Use of high-throughput arrays for profiling differentially expressed proteins in normal and malignant tissues

Gernot Hudelist[a,c], Christian F. Singer[a], Ernst Kubista[a] and Klaus Czerwenka[b]

DNA microarrays have provided researchers with a tool to detect differential expression of thousands of genes in a small sample and have clearly revolutionized the way gene expression analysis is now carried out. These microarrays are, however, confined to the detection of gene transcripts and do not permit the analysis of the translational product. This limits their potential use in research, since, after all, proteins are the business end of gene expression and the usual target of drugs. Until recently, protein detection strategies included ELISAs, Western blotting and immunohistochemistry, and were limited to the detection of a couple of proteins of interest. The recent development of protein microarrays now offers the possibility to simultaneously analyze the protein expression of several hundred proteins. Protein arrays allow us to measure the presence, biochemical characteristics and activation state of a considerable number of proteins in a single experiment. However, the formation of complex tertiary/quaternary structures and the interactions between many proteins still pose a veritable challenge for the development of high-throughput protein analysis, which might ultimately allow for the expression analysis of the whole proteome. In this review, we discuss the principle of antibody arrays and pay specific attention to the methodology of different array types. We also present a number of studies that have already shown the clinical utility of high-throughput protein assays, and which exemplify potential applications for this young and extremely promising technology. *Anti-Cancer Drugs* 16:683–689 © 2005 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2005, **16**:683–689

Keywords: proteomics, high throughput assays, breast cancer

[a]Division of Special Gynecology, Department of Obstetrics and Gynecology, [b]Division of Gynecopathology, Department of Clinical Pathology, University of Vienna Medical Center, Vienna, Austria and [c]Department of Obstetrics and Gynecology, Villach Medical Center, Villach, Austria.

Correspondence to C. F. Singer, Division of Special Gynecology, University of Vienna Medical Center, Waehringer Guertel 18–20, 1090 Vienna, Austria.
Tel: +43 1 40400 2801; fax: +43 1 406 67 49;
e-mail: christian.singer@meduniwien.ac.at

Received 11 March 2005 **Accepted** 29 April 2005

Introduction

Malignant transformation is a complex multistep process that involves a large variety of biological alterations that finally lead to deregulated, uncontrolled and tissue-destructive cell growth. Growing evidence now supports the hypothesis that there are few biological alterations that are pathognomic for a specific tumor entity such as malignant breast or ovarian cancer. Rather, malignant tumors comprise a heterogeneous and individual set of genetic and phenotypic changes. This has led to the systematic analysis of the tumoral genome and proteome in order to identify potential therapeutic targets and to predict susceptibility to potential drug candidates. Among the emerging technologies, cDNA arrays have gained widespread popularity and have already become a standard tool for the simultaneous detection of several hundred genes within one individual biological sample. Based on the hybridization between nucleic acids that derive from reverse transcription and a broad range of arrayed cognate gene fragments, cDNA arrays massively quantify the expression of thousands of genes at the mRNA level. However, the major disadvantage of gene expression profiling via the measurement of differentially expressed mRNAs is the molecular gap between mRNA and the functional protein. Since the functionality of a protein is directed by post-translational modifications such as phosphorylation, sulfation or glycosylation of amino acids and further enzymatic or protein–protein interactions, sole knowledge of only the quantity of mRNA does not necessarily provide information on gene function. It is therefore questionable how far transcriptional assays are truly able to provide information on the action of genes that finally direct the biological behavior of a cell. This is supported by several gene expression studies [1–3]. Protein detection strategies used to date include ELISAs, Western blotting or immunohistochemistry (IHC). Although these methods proved to be effective in terms of detection and validation of certain proteins of interest, they do not allow for the high-throughput analysis of the proteome of a cell. In order to extend genomic to proteomic analysis from gene expression at the mRNA level to the investigation of gene function reflected at the protein level, the latest research efforts led to the development of high-throughput screening tools for proteins. Protein microarrays now offer the possibility to simultaneously detect a network of hundreds of up- or downregulated proteins that represent the cellular status and thereby can provide information on the disease or non-disease state of cellular compounds. Since proteins consist of varying amounts of amino acids, form tertiary and quaternary structures, and interact on the basis of hydrophilic, hydrophobic and electrostatic

0959-4973

interactions, which is in sharp contrast to the more or less simple structure of DNA, it is not surprising that protein arrays also have to overcome several obstacles in order to specifically and sensitively profile the cellular proteome. For example, highly reproducible antibody specificity and affinity is a major precondition to detect lowly abundant proteins in complex biological mixtures. In addition, proteins that are included in the analyte need to be targeted in their native state under optimal lysis conditions in order to avoid denaturing of the epitope. To date, several array formats have been generated, such as protein arrays, tissue arrays and antigen–antibody arrays, for the high-throughput detection of proteins in complex solutions. The present review focuses on the field of antibody array technology, and will discuss the different types and manufacture of antibody arrays, protein detection methods, the preparation of clinical samples, and future directions and applications of this technology.

