)	Anti-rabbit 1:50 (Cy3)	1X PBS/0.5%BSA
Triton 16* SE (Label mask)	BSA Blocking	Anti-mouse 1:50 (Cy5)	Anti-rabbit 1:50 (Cy3)	1X PBS/0.5%BSA
Triton 13 PLL (PapPen after)	BSA Blocking	Anti-mouse 1:50 (Cy5)	Anti-rabbit 1:50 (Cy3)	1X PBS/0.5%BSA
Triton 17 SE (PapPen after)	BSA Blocking	Anti-mouse 1:50 (Cy5)	Anti-rabbit 1:50 (Cy3)	1X PBS/0.5%BSA
Triton 14 PLL (PapPen after)	BSA Blocking	Anti-rabbit 1:50 (Cy5)	blank	1X PBS/0.5%BSA
Triton 18 SE (PapPen after)	BSA Blocking	Anti-mouse 1:50 (Cy5)	blank	1X PBS/0.5%BSA

Table 5. Blocking and conjugations conditions for each slide.
*****slides are discussed in depth in the discussion section

Conjugations using antibody test slides in 1X PBS and 0.05%, 0.01%, 0.001% Triton-100X and 1:50 labeled probe

The altered BSA blocking method was implemented, with an increase in the first wash after blocking to 5mins in 1X PBS, 3 times and the second wash after conjugation to 10mins in 1X PBS, 3 times. 1X PBS/1.0% BSA blocking solution was filtered using a .22µm filter.

The labeled probe volume placed on each array was decreased from 12µl to 9µl. PapPen was placed after blocking in the middle of the slide.

Slide Name	**Blocking Conditions**	**Conjugation Probe**		
		Array 1	**Array 2**	**Array 3**
Triton 19* PLL	BSA Blocking	Anti-mouse 1:50 (Cy5)	blank	1X PBS/0.5%BSA
Triton 28* SE	BSA Blocking	Anti-mouse 1:50 (Cy5)	blank	1X PBS/0.5%BSA
Triton 25* PLL	Nonfat milk blocking	Anti-mouse 1:50 (Cy5)	blank	1X PBS/0.5%BSA
Triton 34* SE	Nonfat milk blocking	Anti-mouse 1:50 (Cy5)	blank	1X PBS/0.5%BSA
Triton 22* PLL	BlockIt buffer	Anti-mouse 1:50 (Cy5)	blank	1X BlockIt buffer
Triton 31* SE	BlockIt buffer	Anti-mouse 1:50 (Cy5)	blank	1X BlockIt buffer
Triton 20* PLL	BSA Blocking	Anti-rabbit 1:50 (Cy5)	blank	1X PBS/0.5%BSA
Triton 29* SE	BSA Blocking	Anti-rabbit 1:50 (Cy5)	blank	1X PBS/0.5%BSA
Triton 26* PLL	Nonfat milk blocking	Anti-rabbit 1:50 (Cy5)	blank	1X PBS/0.5%BSA
Triton 35* SE	Nonfat milk blocking	Anti-rabbit 1:50 (Cy5)	blank	1X PBS/0.5%BSA
Triton 23* PLL	BlockIt buffer	Anti-rabbit 1:50 (Cy5)	blank	1X BlockIt buffer
Triton 32* SE	BlockIt buffer	Anti-rabbit 1:50 (Cy5)	blank	1X BlockIt buffer
Triton 21* PLL	BSA Blocking	Anti-mouse/Anti-rabbit 1:50 (Cy3/Cy5)	blank	1X PBS/0.5%BSA
Triton 30* SE	BSA Blocking	Anti-mouse/Anti-rabbit 1:50 (Cy3/Cy5)	blank	1X PBS/0.5%BSA
Triton 27* PLL	Nonfat milk blocking	Anti-mouse/Anti-rabbit 1:50 (Cy3/Cy5)	blank	1X PBS/0.5%BSA
Triton 36* SE	Nonfat milk blocking	Anti-mouse/Anti-rabbit 1:50 (Cy3/Cy5)	blank	1X PBS/0.5%BSA
Triton 24* PLL	BlockIt buffer	Anti-mouse/Anti-rabbit 1:50 (Cy3/Cy5)	blank	1X BlockIt buffer
Triton 33* SE	BlockIt buffer	Anti-mouse/Anti-rabbit 1:50 (Cy3/Cy5)	blank	1X BlockIt buffer

Table 6. Blocking and conjugations conditions for each slide.

*****slides are discussed in depth in the discussion section

RESULTS & DISCUSSION

Each conjugation was done with three technical repeats. Since each sample was spotted in triplicates, the average of the three spots were taken and plotted along with the corresponding spot concentration.

Spot morphology and quality control test

Slide staining using hand spotted BSA slide

The SYPRO Red gel stain is intended for denaturing and non-denaturing protein gels. The performance of the gel stain was replicated on glass substrates. SYPRO Red gel stain fluoresces at 532nm and 635nm and therefore can offer the ability to perform quality control on printed protein slides.

The protocol for the gel stain was altered for usage on glass substrates. Acetic acid was omitted from the protocol and substituted with H_2O. Extra washes were used to ensure removal of unbound stain. SYPRO Red gel stain was tested on a hand-spotted BSA slide at first to ensure optimal binding efficiency. BSA was hand-spotted on a poly-L-lysine coated slide and incubated with SYPRO Red gel stain. SYPRO Red gel stain was able to bind to the BSA and fluoresce at 635nm and 532nm.

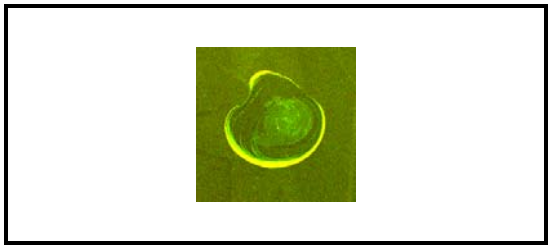


Figure 5. Sypro-1A-500: Hand spotted BSA on PLL slide, incubated with SYPRO Red gel stain visualized at 532nm and 635nm wavelength on an Axon 4000A scanner.

Slide staining using a printed BSA slide

The SYPRO Red gel stain was used on a printed slide after getting positive results from the hand-spotted BSA slide. The printed BSA slide, incubated with Sypro Red gel stain, did not stain efficiently. The background signal intensity was as high as the BSA spot signal intensity. In some parts, higher background signal intensity resulted in black spots on the array. The SYPRO Red gel stain is not efficient in staining proteins on poly-L-lysine slides due to the high non-specific binding of the gel stain to the poly-L-lysine slide.

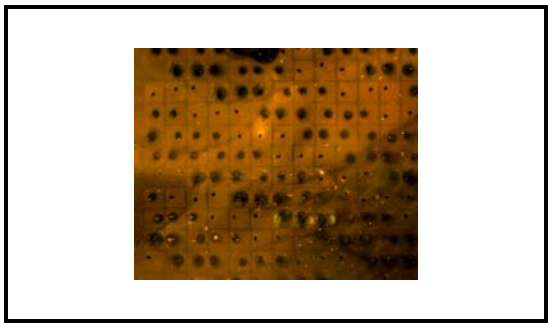


Figure 6. Slide-1-BSA: SMP9 printed BSA on PLL slide, incubated with SYPRO Red gel stain visualized at 532nm and 635nm wavelength on an Axon 4000A scanner.

Pin Comparison

Each type of Telechem Stealth Microarray Pin (SMP) was tested to assess the number of spots printable on poly-L-lysine coated slides from a single uptake of 0.25µl of cyanine labeled anti-mouse and anti-rabbit antibody samples.

The number of spots printed from each pin, along with the spot morphology and spot consistency were collected. The data was tabulated and used to create graphs, showing the relationship between the signal intensity and the number of spots printed for each pin. The spot diameter created by the SMP pins is shown below. The amount of sample delivered to the glass surface increases with the larger pin size and results in a bigger spot diameter.

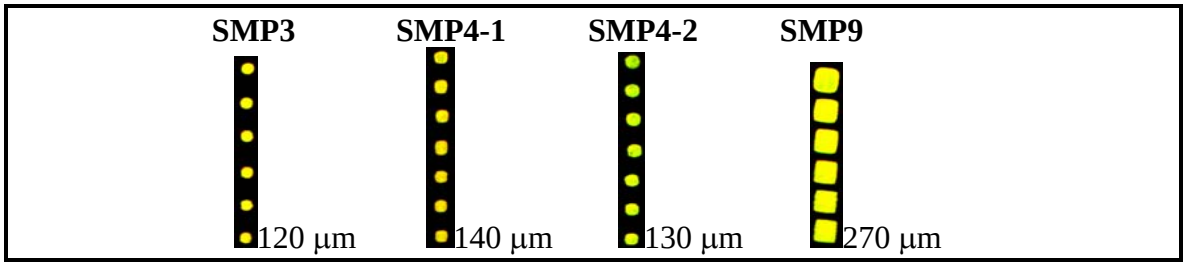


Figure 7. Morphology and diameter of spots created by SMP's on PLL coated slides.

