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# Applications of High Content Antibody Microarrays for Biomarker Discovery and Tracking Cellular Signaling

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## Abstract

Fuelled by advances in our understanding of the human genome and proteome and the increasing availability of pan- and phosphosite-specific antibodies, antibody microarrays have emerged as powerful tools for interrogating protein expression and post-translational modifications, as well as for uncovering interactions with other proteins and small molecules. This economical platform permits ultra-sensitive semi-quantitative measurements of hundreds of proteins simultaneously using only minute amounts of biofluid, tissue and cell specimens. Recent innovations in the design and fabrication of antibody microarrays and in sample preparation have permitted further refinements of the technology to yield significant improvements in data quality. In this review, we described the applications of antibody microarrays for disease biomarker discovery, highlighting how biological complexity and sample handling have compromised earlier work, and how this technology can be exploited for tracking the expression, phosphorylation and ubiquitination of proteins in crude cell and tissue lysate samples. We also suggest how antibody microarrays can be used to uncover protein-protein and protein kinase-drug interactions with unfractionated lysates.

**Keywords:** Antibody Microarray; Biomarker; Cell Signaling; Phosphorylation; Protein Kinase

## Introduction

Antibody microarrays have an unparalleled potential for the simultaneous monitoring of thousands of proteins found in crude unfractionated biofluids and lysates from cells and tissues, with sensitivities that surpass that of any of the competing technologies. Detection of target proteins within the 1-50 pg/ml range has been successfully achieved with antibody microarrays [1-4]. Microarrays with as many as 5264 individual antibody spots have been successfully produced, as demonstrated with our latest generation Kinex™ KAM-1300 slides from Kinexus. Not only can antibody microarrays be used to track changes in protein expression, but also alterations in post-translational modifications, protein-protein, and protein-drug interactions.

The earliest descriptions of antibody arrays in the scientific literature in 1983 were by Tse Wen Chang, when he developed matrices of up to 400 antibody spots printed on glass cover slips to sort and quantify immune cells based on their expression of CD and HLA allotypic antigens [5]. However, it was not until the work of Roger Ekins and his colleagues [6-8] and improvements in microarray printing technologies in the 1990s that general interest in the development and applications of antibody microarrays really began to galvanize in the field. As illustrated in Figure 1, the number of annual publications related to "antibody microarrays" rapidly escalated after 2001, but then plateaued after 2008. During this growth period, several commercial antibody microarrays were launched (Figure 1), and this supported many of the published studies that covered a wide range of applications of antibody microarrays, especially for disease biomarker discovery. However, over the last decade, there has been no further growth in the use of antibody microarrays for biomedical research, and several vendors have since discontinued their sales of high content antibody microarrays (Table 1).

There are several factors that have contributed to limited adoption of antibody microarrays as a research tool for biomarker discovery and other proteomics-based investigations. In this review, we aim to summarize some of the problems that have eroded the confidence of users of this promising technology, and how these may be surmounted so that antibody microarrays can realize their true potential for utility in biomedical research and diagnostics.

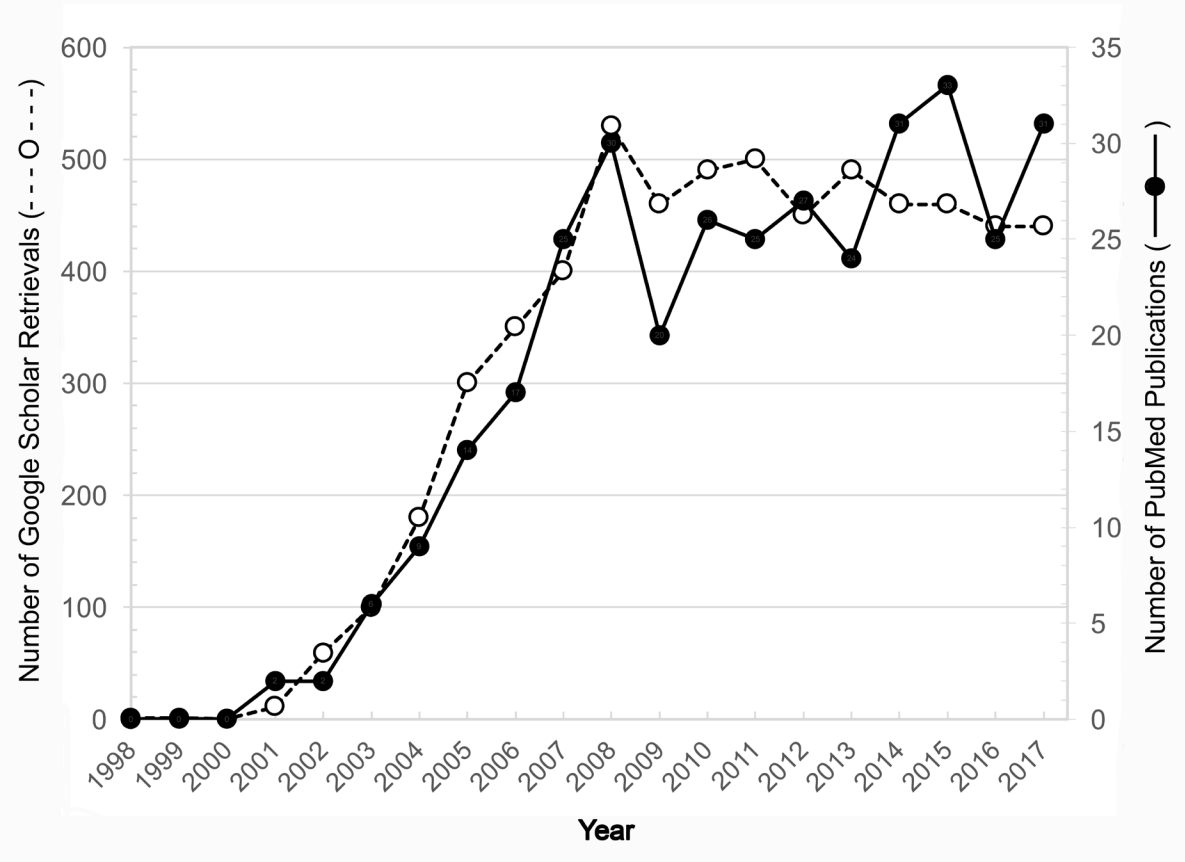


**Figure 1:** Time course of annual rate for antibody microarray publications. The search term was "antibody microarray" and redundant entries from Google Scholar were removed.

| Array Name | Company | Array Type | Unique Pan-Ab | Unique Phospho-Ab | Total Ab Spots/Array | Status |
|---|---|---|---|---|---|---|
| Kinex™ KAM-1300 | Kinexus Bioinformatics | Glass slide | 451 | 865 | 5264 | Available |
| Kinex™ KAM-1150 | Kinexus Bioinformatics | Glass slide | 1150 | 0 | 4600 | Available |
| Cell Signaling Explorer Antibody Array | Full Moon Biosystems | Glass slide | 1358 | 0 | 2716 | Available |
| Phospho Explorer Array PEX100 | Full Moon Biosystems | Glass slide | 630 | 688 | 2636 | Available |
| PlasmaScan | Arrayit Corporation | | 380 | 0 | 2280 | Available |
| Human L1000 Array | RayBiotech | Glass slide | 1000 | 0 | 2000 | Available |

| | | | | | | |
|---|---|---|---|---|---|---|
| Panorama XPRESS Profiler725 | Millipore-Sigma | Glass slide | 738 | 30 | 1536 | Discontinued |
| NF-kB II Phospho-Specific Array | AbNova | Glass slide | 117 | 98 | 1344 | Available |
| Explorer Antibody Array ASB600 | Full Moon Biosystems/ Spring BioScience | Glass slide | 649 | 7 | 1312 | Available |
| Antibody Microarray 500 | Takara Bio –Clontech | Glass slide | 500 | 0 | 1000 | Discontinued |
| LabVision TAA-001 | Thermo Scientific | Glass slide | 720 | 0 | 720 | Discontinued |
| Signal Transduction Antibody Array HM 3000 | Hypromatrix | Nitrocellulose membrane | 395 | 4 | 400 | Available |
| Human RTK Phosphorylation Array C1 | RayBiotech | Nitrocellulose membrane | 71 | 0 | 142 | Available |
| Human Phospho-Receptor Tyrosine Kinase Array Kit | R&D Systems | Nitrocellulose membrane | 49 | 0 | 98 | Available |

**Table 1:** Commercial high content antibody microarrays. "Pan-Ab" refers to antibodies that bind to target proteins independently of their phosphorylation states, whereas "Phospho-Ab" refers to antibodies that are phosphorylation site-specific. In most cases, each antibody was printed with two replicate antibody spots per array, except for the Kinex™ KAM antibody microarrays, which feature four replicates of each antibody.

## Antibodies Probes

Molecular biology-based methods such as DNA amplification with the polymerase chain reaction (PCR), DNA sequencing, site-directed mutagenesis, gene transfection, and recombinant protein expression have provided profound insights into the inner workings of cells. Around 21,300 human genes that encode proteins have become identified, but little is known about the roles and regulation of more than a third of all of these proteins despite their discovery more than 18 years ago. As the main dynamic components that mediate cell structure and function, proteins are highly finicky macromolecules that exhibit diverse structures, enzymatic and other functional activities, and they are subject to a myriad of confounding regulatory controls. A range of immunological techniques that depend on antibodies have been developed to single out proteins so that they can be individually tracked with respect to their expression levels, post-translational modifications, tissue and subcellular locations, and interactions with other proteins. However, this is a daunting task that has actually progressed relatively slowly despite the euphoric adoption of genomic solutions to proteomics problems. While the cost for sequencing entire genomes of humans and other species has plummeted by a million-fold over the last two decades, the pricing of antibodies has not really changed in the last 30 years.

The development of gene microarray technology in which as many as 20,000 complementary oligonucleotide probes for mRNA sequences can be arrayed as individual spots on a 1-inch x 3-inch glass slide actually paved the way for the creation of high density antibody microarrays. In principle, only 10 µg of a particular antibody may be sufficient to be featured as a spot printed with ~0.1 pg of IgG on over 100,000,000 slides. Measurement of a target protein on an antibody microarray requires about 100-times less antibody than required for a typical Enzyme-Linked Immune Sorbent Assay (ELISA) [9]. Consequently, the price of a particular antibody is really secondary to its specificity and potency, and suitability on a microarray.