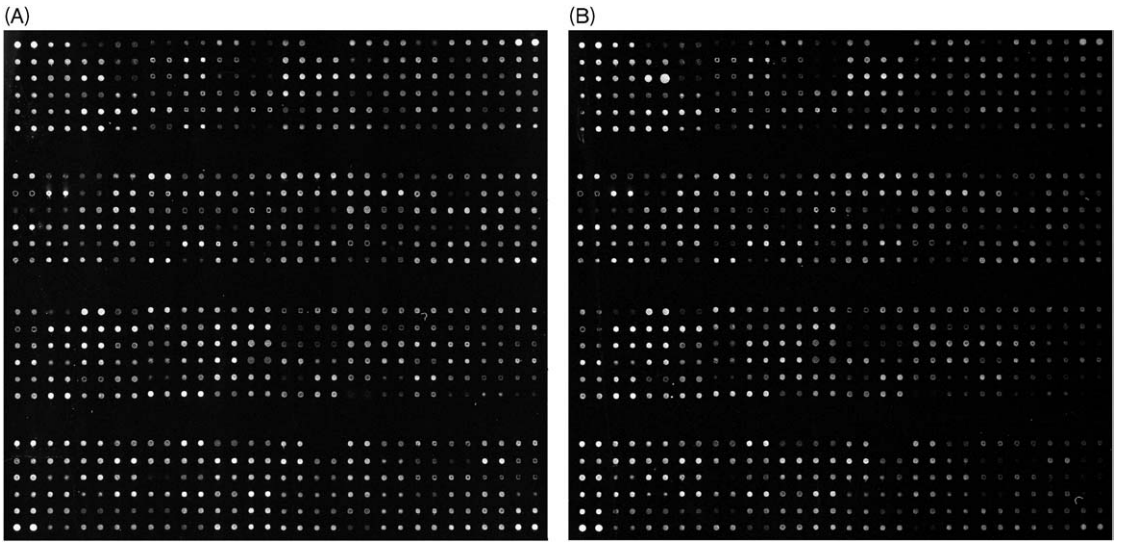
Principle of antibody arrays

In principle, hundreds of immobilized antibodies that act as a 'bait' for the proteins of interest are robotically printed onto a solid surface in an array format, dried and blocked in order to reduce any unspecific background signal. Then a lysed test sample including several proteins of interest is applied onto the array surface. Proteins that are bound by the antibodies included on the array are either identified by a secondary labeled antibody that detects a different epitope of the target protein (sandwich method) or are directly labeled with fluorescence or chemiluminescence dyes. Finally, spot intensities correlate with protein concentrations of the probe and can be measured via scanning methods that are also used for the quantification of cDNA array spot intensities (Fig. 1).

Antibody array surfaces

In order to investigate a large number of proteins simultaneously, antibodies have to be attached to a solid surface at a high density. To achieve this goal, robotic contact printing tools have been used in combination with several materials such as nitrocellulose membranes, and gold, polyvinylidenefluoride (PVDF) and glass surfaces to reach the maximum binding capacity on a small format. Nitrocellulose supports are able to bind bait molecules via non-covalent, non-polar protein surface interactions, which means that antibodies are passively adsorbed by the nitrocellulose layer with high protein binding capacity, but without the need to further modify antibodies for surface attachment [4–6]. One major disadvantage of nitrocellulose membranes is the non-specific binding of proteins that are included in the test sample that results in a high background signal. This can partially be overcome by blocking the area in between the arrayed antibodies with standard blocking reagents such as bovine serum albumin prior to the application of the sample. The second problem that arises using nitrocellulose layers is the disruption of protein–antibody bonds due to the high permeability of the membrane, which has been shown to influence the binding kinetics of the target protein and the antibody [7]. In addition, Lal *et al.*

Fig. 1



Parallel analysis of protein expression in normal and malignant human breast tissue using a commercially available antibody microarray. As depicted, antibody microarrays can measure relative protein abundances between tissue samples. Changes in color intensity are a clear sign that an antigen is more abundant in sample A (left) than in sample B (right).