The uptake of each pin is 0.25 µl, therefore the greater the size of the pin results in a lower number of spots printed from a single uptake. The median total signal intensity of the SMP3 pin remained consistent around 10,000 for 100 spots printed. The two SMP4 pins did not show consistent results. SMP4-1 performed as expected, with 50 spots printed at consistent signal intensity. The median total signal intensity of the SMP4-2 pin was the highest at 5,000. The difference between the two SMP4 pins could be due to insufficient sample in the 384 v-bottom plate. The image of the spot morphology shows that SMP4-2 spots have lower Cy5 signal compared to the other three pins. SMP9 pin generates the spots with the highest signal intensities, but it prints the lowest number of spots from a single sample uptake.

SMP3 pin can generate a high-density array with greater number of spots than compared to a SMP9 pin. But SMP9 has the potential for creating the spots with the largest spot diameter. SMP4 also offer the ability to generate a high-density array and have a slightly bigger spot diameter.

All four pins will be used for the test protein prints and compared to each other in the presence of different samples and printing buffers.

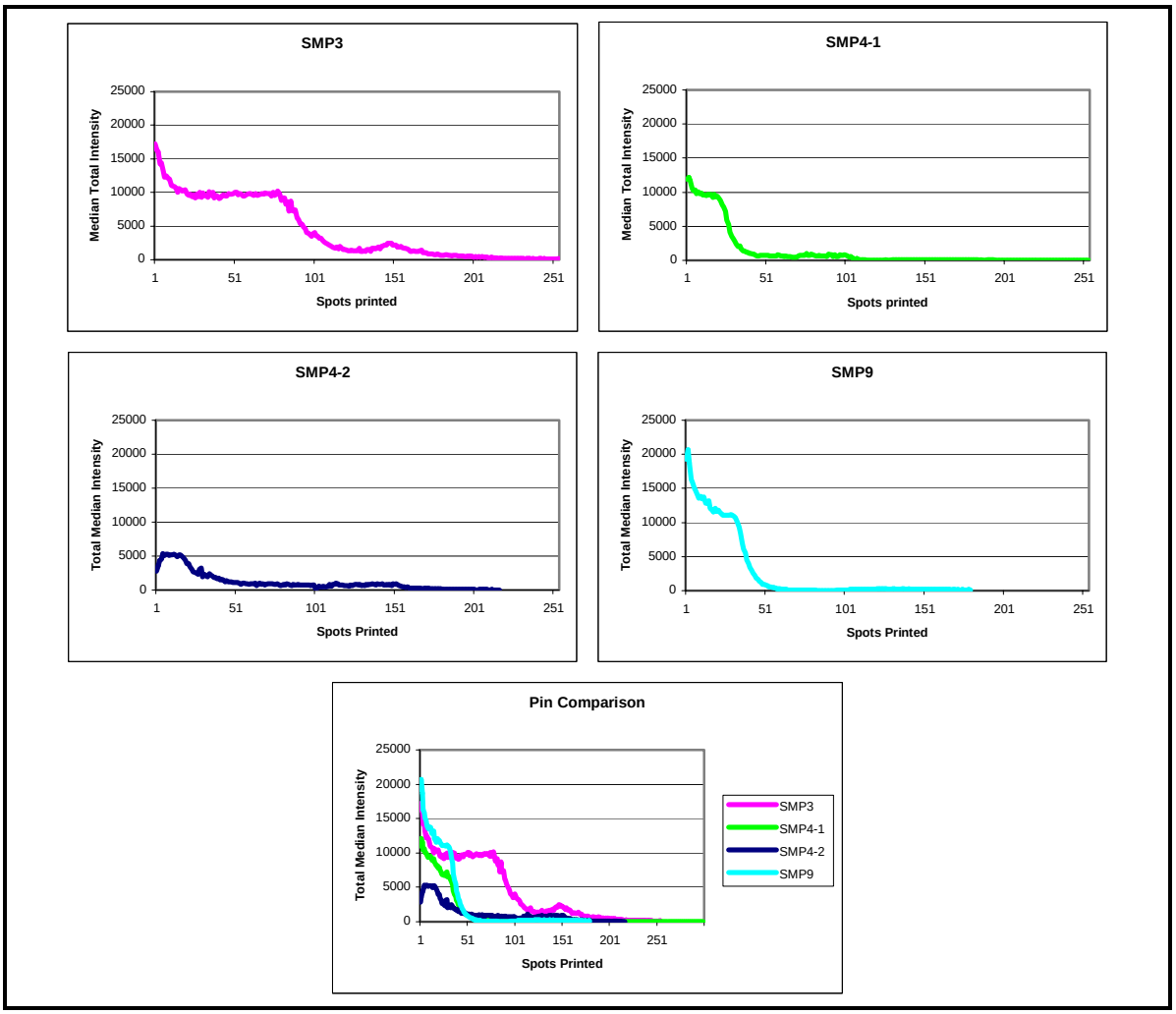


Figure 8. Graphs comparing the median total signal intensity at 532nm and 635nm wavelengths for spots created using SMP3, 4 and 9.

Preventing cross-labeling between arrays

Three arrays were printed on each slide, each with 12500 micron spacing in-between the arrays. The slides are labeled with the probes right after they have been blocked. The slides cannot be dried before conjugation since the air-water interface has to be maintained to keep the proteins stable. It is difficult to place the probes on each array without the fear of cross labeling between arrays. A hydrophobic barrier between each array was established using a PAP pen. The pen provides a thin film-like green tinged barrier when drawn on slides. The Pap pen is drawn between each array but is drawn on a wet slide, due to this there is a chance for the hydrophobic barrier to flow into the array.

Cross labeling between the first and second array occurred on the following slide. The array was hybridized with anti-mouse Cy3 probe but the Cy5 probe from the second array was also detected.

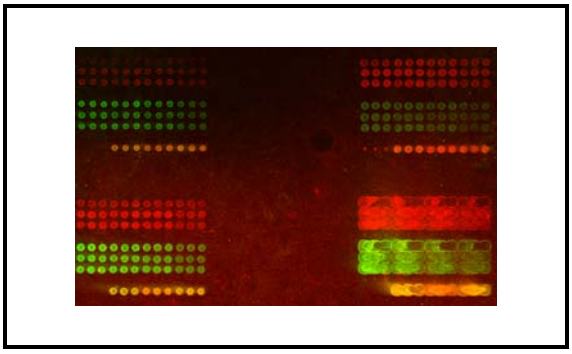


Figure 9. Triton-09 SuperEpoxy (BSA Blocking): Cross labeling between the 1st and 2nd array

Several methods were tried to prevent cross labeling between arrays. The arrays were encircled with Pap pen before they were blocked. After blocking, the labeled probe was placed on the coverslip and inverted onto the array. Placing the hydrophobic barrier around the array before the slide is blocked introduces residual film over the array.