Over two million antibodies are commercially available, although it is very common for the same antibody to be repackaged and offered by multiple vendors. At Kinexus, we have tested over 6000 commercial antibodies by Western blotting, and have determined that less than a quarter of these have sufficient selectivity and sensitivity to be useful for Western blotting in most applications. Others have found similar results and commented on this problem [10-12]. Nevertheless, with so many antibodies on the market, and the ease with which new antibodies can be produced, these are likely to remain the best reagents for selective binding and detection of proteins and other analytes in the foreseeable future.

The reproducibility and supply of "individual" antibodies have been significant factors in the drive to produce monoclonal antibodies or recombinant antibodies. For example, human recombinant single-chain (Fv) antibodies selected from a large phage display library were successfully incorporated into microarrays [13,14]. However, the costs of monoclonal antibody production are typically 5-times higher than polyclonal antibody production, and monoclonal antibodies are much less humane to

produce. Furthermore, in our experience, most of the monoclonal antibodies that we have rigorously tested by Western blotting are no more potent and specific than many of the polyclonal antibodies that are generated against short peptides and affinity-purified. In theory, since the avidity and stability of antibody-antigen complexes is determined by both the affinity of their binding as well as by the valencies of the binding, polyclonal antibodies with similar specificity to monoclonal antibodies should have a better antigen-capturing capacity than monoclonal antibodies due to the formation of more stable complexes with their targets by binding to multiple epitopes. In fact, at least one independent study reported that polyclonal antibodies displayed superiority over monoclonal antibodies in capturing toxins on microarray slides even when they had similar affinities as determined by ELISA [15].

While recombinant antibodies have the potential to be mutated to further improve specificity and potency, in practice, their high costs render their development suitable for primarily therapeutic purposes. These likely accounts for why so few recombinant antibodies are commercially sold for the research market despite the availability of this technology for over 20 years [16]. Nevertheless, some recombinant antibodies have been successfully deployed in low content custom microarrays [17,18].

## Proteome Complexity

Although about 21,300 genes encode proteins in the human genome, the actual number of distinct protein entities or proteoforms in the human proteome may exceed 10 million due to alternative splicing and post-translational modifications. About 95% of the multi-exon genes in the human genome have been estimated to undergo approximately 100,000 intermediate- to alternative splicing events [19,20]. Amongst more than 50 types of post-translational modifications documented in proteins, phosphorylation is the predominant reversible regulatory mechanism in eukaryotic cells. Reputable on-line databases such as PhosphoSite Plus (www.phosphosite.org) and PhosphoNET (www.phosphonet.ca) list over 250,000 non-redundant human phosphosites with approximately 60% on serine residues, 25% on threonine residues and 15.5% on tyrosine residues. A significant portion of the studies that have identified phosphosites by mass spectrometry (MS) used selected enrichment of phosphotyrosine containing peptides with generic anti-phosphotyrosine antibodies. This is because even in Rous sarcoma virus-transformed fibroblasts that overexpress the protein-tyrosine kinase Src in a highly active form, only about 0.3% of the protein phosphorylation by weight is on tyrosine residues (93% on serine and 7% on threonine residues) [21]. Using alternative enrichment strategies such as strong cation exchange chromatography or immobilized metal ion affinity chromatography (IMAC), the ratio of the numbers of phosphoserine: phosphothreonine: phosphotyrosine sites is closer to 19:5:1. Based on predictions algorithms developed at Kinexus [22], we have posted about 960,000 known and predicted human phosphosites on the PhosphoNET website. Since over 44,000 human phosphotyrosine sites have already been confirmed by MS, and there are apparently 24 phosphoserines and phosphothreonine sites for every phosphotyrosine site in the human phosphoproteome, this supports the existence of over a million human phosphosites. However, about three-quarters of the reported phosphosites from MS have only been documented once or twice as evident on PhosphoSite Plus [23]. Only about 1.5% of these phosphosites have been commonly recorded in at least 100 separate MS studies, with about 3% of this particular subset of phosphosites detected in high frequency over a thousand MS experiments. Amongst the most frequently phosphorylated human proteins are: the cytoskeletal proteins actins (ACTA1-Y55, ACTA2-Y55, ACTB-Y53, ACTG1-Y53, ACTG2-Y54),calponins (TAGLN2-Y192, TAGLN3-Y192), paxillin-Y88+Y118, talin 1-Y70, VASP-Y39, vimentin-Y53+Y61, and vinculin-Y822; the scaffolding proteins Crk-Y221,CrkL-Y207, CTNND1-Y228+Y904, DLG3-Y673, Dok1-Y449, Gab1-Y406+Y659, Hrs-Y216, PDLIM5-Y251, N-WASP-Y256, PZR-Y263, Shc1-Y427, and ZO2-Y1118; the metabolic enzymes enolases (ENO1-Y44, ENO2-Y44, ENO3-Y44), LDH-A-Y239, G6PD-Y503, pyruvate dehydrogenases (PDHA1-Y301, PDHA2-Y299), and phosphoglycerate mutases (PGAM1-Y92, PGAM2-Y92, PGAM4-Y92); and the regulatory proteins calmodulin-Y100, eEF1A1-Y29+Y141, eEF1A2-Y29+Y141, phosphoinositide-3-kinase regulatory subunits (PIK3R1-Y467, PIK3R2-Y464), PTPRA-Y798, Rin1-Y36, SHP2-Y62+Y584, and STAT's (STAT3-Y705, STAT5A-Y694, STAT5B-Y699). As pointed out earlier, since MS studies commonly use anti- phosphotyrosine antibodies to enrich phosphotyrosine-containing peptides, the prevalence of phosphotyrosine in these most frequently phosphorylated proteins may be somewhat biased. However, in view of their ubiquitous presence in cells and tissues in high abundance, they are unlikely to be selective biomarkers for diagnostic purposes.

Over 85% of the 21,300 known human proteins have now been reported to be phosphorylated [23]. Consequently, with potentially a million human phosphosites, many proteins will in fact feature more than 50 phosphosites. Most of these phosphosites tend to be clustered in their distribution in proteins, and are most frequently located right next to each other [22]. This has important ramifications with respect to the utility of phosphosite-specific antibodies, since the presence or absence of a phospho moiety in a flanking phosphosite in a protein could interfere with the recognition of the intended phosphosite by the antibody.

In the selection of suitable phosphorylation sites to target for antibody development, priority has been assigned to phosphosites that have been demonstrated to be functionally important in the literature or are likely to be based on high similarity with phosphosites in related, well characterized proteins. Repeated observation of the phosphorylation of a site in numerous MS studies is also desirable, although there are many instances of phosphosites that are well documented to be regulatory but poorly detected by MS. From a query of the PhosphoSite Plus website with 2742 known phosphosites that have been shown to be regulatory in the literature, we found that 602 (22%) have not been reported by random MS studies, and another 434 (16%) had only been documented in one MS experiment. To identify potentially important phosphosites, we have also investigated

the evolutionary conservation of phosphorylation sites in diverse species, since these sites are likely to be retained if they play a regulatory role. The PhosphoNET website provides data on the evolutionary conservation of 960,000 known and predicted human phosphosites [24].

Mass spectrometry remains the method of choice by many in the field for identifying novel phosphorylation sites and quantifying phosphorylation events in exploratory studies despite the high price of equipment and consumable costs associated with this technology. Using stable isotope tag labeling, MS has been very successfully used to monitor fluctuations in the levels of phosphorylated proteins with high specificity. Indeed, the sequencing of the human genome was critical for this, since phosphopeptides can be easily identified from the charge to mass ratio of predicted peptides from protease digested proteins [25]. However, the requirements of sample pre-processing, including protease cleavages and phosphopeptide enrichment, also pose as potential sources of data inaccuracy and bias, as evidenced by the absence of many previously characterized phosphorylation sites in the largest datasets and by the low data reproducibility observed in replicate samples [26,27]. The lack of antibody reagents for following up on promising leads from MS analyses further reduces confidence in these findings without independent validation. By contrast, antibody microarrays not only define potentially important biomarkers, but also the antibody reagents that can serve to further track them by immunoblotting, immunoprecipitation, immunohistochemistry, ELISA and other antibody-based methods.

Mass spectrometry has also been a powerful tool for the discovery of other types of covalent modifications of proteins. While phosphorylation is the most prevalent post-translational modification, at the time of the preparation of this review, the PhosphoSitePlus website had already annotated 77,335 ubiquitination sites, 38,139 acetylation sites, 18,614 methylation sites, 8,171succinylation sites and 10,242 glycosylation sites in proteins found in humans, rats and/or mice by MS [23].

Despite the enthusiasm for application of MS approaches to track changes in the post-translational modifications of proteins, there are serious limitations that compromise the use of this technology for biomarker discovery and tracking cell signaling systems in addition to the high expense in conducting these analyses. While MS is very sensitive, extensive enrichment and fractionation are required to analyze low abundance cell signaling proteins from tissue and cell lysates. The need for substantial amounts of starting sample lysate protein in milligram amounts has particularly curtailed broad adoption of this methodology in phosphoproteomics studies where clinical specimens are to be analyzed. For phosphoprotein profiling, ideally 2 mg or more lysate protein are required. Phosphoproteins are tracked by serendipity, since only a small portion of the phosphoproteome can be monitored within an MS experiment, and the more abundantly expressed proteins are more likely to be detected after enrichment for phosphopeptides prior to MS analyses. This is why the majority of the most frequently detected phosphoproteins are cytoskeletal proteins, scaffolding proteins, or metabolic pathway enzymes as exemplified above. However, the biggest concern is that this technique is unable to distinguish whether any change in phosphorylation detected is due to an alteration in the total concentration of a particular protein or a change in the stoichiometry of phosphorylation of the protein or some combination of both. Ultimately, the observed change must be validated by use of pan-specific (detects both phosphorylated and non-phosphorylated target proteins) and phosphosite-specific antibodies, if these are even available for the given target.

The sheer complexity of the human proteome within a single tissue or cell type poses a serious problem for proteomics analysis of specimens obtained from most of the tissues and cells found in the human body. In one recent study associated with the Human Proteome Atlas Project, across 56 diverse human cell lines that were tested for mRNA transcripts for 19,628 genes, about 11,490 genes were typically expressed as proteins in most cells, and about half of these were “housekeeping” proteins that were commonly found in all of the cell lines [28]. In another report from the Human Proteome Atlas Project, immunohistochemistry studies with 44 human tissues and organs with 24,028 antibodies corresponding to 16,975 protein-encoding genes in addition to mRNA transcription analyses for 32 of the tissues, similarly revealed expression of approximately half of the protein-coding genes in each analyzed tissue [29]. About 60% of all of the enzymes involved in metabolism were expressed in all of the tissues. The largest number of protein-coding genes were expressed in the brain, testis and liver [29].