[8] also describe the problem of light scattering and the incompatibility with protein detection methods such as mass spectroscopy. Therefore, array technology has shifted to the use of glass supports as a platform for antibody attachment. The coupling of proteins onto glass surfaces relies on the covalent binding of amino groups to the pre-treated glass slide which includes cleaning with 1:1 methanol and hydrochloric acid [8] and silanization with certain silane reagents such as 2% (3-mercaptopropyl)trimethoxysilane as described by Rowe *et al.* [9] to further facilitate binding of antibodies to the glass slide. The same effect can also be achieved by coating glass surfaces with immobilized nickel ions that exert high affinity for histidine-tagged antibodies [8], agarose [10], poly-L-lysine [11] or gold [12]. The disadvantage of glass slides is linked to the possibility of unfolding the bait protein due to glass–antibody interactions that are based on the hydrophobic character of the binding partners. As a consequence, the immobilized antibody looses its binding capacity for the proteins of the test sample [8]. In addition, printing of antibodies to the glass surface has to occur in a strictly humidity-controlled environment to favor glass–antibody interactions and attachment [13]. Nevertheless, glass has gained widespread popularity, which can be explained by the fact that it is a low-cost, easily available material. Other surfaces already used as a platform for antibody fixation include polystyrene [14], PVDF [15–17] and gold [18]. Although these materials are equally able to bind bait proteins with high affinity, they proved to be less cost-effective and are limited by their availability. In addition, PVDF confers non-specific protein attachment of test sample molecules, whereas gold-coated silicon is difficult to fabricate and not commercially available.

Generation and validation of antibodies

The successful detection of proteins of interest in a cell lysate or any other complex biological mixture mainly depends on the stability, affinity and specificity of the antibodies included on the array. Therefore, the biggest challenge is the production of specific antibodies that act as protein baits which can be used for high-throughput studies. So far, monoclonal and polyclonal antibodies have been used for protein detection as part of immunohistochemical or Western blotting methods. However, since polyclonal antibodies often lack antigen specificity and confer high expenses, if produced in a large quantity, manufacturers of antibody arrays prefer monoclonal bait proteins. Although methods for the high-throughput generation of monoclonal antibodies, such as hybridomas, are also time consuming and expensive, monoclonal antibodies usually display a higher affinity and specificity for target proteins, a precondition for the use of the array method. In order to overcome this problem, alternative production procedures have been developed. These are aimed at the production of large synthetically generated antibody libraries and include mRNA displays [19] or phage-antibody displays [20]. Independent of the production method, antibodies have to be validated for antigen specificity prior to fixation on an array platform. Therefore, classical protein detection methods, such as Western blotting, are applied where bands at a specified molecular weight indicate the ability of the antibody tested to specifically detect the target protein of interest. Another factor that greatly influences the validity of an antibody array system is the presence of different affinity constants for each antigen–antibody interaction on the array. In other words, antibodies with a high affinity for one certain protein, but a lower affinity for another, which is therefore more prevalent in the biological sample investigated, might react with the low-affinity, but higher concentrated target, as demonstrated by Haab *et al.* [11]. Since the analyte concentration and affinity constants are often unknown in complex biological solutions, several groups have implemented sandwich assays to overcome this problem and alter the specificity of antibody arrays. In principle, the analyte is bound between the arrayed antibody and a secondary labeled antibody binding to a different epitope with a different affinity constant on the same analyte [21]. Although this system precludes the double amount of antibodies, which is laborious and costly, it clearly increases the specificity of the detection process on the array.

Methods for analyte detection

Currently used strategies for detection and visualization of analytes bound on antibody array platforms include fluorescence, chemiluminescence and mass spectrometry techniques. Within this, fluorescence imaging has become the method of choice for antigen detection on antibody arrays due to its high sensitivity and high resolution [6,22]. The biochemical basis for fluorescence visualization is the ability of fluorescent dyes to absorb photons emitted from an external light source, followed by excitation of electrons and emission of light at a characteristic wavelength, which is then measured via fluorescence microscopy. Fluorophores commonly used for analyte detection on protein arrays include indocyanine-derived Cy5 dyes or Alexa probes. Cy5 emits light at 710 nm, thereby exhibiting a red signal on fluorescence microscopy. It does not photobleach, and is characterized by high brightness and a linear relationship between signal intensity and protein concentration up to 1 mg/ml target protein [13,14,23]. Cy5 is usually matched with Cy3 (green) by splitting the analyte in two parts, and then labeling each part with either Cy3 or Cy5 to avoid differences in labeling and antibody-binding efficiencies [24]. Our laboratory tested a commercially available antibody array where two analytes representing the disease state and non-disease state of human tissues were each divided and incubated with Cy3 and Cy5, thus generating four individual samples [24]. Based on the