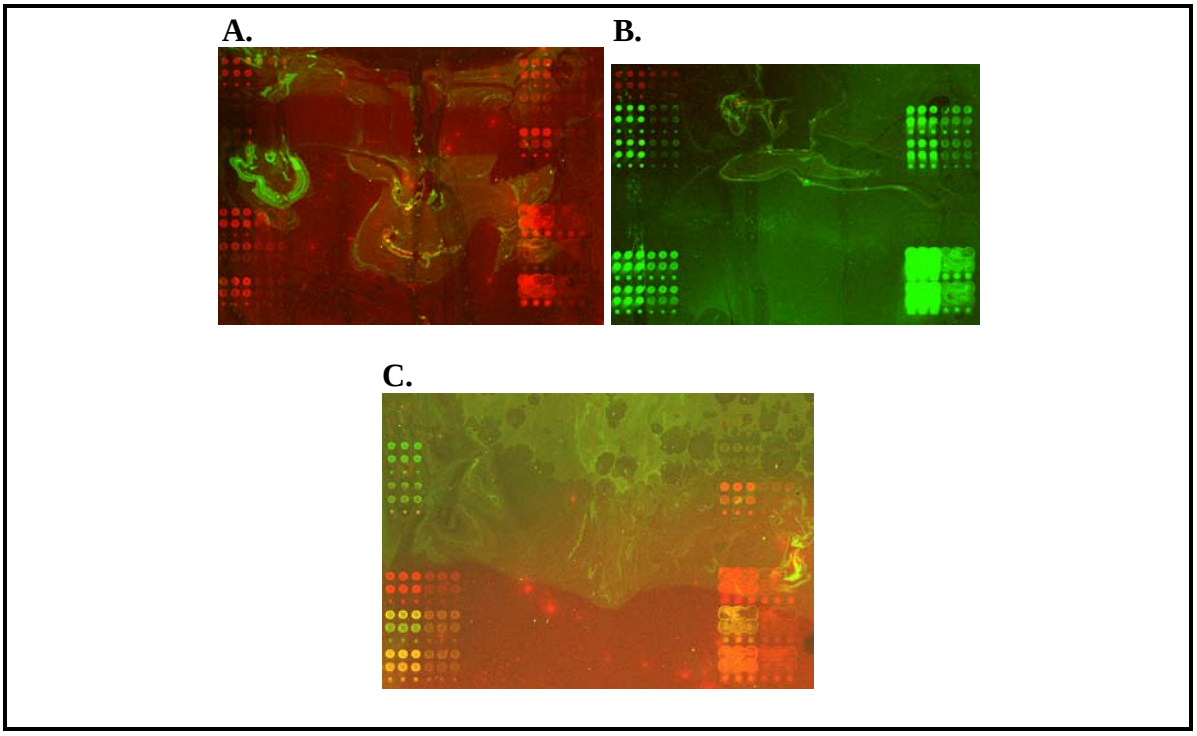


Figure 10. Unsuccessful tries in preventing cross labeling using PapPen and a label mask. A) Triton-15SE: Residual PapPen; B) Triton-15SE: Cross-labeling; C) Triton-16SE: Cross-labeling and adhesive from label mask

Also, by making a hydrophobic barrier around the array before treating the slides does not help in preventing cross labeling. On the 2nd array, Cy5 signal was still detected. An alternate method was to put a label mask around the array. Labeling tape was cut and placed around the array making sure that all edges were overlapping. There were greater numbers of air pockets formed under the array because the label mask was not placed

exactly under the area of the coverslip. The mask did not prevent cross labeling between arrays and the adhesive from the label flowed onto the array.

Testing the effects of blocking buffer and labeled probe concentrations on signal intensity

Conjugations with 1:1000 labeled probe

Anti-GST/Rabbit Ab, GST, 1X PBS/0.5% BSA were hybridized separately on each array using poly-L-lysine coated slides and SuperEpoxy slides using three different blocking and detecting protocols. On all slides, the anti-GST labeled probe detected GST protein printed on the substrate. Neither the labeled GST nor the labeled rabbit antibodies detect their complementary targets printed on the array. According to the labeling reaction, GST protein had a labeling efficiency of 2.06 moles dye/mole protein and showed a prominent peak at 530nm on the absorbance graph (data not shown). Rabbit antibody had a higher labeling efficiency than the GST protein of 2.71 moles dye/mole protein, although its absorbance graph did not show a peak at 530nm (data not shown).
The third array, hybridized with only buffer, was a successful negative control and did not show any conjugation to the array.

All the poly-L-lysine slides used in this test worked well. The SuperEpoxy slides had no signal on any of the three arrays because, as we discovered after the fact, the batch of slides had expired and the coating had lost its binding activity.

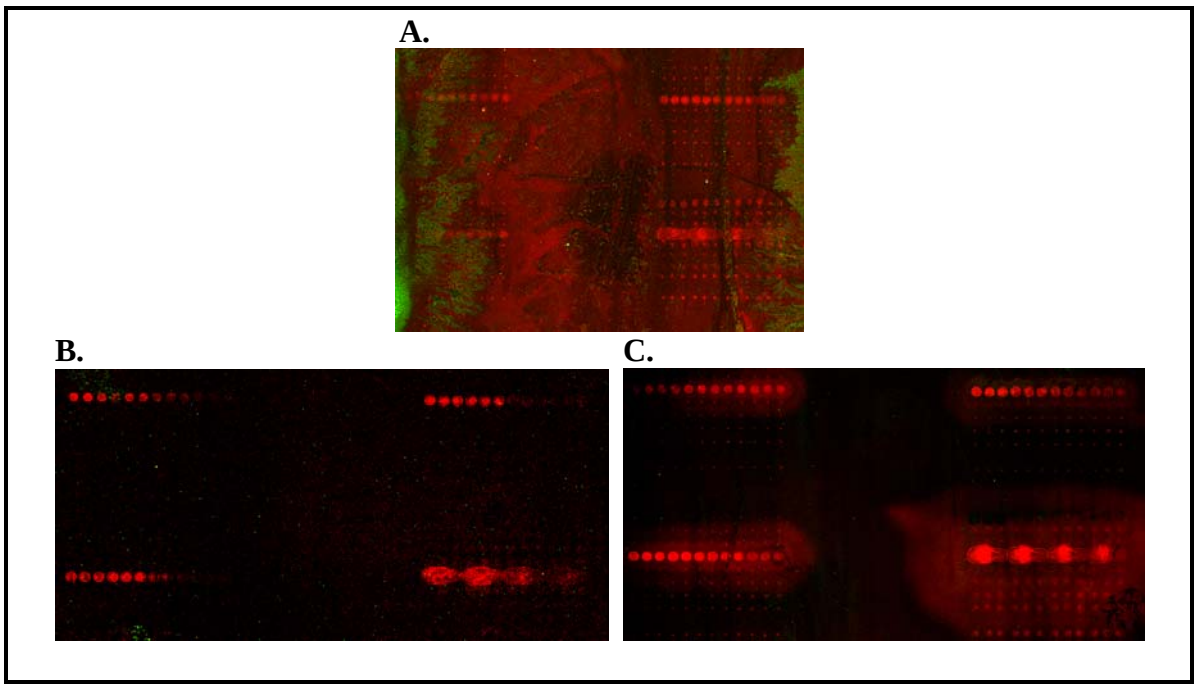


Figure 11. Effect of different blocking solutions on signal and background intensities. GST protein printed on poly-L-lysine slide surface conjugated with Anti-GST antibody 1:1000 dilution. A) AB_PLL 02-Array 1: BSA blocking solution; B) AB_PLL 06-Array 1: TeleChem BlockIt buffer; C) AB_PLL 07-Array 1: nonfat milk blocking solution

The three blocking methods were compared to each other using poly-L-lysine slides with 1:1000 of labeled probe. Anti-GST spots printed with SMP9 pin merged into each other and were not useable. High, uneven background resulted in inconsistent results from BSA blocking on the triplicate slides. Nonfat milk blocking method had consistent results on the three replicate slides. The median signal intensity, however, was under 200. High median signal intensity was obtained using BlockIt buffer. The spot concentration of 1ug/ml had the highest median signal intensity. Increasing probe concentration as well as increasing slide washes may result in higher signal intensity and low background intensity.

