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# Protein arrays as tools for serum autoantibody marker discovery in cancer

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ABSTRACT

Protein array technology has begun to play a significant role in the study of protein–protein interactions and in the identification of antigenic targets of serum autoantibodies in a variety of autoimmune disorders. More recently, this technology has been applied to the identification of autoantibody signatures in cancer.

The identification of tumour-associated antigens (TAAs) recognised by the patient's immune response represents an exciting approach to identify novel diagnostic cancer biomarkers and may contribute towards a better understanding of the molecular mechanisms involved. Circulating autoantibodies have not only been used to identify TAAs as diagnostic/prognostic markers and potential therapeutic targets, they also represent excellent biomarkers for the early detection of tumours and potential markers for monitoring the efficacy of treatment. Protein array technology offers the ability to screen the humoral immune response in cancer against thousands of proteins in a high throughput technique, thus readily identifying new panels of TAAs. Such an approach should not only aid in improved diagnostics, but has already contributed to the identification of complex autoantibody signatures that may represent disease subgroups, early diagnostics and facilitated the analysis of vaccine trials.

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## 1. Introduction

When studying disease processes, the knowledge of alterations on protein level, including protein structure, post-translational modifications or protein–protein interactions, is crucial to elucidate complex biological phenomena. Such insights are difficult to attain due to the enormous complexity of the protein world. Traditional methods for the analysis of proteomes include two dimensional gel electrophoresis and mass spectrometry, however, to compliment the functional analysis of proteins on a large scale, proteins can be studied in array formats. Preferably such arrays would contain functionally active proteins, in all their modified states, immobilised on a surface at high density or in solution in nanowells. Importantly, protein activity is dependant on a wide range of factors (post-translational modifications, cellular localisation, pH, presence/absence of co-factors, etc.) which makes the production of a protein chip containing the whole proteome a daunting task.

Since their introduction over a decade ago [1], protein arrays have been successfully used in a wide range of studies. Large collections of proteins in an array format enabled identification of peptide–protein interactions [2], protein–protein interactions [3] and antibody–antigen interactions [4]. The specificity of these binding interactions was subsequently confirmed by pull-down, yeast two-hybrid, immunoblot and co-immunoprecipitation experiments. In another approach protein arrays were successfully used for identification of novel substrates for arginine N-methyltransferases [5] and for protein kinases [6]. More recent studies identified

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novel substrates for Ubiquitin-protein ligases (E3s) in yeast [7] and novel substrates for Cyclin-dependent kinase (Cdk5) using commercially available protein microarrays [8,9].

In the field of oncology, protein arrays offer huge potential as tools for the identification of cancer biomarkers, in particular auto-antibody/antigen markers. The identification of tumour-associated antigens (TAAs) recognised by the patient's immune system represents an exciting approach to identify novel diagnostic cancer biomarkers and to contribute towards a better understanding of the molecular mechanisms involved. Circulating autoantibodies have not only been used to identify TAAs as diagnostic/prognostic markers and potential therapeutic targets [10,11], they also represent excellent biomarkers for the early detection of tumours and permit monitoring of the efficacy of treatment [12,13].

Protein array technology has already played a significant role in analyzing autoantibodies in a variety of autoimmune disorders [14–16] and cancers [17,18]. The ability to screen an immune response to a large number of proteins in a high throughput technique cannot only aid in improved diagnosis, but has identified autoantibody signatures that may represent disease subgroups, early diagnostics [19] and facilitate the analysis of vaccine trials [20].

This review investigates the current developments in protein array technology and their applications with particular emphasis on the field of tumour-associated antigen (TAA) discovery.