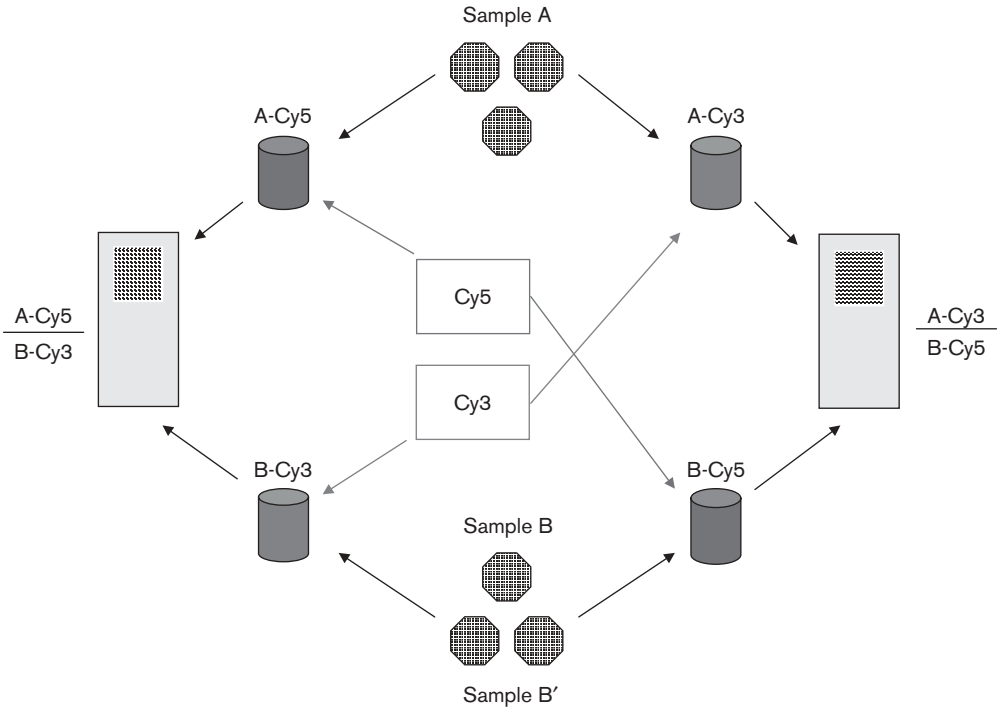
labeling algorithm created by the manufacturer, differences in labeling are overcome even if one of the two dyes (Cy5) reacts more efficiently with a certain target protein than the other one (Cy3). As a consequence, the final detection signal will still be in favor of the analyte labeled with the Cy5 dye because each protein sample is labeled with each dye and a ratio of the two dye signals is calculated for each antibody–antigen pair (Fig. 2). In contrast, Sreekumar *et al.* [25] directly labeled cell lysates from a reference and a test sample with Cy3 and Cy5 fluorophores, and incubated the equally mixed solution on an antibody array. Protein expression levels were successfully calculated in relation to a reference and were validated by immunoblotting.

In accordance with IHC and Western blotting protocols, captured antigens can also be detected via chemiluminescence. In these cases antigen–antibody pairs are visualized by a secondary antibody, conjugated to alkaline phosphatase or horseradish peroxidase (HRP), and are again linked to a chemiluminescent reaction, e.g. the oxidation of luminol [23]. This method was successfully translated into laboratory practice by Huang *et al.* [26], who simultaneously detected differences in cytokine levels by linking biotin-conjugated anti-cytokine antibodies bound to multiple cytokines on a cytokine array with HRP-conjugated streptavidin, which was again coupled with an enhanced chemiluminescence reaction. Non-labeling procedures successfully used for imaging of proteins bound on antibody arrays also include surface-enhanced laser desorption/ionization followed by mass spectrometry (SELDI-MS) to identify proteins. With this method, a laser vaporizes sample proteins, which are bound to arrayed antibodies that are spotted on a metal surface. The evaporation of proteins causes the generation of ionized particles, further by a mass spectrometer. Advantages of these techniques are to avoid biochemical reactions between labeling and antigen molecules, which can affect protein activity, and to enhance the detection sensitivity due to the exact determination of the molecular mass of the target protein [27,28].