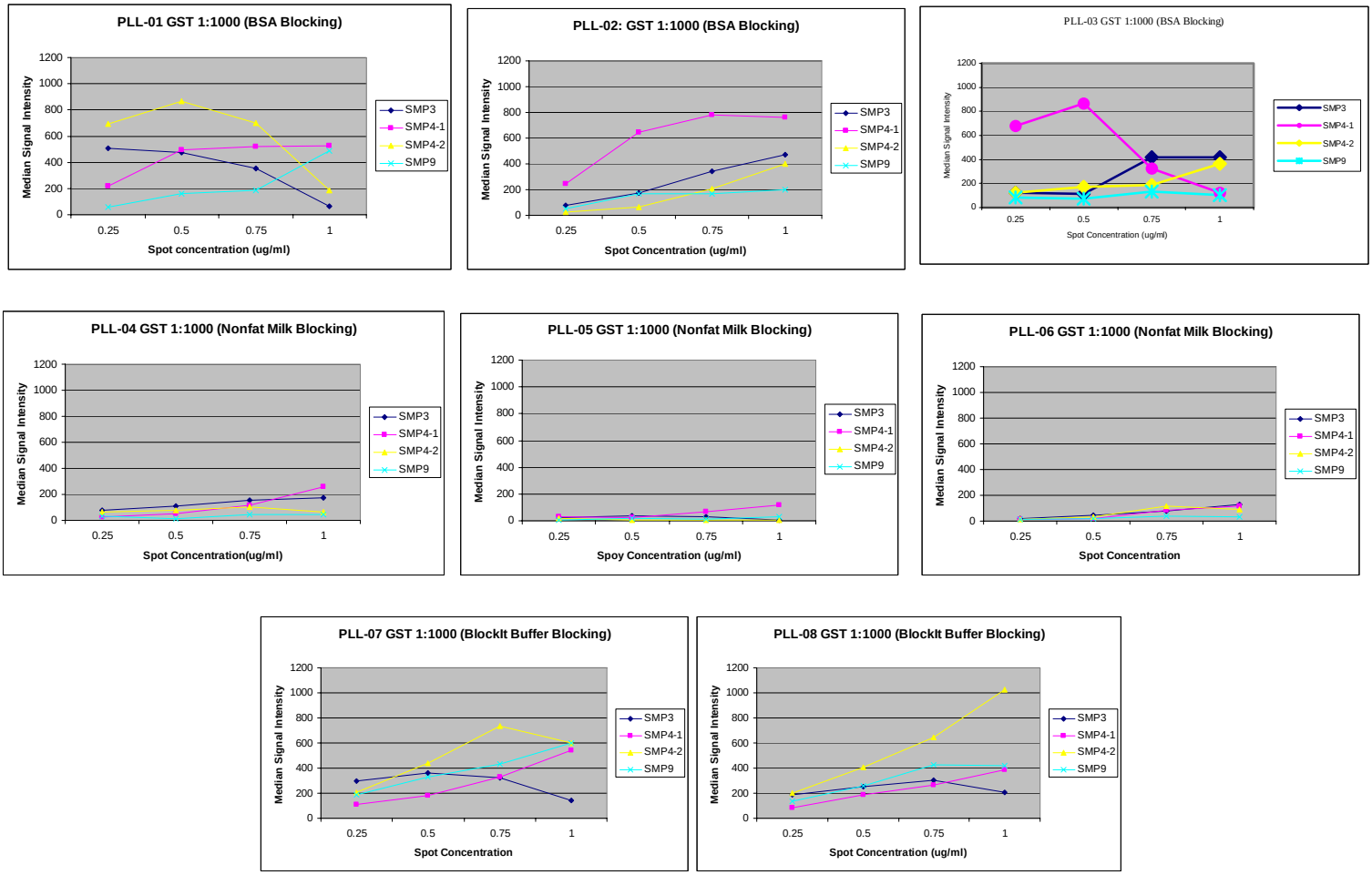


Figure 12. Median signal intensity vs. spot concentration graphs comparing the blocking methods and pin types with 1:1000 labeled probe.

Conjugations with 1:500, 1:100, 1:50 labeled probe.
To attempt to achieve high signal intensity, we tested the effect of varying labeled probe concentration at three different probe dilutions with GST protein and anti-GST. Nonfat milk blocking was used with increased washes after blocking and conjugation.

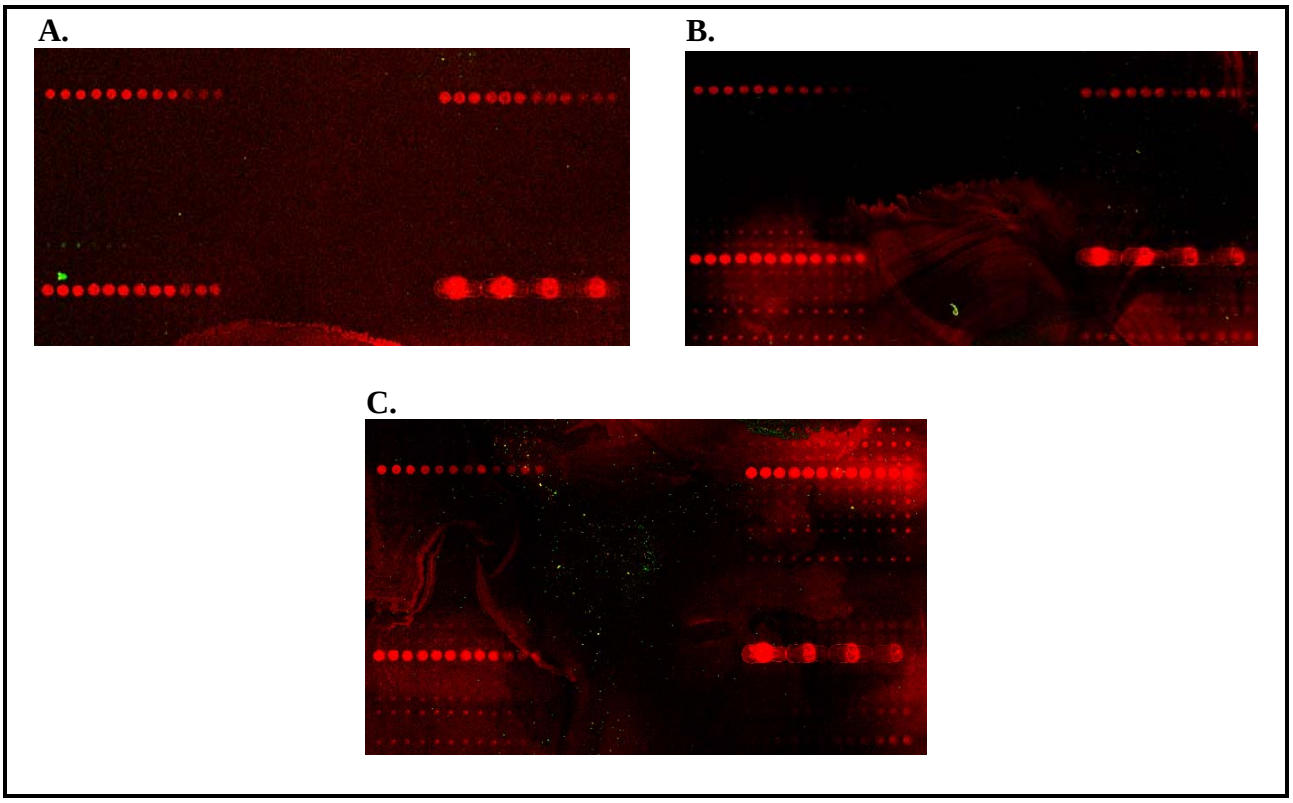


Figure 13. Effect of varying probe concentration using nonfat milk blocking. A) PLL-19-Array 1: 1:500; B) PLL-19-Array 2: 1:100; C) PLL-19-Array 3: 1:50

Median signal intensity was obtained for each spot and plotted against the spot concentration. Data obtained from the SMP9 pin cannot be used due to spot merging. Comparing the three graphs, higher signal intensity is obtained with higher probe concentration. The highest signal intensity is obtained from 1ug/ml spot concentration with 1:50 probe concentration.

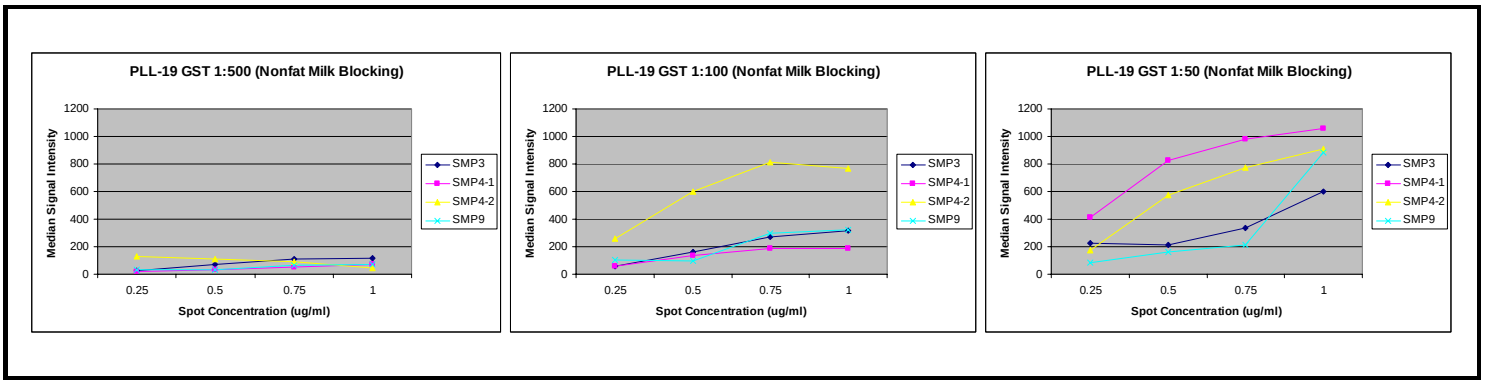


Figure 14. Median signal intensity vs. spot concentration comparing different probe dilutions.

Printing buffer of 1X PBS and 0.05% to 0.25% Triton-X 100
The addition of Triton-X 100 improved spot morphology drastically as compared to the mouse and rabbit IgG, which were spotted in PBS alone. The increasing concentration of Triton in the printing buffer increases the spot diameter. Since SMP9 spots are 300µm in diameter without the addition of PBS, the spots merge together when Triton is added.