Collectively, these studies demonstrate that with alternative splicing and post-translational modifications, more than 100,000 proteoforms may exist within any particular tissue or cell lysate. Under the most optimal conditions, 2D gel electrophoretic techniques such as two-dimensional difference gel electrophoresis (DIGE) [30] can permit the resolution and visualization of under 8,000 protein spots with the most sensitive fluorescent stains. Consequently, tracking changes in protein expression and phosphorylation by 2D gel electrophoresis in earlier proteomics studies was never really up to the challenge and was restricted to primarily monitoring the most abundant of cellular proteins.

Yet another major complicating factor is the high degree of protein-protein interactions that exist within cells and their dynamic nature. With most antibody microarrays, the samples of lysate proteins are incubated with the slides in their native states with only the presence of mild detergents to reduce non-specific interactions (Figure 2). The usage of stronger detergents and other agents that denature or precipitate proteins interferes with capture antibody-antigen interactions on the microarray slides. Any proteins that are in homodimeric or higher homo-oligomeric states are less problematic, since the signal from the label (e.g. biotin or dye) of the lysate proteins will ideally reflect only the target protein, unless the capture antibody also cross-reacts with off target proteins as well. However, it is also possible that many antibody epitopes could be masked when these proteins reside in homo-multimeric complexes.

Of greater concern is the number of proteins that are

bound to other types of proteins in heteromeric complexes. Some of the larger protein complexes include the 40S and 60S ribosomes, proteasomes, nucleosomes, DNA polymerases, RNA polymerases, the TFIIH transcription complex, splicosomes, the anaphase promoting complex, and the mitochondrial respiratory supercomplex. Several enzymes, including many protein kinases and protein phosphatases, are associated with regulatory subunits, and all of the receptor-tyrosine kinases and receptor-tyrosine phosphatases appear to form signalling complexes with other proteins. Capture antibodies on microarrays that target one of the components of these complexes may indirectly retain other proteins, and these interactions within complexes may be subject to regulation. Since these associated proteins are also tagged, they will contribute to the total signal that is detected within an antibody spot on a microarray. Consequently, an apparent increase in the level of a target protein may actually reflect the increased binding of one or more of its partners in a complex, and would represent a false positive event (Figure 2).

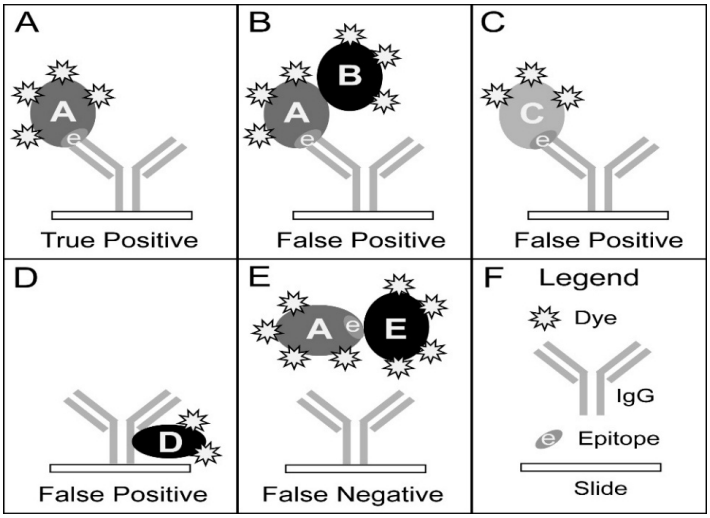


**Figure 2:** Possible issues with antibody microarrays. True positives occur when the target protein that is labeled with a fluorescent dye or another tag specifically binds to the capture antibody in a monomeric form (Panel A), although the target protein can also be bound in a homodimeric or homopolymeric forms. False positives can be generated from signals being generated from associated proteins (Panel B), and cross-reactive off target proteins with the Fab (Panel C) and Fc (Panel D) regions of the capture antibodies. False negative signals can be generated by the binding epitope on the target protein being blocked by interaction with other proteins (Panel E).

Protein phosphorylation, especially on tyrosine residues is well recognized to drive complex formation between signaling proteins by virtue of phosphotyrosine recognition binding domains such as Src-homology-2 (SH2) and protein-tyrosine binding (PTB) domains [31]. Apparently 119 SH2 domains are found in 109 different human proteins and 54 PTB domains reside in another 54 human proteins [22]. Proteins also feature domains that recognize phosphorylated serine or threonine residues for binding. In particular, the seven human 14-3-3 proteins have been reported to interact with over 200 different proteins in complexes [32], and the actual number is likely to be far greater. The formation of these phosphorylation-driven complexes is likely to render the interacting phosphorylation sites inaccessible to capture antibodies on microarrays and generate false negatives.

In addition to problems of protein-protein interactions, the location of proteins embedded in cellular membranes and organelles presents yet another challenge to the detection of proteins of interest with antibodies on microarrays. Approximately 30% of the proteins in the human proteome reside in membranes, and many of these are important therapeutic drug targets [33]. However, in one study, only 1 in 20 commercially available antibodies that were tested were determined to be suitable for targeting detergent-extracted membrane proteins in a microarray [34]. Some proteins, such as protein kinase C isoforms and CTP: Phosphocholinecytidylyl Transferase (PCYT1) can exhibit profound changes in their location between cytosolic and microsomal preparations of lysates depending on how these enzymes are stimulated in cells [35,36]. Likewise, many cytoskeletal proteins may be insoluble in their polymerized forms, but soluble in their monomeric forms. The states of polymerization of many cytoskeletal proteins can change profoundly during the cell cycle in proliferating cells. This is one of the reasons why abundant cytoskeletal proteins such as actins and tubulins are not reliable loading controls for Western blotting studies of lysates prepared for cell signaling research.

Finally, in the case of phosphoproteomics studies, the post-homogenisation instability of phosphorylation sites due to endogenous phosphatases in lysates of cells and tissues is a major problem that is poorly appreciated in the field. We recently demonstrated that most of the phosphorylations of the EGF receptor and other downstream signaling proteins were lost when human A431 epidermoid carcinoma cells, which over-expressed this receptor-tyrosine kinase, were homogenized in buffers that contained a wide range of standard protease and phosphatase inhibitors that are routinely used in most cell signaling studies [37]. We found that these phosphorylation events were preserved if the cells were homogenized directly into SDS-PAGE sample buffer, which contains 1% Sodium Dodecylsulfate (SDS). However, such a concentration of SDS almost completely abolishes antibody-antigen interactions on antibody microarrays. Based on our experiments, we believe that most phosphoproteomics studies performed in the past in other laboratories and ours have substantially under-estimated *in vivo* protein phosphorylation. One exception appears to be in MS studies where cells and tissues were directly homogenized in strong chaotropic agents such as urea and guanidine hydrochloride, and then subjected to trypsin treatment to generate phosphopeptides that were later enriched, resolved and analyzed by MS. These harsh treatments provided better recovery of phosphopeptides.

## Biomarker Discovery

One of the most promising applications of antibody microarrays is for the discovery of biomarkers for disease diagnosis. His-

torically, most of these efforts have been focused on the early detection of cancer, especially in the pancreas, ovaries, and lungs, where prognosis is very poor by the time the cancers are more symptomatic. Table 2 provides a summary of many of the reported studies where antibody microarrays were employed to search for biomarkers of cancer and a few other diseases. Many of these reports utilized biofluids such as serum, Cerebral Spinal Fluid (CSF) and in one study exhaled breath [54].

| Disease | Cancer Type | Biofluid | Tissues | Cells |
|---|---|---|---|---|
| **Cancer** | Bladder | Serum - Sanchez-Carbayo et al. [38] | Srinivasan et al. [39]; Lee et al. [40] | |
| **Cancer** | Breast | Serum - Carlsson et al. [41]; Böhm et al. [17]; Li et al. [42] | | Song et al. [43]; Fares et al. [44] |
| **Cancer** | Colon | | Kit et al. [45] | Sreekumar et al. [46] |
| **Cancer** | Gastric | Serum - Hao et al. [47]; Cui et al. [48] | Cui et al. [48] | |
| **Cancer** | Kidney | Serum - Lukesova et al. [49] | | Reiman et al. [50] |
| **Cancer** | Liver | | | Nonomura et al. [51]; Di Masi et al. [52] |
| **Cancer** | Lung | Serum - Gao et al. [53] Exhaled breath - Kullmann et al. [54] | Bartling et al. [55] | |
| **Cancer** | Lymphomas | Serum - Schröder et al. [56] | | Ghobrial et al. [57]; Díez et al. [58] |
| **Cancer** | Melanoma | | Kaufman et al. [59]; Moschos et al. [60] | |
| **Cancer** | Oral | | Ibrahim et al. [61] | |
| **Cancer** | Osteosarcoma | Serum - Zhu et al. [62] | | |
| **Cancer** | Ovarian | Serum - Loch et al. [63]; Ramirez et al. [64]; Ramirez et al. [65] | | |
| **Cancer** | Pancreatic | Serum - Hamelinck et al. [66]; Orchekowski et al. [67]; Chen et al. [68]; Ingvarsson et al. [69]; Li et al. [70]; Gerdtsson et al. [71]; Mirus et al. [72]; Urine - Schröder et al. [9] | | Shi et al. [73]; Alhamdani et al. [74] |
| **Cancer** | Prostate | Serum - Miller et al. [75]; Shafer et al. [76]; Wan et al. [77]<br>Prostatic fluid - Fujita et al. [78] | | El-Haibi et al. [79] |
| **Cancer** | Rectal | | Yoshikawa et al. [80]; Madoz-Gurpide et al. [81] | |
| **Alzheimer's** | | CSF - Olah et al. [82] | Arnold et al. [83] | |
| **Cystic fibrosis** | | Serum - Srivastava et al. [84] | | |
| **Glaucomas** | | Aqueous humor - Sacca et al. [85] | | |

**Table 2:** Examples of reported studies in the literature to identify biomarkers of disease using antibody microarrays and human clinical specimens.