## 2. General applications of protein arrays

Protein arrays provide a powerful tool to examine interactions between proteins (including antibodies), peptides, DNA/RNA or chemical compounds on a large scale. In one proof-of-principle experiment, a small number of well-defined protein–protein interactions, including an interaction dependent on a small molecule, were demonstrated in a microarray format [21]. As mentioned above, calmodulin- and phospholipids-interacting proteins have been identified by screening almost 6000 yeast proteins, generated by expressing previously annotated open reading frames [22]. Another approach for the identification of protein–protein interactions on protein arrays is the use of short peptides as bait, which can permit studies of proteins that are otherwise difficult to work with in solution. This approach has been successfully applied to identify cytoplasmic interactors of the platelet integrin $\alpha_{IIb}\beta_3$ [2,23]. Protein arrays also make an ideal platform to characterise antibodies. A human protein expression library, involving in situ expression of tens of thousands of recombinant proteins on large membranes, has been successfully screened to identify antibody–protein interactions [4], permitting identification of cross-reacting proteins and potential epitope definition.

Protein arrays can also be used to determine the target of antibodies previously identified as potentially interesting markers in disease. Our group is presently working to identify the antigens of antibodies, initially identified by immunohistochemistry as interesting markers in certain types of cancer. Each antibody can be screened against an appropriate protein expression library. Unlike previous approaches, which could at best identify potential epitopes of a particular antibody, this approach identifies the actual target protein. In addition, potential cross-reacting proteins can also be identified using this method, which is important information for assessing an antibody in disease screening procedures. Similarly, protein arrays could provide a useful screening technique to be introduced into any antibody production process.

Just as it is possible to characterize the binding of a single antibody using protein arrays, it is also possible to characterize many antibodies present in a single sample, such as to profile the antibody repertoire in serum or plasma. One immediate application is the use of "allergen arrays" to screen for the presence of particular IgE molecules in a patient sample [24–27]. In a similar fashion, protein arrays can be used to profile antibodies present in the blood of patients with autoimmune diseases. Initial auto-antigen arrays consisted of almost 200 proteins, peptides and other biomolecules (including several forms of dsDNA and ssDNA) which were known auto-antigens to several well characterized auto-immune diseases, including rheumatoid arthritis and systemic lupus erythematosus [28]. Another approach is to use large arrayed libraries of recombinant proteins as a potential auto-antigen array. In a proof-of-principle experiment, a protein array chip containing almost 2500 purified recombinant human proteins was used to profile the auto-antibodies present in small volume samples from patients with alopecia and rheumatoid arthritis [29]. This approach permitted the identification of previously known auto-antigens and also previously uncharacterized protein auto-antigens. Initial results from screening a large recombinant mouse protein array also indicate the usefulness of this approach to characterize auto-immune disease in a mouse model system for systemic lupus erythematosus [14].

## 3. Autoantibody responses in cancer

Antibody responses to tumour associated antigens in cancer patients have been identified in many cancer types and offer new biomarkers with potentially high levels of specificity and sensitivity (see Table 1). The processes by which such self-proteins become immunogenic are not entirely understood [30]. However, the molecular changes in structure or expression of proteins during tumorigenesis suggest mechanisms by which such proteins could be perceived by the immune system as foreign and initiate antibody production.

The most extensively researched mechanisms of cancer immunity are genetic mutations leading to expression of defective tumour suppressor p53. These frequent mutations in many cancer types, lead to conformational changes in the protein. This in turn results in increased stability of the protein and extended half life time of several hours compared with 20 min for wild-type p53 [31,32]. It is generally accepted that p53 mutations are not directly responsible for priming the antibody production, but rather that it is caused by the high antigenic load resulting from the accumulation of the p53 protein [33]. The disparity between the mutation site and the actual epitope was also observed for Ras p21 in cancer patients [34]. Interestingly, in both cases the accumulation of the protein is required for the antibody response, however, the accumulation of the protein does not always result in antibody