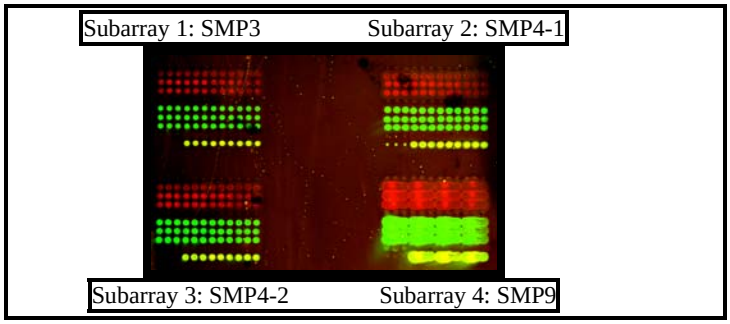


Figure 15. Triton-06PLL-array 3. BSA blocking. Labeled with 1:100 anti-rabbit & anti-mouse

With the addition of Triton to the printing buffer, there is a visible halo around each spot. The halo contributes to background and lowers the signal-to-noise ratio. However, the different concentrations of Triton do not generate drastic differences in spot morphology.

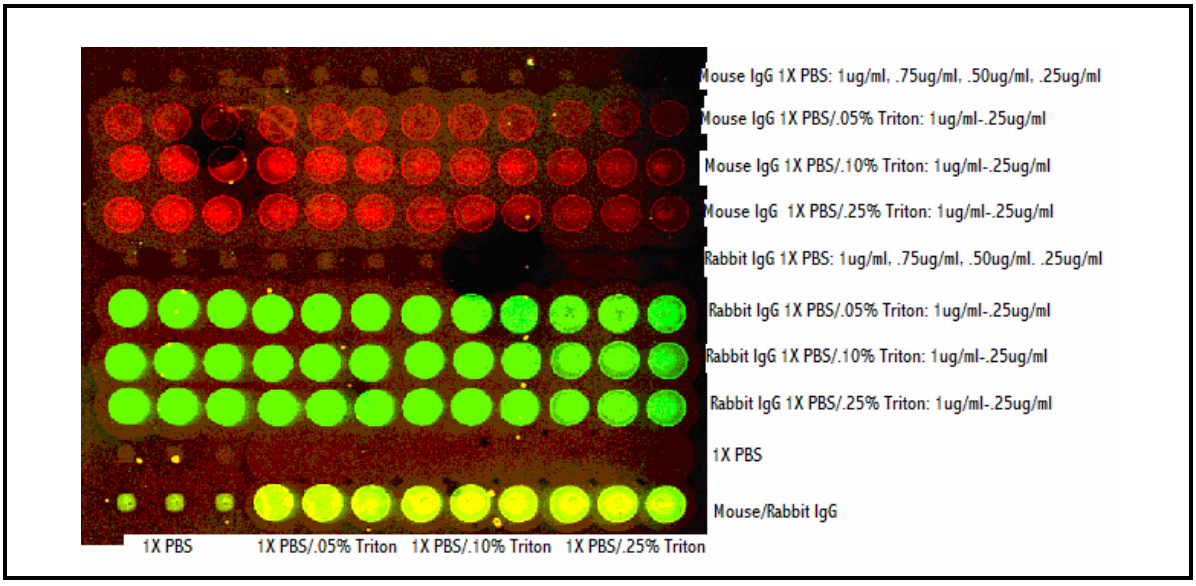


Figure 16. Triton-06 PLL-Array 3 (BSA Blocking) SMP4-1

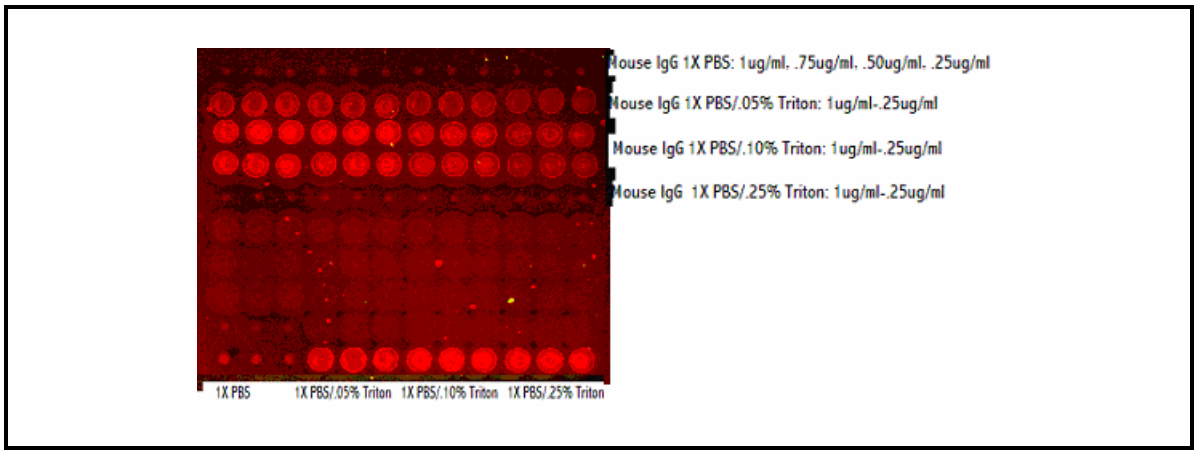


Figure 17. Triton-06 Array 1 (BSA Blocking) SMP4-1

The median signal intensity minus background of each spot concentration (1µg/ml, 0.75µg/ml, 0.50µg/ml, 0.25µg/ml) with the different printing buffers (1X PBS, 1X PBS/0.05% Triton, 1X PBS/0.10% Triton, 1X PBS/0.25% Triton) was obtained. The median signal intensity was plotted against the signal-to-noise ration (SNR) of each spot. This was done to determine which concentration of Triton would give the highest SNR.

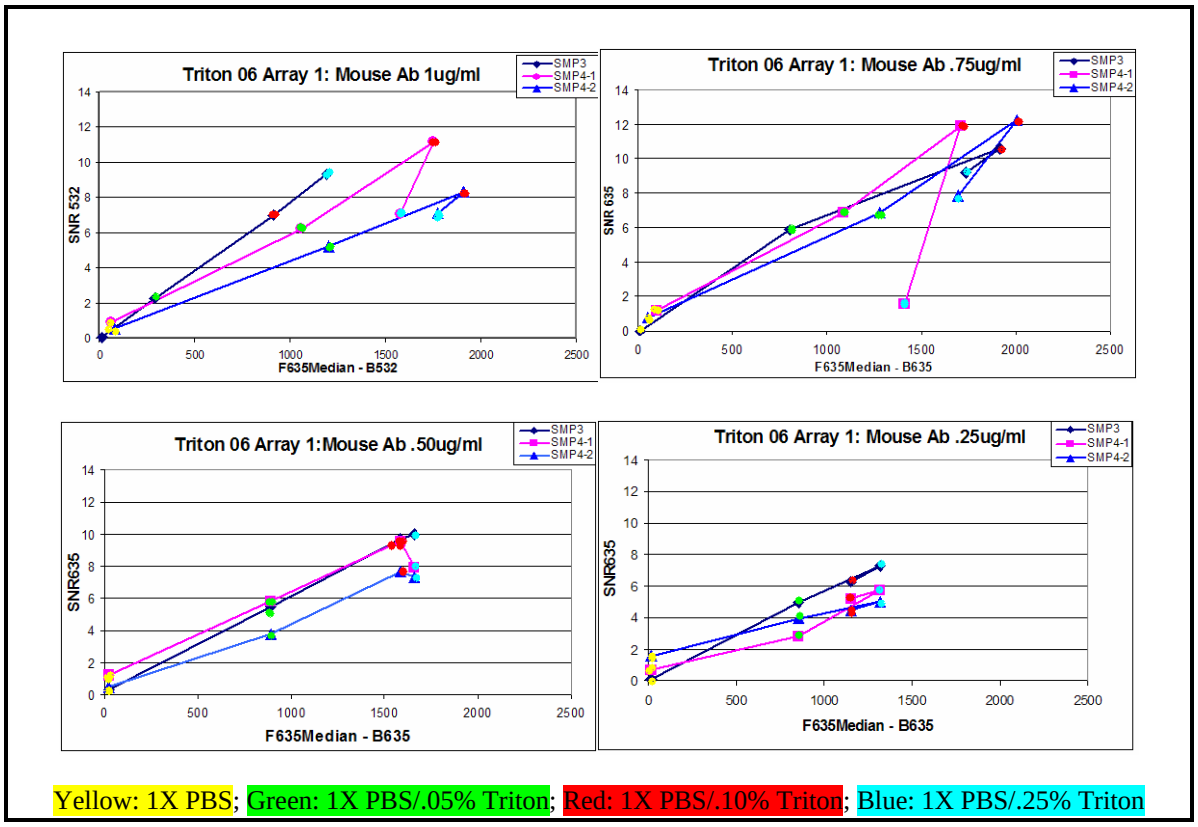


Figure 18. Comparing the effects of varying Triton-X concentrations with different spot concentrations of mouse antibody in Triton 06-Array 1

The highest signal-to-noise ration was achieved with 1X PBS/0.05% Triton. The signal to noise ratio decreased with a decrease in spot concentration. PBS alone in the printing buffer did not produce significant spot intensity.