Due to its large volume, high protein concentration (typically 60-80 mg/ml) and easy access with relatively little discomfort to patients, blood serum has been the biofluid of choice for most studies in the search for disease biomarkers. Serum (after removal of fibrin from plasma) can serve as a systemic mirror of more distantly localized tumors that release proteins or protein-containing exosomes [86]. Plasma may contain as many as 40,000 different proteoforms generated from a thousand distinct gene products, but their individual concentrations can range over a trillion-fold. Only a few proteins actually account for the bulk of the total protein mass in plasma. About 55% of the total protein concentration of human blood plasma comes from albumins, another 38% from globulins such as antibodies, and 7% from fibrinogen. Consequently, these abundant proteins are typically removed to enrich the remaining proteins prior to most proteomics analyses [87]. This can be achieved by using commercial kits [9,72] orcibacron blue chromatography, which has a high affinity for albumin [63]. Significantly, in one antibody microarray study [9] that used the ProteoPrep 20 Plasma Immuno depletion kit from Sigma-Aldrich, which removes 20 of the highly abundant serum proteins with antibodies immobilized on agarose beads, the levels of more than 196 proteins (representing 26% of the detected proteins) were statistically affected, with most of these showing reductions rather than enrichments. While the removal of the most abundant serum proteins may be necessary for MS analyses, unfractionated serum can be directly applied to antibody microarrays due to the insensitivity of most antibodies to these serum proteins. Other human biofluids with proteins such as urine (normally 0-2 mg/ml) [11], CSF (normally 0.2-0.6 mg/ml) [82] and prostatic fluid (~50 mg/ml) [78] have also been successfully interrogated with antibody microarrays.

One of the most commonly used biomarkers for prostate cancer is the prostate specific antigen (PSA), which is the androgen-related serine protease Kallikrein-related peptidase 3 (KLK3). PSA is mainly expressed in the prostate epithelium and is found in seminal fluid at about 1 mg/ml, but its concentration in serum is normally more than 500,000-times lower at 2 ng/ml or less. It is likely that other disease biomarkers also have to be detectable at concentrations in serum that are in the order of 100,000- to a million-fold lower levels than found in the organs from which they originate. Therefore, it may be more instructive to initially identify biomarker proteins in diseased organs and tissues, and then subsequently search for the most promising lead biomarkers in serum specimens. Table 3 identifies several studies with antibody microarrays that utilized tumors, normal tissue, and diverse cancer cell lines to discern potential cancer biomarkers. It is evident from inspection of this list, that a few of the studies were contradictory with respect to some of the observed changes in biomarkers in the same types of cancer (e.g. serum C3, C4, Cathespin D, MCP-1 and VEGF in pancreatic cancer, and serum von Wille brand factor in prostate cancer). However, often the same proteins were similarly affected in diverse cancers. It should be appreciated that no single biomarker, including PSA, is reliable by itself for disease diagnosis. However, monitoring a panel of validated biomarkers can provide diagnosis with high accuracy, which further supports the need for platforms like antibody microarrays that are able to monitor multiple analytes of interest.

| Biomarker Protein | Disease | Specimen Type | Change | Reference |
|---|---|---|---|---|
| Alkaline phosphatase | Pancreatic cancer | Serum | Up | Orchekowski et al. [67] |
| Alkaline phosphatase | Prostate cancer | Serum | Up | Shafer et al. [76] |
| Angiostatin | Bladder cancer | Serum | Up | Sanchez-Carbayo et al. [38] |
| Angiostatin | Pancreatic cancer | Serum | Down | Hamelinck et al. [66] |
| ATT (alpha-1-antitrypsin) | Lung cancer | Serum | Up | Gao et al. [53] |
| ATT (alpha-1-antitrypsin) | Pancreatic cancer | Serum | Up | Orchekowski et al. [67] |
| ATT (alpha-1-antitrypsin) | Pancreatic cancer | Serum | Up | Hamelinck et al. [66] |
| BAK1 (Bcl-2 homologous antagonist/ killer) | Cystic fibrosis | Serum | Up | Srivastava et al. [84] |

| | | | | |
|---|---|---|---|---|
| BAK1 (Bcl-2 homologous antagonist/ killer) | Diffuse large B-cell lymphoma | Plasma | Down | Schröder et al. [56] |
| c-Myc | Colon cancer | Tumors | Up | Kit et al. [45] |
| c-Myc | Colorectal cancer | Tumoural mucosa | Up | Madoz-Gurpide et al. [81] |
| C3 (Complement component 3) | Breast cancer | Serum | Up | Böhm et al. [17] |
| C3 (Complement component 3) | Breast cancer-metastatic | Serum | Up | Carlsson et al. [41] |
| C3 (Complement component 3) | Pancreatic cancer | Serum | Down | Hamelinck et al. [66] |
| C3 (Complement component 3) | Pancreatic cancer | Serum | Up | Ingvarsson et al. [69] |
| C3 (Complement component 3) | Prostate cancer | Serum | Down | Shafer et al. [76] |
| C4 (Complement component 4) | Breast cancer-metastatic | Serum | Up | Carlsson et al. [41] |
| C4 (Complement component 4) | Pancreatic cancer | Serum | Down | Hamelinck et al. [66] |
| C4 (Complement component 4) | Pancreatic cancer | Serum | Up | Ingvarsson et al. [69] |
| C5 (Complement component 5) | Breast cancer-metastatic | Serum | Up | Carlsson et al. [41] |
| C5 (Complement component 5) | Pancreatic cancer | Serum | Up | Ingvarsson et al. [69] |
| CA 19-9 glycan | Pancreatic cancer | Serum | Up | Mirus et al. [72] |
| CA 19-9 glycan | Pancreatic cancer | Serum | Up | Chen et al. [68] |
| CA 19-9 glycan | Prostate cancer | Serum | Up | Shafer et al. [76] |
| Caspase 7 | Bladder cancer | Tumors | Up | Lee et al. [40] |
| Caspase 7 | Colorectal cancer | Tumoral mucosa | Down | Madoz-Gurpide et al. [81] |
| Cathepsin D | Pancreatic cancer | Serum | Down | Hamelinck et al. [66] |
| Cathepsin D | Pancreatic cancer | Serum | Up | Orchekowski et al. [67] |
| CBX2 (Chromobox protein homolog 2) | Alzheimer's disease | Brain tissue | Up | Arnold et al. [83] |

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|---|---|---|---|---|
| CBX2 (Chromobox protein homolog 2) | Cystic fibrosis | Serum | Up | Srivastava et al. [84] |
| CD3 (T-cell receptor subunit zeta) | Gastric cancer | Serum | Down | Hao et al. [47] |
| CD3 (T-cell receptor subunit zeta) | Squamous cell lung cancer | Tissue | Down | Bartling et al. [55] |
| CD44 (Hyaluronic acid receptor) | Lymph node melanomas | Tissue | Up | Kaufman et al. [59] |
| CD44 (Hyaluronic acid receptor) | Prostate cancer | Serum | Down | Shafer et al. [76] |
| CHK1 (Checkpoint kinase-1) | Bladder cancer | Tumors | Up | Lee et al. [40] |
| CHK1 (Checkpoint kinase-1) | Colorectal cancer | Tumoural mucosa | Up | Madoz-Gurpide et al. [81] |
| CRP (C-reactive protein) | Diffuse large B-cell lymphoma | Plasma | Up | Schröder et al. [56] |
| CRP (C-reactive protein) | Lung cancer | Serum | Up | Gao et al. [53] |
| CRP (C-reactive protein) | Pancreatic cancer | Serum | Up | Orchekowski et al. [67] |
| CRP (C-reactive protein) | Pancreatic cancer | Serum | Up | Hamelinck et al. [66] |
| CTNNB1 (Catenin, beta 1) | Colorectal cancer | Tumoral mucosa | Up | Madoz-Gurpide et al. [81] |
| CTNNB1 (Catenin, beta 1) | Cystic fibrosis | Serum | Up | Srivastava et al. [84] |
| ErbB2 (HER2, Neu) | Bladder cancer | Tumors | Up | Lee et al. [40] |
| ErbB2 (HER2, Neu) | Pancreatic cancer | Serum | Up | Mirus et al. [72] |
| Fas (TNFR superfamily, member 6) | Alzheimer's disease | Brain tissue | Down | Arnold et al. [83] |
| Fas (TNFR superfamily, member 6) | Breast cancer | Serum | Up | Li et al. [42] |
| Fas (TNFR superfamily, member 6) | Chronic lymphocytic leukemia | Plasma | Up | Schröder et al. [56] |
| Gelsolin | Lung cancer | Serum | Down | Gao et al. [53] |
| Gelsolin | Pancreatic cancer | Serum | Down | Orchekowski et al. [67] |
| GRO-alpha (Growth-related oncogene-alpha) | Lung cancer | Exhaled breath | Up | Kullmann et al. [54] |

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|---|---|---|---|---|
| GRO-alpha (Growth-related oncogene-alpha) | Osteosarcoma | Serum | Up | Zhu et al. [62] |
| HGF (Hepatocyte growth factor) | Osteosarcoma | Serum | Up | Zhu et al. [62] |
| HGF (Hepatocyte growth factor) | Prostate cancer | Prostatic fluid | Up | Fujita et al. [78] |
| HIF3A (Hypoxia-inducible factor-3alpha) | Ovarian cancer | Serum | Up | Loch et al. [63] |
| HIF3A (Hypoxia-inducible factor-3alpha) | Ovarian cancer | Serum | Up | Ramirez et al. [65] |
| Hsp60 (Heat shock 60 kDa protein) | Breast cancer | Serum | Up | Böhm et al. [17] |
| Hsp60 (Heat shock 60 kDa protein) | Glaucoma (primary open angle) | Aqueous humor | Up | Sacca et al. [85] |
| IgA (Immunoglobulin A) | Bladder cancer | Serum | Down | Sanchez-Carbayo et al. [38] |
| IgA (Immunoglobulin A) | Bladder cancer | Tumors | Down | Lee et al. [40] |
| IgA (Immunoglobulin A) | Pancreatic cancer | Serum | Up | Orchekowski et al. [67] |
| IgA (Immunoglobulin A) | Prostate cancer | Serum | Down | Shafer et al. [76] |
| IGF-1 (insulin-like growth factor-1) | Pancreatic cancer | Serum | Down | Orchekowski et al. [67] |
| IGF-1 (insulin-like growth factor-1) | Prostate cancer | Serum | Up | Shafer et al. [76] |
| IgG (Immunoglobulin G) | Bladder cancer | Serum | Down | Sanchez-Carbayo et al. [38] |
| IgG (Immunoglobulin G) | Prostate cancer | Serum | Down | Miller et al. [75] |
| IgG (Immunoglobulin G) | Prostate cancer | Serum | Down | Shafer et al. [76] |
| IgM (Immunoglobulin M) | Bladder cancer | Serum | Down | Sanchez-Carbayo et al. [38] |
| IgM (Immunoglobulin M) | Pancreatic cancer | Serum | Down | Hamelinck et al. [66] |
| IgM (Immunoglobulin M) | Prostate cancer | Serum | Down | Miller et al. [75] |
| IL-1A Interleukin-1, alpha) | Chronic lymphocytic leukemia | Plasma | Up | Schröder et al. [56] |
| IL-1A Interleukin-1, alpha) | Pancreatic cancer | Serum | Up | Mirus et al. [72] |