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**Table 1 – Examples of both protein (non-antibody) and autoantibody cancer markers**

| Protein (non-antibody) biomarker | Swiss-prot ID | Cancer type | Specificity | Sensitivity | Reference |
|---|---|---|---|---|---|
| Prostate-specific antigen | PSA | Prostate cancer | 92%* | 12%* | [87] |
| Carcinoembryonic antigen | CEA | Colorectal cancer | 90% ** | 34% ** | [88] |
| Cancer antigen CA15-3 | CA15-3 | Breast cancer | 69% | 23% | [89] |
| Cancer antigen CA19-9 | CA19-9 | Gastrointestinal cancer | 86% | 35% | [90] |
| Cancer antigen CA125 | CA125 | Ovarian cancer | 41.5%*** | 80%*** | [91] |
| **Autoantibody biomarker** | **Swiss-prot ID** | **Cancer type** | **Specificity** | **Sensitivity** | **Reference** |
| cAMP-dependent protein kinase A | ECPKA | Several caner types | 90% | 87% | [45] |
| Annexin XI-A | ANXA11 | Breast cancer | 88% | 77% | [92] |
| Tumour suppressor p53 | p53 | Several caner types | 96% | 30% | [93] |
| Huntingtin interacting protein 1 | HIP-1 | Prostate cancer | 73% | 46% | [94] |

*3.1–4.0 ng/ml PSA level **5 ng $ml^{-1}$ CEA level ***330 U/mL CA125.
Autoantibody based biomarkers show promising results and are currently a focus of cancer diagnostic research.

production. Of all cancer patients with p53 mutations, approximately half have detectable serum antibodies specific to the p53 protein.

A recent study by Engelhorn and colleagues investigated the direct potential of mutations to induce antibody-mediated immune responses [35]. The group found in mice experiments, using combinatorial DNA libraries encoding large numbers of random mutations in tyrosinase-related proteins, that truncations of the tyrosinase protein are sufficient to elicit antibody production. Additional amino acid substitutions in the protein can further enhance these immune responses. Antibody responses to mutated gene products were also identified at advanced stages of cancer, including in a mouse model [36]. Since thousands of genes can mutate during the progression of cancer [37], these findings demonstrated the potential of stage-specific immune responses.

An alternative pathway leading to initiation of antibody production is the deregulation of gene expression. The expression of many genes in cancer is deregulated and many genes are overexpressed [38]. For example, the non-secreted surface antigen GA733-2 is over-expressed in over 90% of colorectal cancers and anti-GA733-2 antibodies were detected in 14.5% of the patients [39]. A further example is the HER-2/neu protein, which is overexpressed in approximately 20% of colorectal cancer patients. HER-2/neu antibodies were detected in 46% patients overexpressing the protein and in only 5% patients with no detectable HER-2/neu [40]. The overexpression of genes may lead to an increased antigenic load which in return can be sensed by the immune system and lead to antibody production.

Aberrant cellular localization of antigens caused by malignant transformation of mammalian cells might represent a further potential pathway triggering autoantibody responses in cancer patients. The cAMP-dependent protein kinase A (PKA) is an intracellular enzyme [41,42] which can be secreted by cancer cells into the conditioned medium [43,44]. This PKA, designated as extracellular protein kinase A (ECPKA), is often found to be up-regulated in the serum of cancer patients [43,44] which correlates with the presence of ECPKA specific autoantibodies in sera of corresponding patients [45].

A further potential mechanism by which self-proteins could trigger antibody production is expression of genes that are not usually expressed in a particular tissue. The mRNA binding protein p62 is normally expressed in the foetus and its expression is silenced in adult tissue. In some cases of hepatocellular carcinoma, p62 expression can be reinstated leading to recognition by the immune system and antibody production [46]. Another example is a group of proteins normally expressed in testicular tissue and not recognised by the immune system as foreign (e.g.: NY-ESO-1, SSX2). When, however, aberrantly expressed in tumour cells, these proteins can trigger antibody responses [47].

## 4. Cancer autoantibody discovery in the proteomic age

The vast amount of potential molecular changes during tumorigenesis in different cancer types and the individual variances in immune responses in cancer patients potentially result in complex antibody repertoires. In order to identify a disease specific antibody profile it is required to analyse as many potential antigens as possible.