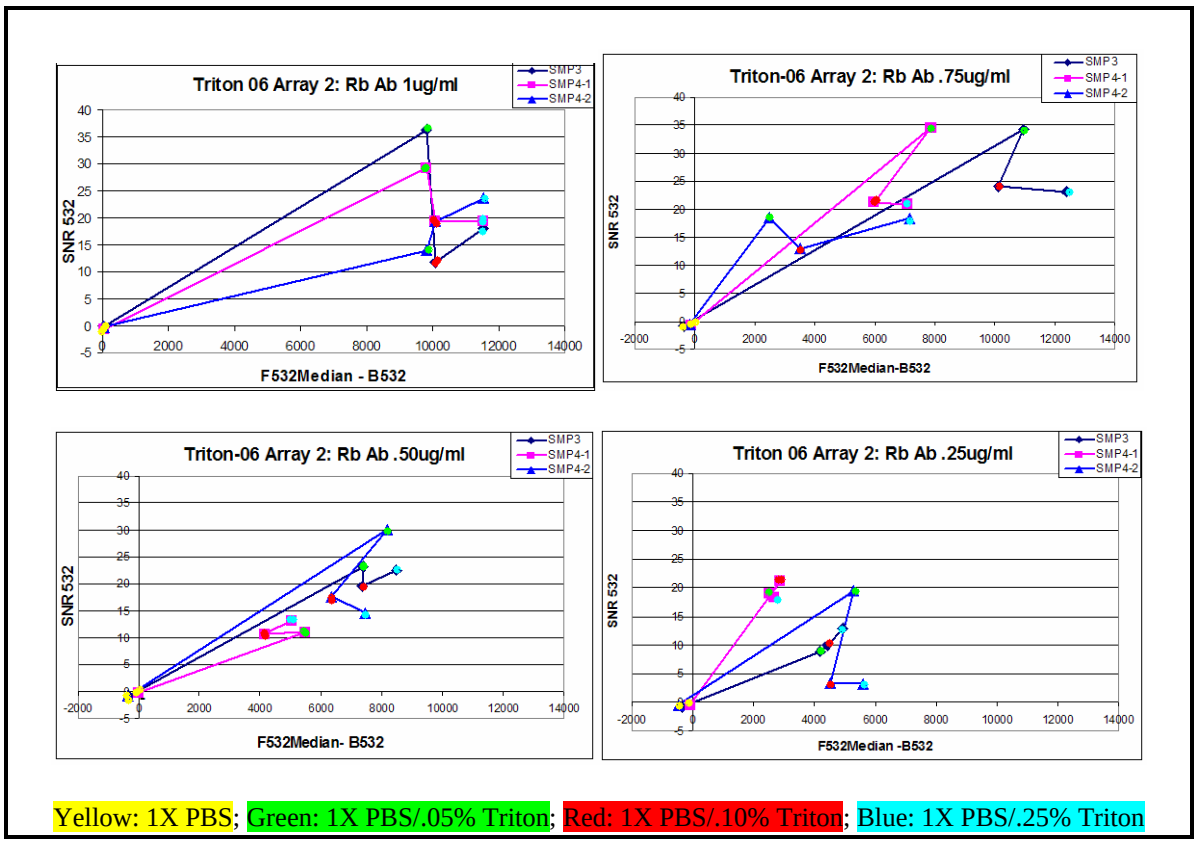


Figure 19. Comparing the effects of varying Triton-X concentrations with different spot concentrations of rabbit antibody in Triton-06 Array 2

Printing buffer containing 1X PBS/0.05% Triton gave the highest signal-to-noise ratio. Although samples containing 1X PBS/0.25% had the highest signal intensity, they did not have as high signal-to-noise ratios. With increasing Triton concentrations, a halo is present around the spot that contributes to low levels of signal-to-noise ratio.

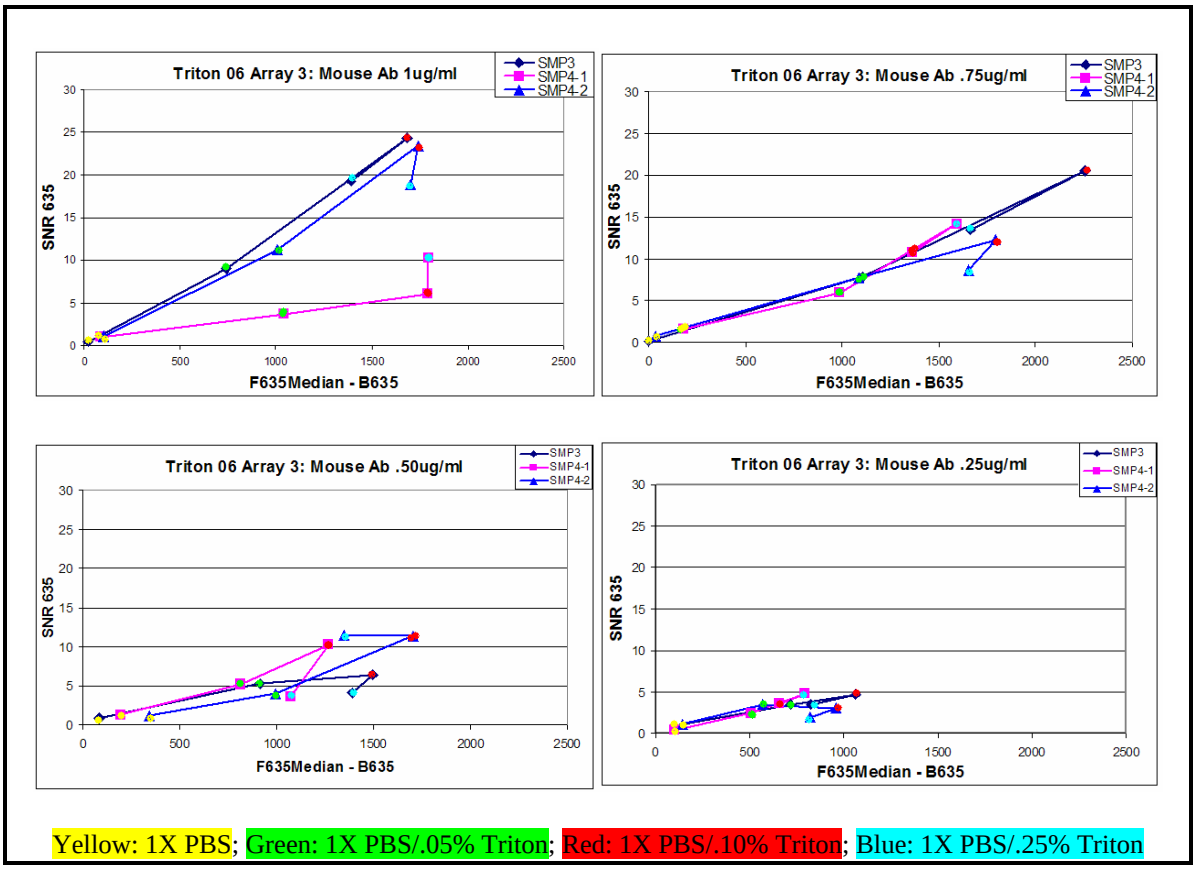


Figure 20. Comparing the effects of varying Triton-X concentrations with different spot concentrations of mouse antibody in Triton 06 Array 3

Samples in 1X PBS/0.10% Triton had the highest signal-to-noise ratio and median signal intensity. The signal intensity decreased with a decrease in antibody concentration.