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|---|---|---|---|---|
| IL-1A Interleukin-1, alpha) | Prostate cancer | Serum | Up | Shafer et al. [76] |
| IL-2R-alpha (Interleukin-2 receptor-alpha) | Pancreatic cancer | Serum | Up | Gerdtsson et al. [71] |
| IL-2R-alpha (Interleukin-2 receptor-alpha) | Pancreatic cancer | Serum | Up | Mirus et al. [72] |
| IL-2R-alpha (Interleukin-2 receptor-alpha) | Prostate cancer | Serum | Up | Shafer et al. [76] |
| IL-4 (Interleukin-4) | Pancreatic cancer | Serum | Up | Gerdtsson et al. [71] |
| IL-4 (Interleukin-4) | Pancreatic cancer | Serum | Up | Ingvarsson et al. [69] |
| IL-5 (Interleukin-5) | Breast cancer-metastatic | Serum | Down | Carlsson et al. [41] |
| IL-5 (Interleukin-5) | Pancreatic cancer | Serum | Up | Ingvarsson et al. [69] |
| IL-6 (Interleukin-6) | Breast cancer | Serum | Up | Böhm et al. [17] |
| IL-6 (Interleukin-6) | Osteosarcoma | Serum | Up | Zhu et al. [62] |
| IL-6 (Interleukin-6) | Pancreatic cancer | Serum | Down | Gerdtsson et al. [71] |
| IL-8 (Interleukin-8) | Breast cancer-metastatic | Serum | Up | Carlsson et al. [41] |
| IL-8 (Interleukin-8) | Chronic lymphocytic leukemia | Plasma | Up | Schröder et al. [56] |
| IL-8 (Interleukin-8) | Osteosarcoma | Serum | Up | Zhu et al. [62] |
| IL-8 (Interleukin-8) | Pancreatic cancer | Serum | Down | Ingvarsson et al. [69] |
| IL-8 (Interleukin-8) | Pancreatic cancer | Serum | Down | Orchekowski et al. [67] |
| IL-10 (Interleukin-10) | Lung cancer | Exhaled breath | Down | Kullmann et al. [54] |
| IL-10 (Interleukin-10) | Osteosarcoma | Serum | Up | Zhu et al. [62] |
| IL-12 (Interleukin-12) | Chronic lymphocytic leukemia | Plasma | Up | Schröder et al. [56] |
| IL-12 (Interleukin-12) | Pancreatic cancer | Serum | Down | Ingvarsson et al. [69] |
| IL-12 (Interleukin-12) | Prostate cancer | Prostatic fluid | Up | Fujita et al. [78] |

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|---|---|---|---|---|
| IL-13 (Interleukin-13) | Pancreatic cancer | Serum | Down | Gerdtsson et al. [71] |
| IL-13 (Interleukin-13) | Pancreatic cancer | Serum | Up | Ingvarsson et al. [69] |
| JNK1 (MAPK8) | Colorectal cancer | Tumoral mucosa | Up | Madoz-Gurpide et al. [81] |
| JNK1 (MAPK8) | Pancreatic cancer | Serum | Down | Gerdtsson et al. [71] |
| LDL (Low-density lipoprotein) | Pancreatic cancer | Serum | Down | Gerdtsson et al. [71] |
| LDL (Low-density lipoprotein) | Pancreatic cancer | Serum | Down | Hamelinck et al. [66] |
| MAP3K12 (ZPK; DLK) | Ovarian cancer | Serum | Up | Loch et al. [63] |
| MAP3K12 (ZPK; DLK) | Ovarian cancer | Serum | Up | Ramirez et al. [65] |
| MAP3K3 (MEK kinase-3; MEKK3) | Ovarian cancer | Serum | Up | Loch et al. [63] |
| MAP3K3 (MEK kinase-3; MEKK3) | Ovarian cancer | Serum | Up | Ramirez et al. [65] |
| MCP-1 (Monocyte chemoattractant protein-1) | Liver cancer | Serum | Up | Lukesova et al. [49] |
| MCP-1 (Monocyte chemoattractant protein-1) | Osteosarcoma | Serum | Up | Zhu et al. [62] |
| MCP-1 (Monocyte chemoattractant protein-1) | Pancreatic cancer | Serum | Down | Ingvarsson et al. [69] |
| MCP-1 (Monocyte chemoattractant protein-1) | Pancreatic cancer | Serum | Up | Gerdtsson et al. [71] |
| MSLN (Mesothelin) | Ovarian cancer | Serum | Up | Ramirez et al. [65] |
| MSLN (Mesothelin) | Ovarian cancer | Serum | Up | Loch et al. [63] |
| MUC1 (Membrane-bound mucin) | Lung cancer | Serum | Up | Gao et al. [53] |
| MUC1 (Membrane-bound mucin) | Pancreatic cancer | Serum | Up | Chen et al. [68] |
| MUC1 (Membrane-bound mucin) | Prostate cancer | Serum | Up | Shafer et al. [76] |
| MUC16 (Mucin-16; CA125) | Ovarian cancer | Serum | Up | Ramirez et al. [65] |
| MUC16 (Mucin-16; CA125) | Ovarian cancer | Serum | Up | Loch et al. [63] |

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|---|---|---|---|---|
| Myoblast determination protein 1 | Alzheimer's disease | Brain tissue | Down | Arnold et al. [83] |
| Myoblast determination protein 1 | Glaucoma (primary open angle) | Aqueous humor | Up | Sacca et al. [85] |
| PAR4 (Protease-activated receptor 4; F2RL3) | Alzheimer's disease | Cerebral spinal fluid | Down | Olah et al. [82] |
| PAR4 (Protease-activated receptor 4; F2RL3) | Colorectal cancer | Tumoral mucosa | Up | Madoz-Gurpide et al. [81] |
| PDGF (Platelet-derived growth factor) | Bladder cancer | Tumors | Up | Lee et al. [40] |
| PDGF (Platelet-derived growth factor) | Liver cancer | Serum | Up | Lukesova et al. [49] |
| PDGF (Platelet-derived growth factor) | Osteosarcoma | Serum | Up | Zhu et al. [62] |
| PDGF (Platelet-derived growth factor) | Parkinson's disease | Serum | Up | Mahlknecht et al. [88] |
| PIVKA II | Prostate cancer | Serum | Up | Shafer et al. [76] |
| PIVKA-II | Pancreatic cancer | Serum | Up | Orchekowski et al. [67] |
| PLCG1 (Phospholipase C-gamma1) | Gastric cancer | Serum | Down | Hao et al. [47] |
| PLCG1 (Phospholipase C-gamma1) | Glaucoma (primary open angle) | Aqueous humor | Up | Sacca et al. [85] |
| Rb (Retinoblastoma protein) | Ovarian cancer | Serum | Up | Loch et al. [63] |
| Rb (Retinoblastoma protein) | Ovarian cancer | Serum | Up | Ramirez et al. [65] |
| RhoGDI (Rho GDP-dissociation inhibitor) | Cystic fibrosis | Serum | Up | Srivastava et al. [84] |
| RhoGDI (Rho GDP-dissociation inhibitor) | Lymph node melanomas | Tissue | Up | Moschos et al. [60] |
| RPS6-phospho (40S ribosomal protein S6) | Ovarian cancer | Serum | Down | Ramirez et al. [65] |
| RPS6-phospho (40S ribosomal protein S6) | Ovarian cancer | Serum | Down | Loch et al. [63] |
| Sialyl Lewis x | Breast cancer-metastatic | Serum | Up | Carlsson et al. [41] |
| Sialyl Lewis x | Pancreatic cancer | Serum | Up | Gerdtsson et al. [71] |
| TGFB1 (Transforming growth factor-beta) | Oral squamous cell carcinoma | Tissue | Up | Ibrahim et al. [61] |

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|---|---|---|---|---|
| TGFB1 (Transforming growth factor-beta) | Osteosarcoma | Serum | Up | Zhu et al. [62] |
| TNFa (Tumor necrosis factor-alpha) | Chronic lymphocytic leukemia | Plasma | Up | Schröder et al. [56] |
| TNFa (Tumor necrosis factor-alpha) | Pancreatic cancer | Serum | Down | Orchekowski et al. [67] |
| TNFa (Tumor necrosis factor-alpha) | Pancreatic cancer | Serum | Down | Gerdtsson et al. [71] |
| TP63 (Tumor protein p63) | Bladder cancer | Serum | Up | Sanchez-Carbayo et al. [38] |
| TP63 (Tumor protein p63) | Colorectal cancer | Tumoral mucosa | Up | Madoz-Gurpide et al. [81] |
| Transferrin | Lung cancer | Serum | Down | Gao et al. [53] |
| Transferrin | Pancreatic cancer | Serum | Down | Hamelinck et al. [66] |
| Transferrin | Pancreatic cancer | Serum | Down | Orchekowski et al. [67] |
| Transferrin | Prostate cancer | Serum | Down | Shafer et al. [76] |
| VEGF (Vascular endothelial growth factor) | Osteosarcoma | Serum | Up | Zhu et al. [62] |
| VEGF (Vascular endothelial growth factor) | Pancreatic cancer | Serum | Down | Hamelinck et al. [66] |
| VEGF (Vascular endothelial growth factor) | Pancreatic cancer | Serum | Down | Ingvarsson et al. [69] |
| VEGF (Vascular endothelial growth factor) | Pancreatic cancer | Serum | Up | Gerdtsson et al. [71] |
| VEGF (Vascular endothelial growth factor) | Prostate cancer | Serum | Up | Shafer et al. [76] |
| vWF (von Willebrand factor) | Pancreatic cancer | Serum | Up | Hamelinck et al. [66] |
| vWF (von Willebrand factor) | Prostate cancer | Serum | Down | Shafer et al. [76] |
| vWF (von Willebrand factor) | Prostate cancer | Serum | Up | Miller et al. [75] |

**Table 3:** Common protein biomarkers of human disease identified using antibody microarrays and clinical specimens. “Change” refers to how the level of the biomarker was affected with the disease relative to biofluid specimens from healthy individuals or from patient-matched control tissues.