Sample preparation and methodology

Antibody array technology was shown to be applicable on body fluid samples [29], cell lines [25,30,31] and even human tissues [24]. In principle, the amount of cells required for protein profiling via an antibody array depends on the sensitivity of the system (moles per volume; s) and the number of molecules included in the test sample (molecules per cell; x) [21]. Therefore, the threshold (T) for cell procurement can be determined by the formula: $T = s\mathrm{A}^*/x$ (A^* is Avogadro's number 6.02×10^{23} molecules/mol).

Fig. 2



Schematic representation of the antibody microarray used for high-throughput analysis of proteomic profiles in normal and malignant human breast tissue samples.

In general, the amount of the biological sample is small in volume and low in concentration of the analyte. Commonly used labeling procedures require about 10^7–10^9 cells, which is equivalent to less than 1 cm^3 or 50–100 mg of tissue. For example, core needle biopsies approximately provide a 2×5-mm core of tissue containing only about 10 000–20 000 cells [21,23,32]. For tissue extracts, 10 µg is found to be sufficient for protein analysis [24].

Another precondition for the successful analysis is the adequate preservation of the test sample. Therefore, formalin-fixed and paraffin-embedded tissue has to be excluded from protein arraying due to denaturing and cross-linking of antigens. As a consequence, snap-frozen tissue or complex biological solutions are stored at $-80°C$ after collection in order to preserve protein stability and minimize protein degradation. Using laser-captured microdissected tissue (LCM), snap-frozen tissue blocks are cut at 5–10 µm, stained with standard solutions (hematoxylin & eosin) and dehydrated with an alcohol gradient prior to the dissection procedure. Protein extraction of cell samples, obtained by LCM, cell cultures or after homogenization of whole tissues, is accomplished by extraction and labeling buffers containing non-denaturing detergents, which do not interact with antibody binding and preserve protein structure [21,33]. In brief, the analyte is incubated with the array after protein labeling and removal of the unbound dye. This can be accomplished by gel-exclusion chromatography (gel filtration) using gel-filtration columns. The labeled proteins included in the biological sample migrate ahead of the unbound dye due to their higher molecular weight. Labeled proteins can be detected visually, whereas excess dye is removed. The protein concentration of the test mixture and the reference sample is usually measured by the bicinchoninic acid method to provide equal quantities of both samples prior to incubation with spotted antibodies on the array. The incubation of the array slides with the labeled protein mixture is performed at room temperature for 30–120 min, depending on the protocol and the manufacturer's experience. Following this, arrays are washed, dried by centrifugation of the slides and finally subjected to the scanning procedure. Arrays can be analyzed using fluorescent scanners, commonly used for cDNA microarray analysis, including those manufactured by companies such as Genetic Microsystems, Axon, Perkin-Elmer Life Sciences or Affimetrix. Analysis of differences in protein expression and cut-off values for determination of protein abundance is still discussed controversially because antigen and background signals often vary from different antibody arrays and inter-laboratory variability. Also, the quantity of a protein does not necessarily correlate directly with the biological consequences of protein function, which means that even proteins with low abundance can theoretically exert high cellular functionality. However, since upregulation of certain molecules is thought to have an impact on cell biology, various cut-off values for significances in protein abundance have been implemented by investigators to quantify up- and downregulation of certain antigens. Our laboratory used a commercially available antibody array for the differential detection and quantification of proteins in a biological sample [24]. According to the manufacturer's protocol, four samples deriving from a test and a reference sample are combined to form two mixes which are incubated with two equally constructed antibody arrays (Fig. 2). An internationally normalized ratio (INR) can be calculated by the formula: $INR = \sqrt{\text{Ratio 1/Ratio 2}}$ (Ratio 1: A-Cy5 relative fluorescence units/B-Cy3 relative fluorescence units and Ratio 2: B-Cy5 relative fluorescence units/A-Cy3 relative fluorescence units).