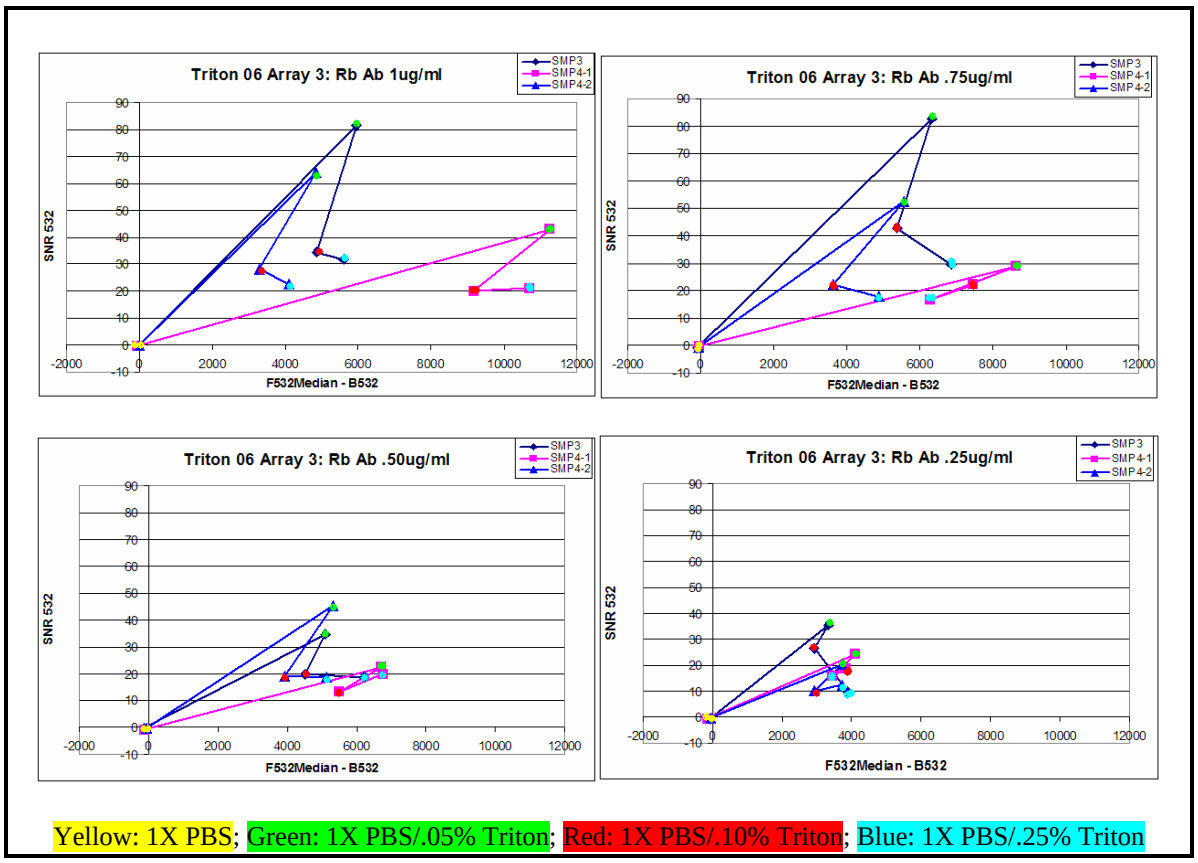


Figure 21. Comparing the effects of varying Triton-X concentrations with different spot concentrations of rabbit antibody in Triton 06 Array 3

The data obtained from different rabbit antibody concentration varied greatly, compared to data obtained from mouse antibody. Rabbit antibody spots generated a signal-to-noise ratio, which was three times greater than signal-to-noise ratio from mouse antibody. Mouse antibody was labeled with Cy5 and rabbit antibody with Cy3. Cy5 is more light sensitive than Cy3 and also is prone to oxidation due to atmosphere changes. Overall, samples in 1X PBS/0.05% Triton performed better. Since the addition of Triton introduces a halo around the spot, lower concentrations of Triton should be tested to achieve high signal intensity along with low background.

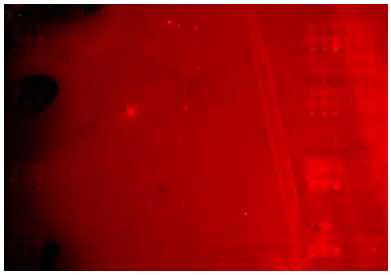
Printing buffer of 1X PBS and 0.001% to 0.05*% Triton-X 100*
Triton concentrations of 0.05%, 0.01% and 0.001% were previously tested on the two different slide substrates, poly-L-lysine and SuperEpoxy, with the three different blocking and detecting assays. From the previous test prints, cross labeling was a major hindrance, along with a halo effect around the spots with the use of 0.25% to 0.1% Triton.

The amount of probe conjugated onto the array was lowered from 12µl to 9µl as a remedy for cross labeling. Since the slide remains wet through out the procedure, the options for successful encasement of each array are limited. The last option would be to only perform one experiment per slide, but due to the cost of the substrates and reagents, it is not feasible.

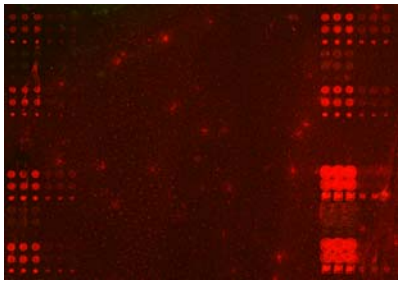
The use of Triton in the printing buffer increases the spot diameter significantly, but it creates a 'do-nut' shaped spot, with a prominent outer ring. The z-axis on the OmniGrid was recalibrated and the depth of the pin contact with the slide decreased. Lower concentrations of Triton were tested since it was hypothesized that Triton crystallizes, therefore causing a ring on the perimeter of the spot.

Only one array was conjugated with the labeled probe, the last array was used as the negative control and PapPen was used in the middle of the slides after they were blocked. Cross labeling did not occur with the lowered probe volume.

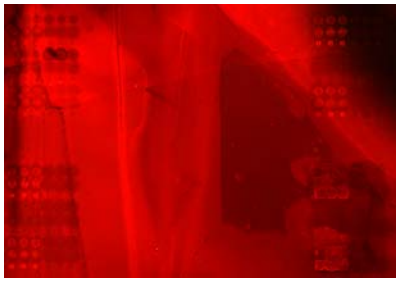
Slide images, along with the highest signal-to-noise ration and median signal intensity are noted for each set of slides. Graphs and data tables comparing the Triton concentration, signal-to-noise ratio, median signal intensity with the three different pins, two slide substrates and three blocking procedures are attached at the end of the paper as supplements.



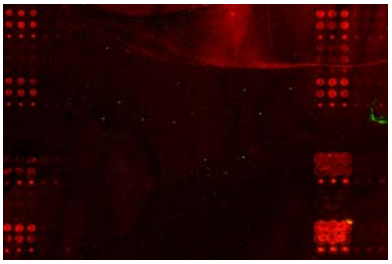
A) Triton 19: Poly-L-Lysine-BSA Blocking
SNR: 3.842, Intensity 625 Triton 0.001%



B) Triton 28: SuperEpoxy-BSA Blocking
SNR: 50.048, Intensity 1081 Triton 0.001%

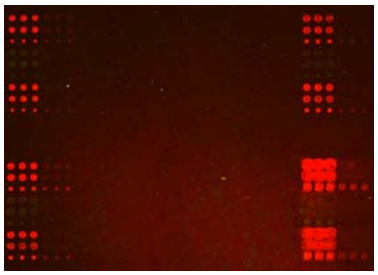


C) Triton 22: Poly-L-lysine-BlockIt buffer
SNR: 5.683, Intensity: 5112 Triton 0.001%



D) Triton 31: SuperEpoxy-BlockIt buffer
SNR: 39.13, Intensity: 443 Triton 0.001%

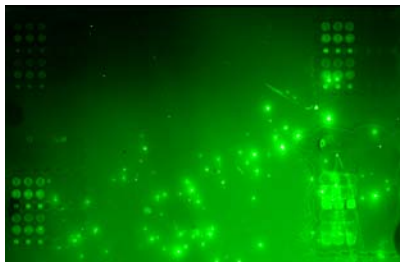
E) Triton 25: Poly-L-lysine-Nonfat milk
SNR: 44.467, Intensity: 5034 Triton 0.001%



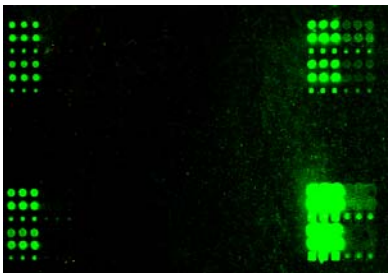
F) Triton 34: SuperEpoxy-Nonfat milk
SNR: 131.341, Intensity: 5095 Triton 0.001%

Figure 22. Poly-L-lysine and SuperEpoxy slides conjugated with 1:50 dilution of anti-mouse IgG with BSA blocking, BlockIt buffer, and nonfat milk blocking.

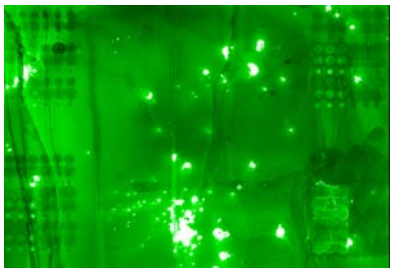
Triton 34 SuperEpoxy slide treated with nonfat blocking solution had the highest signal-to-noise ratio with 0.001% Triton/1X PBS. The spot size with 0.001% Triton was 130µm using the SMP4-2 pin.