Unfortunately, many promising disease biomarkers from proteomics studies ultimately do not pan out in a clinical context, because there are often large differences in individual protein levels even within the healthy, normal population. In one carefully controlled study performed with groups of ten littermate mice of the same gender, we observed fluctuations that ranged in variation by 2-fold in the levels of potential biomarker lung proteins for cigarette smoke particulates exposure amongst the individual animals in the same untreated groups [89]. Pooling lysate specimens from within the same groups and testing the pooled specimens with antibody microarrays is one strategy to find especially robust changes to define biomarker proteins. This can also help to reduce the costs of such analyses. However, for biopsies from lung tumors obtained from the same organ, we have commonly observed profound variations in protein expression and phosphorylation amongst individual patients as determined by both antibody microarrays and by Western blotting.

Another confounding factor in biomarker discovery is the occurrence of what has been referred to as repeatedly identified differentially expressed proteins (RIDEPs), which were originally flagged from primarily 2D gel and MS studies [90-92]. These RIDEPs in human specimens most frequently included enolase 1 and heat shock protein 27 (Hsp27), whilst members of the keratins, annexins and peroxiredoxins were also commonly altered in expression in different experimental model systems. In antibody microarray studies [93], Hsp27, enolase 1, and zyxin were the most commonly observed RIDEPs, and 14-3-3 proteins, BclxL, BID, MAGI3, MyD88, protein kinase C isoforms, SLIPR, Siah2, and Smad4 were also affected in several diverse studies. Most of these proteins are involved in cellular stress responses, and may not be very distinguishing biomarkers for specific pathologies.

The search for serum biomarkers, particularly in the context of cancer, has uncovered a wide range of interesting biomarkers as evident in Table 3. However, the identified proteins have overall proved to have relatively little diagnostic value, because most of the elevated proteins in these studies appear to be derived from activated immune cells or are produced by many different cell types. The same proteins may be produced in response to cancer, autoimmune diseases, and infection with viruses and bacteria. To find more discriminating biomarkers, it may be more sensible to identify proteins that are very tissue-specific in their expression, and then assess how their levels are altered under pathological circumstances. Antibody microarrays can be utilized to rapidly screen for antibodies that uncover such tumor- or tissue-specific proteins, which can then be used to evaluate their reliability as biomarkers in larger trials with more patients.

Most studies with antibody microarrays in the past have focused on tracking changes in protein levels in biofluids, and lysates from tissues and cells. However, the altered phosphorylation status of proteins may provide greater insights as these can serve as indicators of the activity states of these proteins. A few commercial antibody microarrays have been developed to track the phosphorylation status of proteins (Table 2). One of the advantages of commercial antibody microarrays is that with increasing reports of the results of the applications of these microarrays in different model systems, it becomes easier upon comparison of data sets to identify promising biomarkers that are more restricted in the circumstances under which they reveal changes in expression or phosphorylation. Towards this end, Kinexus created the KiNETDataBank (www.kinet-am.ca), which is an open-access website that contains the data from over 2000 different studies performed with Kinex™ KAM antibody microarrays.

## Improving Antibody Microarrays

For antibody microarrays to become better accepted as reliable tools for biomarker identification and the study of cell signaling systems, there are several strategies that can be adopted. These include improving the capture antibodies and microarray slide substrates, the preparation of lysate samples, the detection of captured proteins on microarrays, and the validation of the antibody leads from microarrays.

One of the strategies that we have developed to reduce false positive results on antibody microarrays has been to chemically cleave lysate proteins at cysteine residues by successive treatment with Tris(2-Carboxyethyl) Phosphine Hydrochloride (TCEP) and 2-Nitro-5-Thiocyanatobenzoic Acid (NTCB) [37,94] (Figure 3). Many of the epitope sequences used to generate the antibodies used on antibody microarrays avoid the inclusion of cysteine residues (since this is usually used only at the N- or C-termini of peptides for coupling them to keyhole limpet hemocyanin for immunization and to thio-agarose affinity resins for antibody purification), and so this affords the opportunity to generate peptide fragments that retained the epitopes for these antibodies. While the length of peptides produced from Cysteine Chemical Cleavage (CCC) of the human proteome are predicted to vary markedly, most will feature about 30-40 amino acid residues. These are larger peptides than would be typically generated by treatment with a protease like trypsin, and since many protein phosphorylation sites contain flanking arginine and lysine residues, trypsin digestion would destroy many of the epitopes that phosphosite-specific antibodies will recognize.

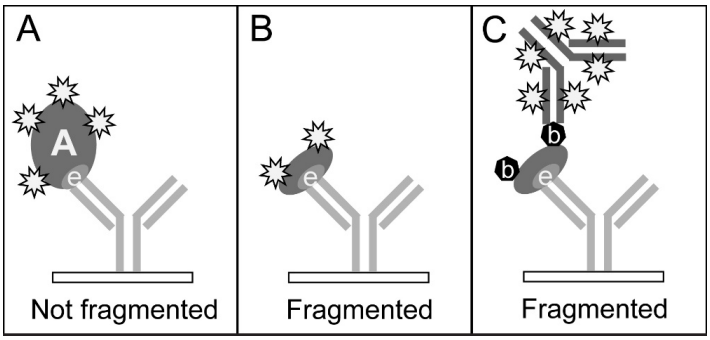


**Figure 3:** Chemical cleavage of proteins and their detection on antibody microarrays. In most published studies, proteins are typically directly dye-labeled in their native form as shown in Panel A. Chemical cleavage at cysteine residues fragments proteins and only the cleaved portion that

retains the epitope is bound to the capture antibody on the microarray. The protein fragment can be directly labeled with fluorescent dye (Panel B) or biotinylated (Panel C). Captured biotinylated proteins on the microarray can be detected with fluorescent dye-labeled avidin or anti-biotin antibody (Panel C).

The CCC procedure has several advantages over commonly adopted protocols, including providing for dissociation of the antibody target protein from associated proteins and facilitating exposure of previously masked epitopes. Epitope-containing peptides tend to be in the same size ranges, so following CCC, the amount of signal recorded for an antibody spot is more reflective of the molar concentration of the target protein and less influenced by its size. The average number of amino acids in the 21,300 native full-length proteins found in the human proteome is about 700 residues, but the sizes of different proteins can vary by several orders of magnitude. The largest human protein is the kinase titin, which features 34,350 amino acid residues.

Lysate protein samples that are subject to CCC are stable at room temperature for over a week. The CCC protocol leads to the destruction of the activities of kinases, phosphatases, proteases and other enzymes that can lead to changes post-homogenization such as alterations in protein phosphorylation. If the CCC procedure is undertaken right at the time of homogenization of tissues and cells, this best preserves the state of protein phosphorylation. In a recent study in which we used the Kinex™ KAM-900 antibody microarray to explore changes in phosphorylation in EGF treated human A431 cervical carcinoma cells, we were able to increase the number of detected lead changes by over 40-fold with the chemical cleavage step performed during lysate preparation as compared to implementing it afterwards [37].

Western blotting has traditionally been the main validation approach to confirm the lead findings from antibody microarray studies. However, antibody microarrays can be several magnitudes more sensitive for detection of target proteins than immunoblots [95] or reverse lysate microarrays. In one antibody microarray study, it was found that 7000-times more cell lysate was required to visualise the lead proteins by Western blotting [18]. With reverse microarrays, the lysate proteins from different tissues or cells are printed as individual spots on slides, and each slide is probed with a different detection antibody. Less than 1 ng of total lysate protein is usually printed as a spot on a reverse microarray, and consequently the sensitivity of antibody detection remains a significant issue. With Western blots, typically 25 µg of lysate protein are deposited in a single lane on an SDS-PAGE gel, but the signal is spread out over the width of the lane. Despite this, Western blotting has successfully confirmed the leads from many different antibody microarray studies [40,63,67,73,95-99].

In our experience with using Western blotting to confirm leads from antibody microarray experiments, we have found that about 40% of the time, the signals from immunoblots performed with the same antibodies used on the microarrays are just too weak for detection. These same antibodies had been previously shown to successfully detect their target proteins in lysates from other cells and tissues, but not in the particular experimental model system under investigation with an antibody microarray. To overcome this issue, especially for validating changes in protein expression, we have tried to incorporate into our microarrays more redundant capture antibodies that target different epitopes in the same protein. When a protein change is observed with multiple antibodies for the same target, this internal cross-validation improves confidence that the change is likely to arise from the intended protein and not an antibody cross-reactive off-target protein. This strategy is more difficult to implement for the analysis of specific phosphosites. In such cases, it may be necessary to enrich the target phosphoprotein first. This might be achieved by subcellular fractionation, or by immunoprecipitation with a pan-specific antibody for the target protein prior to Western blotting with the phosphosite-specific antibody.

Often, from the Western blotting validation exercise, the change observed with an antibody microarray can arise from a cross-reactive protein. In this event, since it is retained by the microarray and detectable on an immunoblot with a capture antibody, it can probably be immunoprecipitated. The immunoprecipitated protein can be subjected to SDS-polyacrylamide gel electrophoresis, and the excised protein from the gel can subsequently be identified by MS. With the availability of an antibody for the identified protein, it is feasible to rapidly follow up with other experiments with larger numbers of specimens to establish how reliable and robust the biomarker may be.

Due to limitations of the amounts of antibodies that are spotted on the antibody microarrays, and the low levels of expression of most cell signaling proteins, the signals generated for many target proteins can be very weak despite the high sensitivity of antibody microarrays. When antibody microarrays were first developed, it was common to directly tag lysate proteins with fluorescent dyes such as Cy3 and Cy5, often in a competitive two dyes format. With this approach, the lysate proteins in a "control" sample might be labeled with Cy3, whereas the lysate proteins in a "treatment" sample would be separately labeled with Cy5. Subsequently, the samples are mixed and incubated on the same field of antibody spots. With equal amounts of a target protein in the control and treatment lysates, effectively half of the antibodies on a spot would bind the Cy3-labeled protein and the other half of the antibodies in the same spot would bind the Cy5-labeled protein. The presence of the two populations of labeled target proteins are monitored separately at the two wavelengths that are optimal for Cy3 and Cy5, but the amplitude of the signals will be reduced in half of what would be achieved with a single dye, non-competitive method. The two dyes, competitive approach reduces the costs of such microarray analyses, but extreme caution needs to be exercised as different proteins do not bind to different dyes uniformly [100,101]. Often, the experiment is repeated by switching the dyes with the two lysate samples undergoing testing, and checking that the same leads for altered expression or phosphorylation between the lysate samples are observed. Where this has been done, it is claimed that most of the leads are still reproducible [45,83,102,103].