Classical laboratory methods, such as Western blotting or enzyme-linked immunosorbent assays (ELISA), have been used to identify a number of tumour antigens. Antibodies to p53, p21 ras or Her/2neu were successfully identified in cancer patients in the previous two decades [34,48,49]. Several novel approaches have been developed to detect new antigens that elicit antibody responses. With the advent of proteomics and recombinant technologies, it is now possible to characterise hundreds of proteins in parallel for potential antibodies in sera of cancer patients. Followed by a short description of methods based on 2D gel electrophoresis, mass spectrometry and phage display we will focus our review on protein arrays as a tool for detection of antibodies associated with cancer.

Proteomics is an alternative approach for identification of novel tumour antigens. Proteomics applies two dimensional gel electrophoresis (2DE), a process that separates large mixtures of proteins from natural sources such as tissue or cell lines. Once the proteins are separated using 2DE and transferred onto protein binding membranes using Western blotting, the autoantibodies can be identified by incubation with serum of cancer patients [50,51]. Although the natural protein source allows analysis of proteins in post-translationally modified states. a disadvantage of 2DE is that very large or

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very small proteins and certain kinds of proteins such as membrane proteins, are difficult or impossible to visualize using 2DE and require further characterisation using mass spectrometry [52].

An alternative proteomics approach combines liquid phase protein separations with microarray technology. Proteins in cell and tissue lysates can be separated using chromatography and hundreds of lysate fractions can be arrayed onto nitrocellulose-coated slides [53]. Incubation of serum samples with lysate arrays may reveal fractions immunoreactive with serum antibodies. A study by Nam and colleagues used a fractionated colon adenocarcinoma cell line and identified ubiquitin C-terminal hydrolase isozyme 3 as an antigen eliciting antibody responses in a group of colon cancer patients [54]. The merit of post-translationally modified antigen detection is reduced by difficulties in characterising the identity of antigens, since each fraction may still contain a complex mixture of proteins.

These proteomics approaches represent a large step towards the identification of novel antigens eliciting antibody responses in cancer patients. However, identification of immune responses to a broad set of antigens in cancer is limited to individual and often abundant proteins. Without doubt, the technical advances in separation methods, image analysis and mass spectrometry will retain proteomics as a vital tool for antibody identification.

In recent years various protein array techniques combined with more classical research methods have been successfully applied to identify cancer associated autoantibody markers. Novel autoantibodies were identified in sera of hepatocellular-carcinoma patients using 2DE proteomic method. The validity of these findings was confirmed using a protein array consisting of the corresponding antigens [55]. Generation of protein arrays based on protein sources from malignant tissue samples is another potentially important step due to advances in protein fractionation methods and mass spectrometry supported by supervised learning analysis of gathered data. Two recent studies focusing on lung and prostate cancer utilized 2D gel electrophoresis and liquid phase chromatography methods respectively as means for generation of highly specialised protein arrays for autoantibody discovery [56,57]. A growing number of current studies strongly indicate that protein arrays are emerging as a tool of choice for research towards deciphering complex protein–protein interactions, in particular for the discovery of novel autoantibody/autoantigen biomarkers. Such work has enormous potential in the discovery and development of panels of such biomarkers, which appear to offer superior accuracy in disease diagnosis compared to individual markers [58].

Phage display offers a powerful platform for identification of tumour associated antibodies [59]. Serological analysis of phage libraries was first introduced in 1995 by Sahin and colleges [60]. Phage display technology allows presentation of peptides and proteins derived from human cDNA libraries on the surface of bacteriophage and testing these for reactivity with serum antibodies. Individual immuno-reactive clones can be enriched in a multi-step biopanning procedure from the random phage libraries and characterized by sequencing. A great advantage of phage display is the very large number of potential antigens encoded by the cDNA libraries. This enables detection of a wide range of cancer-specific antibodies/antigens [61]. However, phage display technology is labour-intensive and peptides expressed by phages are often generated from untranslated DNA sequences and do not correspond to native antigens, thus limiting identification of molecular targets in cancer [11,62].