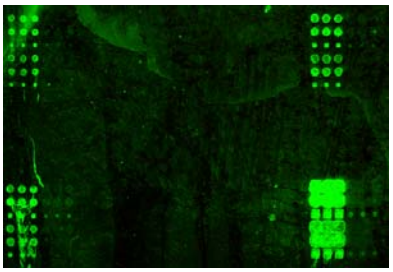
A) Triton-20: Poly-L-Lysine-BSA Blocking
SNR: 114.38, Intensity: 11678 Triton 0.001%



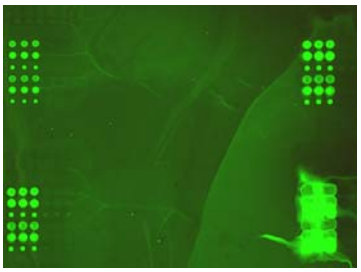
B) Triton 29: SuperEpoxy-BSA Blocking



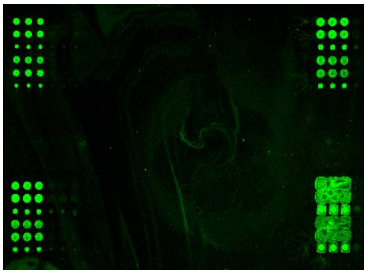
C) Triton 23: Poly-L-lysine-BlockIt buffer
SNR: 7.275, Intensity: 25628 Triton 0.001%



D) Triton 32: SuperEpoxy-BlockIt buffer
SNR: 51.86, Intensity: 2549 Triton 0.001%



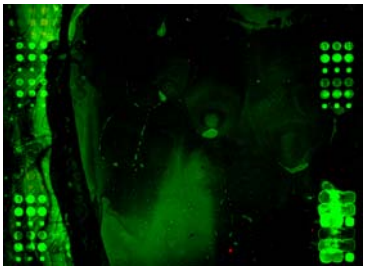
E) Triton 26: Poly-L-lysine-Nonfat milk
SNR: 194.185, Intensity: 31158 Triton 0.001%



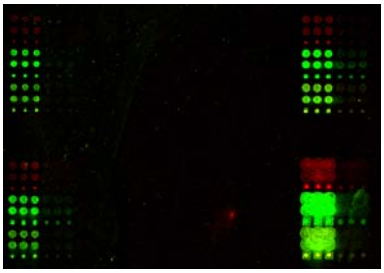
F) Triton 35: SuperEpoxy-Nonfat milk
SNR: 401.459, Intensity: 18088 Triton 0.001%

Figure 23. Poly-L-lysine and SuperEpoxy slides conjugated with 1:50 dilution of anti-rabbit IgG with BSA blocking, BlockIt buffer, and nonfat milk blocking.

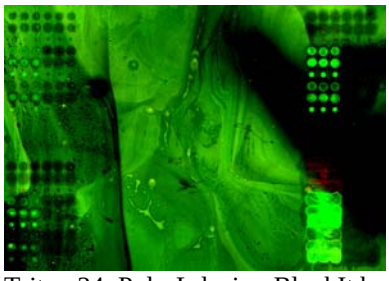
Triton 35 SuperEpoxy slide treated with nonfat blocking solution had the highest signal-to-noise ratio with 0.001% Triton/1X PBS. The spot size with 0.001% Triton was 150µm using the SMP4-1 pin.



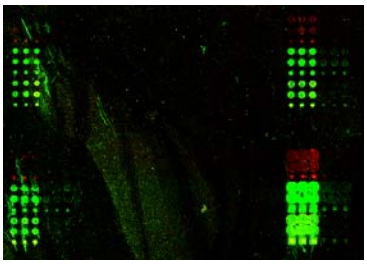
A) Triton-21: Poly-L-Lysine-BSA Blocking
SNR: 28.769, Intensity: 13587 Triton 0.001%



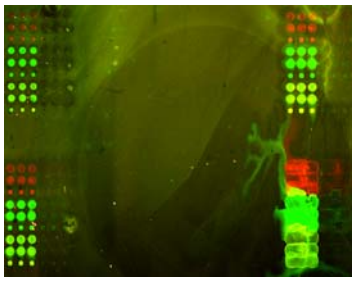
B) Triton 30: SuperEpoxy-BSA Blocking



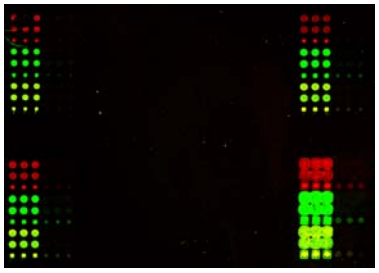
C) Triton 24: Poly-L-lysine-BlockIt buffer
SNR: 26.7, Intensity: 169.73 Triton 0.001%



D) Triton 33: SuperEpoxy-BlockIt buffer
SNR: 33.678, Intensity: 3004, Triton 0.001%



E) Triton 27: Poly-L-lysine-Nonfat milk
SNR: 160.536, Intensity: 31537, Triton 0.001%



F) Triton 36: SuperEpoxy-Nonfat milk
SNR: 420.64, Intensity: 12379, Triton 0.001%

Figure 24. Poly-L-lysine and SuperEpoxy slides conjugated with 1:50 dilution of anti-mouse and anti-rabbit IgG with BSA blocking, BlockIt buffer, and nonfat milk blocking.

Triton 36 SuperEpoxy slide treated with nonfat blocking solution had the highest signal-to-noise ratio with 0.001% Triton/1X PBS. The spot size with 0.001% Triton was 140µm using the SMP4-2 pin.

The three sets of conjugations testing the blocking methods, slide surface and printing buffer concentration produce very consistent results. By comparing the signal-to-noise ratio and signal intensity from each set of spots, triton concentration of 0.001% results in the highest signal intensity. The low concentration of triton results in smaller spot diameter, but the amount of signal is not compromised. The spot uniformity is significantly better than with higher triton concentrations and there is no presence of a ring on the perimeter of the spot.
Nonfat milk blocking consistently gave low nonspecific binding with increased signal intensity on SuperEpoxy slides.

SUMMARY

Significant knowledge and data has been acquired since the start of this project. Key questions have been addressed, but some still remain unanswered. We have been able to test different labeled probe concentrations, along with two slide substrates and three different blocking methods. Optimal printing conditions have been reached, but it has only been focused on mouse and rabbit IgGs. We have yet to test these conditions on the DARPP-32 array due to time limitations.

We have not yet established an optimal way to perform quality control on the antibody slides. Altering protein gel stains for glass substrates has not been successful. It is possible to use labeled anti-mouse and anti-rabbit IgGs for quality control and will be tested on the future DARPP-32 protein arrays.

It has been a challenge to obtain reliable protein concentrations. Even though we have been working with known protein concentrations for all the experiments, we have not had success replicating the protein concentrations using the spectrophotometer. We might have to employ another tool such as the Bradford assay along with the spectrophotometer readings to obtain true protein concentrations.

Future steps

The next step will be to print mouse IgG, rabbit IgG, anti-mouse IgG, anti-rabbit IgG, GST protein, anti-GST antibody, BSA, M6 monoclonal antibody, R911 rabbit polyclonal antibody in .001% Triton/1X PBS. SuperEpoxy slides will be used, along with nonfat milk blocking solution. The sample volume will be kept at 9ul and one array per slide will hybridized with the labeled probe and one array will be used as a negative control. The printed proteins will be detected with combinations of labeled mouse IgG, rabbit IgG, anti-mouse IgG, anti-rabbit IgG, GST protein, anti-GST. With the addition of positive conjugation controls, the binding efficiency of M6 monoclonal antibody with mouse IgG and the binding efficiency of R911 rabbit polyclonal antibody with rabbit IgG will be tested. Once the data is computated from the conjugations, the focus will be placed on DARPP-32.

DARPP-32 protein will be printed on SuperEpoxy slides in its dephosphorylated state and two phosphorylated states along with controls. R500 polyclonal antibody and R911 antibody will be labeled with Alexa 532 and Alexa 647 and hybridized onto the DARPP-32 array. Also, the peptides will be immobilized on the SuperEpoxy slides and detected with the labeled DARPP-32 protein.

Work will continue to be done on the production of a successful DARPP-32 array in the following weeks.

ACKNOWLEDGEMENTS

I would like to thank Dr. Janet Hager for all her help and guidance throughout this project, Dr. Angus Nairn for providing the DARPP-32 protein and peptides, Dr. Eva Sapi for her suggestions with the paper and members of the Keck Microarray Facility for their help and support.

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