To improve the signal to background noise ratio, we prefer using a non-competitive, single dye approach in which a blend of dyes that fluoresce maximally at the same wavelength are used to label all of the different lysate protein samples under investigation. The use of blended dyes increases the opportunity for more uniform labeling of the lysate proteins in view of the differential binding of dyes by proteins. Each sample is tested with a separate field of spotted antibodies, typically with two separate fields per microarray slide, one field for the "control" sample and the other field for the "treatment" sample. Usually replicate printing of each antibody spot is performed in each field to check reproducibility of signals, and the replicate spots are not printed side-by-side to reduce the chances of problems with both replicates due to misprinting, spot mixing, dust, and other background issues.

One of the major issues is the removal of free fluorescent dye remaining even after gel filtration or dialysis of the lysate protein following its labeling step. This free dye can be partly neutralized by the inclusion of ethanolamine or hydroxyurea during the incubation of the dye-labeled lysate samples with the antibody microarray slide. However, there is often still some direct labeling of capture antibodies with remaining free dye that can increase background signals.

To improve sensitivity of detection with antibody microarrays and reduce background noise, initial labeling of lysate protein with biotin instead of fluorescent dyes is an alternative strategy that we and others have adopted [62,73,82,104-106] (Figure 3). The biotinylated lysate proteins captured on the antibody microarray are typically detected with streptavidin that is conjugated to a fluorescent dye. However, avidin exhibits non-specific interactions directly with lysate proteins on the microarray, although NeutrAvidin is a diglycosylated form of avidin that purportedly possesses higher binding specificity and reduced non-specific binding [107]. We prefer using dye-conjugated anti-biotin antibody for detection of biotinylated lysate proteins, because this yields much less non-specific binding than dye-labeled avidin, and since IgG is three times the size of avidin, it binds three times more dye to yield stronger signals [37].

To further improve the detection of captured biotinylated lysate proteins on antibody microarrays, it is possible to use Rolling-Circle Amplification (RCA) for signal generation. In this strategy, the 5'end of an oligonucleotide primer is attached to an anti-biotin antibody. After binding of the oligonucleotide conjugated anti-biotin antibody to the captured biotinylated lysate proteins on an antibody microarray, the oligonucleotide is enzymatically extended using a circular DNA sequence as template. Complementary, fluorescently labeled short oligonucleotides are then hybridised to the extended DNA to decorate each of the bound antibodies with thousands of fluorophores. Using RCA assay, it should be feasible to detect captured proteins in the sub-pg/ml range.

## Expansion of Antibody Microarray Applications

While antibody microarrays have long been used to track the relative differential levels of target proteins and their phosphorylation at specific sites, antibody microarrays can also be adapted to monitor changes in different types of post-translational modifications, protein-protein, and drug-protein interactions. It can additionally be used to monitor changes in protein kinase activities. Figure 4 illustrates some of the ways that different post-translational modifications of proteins can be detected.

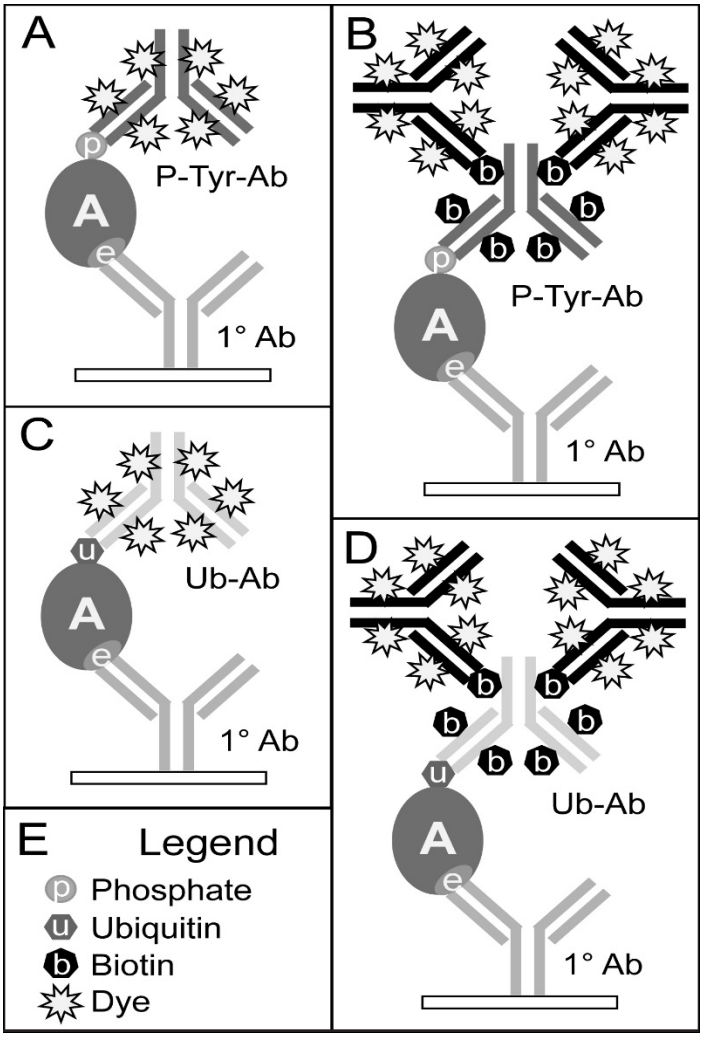


**Figure 4:** Monitoring post-translational modifications of target proteins on antibody microarrays. Following capture of proteins by primary antibodies printed onto antibody microarrays, in a sandwich format, fluorescent dye conjugated reported antibodies that target phosphotyrosine (Panel A) and ubiquitin (Panel C) can be used to track changes in the tyrosine phosphorylation and ubiquitination of proteins, respectively. Biotinylation of the reporter antibodies can be used in combination with dye-labeled anti-biotin antibodies to further amplify signal detection (Panels B and D).

With the availability of generic phosphotyrosine-site antibodies, these have been used as affinity reagents to enrich phosphotyrosine containing peptides prior to MS analyses. However, they can also be used as reporter antibodies to detect differential phosphorylation of lysate proteins on antibody

microarrays. This procedure was initially tested for detection of protein-tyrosine phosphorylation with 35 capture antibodies on a microarray incubated with lysate proteins from Bcr-Abl-expressing RT10+ cells and EGF treated HeLa cells probed with Cy5-labeled custom anti-phosphotyrosine monoclonal antibody [108]. Reductions of at least 45% in the tyrosine phosphorylation signals for 23 of the antibody targets were recorded when the RT10+ cells were pretreated for 2 hours with the protein-tyrosine kinase inhibitor Gleevec, and increases of 2-fold in tyrosine phosphorylation signals were recorded for 16 antibodies following 1 hour of exposure of HeLa cells to EGF after they had been serum-starved previously for 24 hours [108]. We have also detected increased tyrosine phosphorylation of lysate proteins in A431 cells exposed to EGF for 5 minutes using a similar approach with the Kinex™ KAM-1150 antibody microarray and our generic PYK polyclonal anti-phosphotyrosine antibody used as a reporter antibody (L. Yue and S. Pelech, manuscript in preparation). Figure 5 shows an image of identical grids of antibodies spots with captured lysate proteins from untreated and EGF-treated A431 cells probed with fluorescent dye-labeled PYK antibody. We were able to further increase the sensitivity for tyrosine phosphorylation by using biotinylated PYK antibody, and detecting the presence of this reporter antibody with fluorescent dye-conjugated anti-biotin antibody.