Recent technical advances enable generation of phage-based protein/peptide arrays containing several hundred up to several thousand phages [11]. Screening the initial phage library with pooled sera from several patients and pooled sera from non-cancer controls identified in a multi-step biopanning procedure approximately 2000 immunoreactive clones. These clones were then arrayed on glass-slides and re-screened for immuno-reactive clones with non-pooled serum samples. The phage array technology allowed the identification of two panels of antigens specific for prostate and lung cancer [11,63]. Both panels were able to predict cancer with over 80% sensitivity and specificity.

The success rates of phage based screening methods were critically improved with the introduction of protein arrays consisting of antigens identified in concluding steps of phage selection [11]. These recent developments permit screening to focus on several hundred to a few thousand identified leads and validate these in larger cohorts of patients using protein arrays [63].

## 5. Generation of protein arrays for profiling autoantibody repertoires in cancer

The first requirement towards the generation of protein arrays is a source of large numbers of recombinant proteins. A number of strategies are currently employed to provide sources of thousands of proteins for the generation of protein arrays. One approach is high-throughput cloning or amplification using PCR of defined open reading frames coding for the proteins of interest [64,65]. The successful implementation of such an approach relies on the availability of sequence data and its correct annotation in the databases. In particular, the definition of the open reading frames of alternative splice variants of one protein remains difficult with such an approach. Also, previously uncharacterised proteins will be absent, limiting this approach as a discovery tool. For these reasons, this approach has proved most valuable in the production of chips containing proteins from well characterized organisms, such as *Arabidopsis* [64] and *Caenorhabditis elegans* [66].

Another approach is the use of protein expression libraries, which are used for a "shotgun" approach to generate recombinant proteins [1]. Such libraries are generated using mRNA isolated directly from the cell [67,68]. Here, mRNA is isolated from the tissue or organism of interest and directionally sub-cloned into a protein expression vector, suitable for heterologous expression in either a bacterial (e.g. *E. coli*) or eukaryotic organism (e.g. yeast). Several solutions exist for the rapid movement of coding region from one organism to another, such as the GATEWAY system (Life Technologies) [69] or dual expression vectors [67]. Not only does this approach circumvent the cloning of individual open reading frames, readily permitting the expression of tens of thousands of proteins, but also this strategy automatically includes splice variants and previously uncharacterized gene products.

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The Gateway technology has been successfully applied for cloning the Ultimate™ ORF Clone collection resulting in generation of human protein expression clones for protein array applications. Recombinant bacmid DNA were isolated and transfected into Sf9 insect cells in order to generate a recombinant baculovirus that was used for expression experiments. A major advantage of this approach is the generation of protein arrays containing native proteins which are post-translationally modified [18].

Apart from just the question of expressing the recombinant proteins of choice in a particular host system, there is also the question of tagging these proteins. A large number of tags exist, the coding region of which can readily be introduced into vectors. Depending on the approach taken, many options are available, including N-terminal and/or C-terminal tagging, multiple different tags are also an option, in particular in conjunction with proteolytic sites for subsequent removal of the tag, if required. These tags can vary in size from the short $His_6$ tag to the 30 kDa GFP tag. Such tags are required for the detection of the expressed recombinant proteins and, in conjunction with an appropriate affinity separation method, for their purification. For a review of different expression systems see [70].

In one example, a human foetal brain cDNA library was subcloned into a bacterial expression vector, permitting controlled IPTG-inducible expression of $His_6$-tagged recombinant proteins [1]. The *E. coli* clones of this library were then arrayed in high density onto PVDF membranes, grown overnight and recombinant protein expression induced for a controlled length of time. Those clones expressing a human protein are readily detected by means of an anti-$His_6$ antibody. In this fashion, a protein array containing 10–12,000 different human proteins has been generated. Approximately 66% of the proteins expressed in this library are, according to present annotation, full length. Also, such proteins have been shown to be readily expressed and purified in high-throughput using available automated platforms [29,71].