Table 1

Name	Function	*R*/*R* ratio	INR
Casein kinase Ie	utilization/phosphorylation of acidic proteins	6.97	**2.64**
p53	tumor suppression	4.61	**2.15**
Annexin-XI	calcium/phospholipid binding protein	4.20	**2.05**
CDC25C	M phase induction	4.17	**2.04**
eIF-4e	translation initiation	3.94	**1.98**
HDJ-2	heat shock protein	3.72	**1.93**
MAPK kinase 7	protein kinase	3.64	**1.90**
Interleukin 13	cytokine	2.98	**1.72**
MAPK kinase 2	protein kinase	2.71	**1.64**
Caspase 1/ICE	regulation of apoptosis	2.70	**1.64**
Her-2/*neu*	growth factor receptor	2.76	**1.63**
MAPK kinase 3	protein kinase	2.64	**1.62**
Fos	signal transduction/cell proliferation	2.59	**1.61**
$P21^{Cip/Waf}$	inhibition of cellular proliferation	2.54	1.59
c-Myc	regulation of gene transcription	2.47	1.57
MDM 2	oncoprotein (p53 binding)	2.34	1.53
Ki-67	cell cycle progression	1.89	1.37
PI-3 kinase	signal transduction	1.67	1.29
Caspase 7	apoptosis	1.62	1.27
Stat 2	transcription factor	1.50	1.22
P-Cadherin	cell adhesion	1.38	1.17
Caspase 4	induction of apoptosis	1.25	1.11
Bax	induction of apoptosis	1.22	1.10
Bcl-2	apoptosis repressor	1.16	1.08
Smad 4	mediation of signal transduction	1.16	1.07
Cyclin E	cell cycle control	1.00	1.00
Caspase 8	induction of apoptosis	0.98	0.99
c-Cbl	signal transduction	0.95	0.97
Smad 2	transcriptional modulator	0.91	0.95
Cyclin D2	cell cycle control	0.91	0.95
Ha-ras	transcriptional regulation	0.91	0.95
JAK 1	dual specificity kinase	0.83	0.91
Stat 3	regulation of transcription	0.83	0.91
Cyclin D_1	cell cycle control	0.70	0.84
EGFR	growth factor receptor	0.57	0.75
14-3-3e	protein kinase C inhibition	0.35	0.59

An $INR > 1$ indicates an antigen more abundant in sample A than in sample B, whereas an antigen of an $INR < 1$ is considered less abundant in sample A than in sample B. As suggested by the manufacturer, INR values of ≥ 2 or ≤ 0.5 indicate with a very high probability significant differences in protein abundances (Table 1). According to this approach, a single sample is analyzed against itself, thus reducing assay variance significantly. Other authors used mathematical models for calculation

of the cut-off level for differences in protein abundances: Sreekumar *et al.* [17] considered signal intensity values outside the interval of 0.74–1.26 to be differentially expressed with a statistical confidence of 68%, whereas values outside the interval of 0.6–1.4 were considered differentially expressed with a statistical confidence of 86%. Based on these assumptions, the use of cut-off values and INRs does improve the quality of the data obtained by the calculation of different signal intensities. However, since these values only describe relative differences between the test and reference sample, additional procedures, such as Western blotting, IHC or ELISA for certain proteins of interest are recommended to further validate the results of the antibody array analysis.

Clinical applications and future perspectives

Although data are still limited, some preliminary studies already demonstrated, using antibody array technology, that the simultaneous detection of hundreds of proteins has the potency to be used for a large spectrum of applications. One primary aim of this young technology is to join a particular state of disease to a characteristic protein expression pattern in order to define pathophysiological processes and the biological individuality of a disease more precisely. For example, Sreekumar *et al.* [25] analyzed cell lysates of LoVo colon carcinoma cells in response to treatment with ionizing radiation using an antibody array created by the authors and observed high expression levels for five pro-apoptotic proteins known to be induced by radiotherapy. In addition, the authors were able to identify another six upregulated proteins responsive to radiation, thereby linking radiation-induced cell death and arrest of cell growth with these molecules. The detection of differentially expressed proteins in a complex solution via an antibody array-based approach was successfully performed by Huang *et al.* [26]. They spotted capture antibodies against several human cytokines onto a membrane, which was further incubated with patients' sera. Using enhanced chemiluminescence for the visualization of bound antigens, up to 24 different cytokines could be detected, quantified and successfully validated by ELISA. Similarly, Li *et al.* [31] robotically printed antibodies against four mouse cytokines on a modified glass slide in order to measure the release of all four cytokines from cultured mouse monocytes. To demonstrate that all these rather preliminary data possess the potency to be transformed into clinical practice, Belov *et al.* [30] constructed an array containing 48 different anti-cluster of differentiation (CD) antibodies to facilitate immunophenotyping of leukemias. The authors could provide a reproducible dot pattern that allowed the rapid CD-antigen-based discrimination between chronic lymphocytic leukemia, other types of leukemias and normal blood lymphocytes. Finally, Hudelist *et al.* [24] investigated the practicability of a commercially available antibody microarray using comparative fluorescence for the parallel detection and quantification of 378 proteins in normal and malignant human breast tissue specimens. The protein expression pattern of eight representative proteins was validated by immunohistochemical methods to confirm the high specificity and sensitivity of the antibody array system. The study demonstrates the workability of protein expression analysis of whole human tissue blocks by using an antibody microarray, thereby suggesting the use of antibody arrays for a wide range of proteomics-related investigations.