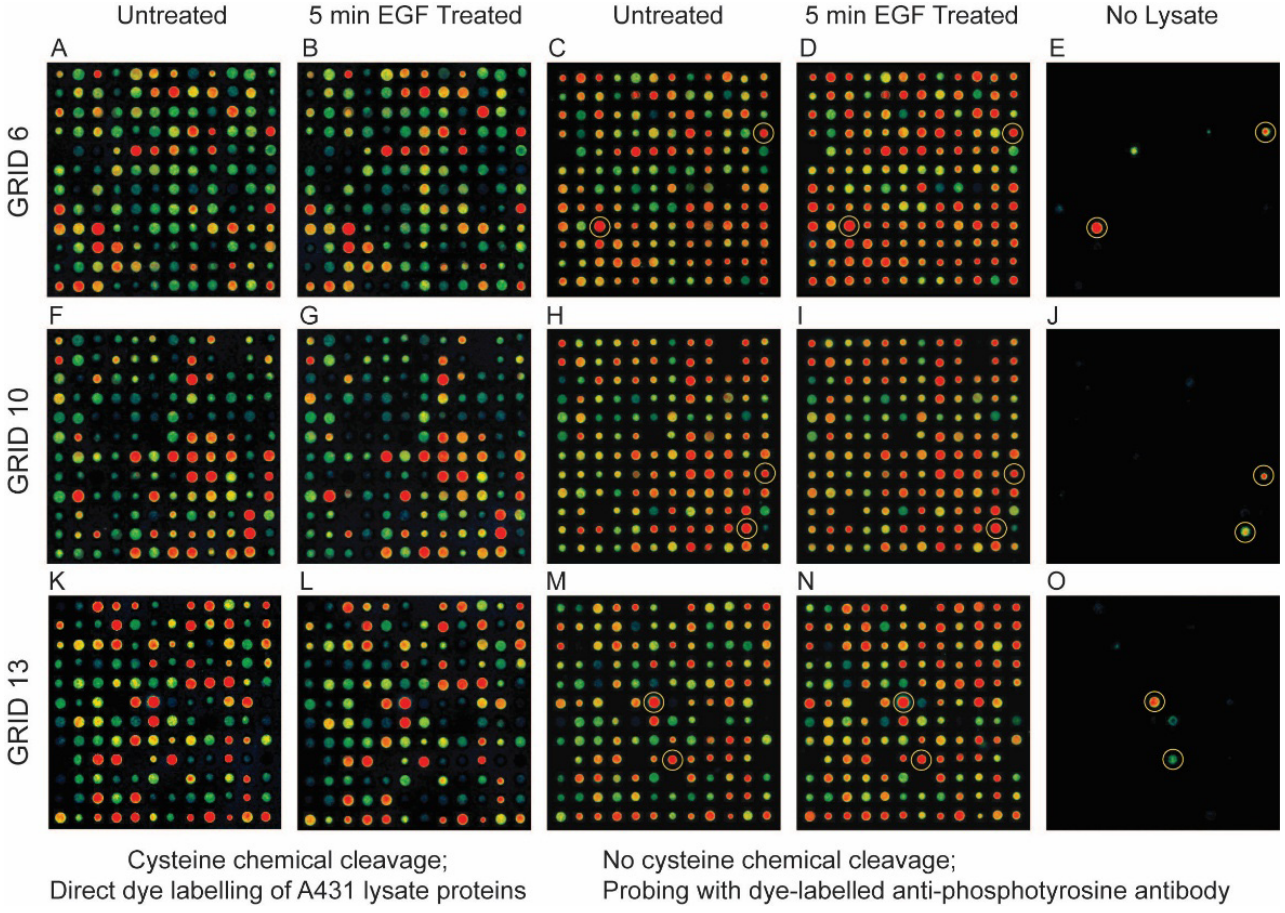


**Figure 5:** Kinex™ KAM-1150 antibody microarray analyses of the expression and tyrosine phosphorylation of signalling proteins in overnight serum-starved human A431 cervical carcinoma cells treated without (Panels A, C, F, H, K, and M) and with 100 ng/ml epidermal growth factor (EGF) for 5 minutes (Panels B, D, G, I, L and N). Cell lysates were prepared with 1.0% Triton X-100 and buffers containing protein phosphatase inhibitors and protease inhibitors and subjected to antibody microarray analyses as described [37]. Approximately 100 µg of each A431 cell lysate were subjected to cysteine chemical cleavage, dye-labeled then and incubated with one of two fields of antibodies printed on each KAM-1150 chip (Panels A, B, F, G, K and L). Other 100 µg aliquots of the same lysates were not chemically cleaved nor tagged, but following capture on one of the grids of a KAM-1150 chip, were probed with dye-conjugated PYK (Cat. No. PG001, Kinexus) generic anti-phosphotyrosine antibody (Panels C, D, H, I, M and N). Only 3 representative grids of the 16 distinct grids of antibodies printed in a single field are shown as scanned images in this figure; each field was replicated four times per array. The strongest signals appear red and the weakest signals are blue. As a control, one field was incubated with only the dye-labeled anti-phosphotyrosine polyclonal rabbit antibody in the absence of cell lysates, and the immunoreactive antibodies are circled (Panels E, J and D). There were little if any changes apparent in protein expression in A431 cells with short term exposure to EGF (Panels A, B, F, G, K and L), but the treatment increased the phosphorylation signals for several of the captured proteins (Panels C, D, H, I, M and N).

Other types of covalent modification of proteins can also be monitored on antibody microarrays using the appropriate reporter antibodies. For example, a custom antibody microarray for 24 target proteins (primarily growth factors and cytokines) was used to investigate their tyrosine nitrosylation in plasma samples from cigarette smokers, those with chronic obstructive pulmonary disease, and non-smokers [109]. In this study, a generic anti-3-nitrotyrosine antibody was used as a biotin-labeled reporter antibody, and this was subsequently detected with fluorescent-dye tagged streptavidin. We have recently begun to monitor changes in the ubiquitination of proteins using the Kinex™ KAM-1150 antibody microarray using dye-labeled anti-ubiquitin antibody as a reporter antibody (Figure 6).

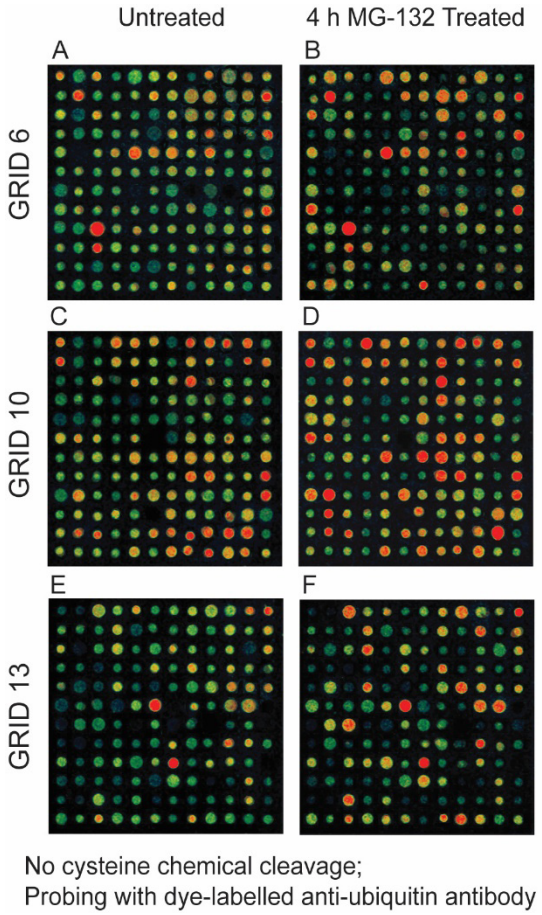


**Figure 6:** Kinex™ KAM-1150 antibody microarray analyses of the ubiquitination of signaling proteins from human HeLa cervical carcinoma cells treated without (Panels A, C and E) and with 50 μM of the proteasome inhibitor MG-132 for 4 hours (Panels B, D and F). Cell lysates with 1.0% Triton-X100 were prepared and 100 μg were incubated with the KAM-1150 chip, which was subsequently probed with anti-ubiquitin antibody (Cat. No. SC-8017, Santa Cruz Biotechnology) that was dye-conjugated. Inhibition of protein degradation with the MG-132 resulted in increased detection of several proteins on the KAM-1150 antibody microarray as shown in 3 representative grids of distinct 16 grids of antibody spots, which were each replicated in four fields on the array.

Many proteins in cells are found in transient complexes with adapter and scaffolding proteins and chaperonins such as heat shock proteins. Reporter antibodies that are specific for these proteins could identify binding partner proteins captured on antibody microarrays and reveal changes in their associations as a consequence of perturbations of experimental model systems (Figure 7A). While this methodology for exploring protein-protein interactions on microarrays has not yet really been exploited, it should prove to be very fruitful.

Finally, antibody microarrays can be adapted to monitor the activity states of proteins, especially as more biosensor molecules become available that selectively detect the inhibited or activated forms of enzymes. One of the most promising opportunities in this direction is for tracking the activation states of protein kinases. Assaying the enzymatic activity of protein kinases in crude cell and tissue lysates is a formidable task without some sort of affinity purification. At least 536 different human protein kinases are documented on our KinaseNET (www.kinasenet.ca) website, and they have highly overlapping substrate specificities. Approximately 484 typical protein kinases of the human kinome feature a common catalytic domain that includes a highly conserved lysine residue in the kinase subdomain II region in the "AXK" motif [110], which is located in the active site of these kinases and is critical for phosphotransferase activity by transferring the gamma-phosphate of ATP on to the hydroxyl group of phosphorylatable residues on protein substrates. Affinity labeling with the ATP analogue 5'-(p-flurosulphonylbenzoyl) adenosine (FSBA) inhibits protein kinases by covalently modifying the kinase subdomain II lysine residue [111]. The active site of protein kinases need to be open and accessible for FSBA to bind, so it can be a useful probe to determine whether kinases are in a state where they may be catalytically active. We have successfully used a biotinylated version of FSBA to interrogate the activation states of protein kinases as illustrated in Figure 7B.

By using preparations of cell lysates in which the endogenous protein kinases have been preactivated in vivo, and able to bind FSBA, it is also feasible to identify subsets of protein kinases that may be sensitive to inhibition by test compounds. As documented in our DrugKiNET website (www.drugkinet.ca), most of the known protein kinase inhibitors bind directly to the active sites of protein kinases, and are partly analogues of ATP. Preincubation of captured protein kinases on an antibody microarray with a kinase inhibitor that occupies the active sites of sensitive kinases will pre-empt the binding of the biotinylated FSBA (Figure 7C). The reduction of biotinylated FSBA binding to the array can be monitored with dye-labeled anti-biotin antibody. Such approaches could be very useful for counter screening promising kinase inhibitor leads with hundreds of kinases for off target effects. It is also possible that the biotinylated FSBA probe may be helpful for identifying possible peptide and protein substrates of kinases, since the binding of these substrates may also interfere with the interaction with the biotinylated FSBA.

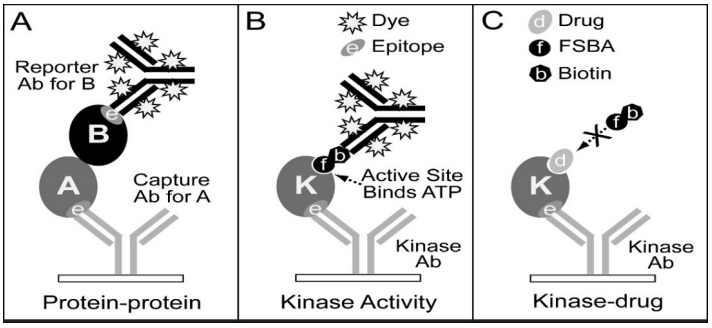


**Figure 7:** Monitoring protein-protein and protein kinase-drug interactions on antibody microarrays. A sandwich antibody microarray format, can be used to detect interactions between two different proteins depicted as A and B (Panel A). The active site of a captured protein kinase on an antibody microarray can become covalently bound to a biotinylated version of the ATP analogue FSBA if the kinase is in an open, active conformation (Panel B). Preincubation of active, captured protein kinases on antibody microarrays with inhibitory drugs can sterically block subsequent access of the FBSA-biotin probe, and through a reduction of signal from dye-conjugated anti-biotin antibody binding observed in the absence of the inhibitor reveal those kinases that are sensitive to the drug (Panel C).

For all of these types of investigations, full length native lysate proteins would be desirable, so the cysteine chemical cleavage process would not be used. Therefore, caution will be necessary in interpretation of the results since the proteins will likely reside in complexes, and some types of covalent modifications may be prone to rapid removal post-homogenisation.

## Conclusion

In this review, we have described many applications of antibody microarray technology in biomedical research while emphasizing its utility and unrealized potential as a quick, sensitive and cost-effective platform that is compatible with various types of biological samples for flexible analysis of different proteomics aspects. Antibody microarrays are burdened by the challenge of availability of high-quality antibodies suitable for the platform, and are also subject to many of the same problems that confound other proteomics assays, such as the nature of the sheer complexity of proteomes, the high degree of protein-protein interactions, and over a million-fold differentially expressed classes of proteins. Nevertheless, antibody microarray technology is superior to mass spectrometry due to the high sensitivity of antibodies as biosensors, affording more quantitative analyses with low sample amount requirements without the need for enrichment of low-abundance proteins. In this review, we have suggested how analyses can be further improved by strategies in sample preparation, detection methods, and also study designs. Ultimately, any findings with the antibody microarrays should be confirmed by alternative methods. However, the high sensitivity, breadth of coverage, economy and speed of antibody microarray analyses will ultimately advance this technology to eventually prevail as the preferred methodology for proteomics investigations into the foreseeable future.

## Conflict of Interest Declaration

S.P. and his family are the majority shareholders of Kinexus Bioinformatics Corporation.

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