In the recent years several novel methods have been developed for cell free *in situ* synthesis of protein arrays [72–75]. *In situ* protein array synthesis uses cell free expression systems to produce proteins directly from nucleic acid templates. The major advantage of these methods is the expression of many proteins in parallel on a chip, avoiding labour intensive and often costly processes of DNA cloning, expression and protein purification. The *in situ* cell free protein generation methods show the capability of high-yield and high-throughput synthesis of hundreds of proteins with minimal variation and good reproducibility [76]. In addition, these methods demonstrate intrinsic purity of synthetized proteins [74] which makes them a desirable tool for TAA detection from complex and polyclonal antibody mixtures present in blood of patients. The high purity of proteins arrayed on such self-assembling chips encourages applications in cancer immunology, as outlined in a recent breast cancer study. The findings show that the identified autoantibody profiles are less prone to false positive results due to highly pure proteins directly assembled on the chip [77]. An extensive review on cell free protein array synthesis methods was recently published by He et al [78].

Once a source of proteins has been established, consideration must be given to the format that a protein microarray can have. Presently, there is a number of possible surfaces available for the manufacture of protein arrays. In general, chip surfaces can be divided into two major categories: flat (planar), and 3D (mostly gel-like) surfaces [79,80]. Planar surfaces have been primarily developed in the area of cDNA arrays. The glass surface of these chips is usually treated to produce a thin layer of a particular chemical group, e.g. an aldehyde group or poly- L-Lysine [81]. The proteins are bound to the surface of these chips by either covalent bonds or simple electrical charge. Another type of planar chip surface available is plastic polymer coated slides, such as the MaxiSorb slides from Nunc — an approach used in ELISAs for many years. The simple adoption of technology from the area of cDNA arrays brings with it a number of major concerns when used with proteins. Firstly, unlike DNA, the surface charge of proteins is highly variable and the use of simple uniform electrostatic interaction for the immobilisation of different proteins results in large variation in the amount of protein bound. Secondly, the structural conformation of proteins deposited onto a planar surface cannot be expected to very closely mimic "native" proteins, but would be more similar to proteins present on membranes, such as in traditional Western blotting or dot blot experiments. Thirdly, there is no control over the orientation of the proteins on the surface, which may result in, for example, the inaccessibility of an active site.

The development of the gel-like 3D chip surface was partly driven in an attempt to minimise the denaturation of the immobilised proteins in the arrays. A number of such surfaces exist based around polyacrylamide and agarose coated on a glass surface, which provide a hydrophilic environment for the proteins. Such surfaces allow the user to adjust various conditions, such as pH and salt concentrations, by incubating the chips in the appropriate buffer. Using a "home made" polyacrylamide surface, a glass chip containing 2413 non-redundant purified human fusion proteins, arrayed at a density of up to 1600 proteins/$cm^2$, has been successfully employed in antibody binding studies, including the screening of human serum samples [29], indicating the maintenance of a degree of structural conformation of the proteins involved. There are also non-gel-like 3D surfaces available, such as the FAST slides from Schleicher and Schuell which have a nitrocellulose surface. Like any 3D surface, these surfaces allow a much higher concentration of proteins per spot.

However, these surface solutions still leave the problem of controlling the orientation of the proteins on the surface of the slides, which is necessary to maximise the activity of these proteins. For example, in order to maximise the binding of antibodies arranged on a surface to their epitopes, it would clearly be an advantage if the heavy chain were attached to, or close to, the surface and the antigen binding site as far away from the surface as possible. One approach has been the use of affinity tags, for example a nickel-coated slide has a natural and specific affinity to $His_6$-tagged recombinant proteins. This approach was used to array 5800 yeast proteins which were screened for their ability to interact with calmodulins and phospholipids [22]. Similarly, successful orientation of antibodies and antibody Fab fragments was achieved by biotinylating such antibodies/Fab fragments and arraying them on a streptavidin-coated surface [82]. A further development of surface chemistry involves the use of a polyethylene glycol layer (PEG)