Conclusions

Currently, high-throughput expression studies are usually based on the use of cDNA arrays. Antibody arrays now offer the opportunity to quantify the proteomic alterations in response to a certain pharmacological agent or as a result of a certain state of disease. In addition, several lines of evidence suggest that differential proteome analysis does reflect cellular biology more accurately than mRNA expression studies since genes do work at the protein level and antibody arrays now allow the analysis of post-translational molecular interactions. Although, still limited in number, some trials have already demonstrated the practicability of antibody arrays for high-throughput protein expression profiling of serum, whole human tissue and microdissected cellular lysates. Data from these studies clearly point out the importance of monitoring proteomic alterations in order to define the biology and specimen-specific individuality of a disease more precisely. The identification of active signal transduction pathways, the determination of the phosphorylation status of signaling proteins, or the presence or absence of well-known prognostic marker proteins that can be monitored with the use of antibody arrays could therefore provide new information on the molecular action of novel therapeutic strategies and help to reveal new target molecules for pharmacological agents. The determination of altered protein expression levels associated with a particular state of disease will help us to better understand the ongoing pathophysiological and biological processes at different stages of a disease, and could allow for the development and use of individual, patient-tailored and stage-specific targeted therapies.

References

1 Anderson L, Seilhamer J. A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis* 1997; **18**:533–537.
2 Celis JE, Kruhoffer M, Gromova I, Frederiksen C, Ostergaard M, Thykjaer T, *et al.* Gene expression profiling: monitoring transcription and translation products using DNA microarrays and proteomics. *FEBS Lett* 2000; **480**: 2–16.
3 Gygi SP, Rochon Y, Franza BR, Aebersold R. Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* 1999; **19**:1720–1730.
4 Zhu H, Snyder M. Protein chip technology. *Curr Opin Chem Biol* 2003; **7**:55–63.
5 Joos TO, Schrenk M, Hopfl P, Kroger K, Chowdhury U, Stoll D, *et al.* A microarray enzyme-linked immunosorbent assay for autoimmune diagnostics. *Electrophoresis* 2000; **21**:2641–2650.

6 Templin MF, Stoll D, Schrenk M, Traub PC, Vohringer CF, Joos TO. Protein microarray technology. *Trends Biotechnol* 2002; **20**:160–166.
7 Steinitz M, Tamir S. An improved method to create nitrocellulose particles suitable for the immobilization of antigen and antibody. *J Immunol Methods* 1995; **187**:171–177.
8 Lal SP, Christopherson RI, dos Remedios CG. Antibody arrays: an embryonic but rapidly growing technology. *Drug Discov Today* 2002; **7**:S143–S149.
9 Rowe CA, Scruggs SB, Feldstein MJ, Golden JP, Ligler FS. An array immunosensor for simultaneous detection of clinical analytes. *Anal Chem* 1999; **71**:433–439.
10 Afanassiev V, Hanemann V, Wolfl S. Preparation of DNA and protein micro arrays on glass slides coated with an agarose film. *Nucleic Acids Res* 2000; **28**:E66.
11 Haab BB, Dunham MJ, Brown PO. Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. *Genome Biol* 2001; **2**:RESEARCH0004.
12 Houseman BT, Huh JH, Kron SJ, Mrksich M. Peptide chips for the quantitative evaluation of protein kinase activity. *Nat Biotechnol* 2002; **20**:270–274.
13 MacBeath G, Schreiber SL. Printing proteins as microarrays for high-throughput function determination. *Science* 2000; **289**: 1760–1763.
14 Silzel JW, Cercek B, Dodson C, Tsay T, Obremski RJ. Mass-sensing, multianalyte microarray immunoassay with imaging detection. *Clin Chem* 1998; **44**:2036–2043.
15 Liu B, Huang L, Sihlbom C, Burlingame A, Marks JD. Towards proteome-wide production of monoclonal antibody by phage display. *J Mol Biol* 2002; **315**:1063–1073.
16 Bieri C, Ernst OP, Heyse S, Hofmann KP, Vogel H. Micropatterned immobilization of a G protein-coupled receptor and direct detection of G protein activation. *Nat Biotechnol* 1999; **17**:1105–1108.
17 Rich RL, Day YS, Morton TA, Myszka DG. High-resolution and high-throughput protocols for measuring drug/human serum albumin interactions using BIACORE. *Anal Biochem* 2001; **296**:197–207.
18 Dong Y, Shannon C. Heterogeneous immunosensing using antigen and antibody monolayers on gold surfaces with electrochemical and scanning probe detection. *Anal Chem* 2000; **72**:2371–2376.
19 Wilson DS, Keefe AD, Szostak JW. The use of mRNA display to select high-affinity protein-binding peptides. *Proc Natl Acad Sci USA* 2001; **98**:3750–3755.
20 Ryu DD, Nam DH. Recent progress in biomolecular engineering. *Biotechnol Prog* 2000; **16**:2–16.
21 Liotta LA, Espina V, Mehta AI, Calvert V, Rosenblatt K, Geho D, *et al.* Protein microarrays: meeting analytical challenges for clinical applications. *Cancer Cell* 2003; **3**:317–325.
22 Haab BB. Advances in protein microarray technology for protein expression and interaction profiling. *Curr Opin Drug Discov Dev* 2001; **4**:116–123.
23 Espina V, Woodhouse EC, Wulfkuhle J, Asmussen HD, Petricoin 3rd EF, Liotta LA. Protein microarray detection strategies. focus on direct detection technologies. *J Immunol Methods* 2004; **290**:121–133.
24 Hudelist G, Pacher-Zavisin M, Singer CF, Holper T, Kubista E, Schreiber M, *et al.* Use of high-throughput protein array for profiling of differentially expressed proteins in normal and malignant breast tissue. *Breast Cancer Res Treat* 2004; **86**:281–291.
25 Sreekumar A, Nyati MK, Varambally S, Barrette TR, Ghosh D, Lawrence TS, *et al.* Profiling of cancer cells using protein microarrays: discovery of novel radiation-regulated proteins. *Cancer Res* 2001; **61**:7585–7593.
26 Huang RP, Huang R, Fan Y, Lin Y. Simultaneous detection of multiple cytokines from conditioned media and patient's sera by an antibody-based protein array system. *Anal Biochem* 2001; **294**:55–62.
27 Hempel G. Strategies to improve the sensitivity in capillary electrophoresis for the analysis of drugs in biological fluids. *Electrophoresis* 2000; **21**: 691–698.
28 Davies H, Lomas L, Austen B. Profiling of amyloid beta peptide variants using SELDI protein chip arrays. *Biotechniques* 1999; **27**:1258–1261.
29 Robinson WH, DiGennaro C, Hueber W, Haab BB, Kamachi M, Dean EJ, *et al.* Autoantigen microarrays for multiplex characterization of autoantibody responses. *Nat Med* 2002; **8**:295–301.
30 Belov L, de la Vega O, dos Remedios CG, Mulligan SP, Christopherson RI. Immunophenotyping of leukemias using a cluster of differentiation antibody microarray. *Cancer Res* 2001; **61**:4483–4489.
31 Li Y, Schutte RJ, Abu-Shakra A, Reichert WM. Protein array method for assessing *in vitro* biomaterial-induced cytokine expression. *Biomaterials* 2005; **26**:1081–1085.
32 Espina V, Mehta AI, Winters ME, Calvert V, Wulfkuhle J, Petricoin 3rd EF, *et al.* Protein microarrays: molecular profiling technologies for clinical specimens. *Proteomics* 2003; **3**:2091–2100.
33 Simone NL, Remaley AT, Charboneau L, Petricoin 3rd EF, Glickman JW, Emmert-Buck MR, *et al.* Sensitive immunoassay of tissue cell proteins procured by laser capture microdissection. *Am J Pathol* 2000; **156**: 445–452.