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[80] or dendrimers [83]. These approaches involve the coupling of the proteins to epoxy groups, which act as spacers preventing direct protein-surface contact and, thus, eliminate the need for blocking reagents to reduce background binding. One further development of this approach has been to link chelating iminodiacetic acid groups to PEG, which in turn can be bound by $Cu^{2+}$ ions and so provide a highly specific binding site for $His_6$-tagged proteins [72]. A number of studies have been carried out comparing the different surfaces available for protein array work, including antibody arrays which assess background noise, sensitivity/detection limits, reproducibility and storage for a variety of experimental designs [79,80].

While the development of 3D surfaces and spacers partially addresses the problems of protein–protein interactions on a chip, many experiments that involve interactions of proteins in a functional state will prove difficult to perform on these chips, which is obviously a major drawback of the current protein arrays. Ideally, we would like to look at protein interactions, where the proteins are in their native state and are functional, i.e. in conditions as close as possible to those in nature. This may be solved by developing a microfluidic chip, which is a series of enclosed micro-channels within a chip format, such as silicon, plastic or glass. The potential of microfluidic chips would include the ability to maintain proteins in their functional conformations and to therefore perform interactions such as protein–protein, protein–peptide, protein–compound, protein–DNA, protein–ligand and protein–antibody interactions in solution. Also, the area of enzyme studies would greatly profit from such a system. In fact the first steps have been taken to use a "lab-on-a-chip" to study some of the reactions in the glycolytic pathway of yeast. Using this system, enzymatic reactions in volumes as low as 6.3–8 nL could be studied [84]. The ability to use such small volumes in microfluidic chips is very important due to the high cost of proteins, antibodies, compound libraries, peptides and even the difficulties in obtaining enough patient samples to screen in high-throughput. Another exciting innovation is the first attempts to establish a lipid bilayer membrane chip that would allow the functional investigation of transmembrane proteins and their interactors [85]. These advances would also permit a more thorough exploitation of the libraries of proteins that currently exist.

The antibody repertoires in humans consist of a complex polyclonal mixture of antibodies with a wide range of specificities, resulting in immunoreactivity with a vast number of potential antigens. Consequently, minute impurities of a protein sample on a protein array may result in a non-specific false-positive signal. To assure the analytical quality of protein microarray experiments, the protein array production and antibody profiling experiments require highest standard and quality. Appropriate quality control and assurance measures are extensively described in an review article by Kricka and Master [86].

## 6. Conclusion

The understanding of molecular events leading to cancer is of the utmost importance to identify effective diagnostic and therapeutic targets. With the complexity of cells and organisms a genome-wide gene expression and protein analyses are essential to elucidate human diseases in terms of dysfunctions of molecular systems. This is especially true for cancer, where multiple key molecular alterations may destabilize essential molecular pathways setting off an avalanche of associated modification on molecular and cellular levels leading to malignancy. As outlined in this review the humoral immune responses to tumour antigens offer an exciting opportunity to identify autoantibody based diagnostic biomarkers. The immune system may serve as a sensitive tool to detect minute amount of cancer specific antigens amplifying autoantibody responses to a measurable level. However, in addition to the complexity of cancer, the vast quantity of antibodies circulating in human blood and the diversity between individuals needs to be taken into account. In this context, individual patients produce an immense repertoire of antibodies of which many might be not cancer related. Therefore, to comprehensively study autoantibodies in patients and to identify cancer specific autoantibody signatures the research relies on large sets of proteins which can serve as potential antigens. The recent developments in protein array technology deliver an excellent tool for analysis of thousand of proteins for their antigenic ability. Supported by current findings, a major future application of high-content protein arrays will contribute to the identification of novel of tumour associated autoantibodies as diagnostic markers for cancer.

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